TRANSCRIPTIONAL REGULATION OF THE SRC1α AND SRC1A PROMOTERS IN HUMAN CANCER CELL LINES

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by

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ABSTRACT

The human SRC gene encodes $pp60^{c\text{-}Src}$ (or c-Src), a 60 kDa, non-receptor tyrosine kinase frequently activated in colon and other tumors. Many studies have demonstrated c-Src activation can be accounted for by overexpression of c-Src protein, and that this overexpression is important for the fully transformed phenotype of cancer cells. The general goal of this thesis, therefore, was to determine the mechanism of this overexpression in human cancer cells. Examination of c-Src expression and activity in human colon cancer cell lines showed that c-Src activation was due to transcriptional activation of the SRC gene. SRC transcription is directed by the ubiquitous, Sp1 regulated SRC1A promoter, and the HNF-1α regulated, tissue restricted SRC1α promoter. To study the mechanism of SRC transcriptional activation in human cancer cell lines, a dual SRC promoter reporter construct was generated with both these promoters in their natural, physiologically linked context. Very low activity of the SRC1α promoter, relative to SRC1A, was consistently observed from this construct, leading to the conclusion that an enhancer element elevates SRC1α promoter activity. Interestingly, the HNF binding site in the SRC1α promoter enhanced SRC1A promoter activity in the dual promoter construct, but only in a colon cancer cell line with activated SRC. These results therefore suggest SRC transcriptional activation results from enhancer action and/or SRC promoter cross-talk in subsets of human cancer cells.

This study has also determined that histone deacetylase inhibitors (HDIs), compounds with documented anti-neoplastic properties, repress transcription from both SRC promoters in various cancer cell lines. To identify the mechanism of this repression, various deletion and mutant SRC promoter constructs were assayed, but HDI

response elements were not identified. However, it was discovered that both promoters shared a common requirement for functional TAF1/TAF(II)250, a component of the general transcription factor TFIID. Compromised TAF1 function impaired SRC transcription, but also blocked SRC repression by HDIs. Experiments with SRC:WAF1 promoter chimeras showed the SRC promoters' TAF1 requirement could be conferred on the heterologous, TAF1-independent promoter for the WAF1 gene, which encodes the cell cycle inhibitor p21. These chimeras were also repressed by HDIs, despite WAF1 normally being strongly induced by these agents. These results therefore provide a potential functional link between promoter architecture, TAF1 dependence, and HDI mediated transcriptional repression.

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Most importantly, I thank my wife, Marlene, for her support, especially when the demands of my graduate program extended past the confines of an average workday. She is responsible for the perfect balance in my life.

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LIST OF ABBREVIATIONS

5' RACE	5' rapid amplification of cDNA ends
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
APL	acute promyelocytic leukemia
AT	acetyltransferase
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
CPSF	cleavage and polyadenylation specificity factor
CSF-1	colony stimulating factor-1
Csk	cellular Src kinase
CTD	carboxyl terminal domain
DEPC	diethyl pyrocarbonate
DH	DNaseI hypersensitive
dNTP	deoxyribonucleotide triphosphate
DPCAT	dual promoter CAT reporter construct
DPE	downstream promoter element
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
FAK	focal adhesion kinase
FAT	factor acetyltransferase
FCS	fetal calf serum
GNAT	Gcn5 related N-acetyltransferase
GSK-3β	glycogen synthase kinase-3β
GTF	general transcription factor
HA	hemagglutin
HAT	histone acetyltransferase
HCCLs	human colon cancer cell lines
HDAC	histone deacetylase
HDI	histone deacetylase inhibitor
HEPES	hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HER1	human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
HGF/SF	hepatocyte growth factor/scatter factor
HMG	high mobility group
HNF-1α	hepatocyte nuclear factor-1α
HNF-1β	hepatocyte nuclear factor-1β
hnRNP K	heterogeneous nuclear ribonuclear protein K
Inr	initiator

LB	Lauria Bertani
MAPK	mitogen activated protein kinase
NaB	sodium butyrate
NAD	nicotinamide adenine dinucleotide
NIB	nuclear isolation buffer
NMT	N-myristoyltransferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol-3-kinase
PIC	preinitiation complex
PMSF	phenylmethylsulfonylflouride
Pol II	RNA Polymerase II
Pol IIA	dephosphorylated form of Pol II carboxyl terminal domain
Pol IIO	Ser2 and/or Ser5 phosphorylated form of Pol II carboxyl terminal domain
P-TEFb	positive transcription elongation factor b
Pu:Py	polypurine:polypyrimidine
RAR	retinoic acid receptor
RSV	Rous sarcoma virus
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SAGA	Spt-Ada-Gcn5 acetyltransferase

SDS	sodium dodecyl sulphate
SH2	Region of Src homology 2
SH3	Region of Src homology 3
SH4	Region of Src homology 4
SIR	silent information regulator
SMASH	small molecular weight activator binding the SrcHNF site
SPy	SRC pyrimidine binding factor
STAT3	signal transducer and activator of transcription 3
TAE	Tris acetate EDTA
TAF	TATA binding protein associated factor
TBE	Tris borate EDTA
TBP	TATA binding protein
TBST	Tris buffered saline Tween-20
Tcf/Lef	Ternary cell factor/lymphoid enhanced factor
TE	Tris EDTA
TRANCE	Tumor necrosis factor-related activation induced cytokine receptor
TSA	trichostatin A
UAS	upstream activator sequences
VEGF	vascular endothelial growth factor
β-Gal	β-Galactosidase

1. REVIEW OF THE LITERATURE

1.1. Introduction

SRC encodes $pp60^{c\text{-}Src}$, a signaling protein involved in many important cellular processes including proliferation, motility, and apoptosis. Activation and/or overexpression of $pp60^{c\text{-}Src}$ have been consistent findings in diverse human cancers, including colon cancer. Therefore, the general goal of this thesis is to investigate the mechanisms that regulate SRC transcription, and define their importance in determining overall $pp60^{c\text{-}Src}$ expression and activity levels in human cancer. The following review of the literature will familiarize the reader with the human SRC gene and gene product, as well as summarize current views of the many levels of eukaryotic transcriptional regulation. These themes will subsequently converge, and the present state of knowledge regarding SRC transcriptional regulation will be discussed.

1.2. Biology of Src

1.2.1. The c-Src Proto-Oncogene and c-Src Gene Product

1.2.1.1. v-Src and the Rous Sarcoma Virus

One of the first glimpses into the modern field of cancer molecular genetics was provided in 1910, when Nobel Laureate Peyton Rous described that a solid tumor could be induced in the common hen by a filterable agent extracted from a fibrosarcoma in another hen (Rous, 1979). At the time, this finding was very controversial, because cancer had been deemed non-infectious. Indeed, it took nearly four decades of findings similar to these, as well as discovery of viruses, before it became readily accepted that certain viruses could induce tumors in appropriate hosts. Eventually, this filterable agent was identified, and now bears the name Rous sarcoma virus (RSV). Being one of the first discovered RNA retroviruses, the findings garnered from early study of RSV comprise one of the major cornerstones in the field of molecular virology.

Subsequent studies with RSV showed that the virus could alter the morphological appearance, or "transform" cultured chick embryo fibroblasts in one of the first described focus-forming assays (Temin and Rubin, 1958). It was concluded that a component of the RSV genome was responsible for transformation, and a major concentration of efforts went into determining its identity. The generation of one particular temperature sensitive RSV mutant was pivotal in identifying the RSV genetic component that caused transformation. In fibroblasts grown at 30^{o}C, this mutant was competent for both replication and transformation. Conversely, when fibroblasts were grown at 39^{o}C, RSV could not elicit transformation, but could still replicate (Martin, 1970). Additional RSV mutants were isolated that were replication competent, but transformation defective (Golde, 1970; Toyoshima et al., 1970). Comparison of the

genomes of these mutants to wild type RSV led to the conclusion that the genomes of transformation defective mutants were smaller. This allowed for the development of markers for the gene hypothesized as responsible for RSV mediated transformation. Inevitably, the eventual identification and sequencing of the v-Src gene was reported (Czernilofsky et al., 1983; Czernilofsky et al., 1980).

At the same time that the v-Src gene was being mapped and cloned, efforts were underway to identify the protein product encoded by this proposed transforming gene, or oncogene. Many approaches were tried *in vitro* to translate the viral RNA, but these attempts were to no avail. Eventually anti-serum was developed to proteins in RSV induced, tumor bearing animals, and a 60 kDa phosphoprotein, termed $pp60^{v\text{-}Src}$ (or v-Src) was immunoprecipitated from these tumor lysates (Brugge and Erikson, 1977). A major breakthrough came when immunoprecipitated complexes were incubated with [γ-^{32}P]-ATP, and it was discovered that v-Src had intrinsic kinase activity (Collett and Erikson, 1978; Levinson et al., 1978). However, it was an unexpected finding that the residues phosphorylated by v-Src were tyrosines, because only serine or threonine phosphorylation had been described at that time (Hunter and Sefton, 1980). As such, the v-Src gene in the RSV genome represented the very first identified oncogene, which encoded the very first protein discovered with tyrosine kinase activity.

1.2.1.2. The c-Src Proto-Oncogene

A stunning finding came in 1976, when Nobel Laureates Michael Bishop and Harold Varmus, along with their colleagues, reported that a cDNA probe to v-Src hybridized to normal avian DNA (Stehelin et al., 1976). The ability of this probe to

hybridize with normal cellular DNA was conserved in vertebrates as well, suggesting a normal cellular counterpart to the v-Src gene. The transforming viral counterpart, v-Src, had previously been coined an oncogene; therefore, to prevent ambiguity, "proto-oncogene" was the term created to describe the normal cellular counterpart. Eventually, this cellular proto-oncogene, aptly named c-Src (cellular-Src), was cloned and sequenced in chickens and humans (Anderson et al., 1985; Shalloway et al., 1981; Takeya and Hanafusa, 1983; Tanaka et al., 1987). This initial landmark discovery led to the identification of additional cellular proto-oncogenes that were very closely related to their oncogenic viral counterparts. Therefore, RNA tumor viruses are thought to have evolved through capture, or transduction, of proto-oncogenes into their genome followed by eventual mutation of these transduced sequences into oncogenes (Bishop, 1983). A revolution in cancer molecular biology soon developed, with the hypothesis that tumors could arise through activation of these proto-oncogenes.

Characterization and comparison of $pp60^{c\text{-}Src}$ and $pp60^{v\text{-}Src}$ (or c-Src and v-Src) identified that they are highly conserved at the level of amino acid sequence (Fig. 1.1). However, the v-Src protein harbors a number of point mutations compared with c-Src as well as mutated and substituted C-terminal sequences, resulting in a truncated version of the c-Src protein. Normal cellular proteins with significant homology to c-Src have also been identified, and have been grouped together to comprise the Src family. Currently, there are at least nine members of this gene family: Src, Yes, Fyn, Fgr, Yrk, Lyn, Blk, Hck, and Lck. Src, Yes, and Fyn expression appears ubiquitous, while most of the other family members differ spatially and temporally in expression patterns (most being restricted to cells of the haematopoietic system) (Thomas and Brugge, 1997).

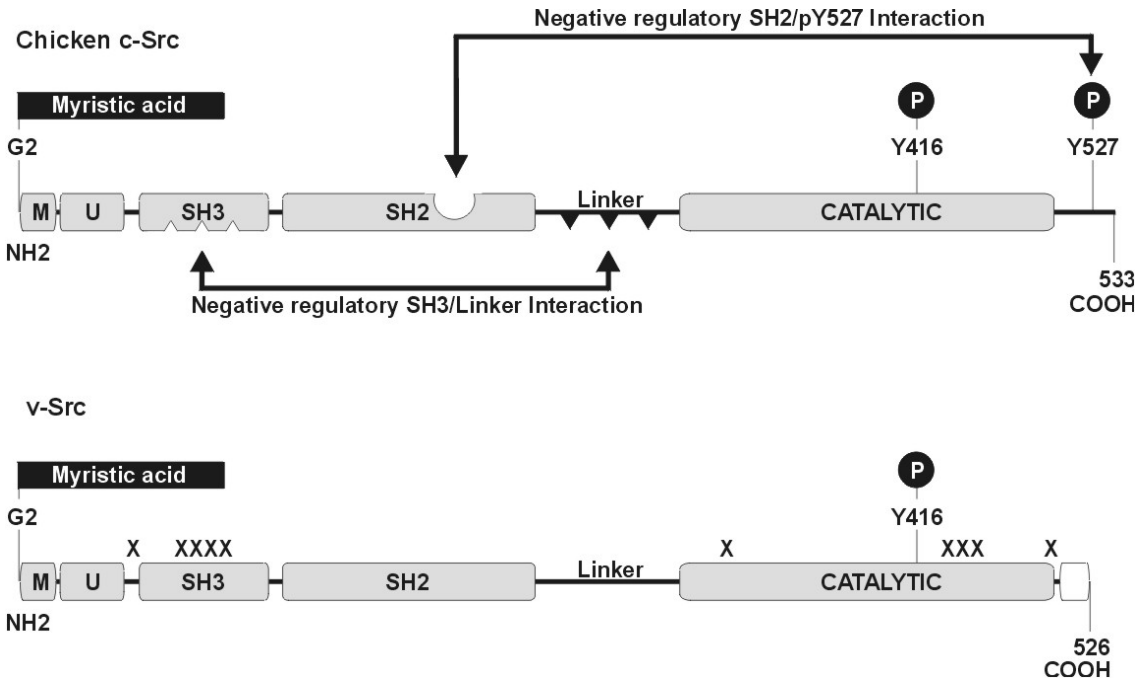

Figure 1.1. Comparison of the c-Src and v-Src proteins. Modular domains in the chicken c-Src and v-Src proteins are shown. M refers to the SH4, or myristoylation domain, and U refers to the unique region. Glycine 2, the myristoylated N-terminal residue, is shown. Important sites of tyrosine phosphorylation are shown. Intramolecular interactions that occur in the closed, inactive conformation of c-Src are illustrated with double-headed arrows. X's along the v-Src protein denote mutations compared to the c-Src protein. The white box at the C-terminus of the v-Src protein refers to amino acids that have been deleted and replaced compared to the c-Src protein.

All c-Src family members share common structural domains (Fig. 1.1). From N to C terminus, they have been defined as the 15 residue region of Src homology 4 (SH4) domain, the poorly conserved 40-70 residue unique domain, the 50 residue SH3 domain, the 100 residue SH2 domain, and the 250 residue catalytic, tyrosine kinase domain (Brown and Cooper, 1996). Interestingly, the SH2, SH3, and kinase domains of c-Src are highly conserved in many proteins involved in signal transduction cascades (Pawson and Gish, 1992; Sudol, 1998). Therefore, c-Src serves as a prototype for many modular proteins, especially tyrosine kinases, that function in signaling pathways. Co-translational processing of c-Src by an aminopeptidase removes the N-terminal methionine residue from the protein, thus exposing glycine 2 within the SH4 domain (Buss et al., 1984). This glycine residue serves as a substrate for the irreversible attachment of myristic acid by the enzyme N-myristoyl transferase (NMT) (Buss et al., 1984). Other Src family members also serve as substrates for N-terminal lipid attachment, but the majority contain palmitic acid rather than myristic acid (Thomas and Brugge, 1997). Lipid modification is essential for Src family activity and localizes the protein to the plasma membrane as well as intracellular membranes such as the endoplasmic reticulum and endosomes (Brown and Cooper, 1996; Courtneidge et al., 1980). The unique domain, located between the SH4 and SH3 domains, is responsible for the specificity in the interactions that occur between Src family members and upstream activators or downstream effectors. The SH3 domain binds the core consensus PxxP (P = proline, x = any amino acid), which forms a left-handed polyproline helix (Ren et al., 1993). SH3 ligands with the PxxP motif fall into two classes; class I ligands bind the SH3 domain in a N to C orientation, while class II ligands bind the SH3 domain in a C to N fashion. For class I SH3 ligands, the amino acids N terminal to the PxxP

core dictate specificity, whereas the amino acids C terminal to this core motif are responsible for class II ligand specificity (Sudol, 1998). The final modular element in the Src family of proteins is the SH2 domain, which binds phosphotyrosine. This SH2 domain contains two discrete binding pockets; one binds phosphotyrosine and the other binds the amino acid in the +3 position, relative to phosphotyrosine (Sudol, 1998). Therefore, the three amino acids immediately C-terminal to the phosphorylated tyrosine residue dictate SH2 specificity (Songyang et al., 1995). The SH2 domains in different proteins have been divided into 4 discrete groups based on the amino acid they preferentially bind in the +1 position of the phosphotyrosine ligand or the amino acids in their binding pockets that interact with the phosphotyrosine ligand (Brown and Cooper, 1996).

1.2.2. Regulation of c-Src Activity

Negative regulation of $pp60^{c\text{-}Src}$ activity occurs primarily through phosphorylation of an important tyrosine residue located at the C-terminus of the c-Src protein (Fig. 1.1). This tyrosine residue has been identified as tyrosine 527 for the chicken c-Src protein, and tyrosine 530 for the human c-Src protein (Bjorge et al., 1996; Brown and Cooper, 1996; Cooper and Howell, 1993). Mutation or deletion of Y527/530 constitutively activates the c-Src kinase, thus conferring transforming ability on $pp60^{c\text{-}Src}$ (Cartwright et al., 1987; Courtneidge, 1985). Phosphorylation of Y527/530 is carried out by cellular src kinase (Csk) (Nada et al., 1991). The importance of this protein in catalyzing Y527/530 modification is highlighted by the observation that inactivation of Csk results in constitutive c-Src activity (Imamoto and Soriano, 1993). Interestingly, the primary defining feature of v-Src is deletion and replacement of sequences encoding the C-

terminal negative regulatory regions of its normal cellular counterpart (Parsons and Weber, 1989). The molecular basis for post-translational regulation has been provided by the X-ray crystallographic structure of c-Src in its inactive conformation, which has demonstrated that Y527/Y530 phosphorylation results in a "closed" conformation of the c-Src protein (Xu et al., 1997). This inactive protein conformation is due to an intramolecular association between phosphorylated Y527/530 and the SH2 domain of c-Src, as well as association between the SH3 domain and a linker region between the SH2 and kinase domains (Fig. 1.1). These intramolecular associations prevent the SH2, SH3, and kinase domains from interacting with other proteins. When Y530 is dephosphorylated, this allows $pp60^{c\text{-}Src}$ to adopt an open conformation and hence interact with and phosphorylate other proteins. The ability to dephosphorylate Y527/530, and subsequently activate $pp60^{c\text{-}Src}$, has been demonstrated for the Shp-2, PTPλ, and PTP1B protein tyrosine phosphatases (Bjorge et al., 2000; Fang et al., 1994; Walter et al., 1999). Once active, $pp60^{c\text{-}Src}$ autophosphorylates within its "activation loop" at Y416, resulting in fully activated protein.

Activation of the c-Src protein is achieved through a combination of SH2 displacement, Y527/530 dephosphorylation, and Y416 phosphorylation. However, the precise order of these events has not been clearly established. The classic model of c-Src activation is through association with growth factor receptors. Ligand binding stimulates the intrinsic tyrosine kinase activity of growth factor receptor proteins by inducing their dimerization. The resulting autophosphorylated tyrosine residues on the intracellular domains of these receptors have been proposed as high affinity binding sites for the c-Src SH2 domain, which displace the negative intramolecular association with

Y527/530 (Schwartzberg, 1998). Once bound to activated growth factor receptors, subsequent Y530/527 dephosphorylation is thought to occur. This allows generation of the "open" conformation of c-Src, which results in Y416 phosphorylation by the activated growth factor receptor, or through an autocatalytic mechanism.

1.2.3. Activation of c-Src Signaling Cascades by Transmembrane Receptors

Activation of c-Src is directly achieved by a large number of transmembrane proteins, including receptor tyrosine kinases (RTKs), immune recognition/major histocompatibility complex receptors, adhesion receptors, G-protein coupled receptors, and cytokine receptors (Thomas and Brugge, 1997). Of these, the best described are RTK and adhesion receptor signaling cascades; therefore, they will be discussed in detail in subsequent sections. Although these signaling pathways are usually presented as discrete, exclusive, and linear, recent evidence has clearly shown that there is extensive cross-talk between them (Uings and Farrow, 2000).

1.2.3.1. Activation of c-Src Signaling Cascades by Receptor Tyrosine Kinases

The majority of studies involving c-Src activation by RTKs have involved mouse fibroblast model systems. While the results garnered from these approaches have revealed much about the signaling pathways that c-Src participates in, it is evident that there are very specific differences between cell types and species. Nevertheless, these studies have identified that the information flow between RTKs and c-Src is two-way, meaning RTKs can bind, phosphorylate, and activate c-Src, and that c-Src can bind, phosphorylate and activate RTKs. One well-studied example is the platelet derived growth factor (PDGF) receptor pathway. The first suggestion that c-Src was involved in

PDGF receptor signaling came from the observation that PDGF stimulation of NIH3T3 mouse fibroblasts resulted in c-Src activation (Ralston and Bishop, 1985). Mutagenesis studies suggested the PDGF receptor/c-Src association is mediated by interactions between the c-Src SH2 domain, and phosphorylated Y579 or Y581 PDGF receptor residues (Mori et al., 1993; Twamley et al., 1992). This PDGF receptor mediated activation is transient, and results in phosphorylation of c-Src on Y416 (Thomas and Brugge, 1997), as well as a novel phosphorylation of c-Src on Y138 (Broome and Hunter, 1997). In turn, the PDGF receptor is phosphorylated directly by c-Src at Y934, which enhances PDGF mitogenic signaling (Hansen et al., 1996).

Another well-described RTK that activates c-Src is the epidermal growth factor (EGF) receptor (EGFR). c-Src overexpression enhances many EGF receptor mediated responses, and EGF treatment increases c-Src catalytic activity 2 to 3 fold (Thomas and Brugge, 1997). An association between c-Src and human EGF receptor 1 (HER1) has been observed in a number of human carcinoma cell lines (Belsches et al., 1997). This association is believed to be EGF dependent, direct, and mediated by c-Src SH2 binding to Y992, Y891, or Y920 of the HER1 protein (Belsches et al., 1997). In addition, c-Src has been shown to phosphorylate two of these residues, namely Y891 and Y920 (Stover et al., 1995). Interestingly, there is much evidence implicating EGFR family members in human breast tumor progression. For example, overexpression and/or amplification of HER1 or HER2 has been observed in 20 to 30% of breast tumors, and HER2 overexpression correlates with a poorer prognosis for the disease (Slamon et al., 1987; Slamon et al., 1989). Although HER1 and HER2 can heterodimerize in an EGF stimulation dependent manner, HER2 homodimerization is ligand independent, and thought to result from overexpression (Tzahar et al., 1996).

1.2.3.2. Activation of c-Src Signaling Cascades by Adhesion Receptors

The integrin adhesion receptors mediate cellular attachment to the extracellular matrix (ECM). The integrin family consists of 15 α subunits and 8 β subunits, which can heterodimerize to generate diverse combinations of functional α/β integrin receptors with specificities for different ECM ligands (Ruoslahti, 1999). The model for c-Src activation following integrin engagement to fibronectin, an ECM component, begins with the recruitment of c-Src and a number of other adhesion signaling proteins to these sites of engagement, termed focal adhesions. This localization has been shown to require the SH3 domain of c-Src as well as N-terminal myristoylation (Thomas and Brugge, 1997). Once localized to these focal adhesions, c-Src is transiently dephosphorylated at Y527/530, and peak c-Src kinase activity can be observed at 15 min (Thomas and Brugge, 1997). Following this transient enhancement in activity, c-Src remains associated with proteins at these sites. In addition to c-Src, another tyrosine kinase, termed focal adhesion kinase (FAK) is recruited to and activated by engaged integrin receptors. Once activated, FAK autophosphorylates on Y397, which serves as a docking site for the c-Src SH2 domain (Ruoslahti, 1999). Interestingly, c-Src and FAK have been suggested to play redundant roles in substrate phosphorylation at these sites of adhesion. As a result of this redundancy, the exact kinase responsible for phosphorylation of proteins at focal adhesions following integrin engagement remains unknown. Indeed, both FAK and c-Src are able to bind and phosphorylate the majority of the structural proteins associated with focal adhesions, including paxillin, $p130^{CAS}$, vinculin, talin, and tensin (Brown and Cooper, 1996). Ultimately, these proteins

generate adhesive contacts with cytoskeletal proteins, thus coupling the ECM to internal cellular architecture.

The other major complex that mediates cellular adhesion is the adherens junction. Like focal adhesions, c-Src and a myriad of other structural proteins localize to these subcellular structures (Aberle et al., 1996; Henderson and Rohrschneider, 1987). Adherens junctions are sites of cell-cell adhesion regulated by Ca^{2+} dependent homotypic interactions between adjacent transmembrane cadherin proteins (Aberle et al., 1996). Proteins with armadillo-like repeat motifs, such as the catenin family, are responsible for transferring cadherin adhesion signals to the cytoskeleton via direct interaction with actin filaments (Brown and Cooper, 1996). In cells transformed with v-Src, increased tyrosine phosphorylation of cadherin and α-, β-, and γ-catenin proteins is observed (Brown and Cooper, 1996). However, cadherin engagement does not activate c-Src kinase activity. Rather, c-Src is thought to be indirectly recruited to adherens junctions by RTKs. This is supported by the observation that EGFR localizes to adherens junctions, and directly interacts with β-catenin (Hoschuetzky et al., 1994).

1.2.4. Cellular Processes Regulated by Src

To summarize the previous section, c-Src is directly activated by various transmembrane receptors. Specific examples of c-Src activation were given for some growth factor and adhesion receptors. Given the diversity in receptors that can activate c-Src, it is not surprising that c-Src is found at the hub of a vast array of important cellular signaling cascades that influence processes such as proliferation, differentiation, motility, architecture, and survival (Fig. 1.2). The precise substrates that c-Src targets

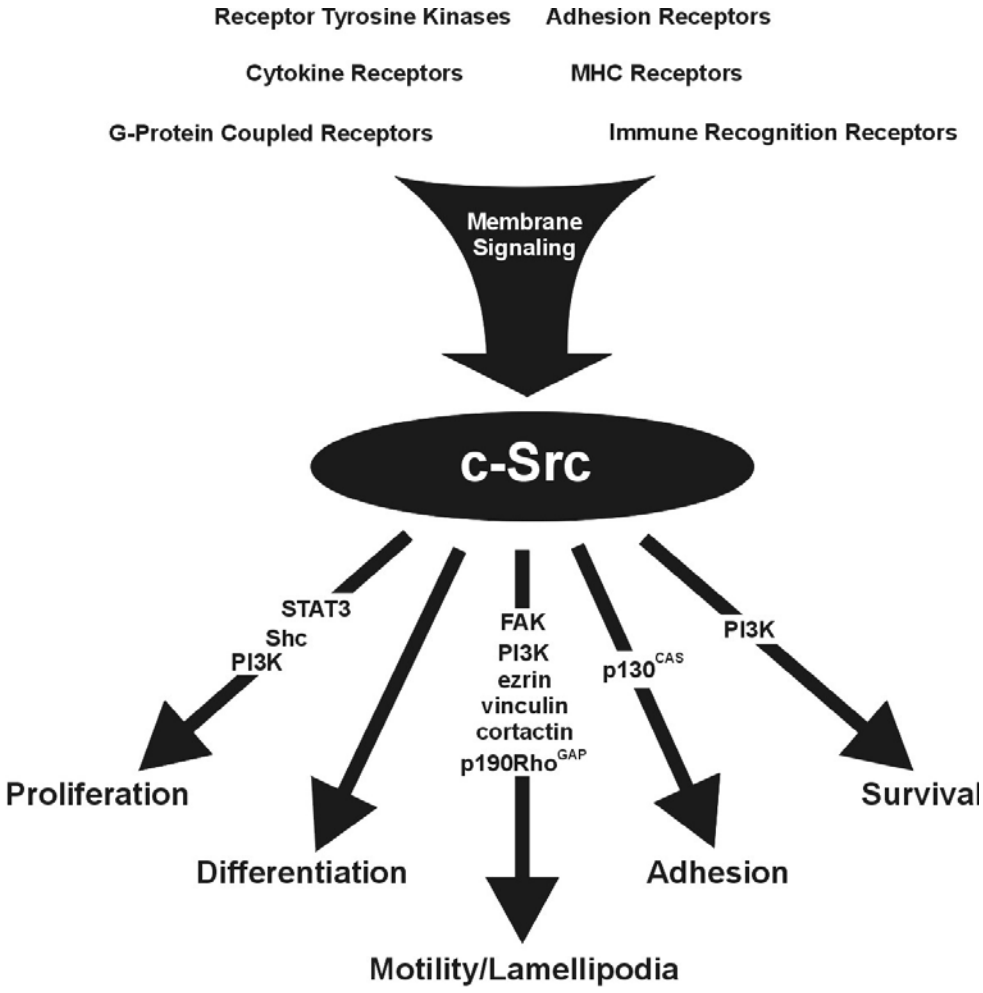

Figure 1.2. Signaling process mediated by c-Src. The various transmembrane receptors that have been implicated in activating c-Src are shown at the top. Effector molecules that are directly affected by c-Src signaling are shown in the middle of the black arrows. The cellular processes affected by activation of these c-Src signal transduction cascades are shown at the bottom.

once activated are a function of the cell type, subcellular localization, as well as the receptor pathway that led to c-Src activation. A comprehensive list of c-Src substrates has been included in a review by Brown and Cooper (Brown and Cooper, 1996).

1.2.4.1. Cell Cycle Progression

c-Src has been shown to influence progression through the cell cycle following growth factor receptor activation. For example, down regulation of c-Src, through microinjection of neutralizing anti-Src antibodies or DNA encoding catalytically inactive c-Src, blocks DNA synthesis mediated by EGF, PDGF, as well as colony stimulating factor-1 (CSF-1) (Roche et al., 1995b; Twamley-Stein et al., 1993). For PDGF, the G1/S arrest caused by blockade of c-Src action is rescued by co-injection of c-Myc (Barone and Courtneidge, 1995). These results therefore implicate c-Src activation, following PDGF receptor stimulation, in controlling the activation of c-Myc, which in turn drives G1/S progression. c-Myc activation is at the level of transcription and the molecular pathway responsible has been dissected. This pathway involves direct phosphorylation and subsequent activation of the STAT3 transcription factor by c-Src, which then binds and activates the MYC promoter (Bowman et al., 2001). Numerous corroborating studies have demonstrated that activation of STAT3 is essential for cellular transformation by activated c-Src (Irby and Yeatman, 2000). Another downstream target of c-Src that is important for the G1/S phase of the cell cycle is Shc, an adapter protein that activates the Ras/MAPK pathway. Shc is phosphorylated by c-Src following engagement of growth factor receptors, immune response receptors, G-protein coupled receptors, cytokine receptors, and integrins (Thomas and Brugge, 1997). In addition, phosphatidylinositol-3-kinase (PI3K) is activated by both the SH2 and SH3

domains of c-Src, and is required for PDGF, but not CSF-1, stimulated DNA synthesis (Thomas and Brugge, 1997).

In addition to the G1/S phase of the cell cycle, increased c-Src kinase activity is observed during mitosis, and is believed to result from N-terminal serine phosphorylation of c-Src by the cyclin dependent kinase, Cdc2 (Taylor and Shalloway, 1993). Indeed, c-Src is required for G2/M progression, as demonstrated by the observation that microinjection of inhibitory c-Src antibodies elicits G2/M arrest (Roche et al., 1995a). A mitosis specific substrate, Sam68, has been identified for c-Src. Sam68 is a heterogeneous ribonuclear protein that may function in RNA transport, splicing, or stability, and is activated by the SH3 and SH2 domains of c-Src (Fumagalli et al., 1994). Interestingly, Sam68 is homologous to *Caenorhabditis elegans* gld-1, which has been shown to negatively regulate G2/M (Lin et al., 1997). In addition to Sam68, c-Src also activates the Ras/MAPK pathway during the G2/M phase of the cell cycle (Brown and Cooper, 1996).

1.2.4.2. Differentiation

The precise role for c-Src in cellular differentiation remains ambiguous. For example, temperature sensitive RSV infection of avian myoblasts, or chondroblasts has shown that v-Src retains these cells in a proliferative state, and prevents their differentiation into myotubes or chondrocytes, respectively (Kim et al., 1992; Nie et al., 1998). Conversely, however, v-Src induces neurite outgrowth of PC12 rat pheochromocytoma cells in a similar fashion to nerve growth factor treatment, demonstrating that v-Src can promote differentiation in these cells (Alema et al., 1985).

In addition, elevated c-Src activity has been associated with more-well differentiated grades of colon tumors (Weber et al., 1992).

1.2.4.3. Motility, Cellular Architecture, and Adhesion

Src-/- mouse fibroblasts display a decreased migration rate. This alteration can be restored upon expression of wild-type c-Src, suggesting c-Src plays an important role in motility (Thomas and Brugge, 1997). This is supported by studies in NBT-11 rat carcinoma cells, where EGF treatment causes cells to dissociate from cell clusters and display increased motility (Rodier et al., 1995). A kinase dead c-Src mutant blocks these effects, demonstrating a direct role for c-Src kinase activity, under the control of EGF signaling, in this process (Rodier et al., 1995). Lamellipodia, also known as membrane ruffles, are essential for this cell motility, as well as the organization of specialized membrane domains (Small et al., 2002). These membrane microstructures rapidly form after v-Src activation (Thomas and Brugge, 1997), suggesting the decrease in mobility in *src*-/- fibroblasts is likely due to their impaired formation. However, the precise molecular targets of c-Src signaling that lead to lamellipodia formation and subsequent increased motility have yet to be determined. Some potential effector candidates include PI3K, ezrin, vinculin, cortactin, and $p190Rho^{GAP}$ (Brown and Cooper, 1996). All these putative effectors are intimately associated with cytoskeletal proteins, and play a role in mediating the architecture of the cell.

In addition to diminished migration, *src*-/- fibroblasts display reduced cellular adhesion and spreading, despite being competent for focal adhesion assembly (Thomas and Brugge, 1997). More detailed studies have shown that $p130^{CAS}$, a c-Src substrate, is excluded from focal adhesion complexes in *src*-/- fibroblasts. Interestingly, restoration

of adhesion and spreading to *src*-/- fibroblasts can be achieved by expression of a kinase dead c-Src mutant, suggesting c-Src may play a role as an adapter protein at focal adhesion sites (Nakamoto et al., 1997). This hypothesis is supported by the observation that adhesion/spreading rescue by kinase dead c-Src is accompanied by recruitment of $p130^{CAS}$ to focal adhesions (Nakamoto et al., 1997).

1.2.4.4. Cell Survival

The earliest studies implicating the importance of c-Src in cell survival demonstrated that v-Src can specifically prevent apoptosis in cells that are deprived of growth factors, cytokines, or ECM components (McCubrey et al., 1993). More recently, c-Src has been deemed essential in transducing survival signals from the TNF-related activation induced cytokine (TRANCE) receptor in dendritic cells and osteoclasts (Wong et al., 1999). In addition, c-Src prevents detachment induced apoptosis (anoikis) in human colon cancer cells (Windham et al., 2002). The mechanism of c-Src mediated cell survival is generally thought to depend upon the ability of c-Src to activate PI3K, which in turn is able to activate the PKB/Akt cell survival pathway (Schlessinger, 2000). This is supported by the observation that PI3K inhibition sensitizes human colon cancer cells with high levels of c-Src activity to anoikis (Windham et al., 2002).

1.2.5. *In vivo* Studies of c-Src Function

Given the importance of c-Src in such critical cellular signaling processes (Fig. 1.2), it was an unexpected finding that despite only a bone remodeling defect, osteopetrosis, mice with targeted disruption of the *src* gene develop normally (Soriano et al., 1991). However, *src-/-* mice do eventually succumb shortly after birth to a wasting

syndrome caused by starvation (Soriano et al., 1991). It was hypothesized that *src-/-* mice appeared to develop normally due to functional redundancy between Src family members. This functional redundancy has historically hampered groups' attempts to assign specific *in vivo* biological functions to c-Src. Indeed, a more severe form of osteopetrosis is seen in *hck-/-:src-/-* double disruption mutants (Lowell et al., 1996), and *fyn-/-;src-/-* or *yes-/-:src-/-* mice die perinatally, although the reason for this lethality is unknown (Stein et al., 1994).

Interestingly, previous to establishment of the *src-/-* mouse model, no role for c-Src had been suggested in bone physiology. Further investigation demonstrated high levels of c-Src expression in osteoclasts, cells involved in bone resorption (Horne et al., 1992). Recent studies have suggested these high levels of c-Src in the osteoclast are likely the result of transcriptional activation during osteoclast differentiation (Higuchi et al., 1999). However it is doubtful c-Src plays a direct role in the differentiation of these cells because osteoclasts are present, and even over-represented, in *src-/-* mice (Hayashi et al., 1998). Rather, these *src-/-* osteoclasts do not form ruffled borders (lamellipodia), structures essential for osteoclast resorption pits, and hence display severe bone resorption defects *in vivo* and *in vitro* (Boyce et al., 1992; Lowe et al., 1993; Soriano et al., 1991). These observations have been expanded, and *src-/-* mice maintained on a liquid diet to prevent starvation present with progressive osteopetrosis of the whole skeleton, leading to odontoma growth which eventually causes complete airway obliteration and suffocation (Amling et al., 2000). The molecular basis for this defect is hypothesized to result from impairment of a number of c-Src mediated signaling pathways. The first is regulated by an important receptor for osteoclast attachment to the ECM, $\alpha_v\beta_3$ integrin (osteopontin). c-Src disruption is thought to abolish

osteopontin/ECM interaction and signaling, and perhaps lead to the defective lamellipodia formation seen in *src-/-* osteoclasts (Chellaiah et al., 1996; Hruska et al., 1995). The other pathways impaired by c-Src disruption in osteoclasts are RTK mediated, and include the CSF-1 and hepatocyte growth factor/scatter factor (HGF/SF) growth factor signaling pathways (Grano et al., 1996; Kurihara et al., 1996; Thomas and Brugge, 1997). Interestingly, the osteopetrotic phenotype of *src-/-* mice can be rescued by transgenic expression of a catalytically inactive c-Src, suggesting c-Src signaling modules other than the kinase domain are essential for osteoclast function (Schwartzberg et al., 1997).

1.2.6. Src and Human Colon Cancer

1.2.6.1. Colon Cancer Progression

Colon cancer is the fourth most common malignancy affecting the North American population, and the second leading cause of deaths due to cancer (Potter, 1999). For no other cancer are the genetic events leading to malignancy better understood than for this disease. This knowledge has allowed for the proposal of stepwise models of colonic tumorigenesis (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). For example, a gatekeeper function has been assigned to the adenomatous polyposis coli (APC) tumor suppressor gene product, APC, implicating this protein in colon tumor initiation (Kinzler and Vogelstein, 1996). Normally functioning APC regulates β-catenin levels by binding cytosolic β-catenin and facilitating its phosphorylation by glycogen synthase kinase 3β (GSK-3β), thus targeting it for ubiquitin-mediated degradation (Behrens, 1999; Ilyas and Tomlinson, 1997). Loss

of APC function (Kinzler and Vogelstein, 1996; Morin et al., 1997) or mutations in β-catenin that render it unresponsive to APC regulation (Dashwood et al., 1998; Morin et al., 1997; Park et al., 1999) results in accumulation of cytosolic β-catenin. Subsequently, β-catenin translocates to the nucleus where it can associate with members of the Tcf/Lef family of transcription factors and up-regulate transcription of genes such as c-Myc and cyclin D1 (He et al., 1998; Tetsu and McCormick, 1999). Further tumor progression is associated with mutations resulting in the loss of function of additional tumor suppressor genes such as p53, DCC, hMLH1, and hMSH2 (Kinzler and Vogelstein, 1996). Another well-characterized event occurring in nearly half of all colon cancers is an activating mutation in the KRAS proto-oncogene, which gives it a potent transforming ability (Bos et al., 1987; Kinzler and Vogelstein, 1996). KRAS encodes K-Ras, a 21 KDa GTP-binding protein that serves as a molecular switch at the centre of a multitude of important and diverse cellular signaling cascades (Khosravi-Far et al., 1998). Mutation locks K-Ras in an active, GTP-bound state, resulting in constitutive activation of downstream effectors, driving proliferation, cytoskeletal rearrangement, and angiogenesis (Khosravi-Far et al., 1998; Rak et al., 1995; Shirasawa et al., 1993).

1.2.6.2. Src Activation in Colon Cancer

In addition to the well-documented mutational events that occur during colon cancer progression, increased activity and/or expression of c-Src has been a consistent early finding in colon tumors and cell lines derived from them (Biscardi et al., 1999; Bolen et al., 1987a; Budde et al., 1994; Cartwright et al., 1994; Cartwright et al., 1990;

Han et al., 1996; Iravani et al., 1998; Staley et al., 1997; Talamonti et al., 1993; Zhao et al., 1990). Tyrosine kinase activity of $pp60^{c\text{-}Src}$ is significantly increased in nearly 80% of colon carcinomas when compared with adjacent normal colon mucosa (Bolen et al., 1987a; Cartwright et al., 1990). In addition, examination of colonic polyps and normal colon epithelia from ulcerative colitis patients has shown a progressive increase in $pp60^{c\text{-}Src}$ activity from benign to malignant disease (Cartwright et al., 1994; Cartwright et al., 1990). Increased $pp60^{c\text{-}Src}$ activity has also been observed in colorectal carcinoma metastasis (hepatic and extrahepatic) compared to normal colonic epithelia and even primary colon tumors (Talamonti et al., 1993). Interestingly, an activating SRC mutation has been described in a small (12%) subset of highly advanced colon cancers (Irby et al., 1999). This mutation, converting glutamine 531 to a stop codon, is activating, transforming, tumorigenic, and promotes metastasis. The importance of SRC mutation in the majority of colon cancers is suspect, however. Follow-up studies have been unable to detect this mutation in a large number of colon and rectal tumors (Daigo et al., 1999; Laghi et al., 2001; Nilbert and Fernebro, 2000). Indeed, in the majority of colon cancer, c-Src is activated through non-mutagenic means, including overexpression of c-Src protein (Biscardi et al., 1999).

Many groups have designed experiments to support the current view that c-Src activation is most often due to increased $pp60^{c\text{-}Src}$ levels, and that overexpression of c-Src is an important oncogenic event in colon tumor progression (Biscardi et al., 1999; Iravani et al., 1998; Park and Cartwright, 1995). For example, immunohistochemical studies have demonstrated strong c-Src expression in 95% of adenomatous colon tumors compared with very weak expression in normal colonic mucosa; these patterns of expression correlated strongly with $pp60^{c\text{-}Src}$ kinase activity (Iravani et al., 1998).

Further, simple overexpression of murine c-Src is able to elicit transformation of mouse fibroblasts, albeit weakly (Lin et al., 1995). However, when c-Src is transfected into the same fibroblasts expressing high levels of EGFR, it can cooperate with this RTK to achieve synergistic levels of transformation (Maa et al., 1995). In addition, specific antisense-mediated down-regulation of c-Src in the HT29 human colon adenocarcinoma cell line results in diminished growth rate and colony forming ability (Staley et al., 1997). When these cells were assayed via nude mouse xenograft, they displayed severely diminished tumor forming ability and vascularization compared with parental cells. The reduction in vascularization appears to be the result of reduced vascular endothelial growth factor (VEGF) production by these cells (Ellis et al., 1998). These cells are also more susceptible to detachment induced apoptosis, or anoikis, than the parental cell line (Windham et al., 2002).

1.2.7. Src and Other Human Cancers

In addition to colon cancer, there are also reports that have suggested c-Src overexpression and/or activation could play an important role in other cancers. For example, a c-Src specific antisense strategy has also been employed in the SKOv-3 ovarian cancer cell line, resulting in diminished anchorage-independent growth and tumor forming ability in a xenograft nude mouse model (Wiener et al., 1999). Similarly, antisense-mediated down-regulation of c-Src expression in NIH3T3 cells engineered to overexpress the EGFR or an EGFR-HER-2 chimera, a common finding in breast cancer, results in reversal of the transformed phenotype of these cells (Karni et al., 1999). Observational studies have also reported increased c-Src expression and/or kinase

activity in other cancers such as breast, lung, pancreas, and liver (Lutz et al., 1998; Masaki et al., 1998; Mazurenko et al., 1992; Verbeek et al., 1996).

1.3. Eukaryotic Transcriptional Control

It is essential for the cell to properly execute its gene expression program. Failure to do so can lead to dire consequences, with the extremes being apoptosis or transformation. The DNA sequence of the human genome has been determined (Lander et al., 2001; Venter et al., 2001), and various estimates of the number of genes contained therein range from 28,000 to 120,000, with the current average consensus being 35,000 to 45,000 (Das et al., 2001; Saha et al., 2002). Significantly, any given cell expresses roughly 2 to 10% of this gene repertoire, alluding to extensive regulation of processes that drive gene expression. One of the most important, and highly regulated gene expression processes is transcription of the DNA template to generate an RNA molecule. In eukaryotes, transcription is carried out by three RNA polymerase enzymes, RNA Polymerase I, II, and III. The vast majority of the genes in eukaryotic genomes encode functional proteins and contain promoters that recruit RNA Polymerase II (Pol II). As such, the regulation of Pol II transcription has been an area of extensive research. Pol II regulatory mechanisms have been described at the level of promoter sequence and architecture, basal transcriptional machinery composition, transcription factor expression and modification, covalent DNA modification, histone modification, and chromatin structure. The protein components associated with each of these levels of regulation are depicted in Figure 1.3, and will be described in detail in subsequent sections. These control mechanisms are all highly interrelated and are believed to co-operate in a unique fashion at each individual promoter in the human genome to allow different cells to execute the expression of the appropriate sets of genes in a spatially and temporally controlled fashion.

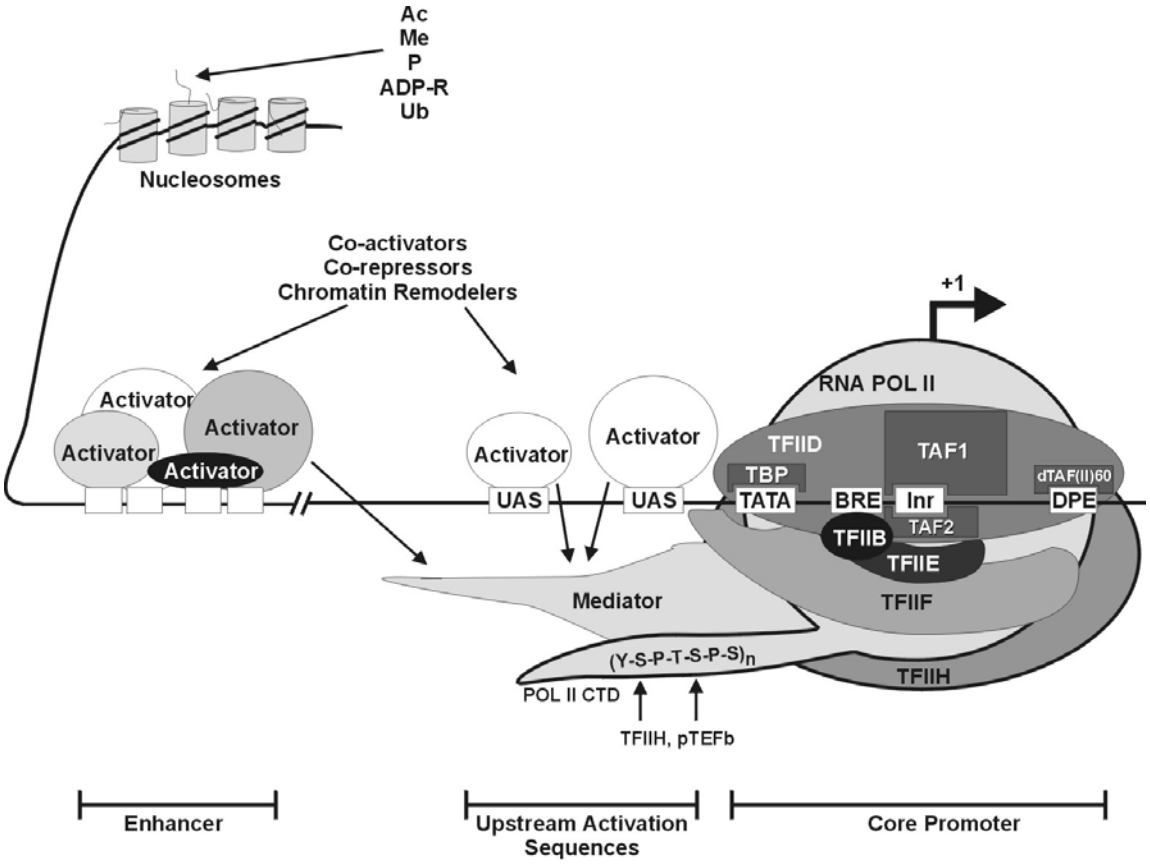

Figure 1.3. Regulation of eukaryotic transcription. This figure summarizes the interactions that occur between DNA elements and proteins that regulate transcription. Potential modifications of histone proteins, such as acetylation (Ac), methylation (Me), phosphorylation (P), ADP-ribosylation (ADP-R), and ubiquitination (Ub), are shown. Elements and factors shown here, as well as the abbreviations used to represent them, are detailed in the text.

1.3.1. Core Promoter and Basal Transcription

1.3.1.1. RNA Polymerase II Transcriptional Machinery

The individual polypeptides that comprise the eukaryotic transcriptional machinery were originally identified by extensive biochemical purification. These polypeptides are components of large protein complexes that have been defined as the general transcriptional machinery. The precise functions assigned to these complexes have primarily resulted from *in vitro* transcriptional reconstitution assays using purified proteins. While it is generally accepted that transcriptional regulation is very different in intact cells compared to these *in vitro* assays, the results garnered from these approaches have provided the current working models for how this bewildering array of polypeptides mediate transcription of a DNA template.

The central component of the transcription process is Pol II, a 12-subunit enzyme that catalyzes the generation of an RNA transcript from a DNA template. The crystal structure of yeast Pol II comprised of 10 of these subunits has been determined at 2.8 Å resolution, as has a 3.3 Å resolution structure of yeast Pol II during the elongation phase of transcription (Cramer et al., 2001; Gnatt et al., 2001). Comparison of these two structures has been crucial for the development of models for the mechanism of transcription. The results generated from these yeast Pol II crystallography experiments will certainly serve as the benchmark for all eukaryotic Pol II structures, owing to the high degree of sequence conservation between Pol II enzymes. For example, the bulk molecular weight of Pol II is accounted for by the Rpb1 and Rpb2 subunits. These subunits make up lower and upper "jaws" of Pol II, with a 25 Å diameter opening between them where the enzyme clamps onto the DNA template. These jaws exist in two conformations; an open conformation is hypothesized to allow entry of melted

promoter DNA, whereas a closed conformation is hypothesized to separate the newly generated DNA/RNA hybrid. Nascent RNA transcripts then exit Pol II through a groove, termed groove 1 (Cramer et al., 2001; Gnatt et al., 2001). Groove 1 terminates just before a 90 residue linker region that separates the Pol II carboxyl terminal domain (CTD) from the active centre of the enzyme (Cramer et al., 2001). The Pol II CTD, situated on the Rpb1 subunit, is of considerable regulatory importance because it plays a central role in the transition of Pol II from the initiation to elongation phases of transcription. In addition, the CTD is a structural and functional element unique to Pol II, as it is not found in RNA Polymerases I or III. The CTD contains a heptapeptide repeat of Tyr-Ser-Pro-Thr-Ser-Pro-Ser, and forms an elongated, unordered structure (Cramer et al., 2001).

Pol II is unable to initiate transcription at promoters or respond to transcriptional regulatory signals on its own. Promoter dependent transcription is achieved by Pol II in the presence the general transcription factors (GTFs) TFIID, A, E, B, H, and F. The general Pol II machinery has therefore been defined as Pol II and GTFs. TFIIB is the only single-polypeptide GTF; the majority of them, rather, are large multi subunit complexes (Lee and Young, 2000; Woychik and Hampsey, 2002). For example, TFIID is made up of TATA binding protein (TBP) and 10 to 12 TBP associated factors (TAFs) (Albright and Tjian, 2000). TFIIF is a heterotetramer comprised of two large RAP74 subunits, and two small RAP30 subunits. TFIIE is also a heterotetramer made up of two large TFIIEα subunits, and two small TFIIEβ subunits. The largest GTF, TFIIH, is comprised of 9 subunits, and has a molecular weight comparable to Pol II. Two of the TFIIH subunits, XPB and XPD, are ATP dependent DNA helicases. Two additional

TFIIH subunits constitute a cyclin/cyclin dependent kinase complex. As a result, TFIIH has been described as two subcomplexes, the TFIIH core, and the cyclin/cyclin dependent kinase complex. The remaining GTF, TFIIA, was originally deemed essential for basal transcription in crude extract systems, but more recent investigation using highly purified preparations has demonstrated that TFIIA is in fact dispensable for basal transcription (Lee and Young, 2000).

1.3.1.2. Framework for the Mechanism of Transcription

Due to the extensive array of individual polypeptides that constitute the general transcriptional machinery, a huge effort has been made to understand the precise roles these proteins play in transcription. For example, how do these polypeptides assemble at the promoter to direct pre-initiation complex (PIC) formation? The transcription pre-initiation complex is defined as the proper assembly of template (promoter) DNA, Pol II and the GTFs TFIID, E, B, H, and F. *In vitro* transcription reconstitution assays have provided a rudimentary framework for understanding the specific mechanistic roles the individual GTFs play in assembly of this complex (Orphanides et al., 1996; Roeder, 1996). However, these assays have provided only the most basic understanding of transcription; therefore, these concepts will be expanded on in subsequent sections. An early observation was that a large number of eukaryotic promoters contain TATA motifs in their core region. As a result, the TATA box was the first identified eukaryotic core promoter element (Butler and Kadonaga, 2002). The core promoter has been defined as an approximately 100 bp region that contains motifs essential for GTF function, such as the TATA box, as well as the transcription initiation site. The TATA box, usually located 25 or 30 bp upstream from the site of transcription initiation, is the binding

recognition site for the TATA binding protein (TBP) component of TFIID (Hoey et al., 1990). TBP has been crystallized alone and in complex with the TATA element, and resembles a "molecular saddle" that straddles the TATA box and confers a sharp bend in the DNA molecule (Kim and Burley, 1994; Kim et al., 1993; Nikolov and Burley, 1994; Nikolov et al., 1995). TBP also provides various interfaces for TAF binding. Therefore, the TATA box plays an important role in directing PIC formation, likely through TFIID recruitment. Following TFIID recruitment, TFIIB enters the PIC and binds a TFIIB recognition element (BRE) found immediately upstream of the TATA box in a small subset of eukaryotic core promoters (Lagrange et al., 1998). This binding is the result of an interaction between a helix-turn-helix motif in the TFIIB core domain and the major groove of the BRE (Bell et al., 1999; Tsai and Sigler, 2000). Interestingly, TFIIB also makes contacts with the minor groove of the DNA helix downstream of the TATA box, and this asymmetric binding is proposed to provide directionality for PIC assembly and subsequent transcription (Tsai and Sigler, 2000). TFIIB is also involved in transcription start site selection, presumably through direct interaction with Pol II. Once a stable complex has formed between the core promoter, TFIID, and TFIIB, Pol II is escorted to the promoter via tight association with TFIIF. The unphosphorylated form of Pol II CTD is required for transcription initiation (Proudfoot et al., 2002). The RAP30 subunit of TFIIF binds on either side of the TATA box, while RAP74 interacts with the core promoter downstream of this motif (Woychik and Hampsey, 2002). TFIIF stabilizes the entire PIC, and is an essential pre-requisite for TFIIE and TFIIH entry into the PIC. As a result, TFIIE is recruited to the core promoter after TFIIF and Pol II have bound. TFIIE contacts DNA within and immediately downstream of sequences that will be melted to generate the transcription bubble (Woychik and Hampsey, 2002). TFIIE

affects the late events in PIC assembly such as TFIIH recruitment and regulating TFIIH activity. The final PIC generates an extensive footprint over the majority of the core promoter, suggesting there are multiple weak DNA-protein contacts that combined constitute a very stable complex. This is supported by the observation that no concise response elements have been defined for the general transcription factors within eukaryotic promoters except for TFIID and perhaps TFIIB.

The helicase activities of TFIIH, once part of the PIC, catalyze ATP dependent promoter melting, thus generating a 12-15 bp transcription bubble, which is essential for transcriptional initiation (Kim et al., 2000). Following bubble formation, a phenomenon called abortive initiation ensues. This continuous process is characterized by the formation of the first few phosphodiester bonds in the transcript, followed by release of the resulting short RNA product (Dvir, 2002). Eventually Pol II is able to exit the abortive initiation phase of transcription, and generate progressively longer transcripts, a process termed promoter clearance (Gnatt, 2002). Once Pol II has cleared the promoter, it pauses 25 to 30 bp downstream and matures to an elongation-competent complex that is able to generate full length RNA transcripts, a transition process called promoter escape (Dvir, 2002; Gnatt, 2002). Although the precise mechanisms that regulate the transition through these early stages of transcription are not completely clear, the composition of proteins interacting with Pol II, as well as the phosphorylation status of the Pol II CTD are tightly associated with each of these individual stages. For example, the Pol II CTD must be unphosphorylated (Pol IIA) in order for transcription initiation to proceed. The cyclin dependent kinase subunit of TFIIH, as well as another cyclin/kinase complex, P-TEFb (positive transcription elongation factor b), are two proteins capable of phosphorylating serines 2 and 5 in the heptapeptide repeat of the Pol

II CTD (Price, 2000) . This phosphorylation prompts Pol II transition to the elongation-competent form (Pol IIO). In early phases of transcription initiation, the Pol II CTD is primarily phosphorylated at Ser 5. Alternatively, serine 2 phosphorylation is associated with later stages of transcriptional elongation (Proudfoot et al., 2002). CTD phosphorylation is postulated to cause transcription initiation factors to release, and new transcription elongation factors to bind. This change in binding of factors to Pol II has been hypothesized to take place during the pause in transcription that occurs with transition from promoter clearance to promoter escape. Interestingly, the majority of the transcription elongation factors are components of the RNA processing machinery, demonstrating that transcription and RNA processing are co-regulated, intertwined events. For example, all three enzymatic activities responsible for capping the 5' end of the nascent RNA transcript are associated with the phosphorylated CTD of Pol II. CTD phosphorylated at Ser 5 even stimulates the guanyltransferase activity of the capping complex (Proudfoot et al., 2002). In addition, phosphorylated CTD enhances RNA splicing events, likely through direct associations with the SR (Ser-Arg) family of splicing factors that play a role in recognizing the 3' and 5' splice sites flanking exonic DNA (Proudfoot et al., 2002). Finally, CPSF (cleavage and polyadenylation specificity factor) is a component of active TFIID that transfers to Pol IIO immediately after its transition from Pol IIA. Phosphorylated Pol II CTD directs the interaction with CPSF and also enhances its activity (Proudfoot et al., 2002).

1.3.1.3. Core Promoter Heterogeneity

The models presented above describing the mechanism of transcription are based on a core promoter containing a TATA motif. Indeed, this motif was the first described

core promoter element and therefore serves as the focal point for a basic understanding of transcription initiation. However, the heterogeneity of core promoters has received much attention in the past few years, and two additional functional elements have been described. These two functional elements have been termed the initiator (Inr) and downstream promoter element (DPE). In *Drosophila*, nearly half of core promoters contain the combination of TATA and Inr, while the other half contains Inr and DPE. However, in mammals, promoters have been catalogued that contain TATA alone, Inr alone, both TATA and Inr motifs, both Inr and DPE motifs, or none of these elements at all. The composition of these elements in a eukaryotic core promoter has been proposed to play a previously unappreciated role in regulating transcription, likely by offering increased combinatorial diversity in promoter structure.

The Inr has been defined as the element encompassing the transcription start site that binds regulatory factors (Lee and Young, 2000). The consensus for the Inr element is YYA(+1)NTYY (Y = pyrimidine, N = any nucleotide), with transcription usually, but not always, arising from the central A residue (Smale et al., 1998). The Inr, much like the TATA box, can direct basic transcription in isolation as assessed by *in vitro* transcription assays (Smale and Baltimore, 1989). This suggests the Inr has similar GTF recruitment properties to the TATA motif. In addition to supporting basal transcription, another similarity to the TATA element is that the Inr can support activated transcription when influenced by activators bound to upstream sequences (Chen and Hampsey, 2002). Although these findings suggest the Inr and TATA motifs may be functionally redundant, many promoters contain both elements. In this situation, synergistic levels of basal transcriptional activity are observed (Lee and Young, 2000). This synergy is only

achieved when the TATA and Inr elements are separated by 25 bp, but not 30, 35, or 40 bp, suggesting a specific architecture is essential (Smale et al., 1998).

Interestingly, for *Drosophila* promoters with disrupted TATA elements, a consensus DPE can functionally compensate for this loss (Burke et al., 1998). The DPE, which was originally identified in *Drosophila* core promoters, is a distinct 7 bp element located 30 bp downstream from the Inr. The consensus for the DPE in *Drosophila* has been defined as RG(A/T)CGTG (R = purine); however, the DPE consensus in mammals has not been determined (Burke et al., 1998). Nevertheless, evidence exists for similar functional elements in mammalian cells, although the exact importance of the DPE in regulating mammalian transcription is still largely not described (Burke et al., 1998).

A unifying feature of these functional core promoter elements is that they all bind distinct TFIID components; therefore, they have been implicated in being able to independently nucleate preinitiation complex assembly. Indeed, the TAF dependence of transcriptional activation is not universal, but rather specified by individual core promoters (Woychik and Hampsey, 2002). Therefore, depending on the make-up of the individual core promoter, different sets of TAFs will be essential for supporting basal transcription. DNA cross-linking experiments have shown that *Drosophila* TAF(II)60 directly interacts with the DPE; therefore, this TAF has been implicated in recognition of this site (Burke and Kadonaga, 1996). In addition, the largest human TAF, hTAF(II)250 or TAF1, has been shown to directly bind the Inr motif in concert with hTAF(II)150 or TAF2 (Chalkley and Verrijzer, 1999). Interestingly, TAF2 can bind DNA on its own; however, its specificity appears to be for DNA structure rather than DNA sequence (Chalkley and Verrijzer, 1999).

1.3.1.4. TATA Binding Protein Associated Factor (TAF)-1

In addition to binding the Inr element and thus providing an important TFIID recruitment function, TAF1 harbours a multitude of enzymatic activities. For example, TAF1 possesses acetyltransferase (AT) activity; however, this activity is very weak towards histone proteins *in vitro* (Wassarman and Sauer, 2001). Interestingly, TAF1 has been shown to acetylate TFIIEβ and the RAP74 subunit of TFIIF (Imhof et al., 1997). The effect acetylation has on the function of these general transcription factors is not known. In addition to its AT domain, TAF1 harbours two distinct N and C terminal Ser/Thr kinase domains, termed the NTK and CTK, respectively. Both the NTK and the CTK can phosphorylate the RAP74 subunit of TFIIF (Dikstein et al., 1996). In addition, the NTK can phosphorylate the largest TFIIA subunit, TFIIA-L (Solow et al., 2001). Although these NTK and CTK substrates were identified *in vitro*, they have been proposed as likely *in vivo* candidates for TAF1 kinase activity. For example, dephosphorylated RAP74 has been shown to inhibit its ability to stimulate transcriptional elongation in reconstituted *in vitro* transcription assays (Wassarman and Sauer, 2001). TFIIA phosphorylation stimulates the generation of a complex between TFIIA, TBP, and a TATA-containing DNA template *in vitro* (Wassarman and Sauer, 2001). TAF1 also possesses E1 ubiquitin activation and E2 ubiquitin conjugation activities. The only definitive substrate for this activity identified thus far is histone H1, as TAF1 has been shown to monoubiqutinate this protein (Pham and Sauer, 2000). The functional consequences of this monoubiquitination have not been described. Finally, TAF1 possesses two tandem bromodomains, which play a role in binding to acetylated histones (Wassarman and Sauer, 2001). The TAF1 bromodomains have been shown to

prefer histone H4 doubly acetylated at lysines 5 and 12 (Jacobson et al., 2000). Therefore, these domains have been proposed to be responsible for targeting TAF1, and hence TFIID, to chromatin packaged promoters (Wassarman and Sauer, 2001).

Because of the myriad of important TAF1 activities, it is not surprising that TAF1 inactivation in *Drosophila* is lethal (Wassarman et al., 2000). This supports the current view that TAF1 serves a vital TFIID recruitment function at promoters with Inr elements, but also contributes essential enzymatic activities at various other classes of core promoters. This theory is strengthened by findings that the transcription of 30% of cellular genes in yeast are absolutely dependent on fully functional TAF1 (Holstege et al., 1998). Tools that have been vital for understanding mammalian TAF1 function are the tsBN462 and ts13 cell lines, which are both derived from BHK-21 (baby hamster kidney) cells and harbor identical G690D mutations in the TAF1 protein (Hayashida et al., 1994). Studies with these cell lines have implicated an essential role for TAF1 in cell cycle progression because they grow normally at 33^{o}C, but undergo G1/S arrest when shifted to the restrictive temperature of 39^{o}C. The cell cycle arrest elicited at 39^{o}C in ts13 cells is rescued by expression of wild-type TAF1, suggesting disruption of TAF1 activity is directly responsible for this temperature sensitive phenotype (Wang and Tjian, 1994). Early studies suggested this temperature sensitive phenotype could be accounted for entirely by abrogation of TAF1 AT activity at the restrictive temperature, because AT activity is compromised *in vitro* at 39^{o}C, but not 33^{o}C (Dunphy et al., 2000). However, a recent report has demonstrated the G690D mutation also abolishes the ability of a TAF1/TAF2 dimer to bind Inr elements *in vitro* (Hilton and Wang, 2003). Combined, the loss of these two important TAF1 functions alters transcription of 18% of cellular genes in these cells (O'Brien and Tjian, 2000). Perhaps most significantly,

temperature shift results in the activation of genes encoding p21 and p27, and reduction in the expression of cyclins D1 and A; these transcriptional responses could very likely be responsible for the observed G1/S arrest (Rushton et al., 1997; Sekiguchi et al., 1996; Suzuki-Yagawa et al., 1997).

1.3.2. Upstream Activation Sequences and Activated Transcription

1.3.2.1. Upstream Activation Sequences

Perhaps the best-defined features of eukaryotic promoters are transcriptional activating sequences. These elements are usually divided into two groups, upstream activating sequences (UASs) and enhancers, depending on their proximity to the core promoter. The UASs are usually discrete elements bound by activators that directly influence the transcription arising from nearby start sites. The second group, the enhancers, are usually clusters of discrete activator binding sites that act in an orientation independent manner and can exert their effects on transcription from great distances away from the start site. The commonality of these two different classes of elements is that they both contain readily identifiable sequences that recruit sequence specific activators, or transcription factors.

Most transcriptional activators are comprised of two modular domains: the DNA binding domain, and the transactivation domain. Based on similarities in DNA binding domain and transactivation domain composition, transcription factors have been grouped into large superfamilies (Paasinen-Sohns and Holtta, 1997). The DNA binding domain is responsible for the ability of these activators to bind their cognate cis-elements, while the transactivation domain has been deemed important for affecting the rate of recruitment of the Pol II transcription initiation complex to the core promoter. Indeed,

direct binding of transcription factor transactivation domains to components of the general transcriptional machinery has been described (Lee and Young, 2000). However, the precise mechanisms by which activators influence the rate of recruitment of the Pol II transcription machinery to the core promoter are still being debated. *In vitro* reconstitution experiments suggest that transcriptional machinery is recruited to the promoter in a factor-by-factor fashion, and that activators directly affect a single rate-limiting step in this process (Buratowski et al., 1989). Conversely, large Pol II-containing complexes have been purified that are competent for directing activated transcription, suggesting the GTFs and Pol II can exist as a single complex *in vivo* that can be recruited to the promoter (Greenblatt, 1997). In this situation, activators have been hypothesized to influence the rate of recruitment of this entire complex to the promoter. It is most likely that these two theories represent the extremes of Pol II machinery recruitment, and that they both occur to varying degrees at each of the individual promoters in the eukaryotic genome. In addition to influencing its rate of recruitment to the promoter, activators have also been described to directly influence the activity of the transcriptional machinery. Examples exist where activators enhance the processivity of Pol II, or increase the rate of Pol II elongation, possibly by directly affecting processes that regulate promoter clearance and promoter escape (Blau et al., 1996). Finally, activators have also been shown to influence the rate of transcriptional re-initiation, thus supporting multiple rounds of transcription from a given promoter. This has been hypothesized to occur through activators being able to stabilize factors that remain promoter bound following promoter escape (Hahn, 1998).

1.3.2.2. The Mediator Complex

Early *in vitro* reconstitution experiments showed that the general transcriptional machinery, consisting of Pol II and GTFs, is sufficient for basal, promoter-specific transcription in a reconstituted system. However, a more current definition of the general transcriptional machinery includes an additional protein complex, termed Mediator. The Mediator complex was originally defined as an activity in crude *Saccharomyces cerevisiae* cell extracts required to bridge the basal transcriptional machinery with upstream activators. This was the result of observations that purified transcription factors could not exert their activation functions on the basic transcriptional apparatus in *in vitro* reconstitution assays without Mediator activity (Flanagan et al., 1991; Kelleher et al., 1990). Indeed, although direct interactions have been demonstrated between transcription factor activation domains and various TAFs, yeast Mediator appears to be the central complex through which transcription factors communicate with the basal transcription apparatus (Myers and Kornberg, 2000). However, the mechanisms of activator communication with the Mediator complex remain obscure. Interestingly, Mediator is not associated with Pol II during the elongation phase of transcription, therefore, the primary role of this complex is likely centred around regulating PIC assembly (Gustafsson and Samuelsson, 2001). Of particular importance to this theory, Mediator alone can stimulate basal transcription, likely through its ability to regulate TFIIH kinase activity towards the Pol II CTD (Woychik and Hampsey, 2002). Since these early studies in *S. cerevisiae*, Mediator has been identified in *Drosophila* and mammalian cells, therefore, it has become very evident that this complex is essential for regulating all activated eukaryotic transcription (Lee and Young, 2000; Myers and Kornberg, 2000; Woychik and Hampsey, 2002).

Yeast Mediator has been the most extensively characterized, and is composed of 23 subunits, with two evident and functionally distinct subcomplexes, termed the Srb4 and Rgr1 modules. SRB genes were originally identified as suppressors of yeast growth defects caused by partial RNA Pol II CTD truncations (Nonet and Young, 1989). Their gene products, the Srb proteins, were subsequently isolated in a complex with RNA Pol II (Thompson et al., 1993). These Srb proteins confer Pol II, TBP, and TFIIB binding capacity on the Srb4 module (Myers and Kornberg, 2000). Low-resolution structure analysis has shown that Mediator adopts a crescent shaped structure when complexed with RNA Pol II. The Srb4 module represents the head, and the Rgr1 module makes up the middle and tail portions of this structure (Dotson et al., 2000).

1.3.3. Transcription and Chromatin

1.3.3.1. Chromatin Structure and Transcriptional Control

The diversity in architecture of UASs, enhancers, and core promoters, coupled with the abundance of transcriptional activators and components of the general transcriptional machinery offer a bewildering number of combinatorial controls to regulate the appropriate expression of all genes in the eukaryotic genome. However, even the complicated interactions between all these factors represent a reductionist view of transcriptional regulation. An additional layer of complexity is provided by the regulation of the packaging of the DNA template into higher order structures. In eukaryotes, DNA typically exists *in vivo* as a highly ordered structure based on arrays of repeating nucleosomes (Kornberg and Lorch, 1999). This nucleosomal structure is conferred on DNA by histone proteins, and provides the best-defined level of regulation of chromatin structure. A single nucleosome can be defined as a 146 bp segment of

DNA wrapped twice around a histone core octamer, which is composed of two of each of the histone proteins H2A, H2B, H3, and H4. Other chromatin associated proteins, such as the linker histone H1, the high mobility group (HMG), and silent information regulator (SIR) proteins are responsible for mediating the packaging of these simple nucleosomes into higher ordered chromatin structures. The two extremes of these higher-order structures are represented by the decondensed, accessible euchromatin structure, and the most highly condensed, inaccessible heterochromatin structure (Naar et al., 2001). In general, nucleosomal structure is considered repressive to transcription, likely due to its masking of important DNA elements from transcription factors and basal transcriptional machinery (Grunstein, 1990). As a result, an important step in transcription initiation is the generation of a nucleosomal organization at promoters that is amenable to factor binding and communication. This regulation is provided by a subset of co-activator proteins, which are recruited to promoters during transcriptional activation (Naar et al., 2001). Recently, co-activators have been broadly grouped into two categories, but were originally solely defined as proteins that transcriptional activators required to elicit activated transcription (Featherstone, 2002). The first group of co-activators includes proteins that are components of, or interact with, the general transcriptional machinery. Examples from this class of co-activators include TAFs and various components of the Mediator complex. The second group of co-activators are capable of modifying the structure of chromatin. This class of co-activators is largely comprised of multi-protein complexes that may or may not directly interact with transcriptional activators. Examples of these chromatin modifiers include the ATP-dependent chromatin-remodeling enzyme SWI/SNF and the p300 histone acetyltransferase (HAT) enzyme, respectively.

1.3.3.2. ATP Dependent Chromatin Remodeling Enzymes

ATP dependent chromatin remodeling enzymes are parts of large, multi-subunit complexes that utilize ATP hydrolysis to increase the accessibility of nucleosomal DNA. Chromatin remodeling is a general term used to describe a collection of documented biochemical, ATP-dependent activities that all of these enzymes possess. The *in vitro* assays developed to demonstrate these activities have been extensively described (Narlikar et al., 2002). These activities include the ability to alter DNaseI cleavage patterns, move histone octamers relative to a specific DNA position, and change the negative coiling of closed circular arrays catalyzed by topoisomerase enzymes (Narlikar et al., 2002). Chromatin remodeling enzymes have been grouped into three families based on the identity of their central ATPase subunit. It is important to note that this central ATPase subunit has remodeling activity in isolation; therefore, the remaining subunits in these large complexes are thought to affect substrate specificity as well as the efficiency and outcome of remodeling (Narlikar et al., 2002). These families include the SWI2/SNF2 family, the ISWI family, and the Mi-2 family. Homologues of the yeast SWI2/SNF2 central ATPase are the Brahma family of *Drosophila* and human ATPases, BRG1, and hBRM, respectively (Naar et al., 2001). Examples of the ISWI family of chromatin remodelers include yeast ISW1, human RSF, and *Drosophila* NURF. The human NuRD chromatin-remodeling complex is the prototype for the Mi-2 family. A common generalization is that the chromatin modifications exerted by these enzymes always occur before factors bind to promoters. This arises from the theory that a localized chromatin structure appropriate for the binding of transcriptional activators and the general transcriptional apparatus must be provided as the initial step in

transcriptional activation. However, the results of experiments with steroid receptor and other transcription factors have demonstrated direct activator interaction can occur with the SWI/SNF complex, thus suggesting remodeling activity is recruited to specific promoters by transcriptional activators (Naar et al., 2001). These two competing views probably represent the extremes, and most likely, the final temporal and spatial outcome of chromatin remodeling activity *in vivo* is a function of the specific signals exerted on the individual complex by transcriptional activators, basal transcriptional machinery, and local DNA architecture. Potential for such intricate specificity is highlighted by results from large-scale gene expression studies in yeast that have demonstrated the SWI/SNF complex only participates in the transcriptional regulation of 6% of cellular genes (Sudarsanam et al., 2000)

1.3.3.3. Enzymes that Covalently Modify Histone Proteins

There are numerous enzymes that are able to covalently modify histone proteins. Histone modifications described to date are phosphorylation, ubiquitination, acetylation, methylation, and ADP-riboslylation. The majority of these modifications occur within each of the histones' basic N-terminal tail regions, which are rich in highly conserved lysines, and extend from the globular histone core (Roth et al., 2001). The strong net positive charge associated with the histone tails is thought to mediate strong histone/DNA interactions and internucleosomal interactions. Early interest in histone modifications therefore focused on acetylation, because this particular modification was postulated to neutralize the overall positive charge on the histone tails. This neutralization is thought to result in disrupted histone/DNA and internucleosomal interactions, thus allowing factor access to the DNA template. This theory is strongly

supported by the historical tight correlation between acetylated histones and transcriptionally active chromatin (Hebbes et al., 1988). Further support comes from observations that many co-activator complexes recruited to active promoters possess histone acetyltransferase (HAT) activity while many co-repressor complexes recruited to repressed promoters possess histone deacetylase (HDAC) activity (Pazin and Kadonaga, 1997). However, because these associations between HAT activity, histone acetylation, and transcriptional activity are not always observed, an alternative to this generalization, termed the histone code, has been proposed (Agalioti et al., 2002). This newly emerging hypothesis suggests the combination of modifications on histone tails, in conjunction with direct CpG methylation patterns on the DNA template, constitute a code that specifies gene expression patterns (Turner, 2002). The identification of bromodomains and chromodomains on nuclear proteins, which are binding motifs specific for acetylated and methylated histones, respectively, increases the validity of this theory (Turner, 2002).

In light of the controversy surrounding these competing theories, and the apparent importance of histone acetylation and deacetylation dynamics for transcriptional regulation, the HAT and HDAC enzymes that catalyze these reactions have been the focus of intense study. HAT enzymes utilize acetyl-CoA as a substrate to acetylate specific lysine residues on histone as well as other proteins. In situations where histone proteins are not the target of acetylation, the generic term acetyltransferase (AT) or factor acetyltransferase (FAT) have been employed to describe these enzymes. Much confusion stems from the observations that many HAT enzymes possess more general AT, or FAT, activity. Examples of non-histone nuclear proteins that can be acetylated by these AT enzymes include DNA-binding activators such as p53 (Gu and Roeder,

1997), Sp3 (Braun et al., 2001), EKLF (Zhang and Bieker, 1998), and GATA-1 (Boyes et al., 1998), the general transcription factors TFIIE and TFIIF (Imhof et al., 1997), and architectural proteins such as HMG-I and HMG-17 (Lee and Young, 2000) The HAT enzymes have been divided into two types: type A HATs are nuclear, whereas type B HATs are cytoplasmic. Of the type A HATs, there are the GNAT, MYST, p300/CBP, and nuclear receptor coactivator families. The TAF(II)250 HAT domain is very different from the other type A HAT proteins; therefore this TAF is often catalogued as "other" with respect to family membership. These HAT activities are recruited to specific genes in part through interactions with sequence specific transcriptional activators (Sterner and Berger, 2000). The best-defined and comprehensively studied set of ATs is the GNAT (Gcn5-related N-acetyltransferase) superfamily, which is comprised of the Gcn5, PCAF, Hat1, Elp3, and Hpa2 HAT enzymes (Sterner and Berger, 2000). Yeast Gcn5 was the first discovered HAT enzyme; therefore it serves as the prototype for this family. *In vitro*, recombinant Gcn5 has HAT activity specific for lysine 14 on histone H3, and lysines 8 and 16 on histone H4 (Kuo et al., 1996). However, in these *in vitro* assays, Gcn5 is unable to acetylate nucleosomal histone proteins, the physiological substrate, unless assayed at high concentration under specific reaction conditions (Sterner and Berger, 2000). Therefore, it is significant that yeast Gcn5 exists in a larger HAT complex *in vivo*, termed the SAGA complex (Spt-Ada-Gcn5 acetyltransferase). Similarly, MYST (named after its family members: MOZ, Ybf2, Sas2, Tip60) containing HAT complexes have been identified in yeast. Yeast SAGA has been sized at approximately 1.8 MDa, and 15 of its subunits have been identified. Interestingly, many of these subunits include factors that have previously been identified as intimately involved in transcription, including the Sin4 component of

the Mediator complex, various TAFs, and the Ada and Spt transcriptional adapter proteins (Sterner and Berger, 2000). This suggests that various subunits are shared between TFIID, Mediator, and HAT complexes such as SAGA at promoters *in vivo*. It is therefore likely that the exact compositions of these mega-complexes offer significant degrees of combinatorial control to individual eukaryotic promoters.

Balancing the activities of the HAT enzymes are the HDAC enzymes. Like HAT enzymes, there are numerous mammalian HDACs, which have been divided into three classes based on their functional homology to yeast HDACs. The first group of mammalian HDAC enzymes, class I HDACs, are homologous to yeast RPD-3, and include HDAC1, 2, 3, and 8 (Emiliani et al., 1998; Hu et al., 2000; Laherty et al., 1997; Taunton et al., 1996). Class II mammalian HDACs are homologous to yeast HDA-1, and include HDAC4, 5, 6, and 7 (Grozinger et al., 1999). Class II HDACs are much larger proteins than the class I HDACs; however, the catalytic activities of HDACs within these two classes are very similar in that they all require a Zn^{2+} cofactor. The yeast SIR-2-like Class III HDACs are quite different from classes I and II HDACs; there are seven enzymes within this family (SIR1-7), and all require nicotinamide adenine dinucleotide (NAD) as a co-factor or substrate (Imai et al., 2000). An additional similarity to the HAT enzymes is that HDACs are associated with large, multi-protein complexes *in vivo*. Two co-repressor complexes, termed Sin3 and NuRD, have been characterized to date, and each contains HDAC1 and HDAC2 in their core domain. Sin3 was first characterized as a co-repressor for the transcriptionally repressive Mad-Max heterodimer (Ayer et al., 1995). Interestingly, the HDAC1 and 2 containing NuRD complex is actually a Mi-2 family ATP-dependent chromatin remodeler, thus providing

an HDAC-chromatin remodeling link and additional complexity to regulation of nucleosome dynamics.

1.3.3.4. Histone Deacetylase Inhibitors

Aberrant HDAC-mediated transcriptional repression has been implicated in the development and progression of various human cancers, especially haematological malignancies such as acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML) (Vigushin and Coombes, 2002). For example, a subset of APL is characterized by the t(15;17) chromosomal translocation, which results in a gene fusion that encodes a retinoic acid receptor (RAR)-α:PML transcription factor chimera (Grignani et al., 2000). The RAR-α:PML chimera is a strong transcriptional repressor and blocks transcription of RAR-regulated genes through recruitment of the HDAC-containing Sin3 co-repressor complex (Grignani et al., 1998). The resulting prevention of myeloid maturation plays a direct role in the subsequent development of APL (Grignani et al., 2000). In light of these observations, it is very significant that histone deacetylase (HDAC) inhibitors (HDIs) display chemotherapeutic and chemopreventive properties towards transformed cells in culture and animal models (Marks et al., 2000). Interestingly, these agents' anti-cancer effects have been observed in a large number of haematological malignancies and solid tumors; therefore these agents have sparked a great deal of interest as general anti-neoplastics.

The HDIs have been divided into five classes according to the relatedness of their chemical structures. These classes include the hydroxamic acids, cyclic tetrapeptides with an epoxyketone moiety, cyclic peptides without an epoxyketone moiety,

benzamides, and short chain fatty acids (Vigushin and Coombes, 2002). The two most commonly studied HDI classes are trichostatin A (TSA) and butyrate, which belong to the hydroxamic acid and short chain fatty acid classes of HDIs, respectively (Fig. 1.4). These agents both effectively and reversibly inhibit HDAC enzymes. Specifically, both butyrate and TSA have been kinetically characterized as non-competitive inhibitors of HDAC enzymes (Marks et al., 2000; Yoshida et al., 1990). However, more recent structural studies have shown that TSA is able to directly bind the HDAC active site, thus suggesting it would be more likely to act as a competitive inhibitor (Finnin et al., 1999). In addition, TSA is much more potent than butyrate, achieving HDAC inhibition at nanomolar concentrations (Marks et al., 2000). *In vivo* studies have demonstrated direct anti-tumor activity of hydroxamic acids in a rat breast cancer model, as well as human tumor xenograft experiments with cancer cells derived from melanoma, androgen refractive prostate cancer, gastrointestinal tract, lung, and breast (Cohen et al., 1999; Komatsu et al., 2001; Marks et al., 2000; Qiu et al., 1999). Prevention of carcinogen induced rat mammary and mouse lung tumors have also been observed for the hydroxamic acids (Cohen et al., 1999; Marks et al., 2000). The general dogma surrounding these agents is that they specifically inhibit histone deacetylation, and therefore elicit transcriptional induction of cellular genes. This is illustrated by the most well-characterized response to HDI treatment: the p53-independent transcriptional induction of the WAF1 gene, which encodes the cell cycle inhibitor $p21^{WAF1}$ (Huang et al., 2000; Nakano et al., 1997). Induction of WAF1 has been demonstrated as essential for the growth inhibitory effects of these agents (Archer et al., 1998b). However, the common beliefs regarding HDAC action do not take into account the inhibition of deacetylation of non-histone proteins. Indeed, large scale gene expression studies have

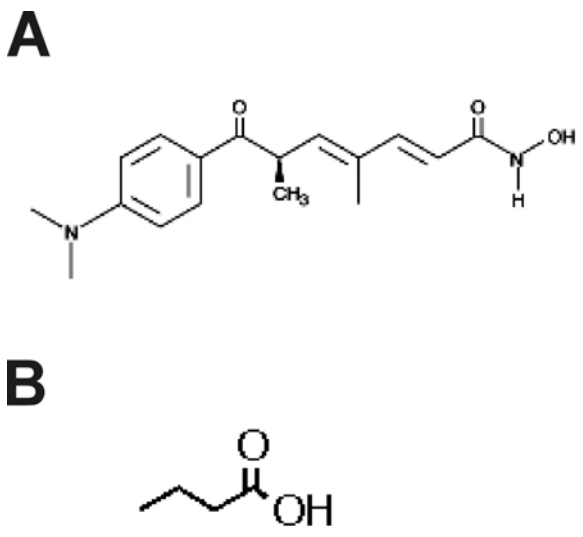

Figure 1.4. Chemical structures of HDAC inhibitors. The structures of the hydroxamic acid-based compound, TSA **(A)**, and the short chain fatty acid, butyric acid **(B)**, are shown.

clearly demonstrated that HDIs also elicit direct repression of just as many genes as are induced (Mariadason et al., 2000). Therefore, the anti-cancer effects of HDIs can be more accurately attributed to the reprogramming of cellular gene expression, resulting in both the induction and repression of a very specific subset of cellular genes (Mariadason et al., 2000). Clearly, repression of growth promoting genes such as cyclin D1 (Lallemand et al., 1996) and c-Myc (Heruth et al., 1993; Souleimani and Asselin, 1993) also offers valid explanation for the anti-cancer effectiveness of HDIs. Thus, the molecular pathways that lead to activation and repression of transcription following HDI treatment are currently an area of intense study.

1.4. Regulation of SRC Transcription

The human SRC gene is comprised of 15 exons (Anderson et al., 1985; Bonham and Fujita, 1993; Bonham et al., 2000; Gibbs et al., 1985; Parker et al., 1985) (Fig 1.5). Exons 2-12 encode $pp60^{c\text{-}Src}$ as well as the 3' untranslated region of the c-Src mRNA. Exons 1B, and 1C are located within the 5' untranslated region of c-Src mRNA. Two distinct promoters, each associated with separate exons, are located at the extreme 5' end of the SRC gene. These promoters have been termed SRC1α and SRC 1A, and their associated exons Exon 1α and Exon 1A, respectively (Bonham and Fujita, 1993; Bonham et al., 2000; Ritchie et al., 2000). The SRC promoters are very close physically, separated by a distance of roughly 1 kb. When transcription arises from the SRC1α promoter, mature c-Src mRNA transcripts contain Exon 1α spliced to Exon 1B (Fig. 1.5). Conversely, when transcription arises from the SRC1A promoter, c-Src mRNA transcripts contain Exon 1A spliced to 1B (Fig. 1.5). Therefore, differential promoter usage results in two different c-Src transcripts that are identical in coding capacity, but differ in their 5' non-coding extremity (Fig. 1.5).

1.4.1. SRC1A Promoter

The SRC1A promoter closely resembles a typical housekeeping promoter; it lacks TATA or CAAT regulatory sequences, has an extremely high GC content, and transcription is initiated from multiple sites (Bonham and Fujita, 1993). Detailed analysis of this promoter has revealed regulation of SRC1A transcription occurs primarily through the Sp-family of transcriptional activators (Ritchie et al., 2000). Sp1, or specificity protein-1, is the prototype member for this well-studied transcription factor

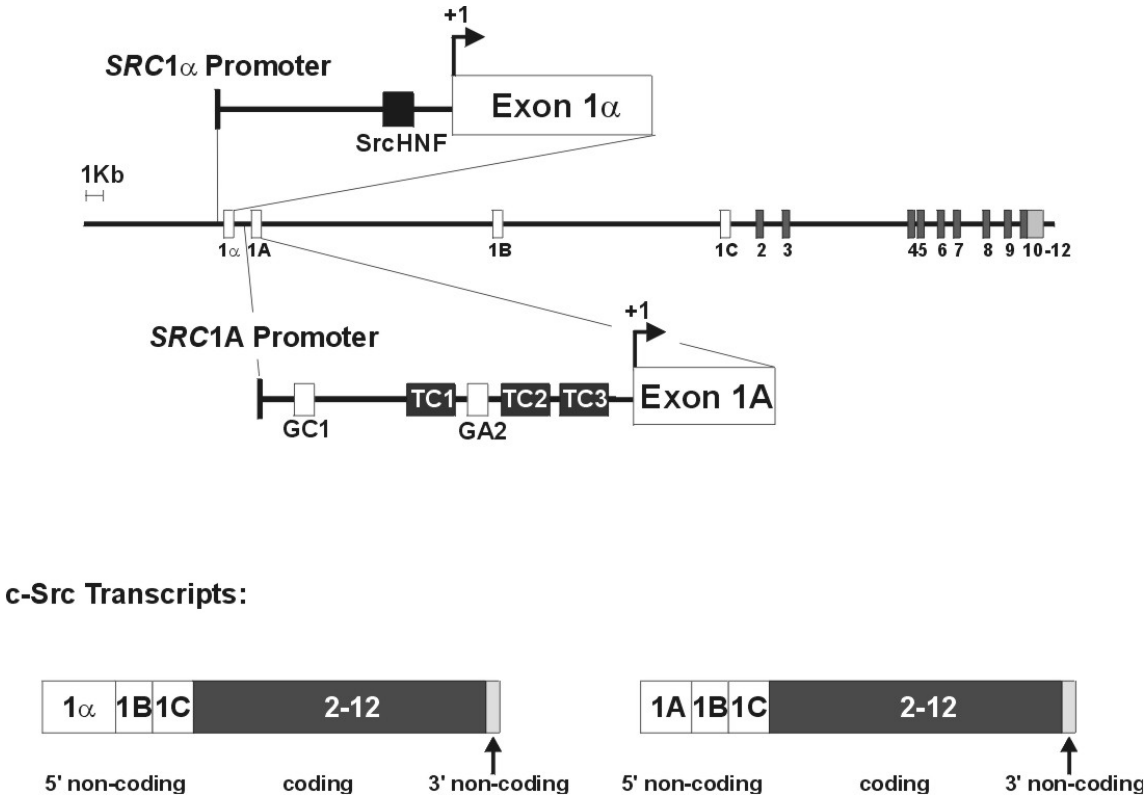

Figure 1.5. Organization of the human SRC gene. The exon/intron boundaries of the human SRC gene on chromosome 20q12.3 are shown. Exons 1A, 1α, 1B, and 1C constitute the 5' non-coding region (white boxes), and Exons 2-12 constitute the open reading frame and 3' non-coding region of c-Src mRNA (dark and light grey boxes, respectively). Two separate promoters are associated with Exons 1α and 1A, and are termed the SRC1α and SRC1A promoters, respectively. The primary cis-acting DNA elements in these promoters that have been identified and characterized are shown, and are discussed in detail in the text. Alternative promoter use results in two distinct c-Src mRNA species that are shown at the bottom. These c-Src transcripts are identical in their coding capacity, but differ in their extreme 5' ends.

family, which also includes Sp2, Sp3, and Sp4 (Suske, 1999). The Sp-family members all contain glutamine-rich transactivation domains, and specifically bind GC-rich elements through zinc finger motifs in their DNA binding domains (Philipsen and Suske, 1999). The Sp-family members, especially Sp1 and Sp3, are ubiquitously expressed, versatile in their activities, and obligatory factors at the majority of eukaryotic promoters and enhancers (Suske, 1999). In terms of the SRC1A promoter, both Sp1 and Sp3 are capable of binding two Sp-family recognition sites, termed GC1 and GA2 (Fig. 1.5). Co-transfection experiments in *Drosophila* SL2 cells has shown that Sp1 is capable of transactivating a SRC1A promoter reporter construct, whereas Sp3 can repress this Sp1-dependent transactivation, presumably through binding site competition (Ritchie et al., 2000). Mutagenesis of the GC1 and GA2 sites reduces SRC1A activity approximately 90 %, thus demonstrating these sites to be critical for transcription from this promoter. In addition to GC1 and GA2, there are also three perfect polypurine:polypyrimidine (Pu:Py) tracts within the SRC1A promoter, termed TC1, TC2 and TC3 (Fig. 1.5). Pu:Py sequences such as these have previously been suggested to form non-B-DNA triple-helical structures, or H-DNA (Mirkin and Frank-Kamenetskii, 1994). A nuclear factor, originally named SPy (Src pyrimidine binding factor), binds these Pu:Py tracts with interesting double- and single-stranded affinity. For example, SPy is capable of binding to double stranded CTTCC motifs located within TC1 and TC2. However, SPy binds with a higher affinity to the single-stranded pyrimidine tracts of TC1, TC2, and TC3. Recently, a DNA affinity purification approach identified SPy as heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Ritchie et al., 2003). hnRNP K is a well-characterized RNA binding protein and component of hnRNP complexes (Bomsztyk et al., 1997). However, further versatility for this protein has been highlighted by the observation that

hnRNP K is also a transcription factor that binds Pu:Py tracts in the MYC promoter (Michelotti et al., 1996). Indeed, mutations in the SPy binding motifs within TC1 and TC2 that abolish SPy double or single stranded binding individually reduce SRC1A promoter activity approximately 20 to 50% (Ritchie et al., 2000; Ritchie et al., 2003). Interestingly, complete inhibition of SPy single stranded binding to both TC1 and TC2 reduced SRC1A promoter activity two fold further than complete inhibition of SPy double stranded binding to both these tracts (Ritchie et al., 2003). These results therefore signify an important functional role for SPy single stranded binding at the SRC1A promoter. Combinations of mutations in Sp-family and SPy binding sites has revealed that the TC1 and GC1 sites act in a cooperative or additive manner, and that the individual GC1, GA2, TC1, and TC2 sites all interact in a complex fashion to affect transcriptional activity from the SRC1A promoter.

1.4.2. SRC1α Promoter

The SRC1α promoter was discovered following the observation that many c-Src mRNAs from human colon cancer cell lines did not contain Exon 1A in their 5' extremity (Bonham et al., 2000). Preliminary experiments showed SRC1α transcripts arise from a single major start site in HepG2 liver carcinoma and HT29 colon carcinoma cells. Analysis of promoter use in various human cancer cell lines demonstrated that the SRC1A and SRC1α promoters are both utilized in the same cell line, but the ratios of promoter use are highly variable. A single DNA element was identified that resembled a binding site for the hepatocyte nuclear factor (HNF)-1 transcription factor, and subsequent deletion and mutagenesis experiments verified this site is absolutely essential

for SRC1α promoter activity (Fig. 1.5). The HNF-1 transcription factor is a liver enriched transcription factor that was initially found responsible for liver specific transcription of β-fibrinogen, albumin, and α1-antitrypsin promoters (Cereghini, 1996). Further analysis determined this factor is in fact a homo- or heterodimer comprised of distinct HNF-1α and/or HNF-1β subunits (Hayashi et al., 1999). Each of these HNF-1 components contain similar dimerization domains, and DNA binding homeodomains, but differ significantly in the composition of their transcriptional activation domains. The HNF-1α transactivation domain actually contains three distinct regions that have all been deemed important for activation of different genes; these include the serine rich activation domain I, the proline rich activation domain II, and the glutamine rich activation domain III (Cereghini, 1996). Transient transfection assays have demonstrated that both HNF-1α and HNF-1β have transactivation potential, depending on the promoter being studied (Hayashi et al., 1999). Determination of the factors bound to the SRC1α promoter in HepG2 cells showed that HNF-1α was the primary HNF-1 component interacting with the HNF site. Co-transfection experiments in HT29 cells verified that HNF-1α, but not HNF-1β, transactivates the SRC1α promoter (Bonham et al., 2000). Expression analysis of the SRC1α promoter demonstrated that SRC1α-derived transcripts are tissue-restricted in their pattern of expression. While c-Src expression appears to be ubiquitous (although at different levels) in nearly all tissues, c-Src transcripts containing Exon 1α are more restricted to tissues such as stomach, kidney, pancreas, and fetal lung. Lower levels of SRC1α-derived transcripts are seen in colon, liver, prostate, fetal kidney, and fetal liver (Bonham et al., 2000). Interestingly, these are the same cell types that HNF-1α expression is restricted to.

Therefore, these findings support the notion that SRC1α promoter use is regulated in normal tissues primarily by HNF-1α expression.

2. SPECIFIC AIMS AND HYPOTHESIS

A frequent finding in diverse human cancers, including colon cancer, is activation of c-Src. Often, this activation is accounted for by a concomitant increase in c-Src protein levels compared with surrounding normal tissue. These findings suggest overexpression of c-Src protein could play an important role in the development and progression of human cancer. One of key observations that led to the studies detailed in this thesis was that c-Src mRNA expression was very high in some cancer cell lines and very low in others. This observation bridged the interests of those studying c-Src activation and those of Dr. Bonham's laboratory, which focuses on the regulation of SRC transcription in normal and cancerous cells. SRC transcription is regulated by an interesting system of two promoters, which are separated by approximately 1 kb. Preliminary analysis of the use of these promoters in normal tissues has demonstrated the upstream SRC1α promoter is tissue-restricted in its expression, while the downstream SRC1A promoter is ubiquitously expressed. Combined, these observations constitute the basis for the general hypothesis and specific aims of this thesis, which are detailed below.

HYPOTHESIS: The mechanisms regulating SRC transcription are important determinants of c-Src expression and activity in human cancer cells.

SPECIFIC AIMS:

Specific Aim #1: To determine the contribution of SRC transcriptional activity to the overexpression and activation of c-Src in human colon cancer cell lines.

Specific Aim #2: To study transcriptional regulation of both SRC promoters in their natural, physiologically linked context, with emphasis on the mechanism(s) of SRC transcriptional activation in human cancer.

Specific Aim #3: To evaluate the importance of SRC promoter architecture in SRC transcriptional repression mediated by histone deacetylase inhibitors.

3. MATERIALS AND METHODS

3.1. Reagents and Suppliers

The materials and reagents used in this study are listed in Table 3.1. All were molecular biology or reagent grade. In addition, a number of commercially available kits were directly utilized in this study, and are listed in Table 3.2. Table 3.3 is a list of companies that supplied reagents and kits.

Table 3.1. A List of Reagents and Suppliers

Reagent	**Supplier Name**
[γ^{32}P]-ATP (6000 Ci/mmol)	NEN Research Products
[α^{32}P]-dCTP (6000 Ci/mmol)	NEN Research Products
α-MEM	Invitrogen Life Technologies
β-mercaptoethanol	BDH
λDNA	Amersham Biotech
Actinomycin D	Sigma
agarose	EM Science
alkaline phosphatase	Invitrogen Life Technologies
ampicillin	Fisher
anti-AcH3 (Lys9/14) antibody	Upstate Biotechnologies
anti-HNF1α antibody	Santa Cruz
anti-Sp1 antibody	Santa Cruz
anti-Sp3 antibody	Santa Cruz
anti-Src antibody	Oncogene Research Products
aprotinin	Sigma
APS	EM Science
ATP	Amersham Biotech

bacto-agar	Invitrogen Life Technologies
bacto-tryptone	Difco Laboratories
bacto-yeast extract	Difco Laboratories
borate	BDH
bovine insulin	Eli Lilly
Bradford protein reagent	BioRad
bromophenol blue	BDH
$CaCl_2$	BDH
Coomassie protein stain	BioRad
DEPC	Sigma
DMEM	Invitrogen Life Technologies
DMSO	Sigma
dNTP mix	Invitrogen Life Technologies
DTT	BioRad
EDTA	BDH
ethidium bromide	Sigma
ExpressHyb	Clontech
fetal calf serum	CanSera
formaldehyde	BDH
formamide	BDH
G418	Invitrogen Life Technologies
GeneScreen Plus nylon membrane	NEN Research Products
glycerol	BDH
glycine	EM Science
guanidinium thiocyanite	BDH
HCl	EM Science
HEPES	EM Science
KCl	BDH
Klenow Fragment (DNA Polymerase I)	Amersham Biotech
leupeptin	Sigma
Lowry protein assay kit	Sigma
methanol	BDH
$MgCl_2$	BDH
MOPS	Sigma
N,N-methylene-bis-acrylamide	BioRad
NaCl	BDH
NaH_2PO_4	BDH
$NaHPO_4$	BDH

NaOH	BDH
nitrocellulose	Sigma
non-fat dry skim milk powder	Carnation
NP-40	BDH
ONPG	Sigma
penicillin/streptomycin 100X mix	Invitrogen Life Technologies
Pfu DNA Polymerase	Stratagene
phenol	BDH
PMSF	Sigma
poly(dI-dC)	Amersham Biotech
polyacrylamide	EM Science
prestained molecular weight markers	Sigma
Protease Inhibitor Cocktail	Sigma
proteinase K	Qiagen
RPMI-1640	Invitrogen Life Technologies
SDS	Sigma
sodium acetate	BDH
sodium azide	Sigma
sodium butyrate	Sigma
sodium carbonate	BDH
sodium citrate	BDH
sodium deoxycholate	Sigma
sodium orthovanadate	Sigma
SRC Optimal Peptide	Oncogene Research Products
Superfect	Qiagen
SuperSignal West chemiluminescent reagent	Pierce
T4 DNA Polymerase	Amersham Biotech
Taq DNA Polymerase	Qiagen
TEMED	EM Science
Trichostatin A	Sigma
Tris	Fisher
Trypan Blue	BDH
trypsin	Invitrogen Life Technologies
Tween-20	Sigma
Urea	BDH
xylene cyanol FF	BDH

Table 3.2. Commercially Available Kits

Commercial Kit	**Supplier**
5' RACE Kit	Invitrogen Life Technologies
CAT ELISA kit	Roche
Chromatin Immunoprecipitation (ChIP) Kit	Upstate Biotechnologies
EndoFree DNA Plasmid Maxi Kit	Qiagen
Lowry Protein Assay Kit	Sigma
QIAquick Gel Extraction Kit	Qiagen
QIAquick Nucleotide Removal Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
S1 Nuclease Protection Assay Kit	Ambion
T4 Quick Ligation Kit	New England Biolabs
T7 DNA Sequencing kit	Amersham Biotech
Thermoscript RT System	Invitrogen Life Technologies

Table 3.3. Names and Addresses of Suppliers

Supplier	**Address**
Ambion, Inc	Ambion Inc., Austin, TX, USA
Amersham Biotech, Inc.	Amersham Biotech, Inc., Baie d'Urfe, Quebec,
BDH	BDH Inc., Toronto, Canada
Beckman-Coulter	Beckman-Coulter, Miami, FL, USA
BioRad	BioRad Laboratories, Inc., Mississauga, Canada
CanSera	CanSera International Inc., Rexdale, Canada
Clontech	Clontech, Palo Alto, CA, USA
Difco Laboratories	Difco Laboratories, Detroit, MI, USA
EM Science	EM Science, Gibbstown, NJ, USA
Fisher	Fisher Scientific Ltd., Nepean, Canada
Invitrogen Life Technologies	Invitrogen Technologies, Burlington, Canada
Millipore	Millipore Ltd., Nepean, Canada
NEN Research Products	DuPont NEN Research Products, Boston, MA, USA
Oncogene Research Products	Oncogene Research, San Diego, CA, USA
Pierce	BioLynx, Brockville, Canada
Promega	Promega Corporation, Madison, WI, USA

Qiagen	Qiagen, Mississauga, Canada
Roche Diagnostics	Roche, Indianapolis, IN, USA
Santa Cruz Biotechnology	Santa Cruz Biotechnology, Santa Cruz, CA, USA
USB	USB Corporation, Cleveland, OH, USA

3.2. Cell Lines and Tissue Culture

3.2.1. Cell Lines and Standard Culture Conditions

All of the human colon cancer cell lines used in this study were obtained from the American Type Culture Collection (ATCC). All tissue culture media was obtained from Invitrogen. HT29, WiDr, SW480, SW620, LS174-T, and KM12C colon cancer cell lines were all grown in DMEM media and 10% fetal calf serum (FCS, CanSera). Colo 201, Colo 205, Colo 320, DLD-1, and HCT-15 colon cancer cell lines were grown in RPMI-1640 media with 10% FCS. The HCT-116 colon cancer cell line was grown in McCoy's 5A media with 10% FCS. The Hke-3 and Hkh-2 cell lines were obtained from Dr. S. Shirasawa (Kyushu University, Japan) and maintained in HCT-116 growth media supplemented with 600 μg/mL G418. DKO-4 and DKS-8 were also obtained from Dr. Shirasawa and grown in DLD-1 growth media containing 600 μg/mL G418. HeLa cervical carcinoma cells were obtained from ATCC and grown in RPMI-1640 with 10% FCS. Molt-4 T-cell leukemia cells were obtained from ATCC and grown in RPMI-1640 with 10% FCS. The breast cancer cell lines MDA-MB-231, T47D, and HS578-T were obtained from Dr. S. Carlsen (Saskatchewan Cancer Agency) and grown in RPMI-1640 with 10% FCS. T47D and HS578-T media was further supplemented with 0.2 IU/mL bovine insulin (Eli Lilly). The SV40 immortalized normal breast cell line, HBL100, was also obtained from Dr. Carlsen and grown in α-MEM media with 10% FCS and 0.2

IU/mL bovine insulin. BHK-21 hamster cells and their derivative, tsBN462, were obtained from Dr. T. Sekiguchi (Kyushu University, Japan), and grown in DMEM media with 10% fetal calf serum. All cells were maintained at 37°C and 5% CO_2, except for tsBN462 cells, which were maintained at 33°C and 5% CO_2.

3.2.2. Actinomycin D Treatments

For mRNA half-life studies, Actinomycin D was added to semi-confluent cells to a final concentration of 5μg/mL. Following Actinomycin D addition, cells were harvested at various time points.

3.2.3. Histone Deacetylase Inhibitor Treatments

For HDAC inhibitor studies, exponentially growing cells were trypsinized, seeded at 50% confluence, and then allowed to grow for 24 hours. Cells were then treated with 5 mM sodium butyrate (NaB, Sigma) or 1 μM trichostatin A (TSA, Sigma) for various times prior to harvesting.

3.3. Bacterial Strains

Escherichia coli (*E. coli*) strain DH5α was used as the host for propagation of all plasmid vectors. Transformed DH5α cells were grown at 37°C in a shaking incubator in Lauria-Bertani (LB) broth consisting of 1.0% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, and 1.0% (w/v) NaCl with 100μg/mL ampicillin. Selective LB-agar plates for isolation of transformed DH5α colonies consisted of LB broth with 1.5% (w/v) agar and 100 μg/mL ampicillin. Plates were incubated upside-down at 37°C.

3.4. General Molecular Techniques

The majority of the molecular biology techniques utilized in this study are based on those described by Sambrook (Sambrook et al., 1989).

3.4.1. Molecular Cloning Techniques

3.4.1.1. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed using the buffers supplied with heat stable *Pfu* (Stratagene) or *Taq* (Qiagen) DNA Polymerases, dNTPs (Invitrogen), template DNA, and specifically designed forward and reverse primers. Components were added to achieve the final reaction concentrations recommended by the PCR enzyme manufacturer's protocol. Reaction annealing temperatures were determined using MacVector software and input sequences for the template and primers. Reactions were typically performed for between 25 and 35 cycles in a thermocycler (PE Biosystems), with a standard melting temperature of 95^{o}C and an extension temperature of 68^{o}C (*Pfu*) or 72^{o}C (*Taq*). Extension times were adjusted for the expected product size, utilizing an average extension rate of 0.5 kb/min (*Pfu*) or 2.0 kb/min (*Taq*).

3.4.1.2. Restriction Enzyme Digestion

All restriction enzymes used were obtained from New England Biolabs. Restriction enzyme digestions were performed on 0.1 μg/μL target DNA using restriction enzymes (1 unit per 1 μg DNA) with the buffer supplied by the manufacturer. When DNA was digested to completion, reactions were allowed to proceed for 2-3 h at 37^{o}C. For partial digestions, reactions were performed for 20 min at room temperature.

3.4.1.3. Generation of Blunt Ends from Recessed or Protruding 3' Termini

Recessed 3' termini on double stranded DNA fragments were extended by directly adding 1 μL of a 10 mM dNTP mixture, and 5 units of DNA Polymerase I Klenow fragment (Amersham) to 20 μL of a restriction enzyme digest reaction. Reactions proceeded for 15 min at room temperature. To remove protruding 3' termini from double stranded DNA fragments, 1 μL of a 10 mM dNTP mixture and 5 units of T4 DNA Polymerase (Amersham) were added directly to 20 μL of a restriction enzyme digest reaction. Reactions proceeded for 15 min at 12^{o}C, and were terminated by heating at 75^{o}C for 10 min.

3.4.1.4. Removal of 5' Terminal Phosphate

To dephosphorylate the 5' termini of vector DNA prior to ligation reactions, 1 unit of calf intestinal alkaline phosphatase (Invitrogen) was added to restriction enzyme digests and incubated at room temperature for 30 minutes.

3.4.1.5. Agarose Gel Electrophoresis

DNA fragments were loaded onto agarose gels in gel loading buffer (0.015% (w/v) bromophenol blue, 0.015% (w/v) xylene cyanol FF, 10% (v/v) glycerol, and 10mM ethylenediamine tetracetic acid (EDTA)). Gels consisted of 1-2% agarose (w/v) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH. 8.0) or TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH. 8.0). Electrophoresis was performed at 100 V for approximately 45 min using a TAE or TBE running buffer.

3.4.1.6. Purification of Linear DNA Fragments

Linear DNA fragments generated for ligation reactions were isolated from agarose gels and purified using a QIAquick gel purification kit (Qiagen).

3.4.1.7. DNA Ligation

DNA ligation reactions consisted of 50 ng of purified, dephosphorylated vector DNA, and 200 ng of purified insert DNA in a 20 μL reaction volume with 1 μL of Quick T4 DNA ligase (New England Biolabs) in the supplied buffer. Reactions proceeded for 15 min at room temperature. For re-circularization of linear DNA, 100 ng of purified vector DNA was used in the ligation reaction.

3.4.2. Site Directed Mutagenesis

All mutant plasmids were generated using the QuickChange site directed mutagenesis protocol (Stratagene) with complementary primers spanning the mutation site.

3.4.3. DNA Sequencing

Manual DNA sequencing was performed using a T7 Polymerase Sequencing kit (Amersham). Sequencing reactions were separated by electrophoresis on denaturing 6% polyacrylamide gels (6% acrylamide:N;N-methylene bis-acrylamide (19:1), 8 M urea, 1X TBE) using a TBE running buffer. Alternatively, automated DNA sequencing was performed by Annette Kerviche at the Saskatchewan Cancer Agency DNA Sequencing Facility using an ABI Prism 310 Genetic Analyzer.

3.4.4. Preparation and Transformation of Competent Bacterial Cells

E. coli DH5α cells were rendered competent for plasmid transformation according the procedure originally described by Hanahan (Hanahan, 1983). Competent cells were transformed as described (Hanahan, 1983), plated onto LB-ampicillin plates, and grown for 18 hours at 37^{o}C.

3.4.5. Isolation of Plasmid DNA from Bacterial Cells

3.4.5.1. Small Scale Plasmid Purification

Isolated, transformed, bacterial colonies were picked from LB-ampicillin plates and transferred to LB-ampicillin liquid culture media. Cultures were grown overnight, and then bacteria were pelleted by centrifugation for 1 minute at top speed in a microcentrifuge. Plasmid DNA was subsequently isolated as described (Birnboim and Doly, 1979), and re-suspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1mM EDTA) containing 20 μg/mL RNase A.

3.4.5.2. Large Scale Plasmid Purification

Large scale, endotoxin free, plasmid DNA was prepared from 100 mL bacterial cultures using an EndoFree DNA Plasmid Maxi Kit (Qiagen) as per the manufacturer's protocol.

3.4.6. Isolation of RNA from Cultured Eukaryotic Cells

RNA was isolated from semi-confluent tissue culture plates according to the denaturing guanidinium thiocyanite method described by Chomczynski and Sacci (Chomczynski and Sacchi, 1987). Purified RNA was re-suspended in RNA Storage Buffer (0.1 mM EDTA, 0.1 % (v/v) diethyl pyrocarbonate (DEPC)). RNA concentration and purity was determined by A_{260}/A_{280} followed by verification of integrity on ethidium bromide stained agarose gels. RNA with an A_{260}/A_{280} ratio above 1.7 was considered pure.

3.4.7. Isolation of Genomic DNA from Cultured Eukaryotic Cells

Semi-confluent 150 mm plates were washed with an ice cold phosphate buffered saline solution and lysed in 5 mL of proteinase K buffer (10 mM Tris-HCl pH 7.4, 10

mM EDTA, 150 mM NaCl, 0.4 % (w/v) sodium dodecyl sulphate (SDS)). Cell lysates were incubated at 37°C with 5 mg of proteinase K followed by several organic extractions with equivalent volumes of phenol saturated with Tris-HCl, pH 7.4, and chloroform/isoamyl alcohol (49:1, v:v). Genomic DNA in the aqueous partition was precipitated with 2.5 volumes of 95% ethanol, washed, and dissolved in 1 mL TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) with 100μg RNase A, and incubated at 37°C for 1 hour. RNase A was removed by subsequent organic extractions; pure genomic DNA in the aqueous fraction was ethanol precipitated, washed, and dissolved in 1 mL of TE buffer. DNA concentration and purity was determined by A_{260}/A_{280}. DNA with a ratio above 1.6 was considered pure. This protocol was adapted as required to purify genomic DNA from a greater or smaller starting cell number.

3.4.8. Annealing of Complementary Single Stranded DNA Oligonucleotides

Equal molar amounts of complementary single stranded DNA oligonucleotides were dissolved in STE buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA) at a concentration of approximately 1-2 A_{260} units/100 μL for each oligonucleotide. The mixture was heated to 95^{o}C, and allowed to gradually cool to room temperature.

3.4.9. Generation of DNA Size Markers

λ DNA digested with *Eco*R I or *Eco*R I/*Hind* III was used as a DNA size marker for fragments ranging from 1 to 20 kb. For smaller DNA fragments, pBluescript digested with *Hpa* II was used as a size marker.

3.4.10. Generation of Labeled DNA Probes

3.4.10.1. Random Prime Method

Uniformly [^{32}P]-labeled DNA probes for Northern and Southern hybridization procedures were created from double stranded DNA fragments using an oligolabeling kit (Amersham) according to the manufacturer's recommendations. Unincorporated radioactivity was removed using a QIAquick nucleotide removal kit (Qiagen).

3.4.10.2. In-Fill Method

Approximately 1 nmol of double stranded DNA with 5' overhangs was labeled for bandshift reactions via an in-fill reaction in one-phor-all buffer (Invitrogen) with 5 units of DNA Polymerase I Klenow fragment (Amersham) in the presence of 50 μM each of dATP, dGTP, dTTP, and 50 μCi [α^{32}P]-dCTP. Reactions proceeded for 15 min at room temperature. Labeled DNA was precipitated and re-suspended in TE buffer.

3.4.10.3. Linear Amplification Method

[^{32}P]-labeled, single-stranded DNA probes for S1 nuclease protection assays were generated from 100 ng of double stranded, linear DNA templates using a gene-specific primer and *Taq* DNA Polymerase. Reactions were set up exactly as for PCR except cold dCTP was substituted with 50 μCi [α^{32}P]-dCTP. Following an initial incubation at 95^{o}C for 3 min., reactions proceeded through 25 cycles in a thermocycler (PE Biosystems, cycling parameters: melt 95^{o}C, 30 sec; anneal 55^{o}C, 30 sec; extend 72^{o}C, 90 sec.).

3.4.11. Determination of Protein Concentration

3.4.11.1. Bradford Method

The Bradford method of protein quantitation was utilized for analysis of transfected cell lysates as well as determination of nuclear extract concentrations.

Protein extracts were mixed with 500 μL of Bradford Reagent (BioRad) and incubated for 15 min at room temperature. The intensity of blue colour development was measured spectrophotometrically at A_{595}. Extract volumes were used that gave an A_{595} reading in the linear range of the assay (0.2 to 1.0 absorbance units). Various concentrations of bovine serum albumin (BSA) were used as protein standards.

3.4.11.2. Lowry Method

The Lowry method of protein quantitation was utilized for lysates prepared for Western blot analysis or *in vitro* Src kinase assays. The Lowry method was employed only for these applications because the buffers used were not compatible with the Bradford method of protein quantitation. This technique was performed using a Lowry Assay Kit (Sigma).

3.5. Plasmid Construct Details

3.5.1. Expression Vectors and cDNAs

c-Src cDNA clones have been described previously (Lin et al., 1995). HHC189 containing a 1.1 kb insert of β-Actin and pGD-P-25A containing a 1.5 kb insert of glucose-6-phosphate dehydrogenase were obtained from the ATCC. pMI containing exons 2 and 3 of the human c-Myc gene was a gift of Dr. Zheng (Pathology, University of Saskatchewan). pRibo containing a 5.8 kb insert of 18S ribosomal RNA cDNA, as well as pCMV-βGAL were a gift of Dr. W. Roesler (Biochemistry, University of Saskatchewan). The GAL4 DNA binding domain/transcription factor fusion constructs HNF-1α/GAL4 and HNF-1β/GAL4, as well as their parental GAL4 fusion vector, pSG424, were obtained from Dr. R. O'Brien (Vanderbilt University, Tennessee)

(Streeper et al., 2000). GAL4/Sp1 and GAL4/Sp3 fusions, as well as their parental GAL4 fusion vector, pM, were obtained from Dr. T. Sakai (Kyoto University, Japan) (Nakano et al., 1997). GAL4/VP16 was obtained from Dr. D. Anderson (Saskatchewan Cancer Agency). pCMV-hTAF(II)250 was a gift of Dr. R. Tjian (Howard Hughes Medical Institute and University of California, Berkeley). pBJ5-HNF-1α and pBJ5-HNF-1β were obtained from Dr. Gerry Crabtree (Stanford University) (Cereghini, 1996).

3.5.2. CAT Reporter Constructs

The 0.38 SRC1A-CAT and 0.54 SRC1A-CAT (CAT = chloramphenicol acetyltransferase) promoter constructs with Gem2CAT (Promega) reporter construct backbones, as well as the SRC1A promoter:cDNA chimera, pSRC1A-chimera have been described (Bonham and Fujita, 1993; Ritchie et al., 2000). The pCAT3 (Promega) based SRC1α constructs, -145 SRC1α-CAT, -777 SRC1α-CAT, and -2852 SRC1α-CAT, as well as the SRC1α promoter:cDNA chimera, pSRC1α-chimera, were all obtained from Mark Boyd (Bonham et al., 2000).

3.5.3. SRC Dual Promoter Reporter Study

3.5.3.1. SRC Dual Promoter CAT Reporter Construct and Related Plasmids

A 2.4 kb *Eco*R V/*Bam*H I fragment containing the two SRC promoters was liberated from pBam4.8 (Bonham and Fujita, 1993) and ligated into a *Eco*R V/*Bam*H I cut pBlue KS+ shuttle vector to form the construct p2.4SRCBlue. A 1.1 kb DNA fragment downstream from the SRC promoter region was isolated from cos11B (Bonham and Fujita, 1993) via PCR using *Pfu* DNA Polymerase and primers FWD: 5'-GCCTTTTGTTGTGATGCAGCG and REV: 5'-CTTCTAGAATGAATTCGCCAGC.

The resulting PCR fragment was digested with *Bam*H I/*Xba* I, and ligated into *Bam*H I/*Xba* I cut p2.4SRCBlue, to create p3.5SRCBlue, a shuttle vector harbouring a contiguous 3.5 kb fragment of the SRC promoters and downstream sequences. This 3.5 kb promoter fragment was isolated from p3.5SRCBlue using *Sac* I/*Kpn* I, and ligated with a *Sac* I/*Kpn* I cut version of pCAT3 Basic (Promega) lacking a 223 bp *Hind* III fragment (intronless pCAT3-Basic), thus creating p3.5SRC-CAT. A 1.3 kb genomic DNA fragment containing part of SRC Exon 1B and accompanying upstream sequences was generated using PCR with *Pfu* DNA Polymerase, a cos11B template and the primers FWD: 5'-GGGAGAGGAATTCTGCTAATG, and REV: 5'-AGCTGGGCAAGTTGCTTCACTTC. This PCR fragment was digested with *Eco*R I, and cloned into p3.5SRC-CAT that had been prepared by *Sac* I digestion, removal of the 3' overhang, and subsequent *Eco*R I digestion. This construct was termed -560crypticDPCAT. The -560 DPCAT construct was created by removing a 1 kb, *Eco*R I/*Bbv*C I fragment between SRC Exons 1A and 1B from -560crypticDPCAT, generation of blunt ends, and subsequent re-ligation. To create -2852 DPCAT, a 2.2 kb *Eco*R V/*Kpn* I fragment upstream of the SRC1α promoter was isolated from -2852 SRC1α-CAT (Bonham et al., 2000), and cloned into *Eco*R V/*Kpn* I digested -560 DPCAT. To create -145 DPCAT, a complete digest with *Eco*R V followed by partial digest with *Msc* I was performed on -560 DPCAT, and a 7.3 kb product from this reaction was gel purified, blunt ended, and re-ligated. Δ1α DPCAT was generated by partial *Sal* I digestion of -560 DPCAT, followed by isolation and re-ligation of the 6.0 kb product of this reaction. Δ1A DPCAT was created by removing a *Sac* II/*Sac* I fragment from -560 DPCAT, generation of blunt ends, and subsequent re-ligation. To create HNFmut

DPCAT, p2.4SRCBlue was digested with *Hind* III/*Xho* I, blunt-ended, and re-ligated in order to remove a small fragment containing an unwanted *Hinc* II site, thus creating p2.4SRCBlueΔHinc2. A *Pst* I/*Hinc* II fragment from -777 mutSRC1α (Bonham et al., 2000), containing a mutant Src HNF site in the SRC1α promoter, was cloned into *Pst* I/*Hinc* II digested p2.4SRCBlueΔHinc2, creating p1.4HNFmutSRCBlue, a variant of p2.4SRCBlue missing a 1 kb *Hinc* II fragment. This 1 kb *Hinc* II fragment from p2.4SRCBlue was re-introduced into *Hinc* II digested p1.4HNFmutSRCBlue, thus generating p2.4HNFmutSRCBlue. A 2.1 kb *Eco*R V/*Rsr* II fragment from p2.4HNFmutSRCBlue was finally cloned directly into *Eco*R V/*Rsr* II digested -560 DPCAT to generate HNFmut DPCAT. All constructs were mapped with restriction enzymes to verify their integrity, and sequenced to verify the incorporation of correct PCR and restriction fragments.

3.5.3.2. CAT Constructs Harbouring Proposed Enhancer

A 1.65 kb *Bam*H I/*EcoR* I fragment from cos11B (Bonham and Fujita, 1993) containing SRC genomic sequence upstream of Exon 1α was cloned into a *Bam*H I/*Eco*R I digested pBlue shuttle vector, creating p-4496/-2852Blue. This insert was removed via *Sac* I/*Eco*R V digestion, and cloned into -2852 SRC1α-CAT that had been *Kpn* I digested, blunt ended, and subsequently *Sac* I digested, thus generating pBamEco-2852SRC1α-CAT. To create -4496 SRC1α-CAT, a 50 bp *Bam*H I fragment was removed from pBamEco-2852SRC1α-CAT, and the vector was re-ligated. To derive -6882 SRC1α-CAT, a 2.4 kb *Eco*R I fragment was liberated from cos11B, and cloned directly into *Eco*R I digested -4496 SRC1α-CAT. A fragment harbouring the HepG2 DNaseI hypersensitive site, DH3, was prepared from -6882 SRC1α-CAT via *Kpn* I/ *Xho*

I digest, followed by generation of blunt ends. This fragment was cloned into *Kpn* I digested, blunt-ended -145 SRC1α-CAT to create -145 SRC1α/DH3-CAT. This fragment was also cloned into *Kpn* I digested, blunt-ended -560 DPCAT to create -560 DH3 DPCAT.

3.5.3.3. S1 Probe Vectors

pSRC1A chimera has been described previously (Bonham and Fujita, 1993). A 827 bp PCR product from pSRC1A chimera was generated using *Pfu* DNA Polymerase and the primers FWD: 5'-GGGGTACCAGGGATGTTTTGC, and REV: 5'-AGCTGGGCAAGTTGCTTCACTTC. To derive the SRC1A-CAT S1 probe vector, pSRC1A-CAT-chimera, this product was digested with *Kpn* I, and subsequently ligated directly into intronless pCAT3-Basic that had been digested with *Sac* I, blunt-ended, and then *Kpn* I digested. A 1.4 kb PCR product from pSRC1α chimera (Bonham et al., 2000) was generated using *Pfu* DNA Polymerase, a forward primer, FWD: 5'-GGGGTACCAGGGATGTTTTGC, and the same reverse primer used to isolate the pSRC1A chimera fragment. To create the SRC1α-CAT S1 probe vector, pSRC1α-CAT-chimera, this fragment was digested with *Kpn* I, and cloned into intronless pCAT3-Basic that had been digested with *Sac* I and blunt-ended, followed by *Kpn* I digestion. All constructs were sequenced to verify the integrity of the chimeric promoter-cDNA inserts.

3.5.4. GAL4 Based SRC1A and SRC1α CAT Constructs

A *Kpn* I site was introduced into 0.38 SRC1A-CAT, downstream of the GC1 Sp-family binding site, using site directed mutagenesis with mutagenic primers FWD: 5'-GCTTCTGTGCCCGGTACCCCCACCCCGCCC and REV: 5'-

GGGCGGGGTGGGGGTACCGGGCACAGAAGC. This mutant vector was digested with *Nar* I/*Kpn* I, and a synthetic double stranded DNA cassette created by annealing sense (GC1-GAL4 plus, 5'-CGCCCTGGCGTCGGAGTACTGTCCTCCGATCGGCCCGGTAC, GAL4 DNA recognition site underlined), and antisense (GC1-GAL4 minus, 5'-CGGGCCGATCGGAGGACAGTACTCCGACGCCAGGG) oligonucleotides was inserted into this *Nar* I/*Kpn* I site. This resulted in the generation of SRC1AΔGC1-GAL4-CAT, a 0.38 SRC1A-CAT variant where the GC1 Sp-family binding site had been replaced by the core recognition site for the GAL4 yeast transcription factor. The GA2 Sp-family binding site was also replaced with a GAL4 binding site by removing a *BssH* II fragment from SRC1AΔGC1-GAL4-CAT, and inserting a double-stranded cassette created by annealing single-stranded sense (GA2-GAL4 plus, 5'-CGCGCTTCCTCCTTCCTCCTCCTCCCGGCTGCTCGGAGTACTGTCCTCCGATCGCG, GAL4 recognition sequence underlined), and antisense (GA2-GAL4 minus, 5'-CGCGCGCGATCGGAGGACAGTACTCCGAGCAGCCGGGAGGAGGAGGAAGGAGGAAG) oligonucleotides. The resulting vector was termed SRC1AΔGC1/GA2-GAL4-CAT. The entire promoter region from this construct was isolated via *Nar* I/*Sac* II digestion, and re-introduced into *Nar* I/*Sac* II digested wild-type 0.38 SRC1A-CAT to ensure the absence of unwanted mutations that could have been generated within the plasmid during the mutagenesis protocol. The Src HNF site was replaced with a GAL4 binding site in the -145 SRC1α-CAT vector using site directed mutagenesis and the primers HNF-GAL4mut sense (5'-GCTGGGGGCCCGCCCTGAGCCCCTGGGAATCGGAGTACTGTCCTCCGATCGG

CCTTGCAAACAAGTGCGGCCATTTCAC, GAL4 site underlined), and HNF-GAL4mut antisense (5'-GTGAATGGCCGCACTTGTTTGCAAGGCCGATCGGAGGACAGTACTCCGATTCCCAGGGCTCAGGGCGGGCCCCCAGC), thus creating -145 SRC1αΔHNF-GAL4-CAT. The mutant SRC1α promoter region was subcloned back into wild-type -145 SRC1α-CAT using *Kpn* I/*Pst* I, ensuring solely the GAL4 mutation in this plasmid construct. All mutations were verified by DNA sequencing.

3.5.5. SRC1A 5' Promoter Deletion CAT Constructs

Constructs based on 0.38 SRC1A-CAT, and harbouring various deletions in TC1, TC2, and TC3, were described previously (Ritchie and Bonham, 1998; Ritchie et al., 2000), and obtained from Dr. Shawn Ritchie. The construct 0.2 SRC1A-CAT was created by *Nar* I/*Bss*H II digestion of 0.38 SRC1A-CAT, generation of blunt ends, and subsequent re-ligation of the plasmid vector.

3.5.6. SRC1A and SRC1α 3' Promoter Deletion CAT Constructs

The SRC1A promoter reporter construct, 0.54 SRC1A-CAT, has been described (Bonham and Fujita, 1993). A *Hinc* II/*Sal* I fragment from this construct, containing the SRC1A promoter cassette, was blunt-ended and subcloned into *EcoR* V/*Xho* I digested, blunt ended pCAT3-Basic. All SRC1A 3' deletion constructs were based on 0.38 SRC1A-CAT3-Basic, which was generated by deleting a 160 bp *Nar* I promoter fragment from 0.54 SRC1A-CAT3-Basic, as well as a 223 bp *Hind* III fragment from the CAT3 vector backbone. SRC1A3'ΔSac2-CAT was derived by digesting 0.38 SRC1A-CAT with *Sac* II/*Hind* III, creation of blunt ends, and re-ligation. The remaining SRC1A 3' promoter deletions were isolated as PCR fragments from 0.38

SRC1A-CAT using *Pfu* DNA Polymerase, and a common forward primer (SRC1A/1α3'ΔFWD, 5'-GGTACCGAGCTCTTACGCGTGC) in conjunction with specific reverse primers (SRC1AΔ+13REV, 5'-CCGCTCAAGCTTCCAGGCCGG; SRC1AΔ-26REV, 3'-AGAAAGCTTGAGAGAGAAAGGG; SRC1AΔ-60REV, AGGAAGCTTCGGCGGCCCGGG). SRC1α 3' promoter deletion fragments were isolated from -145 SRC1α-CAT as PCR fragments using *Pfu* DNA Polymerase, and a common forward primer (SRC1A/1α3'ΔFWD), paired with specific reverse primers (SRC1αΔ+99REV, GGTAAGCTTGTGCTAGATGAATGG; SRC1αΔ+41REV, GGGAAGCTTGAGGTGCCACAGC; SRC1αΔ-20REV, GGCCAAGCTTGTTTGCAAGGC). PCR products were digested with *Sac* I/*Hind* III, cloned directly into *Sac* I/*Hind* III digested pCAT3-Basic, and sequenced to verify their integrity.

3.5.7. WAF1 Promoter CAT Constructs

A p21/WAF1 promoter-luciferase construct, pWWP-Luc, was a gift of Dr. B. Vogelstein (el-Deiry et al., 1993). The 2.3 kb WAF1 promoter fragment was isolated from this construct via *Hind* III digestion, and subcloned into a *Hind* III digested pBlue shuttle vector. The WAF1 promoter was deleted to -210, relative to the transcription start site, via *Pst* I digestion and re-ligation. To create -210 WAF1-CAT, the truncated WAF1 promoter was removed from pBlue with *Sac* I/*Sal* I, and cloned into *Sac* I/*Sal* I digested pCAT3-Basic. To allow for further truncation of the WAF1 promoter, site directed mutagenesis was employed to introduce a *Sac* I site at the -101 position using the mutagenic primers sense: 5'-GGGCGGTCCCGGGCGGAGCTCTGGGCCGAGCGAGGGTCCC, and antisense 5'-

GGGACCCGCGCTCGGCCCAGAGCTCCGCCCGGGACCGCCC. This WAF1 *Sac* I mutant construct was subsequently digested with *Sac* I, and re-ligated to create -101 WAF1-CAT.

3.5.8. SRC1A/1α and WAF1 Promoter Chimeras

3.5.8.1. SRC1A/1α : WAF1 Core Promoter Chimeras

A -145 SRC1α-CAT variant, harbouring a *Sac* II recognition site at -10, was created by site directed mutagenesis and the primers 1αSac FWD (5'-GCAAACAAGTGCGGCCATTTCCGCGGCCCAGGCTGGCTTCTGC) and 1αSac REV (5'-GCAGAAGCCAGCCTGGGCCGCGGAAATGGCCGCACTTGTTTGC). A similar variant of 0.38 SRC1A-CAT3-Basic, with a *Sac* II recognition site engineered at -10, was created using the primers 1ASac FWD (5'-CGATCTGTCTCTCCCGGCCCGCGGTCCATTCCGGCCTGGGAGC) and 1ASac REV (5'-GCTCCCAGGCCGGAATGGACCGCGGGCCGGGAGAGACAGATCG). The WAF1 core promoter was amplified from -210 WAF1-CAT, using PCR and primers WafSacFWD (5'-GGCGCCGCGGTTGTATATCAGG) and CAT3NcoREV (5'-TTTCTCCATGGTGGCTTTACC). The chimeric promoter constructs, 0.38SRC1A:WAFcore-CAT and -145SRC1α:WAFcore-CAT, were derived by digesting the WAF1 PCR product with *Sac* II/*Nco* I, and cloning it into the *Sac* II engineered, *Sac* II/*Nco* I digested SRC1A or SRC1α CAT constructs, respectively.

3.5.8.2. WAF1 : SRC1A/1α Core Promoter Chimeras

A similar strategy was employed to engineer WAF1 and SRC1A or 1α promoter chimeras containing WAF1 upstream sequence fused to the SRC1A or 1α core promoters. To this end, *Eco*R I recognition sites were engineered in the -101 WAF1-

CAT and -210 WAF1-CAT vectors using site directed mutagenesis with the primers EcoWaf FWD (5'-CGGGCGGGGCGGTTGGAATTCAGGGCCGCGCTGAGC) and EcoWaf REV (5'-GCTCAGCGCGGCCCTGAATTCCAACCGCCCCGCCCG). The SRC1A and SRC1α core promoters were isolated from 0.38 SRC1A-CAT-Basic and -145 SRC1α-CAT via PCR using a common reverse primer, CAT3NcoREV, paired with the appropriate 1A or 1α specific forward primer (1AEcoFWD, 5'-CTCCGAATTCTCCCTTTCTCTCTCG; 1αEcoFWD, 5'-GGTTAGAATTCAAGCCAGCCTTGC). The SRC1A core promoter PCR product was digested with *Eco*R I/*Nco* I, and cloned directly into *Eco*R I/*Nco* I digested -101 WAF1-CAT or -210 WAF1-CAT, to create -101WAF1:SRC1Acore-CAT and -210WAF1:SRC1Acore-CAT, respectively. The SRC1α core promoter PCR product was similarly digested and ligated with -210 WAF1-CAT or -101 WAF1-CAT, thus generating -101WAF1:SRC1αcore-CAT and -101WAF1:SRC1αcore-CAT.

3.6. Transfection of Eukaryotic Cell Lines

3.6.1. Standard Transfection Reaction and Conditions

All plasmid constructs used in transfection experiments were isolated and purified using an EndoFree Plasmid Maxi Kit from Qiagen. In a typical transfection experiment, 1.5 μg of a promoter CAT construct, 0.5 μg of pCMVβ-Gal, and 10 μL of Superfect reagent (Qiagen) were mixed together in 85 μL of serum free DMEM. After a 20 minute incubation at room temperature, the DNA-Superfect mixture was further diluted with 600 μL of cell culture media with 10% FCS. This transfection mix was then added directly to plates seeded the previous day at a density of $3X10^5$ cells per 35 mm tissue

culture plate. Transfections were allowed to proceed for 3 hours, followed by the addition of 2 mL of the appropriate growth media containing 10% FCS. Cells were grown under standard conditions for an additional 48 h prior to harvesting.

3.6.2. Other Transfection Reactions and Conditions

3.6.2.1. Co-transfection Studies

To assess the effects of HNF-1α or HNF-1β expression on the expression of various DPCAT constructs, DNA mixtures consisted of 0.75 μg CAT reporter, 0.5 μg pCMV-βGAL, and 0, 21, 125, or 750 ng of pBJ-HNF-1α or -1β. For SRC1A GAL4-transcription factor fusion studies, DNA mixtures consisted of 1.0 μg CAT reporter, 0.5 μg pCMV-βGAL, and 0.25 μg Sp1/Sp3 GAL4 fusion or 1.0 ng VP16/GAL4. For SRC1α GAL4-transcription factor fusion studies, DNA mixtures consisted of 0.5 μg CAT reporter, 0.5 μg pCMV-βGAL, and 1.0 μg of HNF-1α/1β GAL4 fusion or 0.5 ng VP16/GAL4. For TAF1 rescue experiments in tsBN462 cells, DNA mixtures consisted of 0.75 μg CAT reporter, 0.5 μg pCMV-βGAL, and 0, 47, 94, or 188 ng of pCMV-hTAF(II)250. pBlue was always added to transfection mixtures as required to ensure a final DNA mass of 2.0 μg.

3.6.2.2. HDAC Inhibitor Studies

Following incubation with DNA-Superfect complexes, cultured cells were supplemented with 2 mL of normal growth media containing 10% FCS, and allowed to grow under standard culture conditions. After 24 hours, the cells were either exposed to NaB (5 mM), TSA (1 μM), or left untreated. Cells were harvested after an additional 24 hours.

3.6.2.3. tsBN462 and BHK-21 Transfections

For tsBN462 and BHK-21 transfection experiments, cells were transfected at 33°C. Following transfection, fresh media was added to cells, and they were allowed to grow at 33°C for an additional 24 h. At this time, cells were either maintained at 33°C or shifted to 39 °C for 24 h of growth prior to harvesting. To assess the effects of HDAC inhibitors on reporter gene activity, tsBN462 and BHK-21 cells were allowed to grow at 33 °C for 36 h following transfection. Cells were then left untreated or exposed to TSA (1 mM) or NaB (5 mM) for 18 h at 33 °C or 39 °C before harvesting.

3.7. Detection of Reporter Gene Activity

3.7.1. CAT ELISA

Cells transfected on 35 mm plates were washed twice with ice-cold PBS, then lysed in 500 μL of 1X Lysis buffer provided with a CAT ELISA kit (Roche). Levels of CAT expression in 200 μL of lysate were subsequently determined by enzyme linked immunosorbant assay (ELISA) as per the manufacturer's protocol.

3.7.2. β-Galactosidase Assay

A colorimetric assay was utilized to measure β-Galactosidase (β-Gal) activity in transfection lysates exactly as described (Hall et al., 1983).

3.8. Northern Blot

3.8.1. Northern Procedure

RNA samples were prepared for electrophoresis by mixing equal amounts (15 μg) of RNA with 15 μL of Northern Gel Loading Buffer (20 mM Morphpropane sulfonic

acid (MOPS), 5.6 mM sodium acetate, 1.1 mM EDTA, 50 % (v/v) formamide, 6.7 % (v/v) formaldehyde, 5.6 % glycerol) in a final volume of 18 μL, boiling for 2 min, then plunging quickly on ice. Ethidium bromide (5 μg) and gel loading dye (1.5 μL) were subsequently added. Samples were fractionated in 1% denaturing formaldehyde-agarose gels. Gels were photographed, denatured in 50 mM NaOH for 15 min, neutralized in 100 mM Tris-HCl pH 7.4 for 15 min, and transferred to charged nylon membranes (NEN) by capillary elution in 50 mM sodium phosphate pH 7.0. RNA-bound membranes were UV cross-linked using a UV Stratalinker (Stratagene). Membranes were incubated for 30 min at 65°C in ExpressHyb solution (Clontech), prior to addition of specific [α^{32}P] dCTP-labeled probes diluted in ExpressHyb. Hybridizations proceeded 2 h at 65°C followed by 20 min washes at 65°C in Wash Buffer 1 (0.3 M NaCl, 30 mM sodium citrate, 1 % SDS), Wash Buffer 2 (75 mM NaCl, 7.5 mM sodium citrate, 1% SDS), and Wash Buffer 3 (30 mM NaCl, 3 mM sodium citrate, 1% SDS). Membranes were exposed to Storage Phosphor Screens (Kodak) overnight. Phosphorimager analysis was subsequently performed on the screens using a BioRad Molecular Imager FX (BioRad). Alternatively, autoradiography was performed at -80^{O}C using an intensifier screen (Kodak).

3.8.2. Probes Used for Northern Blot Hybridization

A 900bp *Nco* I/*Kpn* I fragment from c-Src cDNA was utilized to generate Northern probe specific for c-Src mRNA expression. For detection of β-actin mRNA, a 700 bp *Eco*R I fragment from the β-actin cDNA clone, HHC189, was employed. To detect c-Myc mRNA expression, a 500 bp *Pst* I fragment from pMI, a c-myc cDNA clone, was used. All probes were labeled with an oligolabeling kit (Amersham).

3.9. Detection of DNaseI Hypersensitive Sites

3.9.1. Nuclei Isolation and DNaseI Treatment

Cells were grown to 80-90% confluence, washed, trypsinized, and pelleted. Cells were re-suspended in phosphate buffered saline (PBS, 0.15 M NaCl, 20 mM sodium phosphate, pH 7.4), counted, and 14 X 10^6 cells were pelleted, washed in nuclei isolation buffer (NIB, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 15 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 0.4 mM dithiothreitol, 0.3 M sucrose), pelleted again, and re-suspended in a volume of NIB approximately equal to 5 times the pelleted cell volume. Cells were gently lysed on ice by addition of Nonidet-P40 to a final concentration of 0.5% (v/v). Cellular lysis was assessed by staining aliquots of the lysis reaction with trypan blue. When nuclei were clearly visible, nuclei were pelleted by centrifugation at 500Xg, and re-suspended in NIB with 5% glycerol to a final concentration of 2 X 10^6 cells per 100 μL. Aliquots of 100 μL of nuclei were then digested with increasing concentrations of DNaseI for 3 min at room temperature. The optimal range of DNaseI concentrations was determined previously by agarose gel analysis, and was typically in the range of 0-2.0 μg/100 μL nuclei for all cell lines studied. DNaseI digests were terminated by addition of 50 μL of proteinase K buffer. Genomic DNA was subsequently isolated.

3.9.2. Southern Blot

Genomic DNA (20 μg) was digested with the appropriate restriction enzymes and buffer compositions in 200 μL reactions. Digest completion was assessed by electrophoresis of 5 μL aliquots of in 1 % agarose gels. Digested genomic DNA was ethanol precipitated, washed, and re-suspended in 20 μL of gel loading buffer. Restriction fragments were resolved by fractionation through 0.8 % agarose TAE gels.

Subsequently, gels were stained, photographed, depurinated for 10 min in 0.2 M HCl, denatured for 45 min in 0.5 M NaOH, 1.5 M NaCl, and neutralized for 30 min in 1 M Tris-HCl pH 7.4, 1.5 mM NaCl. Treated gels were transferred to charged nylon membranes (DuPont) via capillary elution in 10X SET (1.5 M NaCl, 0.3 M Tris-HCl pH 8.0, 20 mM EDTA), and cross-linked in a UV Stratalinker (Stratagene). Membranes were incubated for 30 min at 65°C in ExpressHyb solution (Clontech), prior to addition of specific [α^{32}P] dCTP-labeled probes diluted in ExpressHyb. Hybridizations proceeded for 2 h at 65°C followed by 20 min washes at 65°C in Wash Buffer 1 (2 X SET, 1 % SDS), Wash Buffer 2 (1 X SET, 1% SDS), and Wash Buffer 3 (0.5 X SET, 1% SDS). Membranes were exposed to Storage Phosphor Screens (Kodak) overnight. Phosphorimager analysis was subsequently performed on the screens using a BioRad Molecular Imager FX (BioRad).

3.9.3. Probes used for Southern Blot Hybridization

For all Southern Blot experiments, probes were designed that would hybridize to the extreme 5' or 3' ends of genomic DNA restriction fragments. For detection of the 13.9 kb *Eco*R V restriction fragment immediately upstream of the SRC promoters, a 259 bp PCR fragment was generated using *Taq* DNA Polymerase and PCR primers Eco13.9FWD (5'-TAGCAAAAGCCCAGAGGGGTAG) and Eco13.9REV (5'-TGAAATGAGGTGATGCCCGC). This PCR product was purified and labeled using an oligolabeling kit (Amersham).

3.10. S1 Nuclease Protection Assays

3.10.1. Determination of SRC DPCAT Promoter Use

[^{32}P]-labeled SRC1A-CAT and SRC1α-CAT specific single stranded DNA probes were generated by primer extension using a CAT-specific reverse primer (5'-AAGGCCGGATAAAACTTGTGC) with a *Stu* I-digested pSRC1A-CAT-chimera template or a *Nar* I-digested pSRC1α-CAT-chimera template. Full-length primer extension products were obtained by purifying labeled species of the desired lengths from denaturing 4% polyacrylamide sequencing gels. RNA isolated from transfected or untransfected cells was thoroughly digested with DNaseI prior to S1 nuclease protection assays. Hybridization of labeled complementary probes to target RNA species and S1 nuclease digestions were performed using an S1 Assay kit (Ambion). Probe:target hybridizations were carried out overnight at 46^{o}C for the 1α-CAT specific probe, and 56^{o}C for the 1A-CAT specific probe. Protected species were separated using denaturing 6% polyacrylamide sequencing gels, and exposed to a Storage Phosphor Screen (Kodak). Screens were scanned using a Molecular Imager FX (BioRad). Alternatively, autoradiography was performed at -80^{o}C using an intensifier screen (Kodak).

3.10.2. Determination of Endogenous SRC Promoter Use

S1 nuclease protection assays were performed essentially as described for determining SRC DPCAT promoter usage with the following modification. A [^{32}P]-labeled SRC1α specific S1 primer was generated from a *Stu* I digested pSRC1α-chimera vector by primer extension reaction with an exon 1C-specific reverse primer (5'-GAGTCAGGGGTCTCGAAATAGAG).

3.10.3. Mapping of SRC1A Transcription Start Sites

SRC1A promoter transcription start sites were mapped using a S1 nuclease protection approach in HepG2, HT29, and SW480 cells exactly as described previously (Bonham and Fujita, 1993).

3.11. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from HepG2 cells transfected with -560 DPCAT, and thoroughly digested with DNaseI. RNA was reverse transcribed using the Thermoscript RT System (Invitrogen), and a CAT specific reverse primer (5'-TCACCGTAACACGCCACATC). Subsequently, 2 μL of the reverse transcription reaction was amplified by PCR using forward primers specific for SRC1α (5'-CCTCTAGCCTCAGTTTATCACCGC) or SRC1A (CTCCCGTGCGTCCGTCTGCC), paired with a nested, CAT-specific reverse primer (5'-CGGAAATCGTCGTGGTATTCACTC). PCR products were analyzed via agarose gel electrophoresis.

3.12. 5' Rapid Amplification of cDNA Ends (RACE)

HepG2 RNA was isolated, DNaseI digested, and reverse transcribed exactly as described for RT-PCR. cDNAs generated by reverse transcription were subsequently purified using QIAquick columns (Qiagen). Purified cDNAs were tailed using a 5' RACE kit (Invitrogen). First round PCR was performed for 30 cycles on tailed cDNA using the forward Abridged Anchor Primer supplied with the 5' RACE kit, paired with a CAT specific reverse primer (5'-CGGAAATCGTCGTGGTATTCACTC). PCR

products were diluted 1 in 1000, and 1 μL of this dilution was used as a template for a second round, 30 cycle, PCR reaction using a forward nested AUAP primer supplied with the 5' RACE kit and a nested CAT reverse primer (5'-AAGGCCGGATAAAACTTGTGC). Second round PCR products were analyzed by agarose gel electrophoresis. PCR bands were gel-purified, digested with *Sal* I/*Hind* III, cloned into *Sal* I/*Hind* III digested pBlue, and sequenced.

3.13. Chromatin Immunoprecipitation-PCR

Semi-confluent HT29 and SW480 cells were treated with 1 μM TSA for various times. Formaldehyde was then added directly to the growth media to a final concentration of 1%, and cells were incubated at 37^{o}C for 10 min. Cells were washed twice in ice cold PBS containing freshly added protease inhibitor cocktail (Sigma), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1mM dithiothreitol (DTT). Cells were then scraped off the plate, pelleted, and lysed in 350 mL of a lysis buffer supplied with a chromatin immunoprecipitation (ChIP) kit (Upstate Biotech), supplemented with freshly added protease inhibitors, PMSF, and DTT. Genomic DNA in the lysates was sheared to lengths between 200 and 500 bp by sonication (Branson Sonifier 450, output control set to 3, 60% duty cycle). Sheared lysates were cleared by centrifugation, and 50 μL was set aside as an input fraction. The remaining lysate was split; one half was immunoprecipitated with 5 μg of anti -acetyl histone H3 (9/14) antibody (Upstate Biotech), and the other half was immunoprecipitated with pre-immune rabbit IgG using reagents and protocols supplied with the ChIP kit. Following the ChIP protocol, cross-links were reversed and genomic DNA was isolated from the immunoprecipitated

samples as well as the input fraction. These samples were then subjected to PCR using primers specific for the SRC1α promoter (FWD, 5'-CACTGGGTAAATGTCCCTGCC; REV, 5'-CAACAGCAGAAGCCAGCCTG) or the SRC1A promoter (FWD, 5'-GTGCCCAGCCCCAAAAGG; REV, ATTCCGGGCCGGGAGAGAC). PCR products were analyzed via agarose gel electrophoresis.

3.14. Nuclear Run-On Assay

This experiment was performed in collaboration with Dr. Keith Bonham at the Saskatchewan Cancer Agency. Nuclear run-ons were performed essentially as described (Fei and Drake, 1993) with minor modifications (Dehm et al., 2001).

3.15. Electrophoretic Mobility Shift Assay

HepG2 cells were harvested at various time points following treatment with 1μM TSA, and nuclear extracts were prepared by the method of Andrews (Andrews and Faller, 1991). A [^{32}P]-dCTP labeled probe encompassing the -145 to +19 region of the SRC1α promoter was prepared via an in-fill reaction of a *Cla* I/*Hinc* II promoter restriction fragment derived from a SRC1α promoter construct (Bonham et al., 2000). The SRC1A EMSA probes A and B were prepared simultaneously by digestion of 0.38 SRC1A-CAT with *Nar* I and *BssH* II, followed by a Klenow in-fill reaction with [α^{32}P]-dCTP, and subsequent agarose gel purification. Electrophoretic mobility shift assay (EMSA) reactions were carried out by initially incubating 5 μg of nuclear extract in Binding Buffer (25 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0, 4 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM

DTT), 3 μg of poly(dI-dC), and 10 μg of bovine serum albumin for 15 minutes on ice. Following this incubation period, 4 X 10^5 cpm of the appropriate probe was added, thus making the final EMSA reaction volume 20 μL, and incubated at 25°C for 30 min. Bound and unbound probe were resolved in 4% polyacrylamide gels with an electrophoresis buffer containing 50 mM Tris, 380 mM glycine, and 2 mM EDTA at 150 V for 3 h at 4 °C. Gels were dried and exposed to Storage Phosphor Screens (Kodak) overnight. Phosphorimager analysis was subsequently performed on the screens using a BioRad Molecular Imager FX (BioRad).

3.16. TAF1-TAF2 Binding Assays

These assays were performed in collaboration with Dr. Edith Wang at the University of Washington in Seattle, Washington, USA. Recombinant baculovirus vectors were used to express HA-tagged wild-type or G690D mutant TAF1 and FLAG-tagged TAF2 in Sf9 cells. HA-TAF1 and FLAG-TAF2 were immunoaffinity purified and assembled into heterodimers *in vitro* as described (Chen and Tjian, 1996). Double stranded, [^{32}P]-labeled probes encompassing the SRC1A (5'-CCGCTCCGGCTCCCAGGCCGGAATGGATTCCGGGCCGGGAGAGACAGAT), or SRC1α (5'-ACAAGTGCGGCCATTTCACCAGCCCAGGCTGGCTTCTGCTGTTGACTGG), core promoters were generated. For EMSA reactions, 5 μg of TAF1/2 heterodimer was incubated in a binding buffer (Hilton and Wang, 2003) with 1 μg poly dG-dC and 300 μg/mL BSA at 25°C or 37°C for 20 min. Labeled, double stranded probes were subsequently added, and the reaction proceeded at the same temperature for an

additional 20 min. For competition experiments, double stranded competitors representing wild-type SRC1A or SRC1α core promoters, or SRC core promoters harbouring mutations in the Inr elements (mutant SRC1A, 5'-ATCTGTCTCTCCCGGCCCGGCCTCCGGGCCGGCCTGGGAGCCGGAGCGG; mutant SRC1α, 5'-ACAAGTGCGGCCATTTCACCAGCCCGGGCTGGCTTCTGCTGTTGACTGG) were added to the initial pre-incubation reaction with the TAF1/2 heterodimer prior to the addition of [^{32}P]-labeled probe. Bound and unbound core promoter probes were fractionated on agarose gels as described (Hilton and Wang, 2003), and visualized via autoradiography.

3.17. Western Blot

Cells were lysed directly in a loading buffer containing 65 mM Tris-HCl (pH 7.0), 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) bromophenol blue. Equal amounts (typically 30 μg per lane) of protein were resolved on a 10% SDS-polyacrylamide gel, followed by transfer to nitrocellulose. Membranes were washed in a TBST buffer (10 mM Tris, 15 mM NaCl, 0.5% (v/v) Tween-20), and blocked at room temperature in a blocking buffer consisting of 5% (w/v) fat-free skim milk powder (Carnation) in TBST. Membranes were incubated with anti-Src (Oncogene Research) at 1 μg/mL, anti-Sp1 (Santa Cruz) at 2ug/mL, anti-Sp3 (Santa Cruz) at 2ug/mL, or anti -HNF-1α (Santa Cruz) at 2ug/mL, for 2 h at room temperature. All antibodies were diluted in blocking buffer. Following primary antibody incubation, membranes were washed in TBST, and incubated with the appropriate secondary anti-

host -horseradish peroxidase conjugate (Santa Cruz) diluted 1:2000 in blocking buffer for 1 hr at room temperature. Membranes were washed in TBST, immersed in chemiluminescence reagents (Pierce) and exposed to Kodak X-Omat Blue XB-1 film for signal detection.

3.18. Src Kinase Assay

Cells were lysed in ice-cold, modified RIPA buffer (25 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1mM EDTA) with protease inhibitors (aprotinin, leupeptin, and pepstatin A, each at 1 μg/mL) and 100 μg/mL PMSF freshly added. Immunoprecipitation and *in vitro* c-Src kinase assays were subsequently performed as described (Bjorge et al., 1995). Briefly, lysate containing 500 μg of total cellular protein was incubated with excess anti -Src antibody (Oncogene Research) for 1h at 4°C, and centrifugation at 10,000 g for 1 min at 4°C. Immunoprecipitate pellets were re-suspended in 40 μL of a kinase reaction buffer containing 0.25 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), pH 7.8, 25 mM $MgCl_2$, 0.75 M NaCl, 1μCi [γ-^{32}P]ATP, 10 μM ATP, 300 μM Src optimal peptide (AEEEIYGEFEAKKKK) (Zhou et al., 1995), 200 μM sodium orthovanadate, and 4 mg/mL p-nitrophenol phosphate for 15 min at 30ºC. Reactions were terminated by the addition of 25 μL of 50% (v/v) acetic acid. Aliquots from each reaction were spotted onto p81 phosphocellulose squares, which were subsequently washed three times in 0.5% (v/v) acetic acid, dried and counted in a scintillation counter.

4. RESULTS AND DISCUSSION

4.1. SRC TRANSCRIPTIONAL ACTIVATION IN HUMAN COLON CANCER CELL LINES

4.1.1. c-Src mRNA Expression in Human Cancer Cells

To better understand the mechanism(s) of c-Src activation in human cancer, the expression of c-Src mRNA was examined in various human cancer cell lines by Northern blot (Fig. 4.1). To control for RNA loading, the expression of β-actin was also examined on the same membrane. The HeLa cervical carcinoma and Molt-4 T-cell leukemia cell lines displayed very low levels of c-Src mRNA expression. Similar low levels of c-Src were also noted in colon cancer cell lines such as SW480, SW620, KM12C, as well as the HS578T breast cancer cell line. However, much higher levels of c-Src mRNA expression were observed in the HT29, LS174T, LS180, WiDr, Colo 201, Colo 205, and Colo 320 colon cancer cell lines, HepG2 hepatoma cell line, and T47D, MDA-MB-231, and HBL100 breast cancer cell lines. Previous studies have shown that the activation of c-Src in a subset of human colon cancer cell lines (HCCLs) can be accounted for by increased expression of c-Src protein (Park et al., 1993). Therefore, these findings led to the hypothesis that overexpression of c-Src mRNA could play a role in $pp60^{c\text{-}Src}$ activation in human cancer cells.

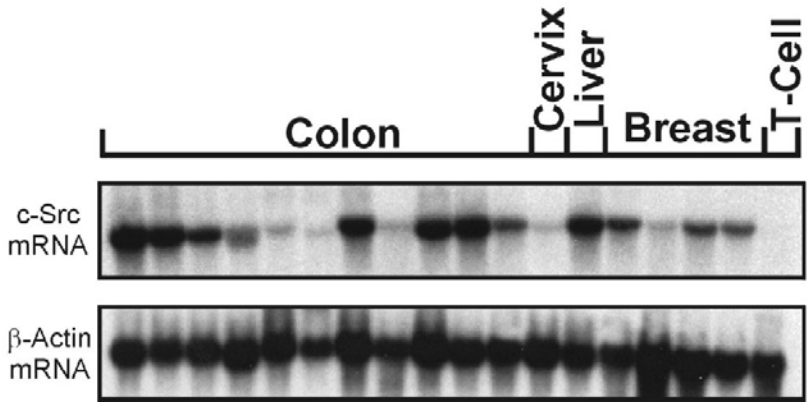

Figure 4.1. c-Src mRNA expression in human cancer cell lines. Total cellular RNA was isolated from (L to R) HT29, HT29, LS174T, LS180, SW480, SW620, WiDr, KM12C, Colo 201, Colo 205, and Colo 320 colon cancer cells, HeLa cervical carcinoma cells, HepG2 hepatoma cells, T47D, HS578T, MDA-MB-231, and HBL100 breast cancer cells, and Molt-4 T-cell leukemia cells and subjected to Northern blot analysis using probes specific for c-Src and β-Actin.

4.1.2. c-Src Expression and Activity in HCCLs

To examine whether increased c-Src mRNA levels could explain c-Src protein overexpression in HCCLs, Western and Northern blots were generated (Figs. 4.2 A and B). Cell lines that were found to constitutively overexpress $pp60^{c\text{-}Src}$ (HT29, Colo 201, Colo 205, WiDr, and LS174-T) also expressed high levels of c-Src mRNA. Conversely, cell lines displaying lower $pp60^{c\text{-}Src}$ levels (HCT-15, DLD-1, KM12C, SW620, SW480, and HCT-116) also displayed lower c-Src mRNA expression. Therefore, a strong positive correlation existed for c-Src mRNA and protein expression. The only cell line that did not follow this tight correlation was the Colo 320 cell line. These cells displayed intermediate steady-state c-Src mRNA levels and low protein levels. Interestingly, this particular cell line also displayed unusually high levels of c-Myc mRNA due to a massive amplification of the MYC gene in the form of double minute chromosomes (Bianchi et al., 1991).

To expand this correlation between c-Src mRNA and protein levels, *in vitro* c-Src kinase assays were performed (Fig. 4.2 C). Low $pp60^{c\text{-}Src}$ activity was observed in HCT-15, DLD-1, Colo 320, KM12C, SW620, SW480, and HCT-116 cell lines. These were the same cell lines that displayed low c-Src protein levels. High $pp60^{c\text{-}Src}$ was demonstrated in the same cell lines that displayed high c-Src mRNA and protein levels (HT29, Colo 201, WiDr Colo 205, LS-174T. These data confirmed that elevated $pp60^{c\text{-}Src}$ activity in HCCLs could be explained by overexpression of c-Src mRNA.

4.1.3. c-Src Transcriptional Activity in HCCLs

To address whether the observed increase in c-Src mRNA levels resulted from increased transcription of the SRC gene, nuclear run-on analysis was performed by Dr.

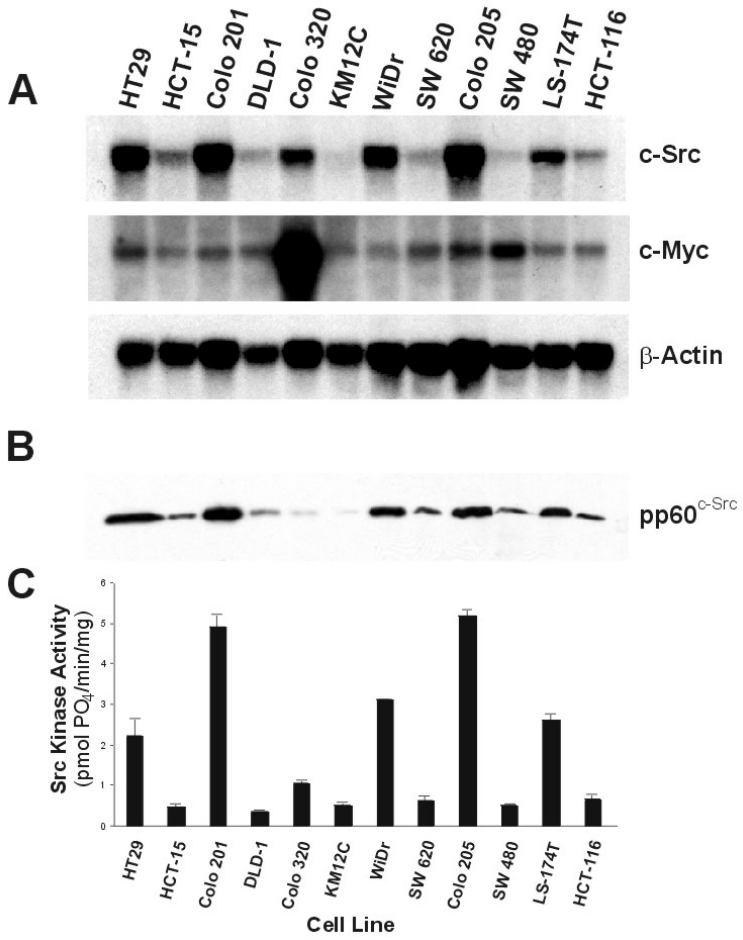

Figure 4.2. c-Src mRNA, protein, and kinase activity levels in HCCLs. **(A)** Northern blot of total RNA from semi-confluent cells sequentially probed for c-Src, c-Myc, and β-Actin. The figure shown is representative of several repeated experiments. **(B)** Western blot of total cellular proteins from the same cell lines harvested at the same time as in (A), using a specific anti-pp60$^{c\text{-}Src}$ monoclonal antibody. **(C)** Evaluation of c-Src kinase activity in anti-pp60$^{c\text{-}Src}$ immunoprecipitates using a synthetic peptide (AEEEIYGEFEAKKK) substrate. Kinase activity is expressed as pmol of radioactive phosphate incorporated per minute per mg of total cellular protein. The bar graph represents the mean +/- the standard deviation from three replicate experiments.

Keith Bonham (Saskatchewan Cancer Agency). A higher signal for c-Src transcription was detected in cell lines that displayed elevated c-Src mRNA levels (HT29, Colo 205) when compared to cell lines with lower levels of c-Src mRNA expression (Fig. 4.3). These results suggested that the SRC gene was transcribed at relatively higher rates in the c-Src overexpressing cell lines compared to the low expressing cell lines.

4.1.4. Determination of c-Src mRNA Stability in HCCLs

The level of SRC transcription measured in HT29 and Colo 205 cells by the nuclear run-on approach was at the lower level of the detection limit of the assay. Because of this, and the possibility that steady state levels of mRNA detected in the Northern blots could also be influenced by differential mRNA stability, a study of c-Src mRNA half-life in these HCCLs was necessary. Cellular transcription in HT29, Colo 201, Colo 205, SW480 and SW620 cells was inhibited by treatment with Actinomycin D, and isolated RNA was analyzed by Northern blot (Fig. 4.4). A consistent observation was the rapid degradation of c-Myc transcripts, confirming Actinomycin D was exerting a similar level of transcriptional inhibition in the HCCLs studied. Conversely, β-Actin mRNA levels were steady throughout the course of the experiment. In cells that express low levels of c-Src (SW480, SW620), the c-Src transcripts were stable over the time course of the study. However, in high c-Src expressing cell lines, the c-Src transcripts were rapidly degraded, with an estimated half-life of less than 2 hours. Decreased c-Src mRNA stability in cell lines with elevated c-Src mRNA levels suggests an even higher level of SRC transcription necessary to account for steady-state c-Src mRNA levels than previously anticipated. Taken together, the data from mRNA stability studies and

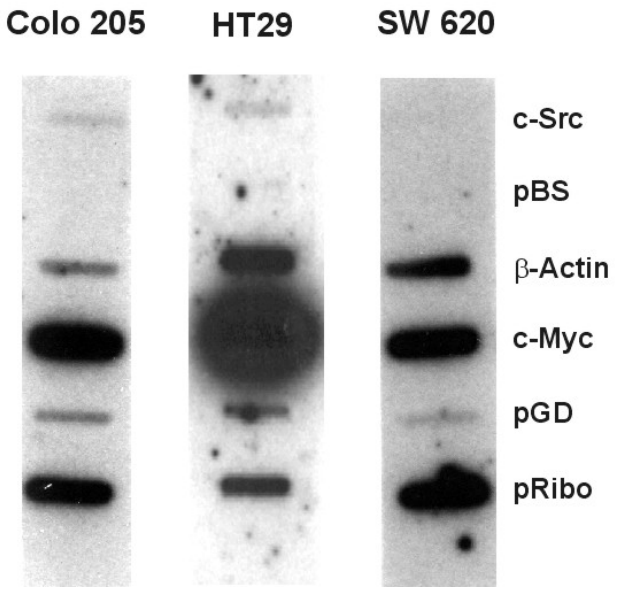

Figure 4.3. SRC transcriptional activity in HCCLs. Nuclei were prepared from HT29, Colo 205, and SW620 cell lines and run-ons performed. [^{32}P]-labeled RNA from these nuclei was then hybridized to nylon membranes containing 10 μg slot-blots of denatured c-Src, pBS (pBluescript), β-Actin, c-Myc, pGD (glucose-6-phosphate dehydrogenase), and pRibo (18S ribosomal RNA cDNA) cDNAs as indicated.

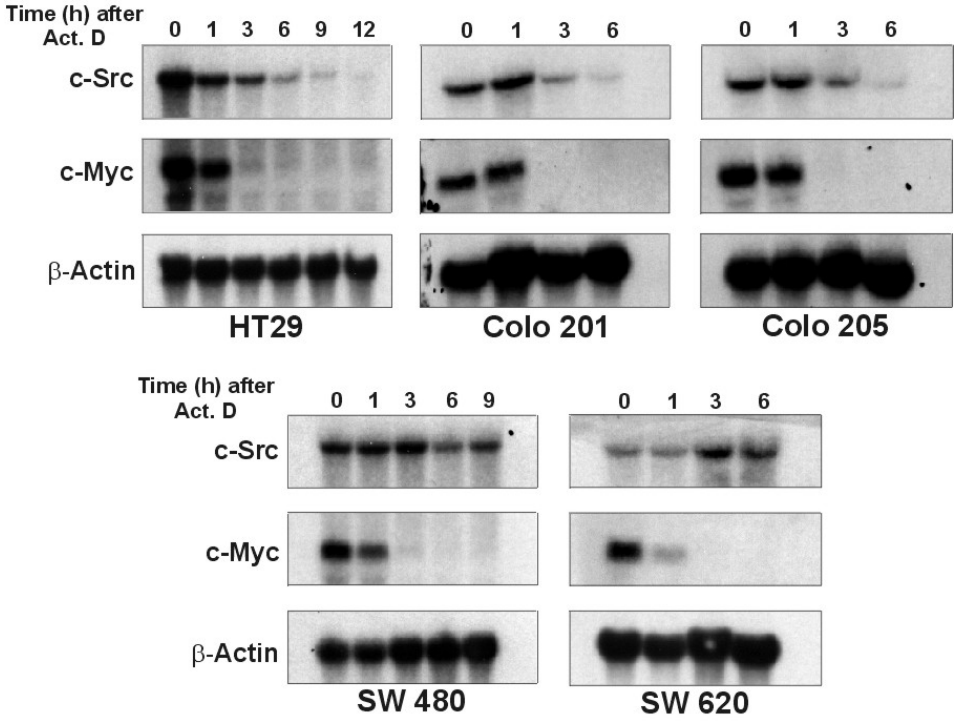

Figure 4.4. c-Src mRNA half-life determination in HCCLs. Total RNA was isolated from five colon cancer cell lines at various times following exposure to 5 μg/mL Actinomycin D. Total RNA was blotted to charged nylon membranes and hybridized sequentially with probes specific for c-Src, c-Myc and β-Actin. Lane numbering refers to hours after initial Actinomycin D exposure that cells were harvested. The Northern blots shown are representative of several repeated experiments. Much longer exposures to film were necessary to obtain signals for c-Src mRNA expression in SW480 and SW620 (4 to 5 days) compared with HT29, Colo 201, and Colo 205 (overnight).

nuclear run-on experiments strongly implicate that the mechanism of c-Src overexpression in this subset of HCCLs is at the level of transcription.

4.1.5. c-Src Expression Levels vs. KRAS Gene Status

Activating mutations in the KRAS gene have been well documented as a frequent and important event in colon cancer (Bos et al., 1987; Burmer and Loeb, 1989; Kinzler and Vogelstein, 1996). Interestingly, SRC transcription was activated only in HCCLs containing two normal KRAS alleles (Table 4.1). Conversely, those cell lines that expressed low levels of c-Src harboured a mutant KRAS allele encoding an activated K-Ras protein (Nagasu et al., 1995). The only exception to this pattern was the Colo 320 cell line, which expresses unusually high levels of c-Myc from the amplified MYC locus (Bianchi et al., 1991). This interesting correlation therefore leads to the proposal that mutational activation of the KRAS gene or transcriptional activation of SRC may represent two parallel and mutually exclusive pathways that ultimately contribute to colon cancer progression.

Table 4.1. Relative c-Src Expression Levels and KRAS Gene Status in Human Colon Cancer Cell Lines

Cell Line	c-Src Expression (Northern, Western)	KRAS Status
HT29	+++++[a]	wild-type
Colo 201	+++++	wild-type
Colo 205	+++++	wild-type
WiDr	+++++	wild-type
LS-174T	+++++	not determined
Colo 320	++	wild-type
SW480	+	K12V
SW620	+	K12V
HCT-15	+	K13D
HCT-116	+	K13D
DLD-1	+	K13D
KM12C	+	not determined

[a] (+++++) represents high and (+) represents low levels of c-Src expression as visually determined by Northern and Western blot analysis.

4.1.6. c-Src Expression and Activity in Mutant KRAS Knock-Out Cell Lines

In order to initially address the apparent reciprocal relationship between SRC transcriptional activation and activating KRAS mutations, c-Src expression and activity were assessed in HCCLs with the mutant K13D KRAS allele disrupted by homologous recombination. Hke-3 and Hkh-2 are two such HCT-116 derivatives, and DKO-4 and DKS-8 are two such DLD-1 variants (Okada et al., 1998; Shirasawa et al., 1993). As shown in Fig. 4.5 C, c-Src kinase activity was elevated 2 to 5 fold in Hke-3 and Hkh-2 cell lines relative to HCT-116. A similar level of increased c-Src kinase activity was observed in DKO-4 and DKS-8 cell lines relative to DLD-1. The increase in c-Src kinase activity was matched by a slight increase in c-Src protein levels in knock-out vs. parental HCCLs (Fig. 4.5 B). However, when c-Src mRNA levels were measured by Northern blot, only the Hke-3 cell line displayed an increase relative to the parental cell line (Fig. 4.5 A). These data suggest that the elevation in c-Src kinase activity in knock-out cell lines is compensatory and necessary for the cells to overcome the growth inhibition caused by targeted disruption of the mutant K13D allele.

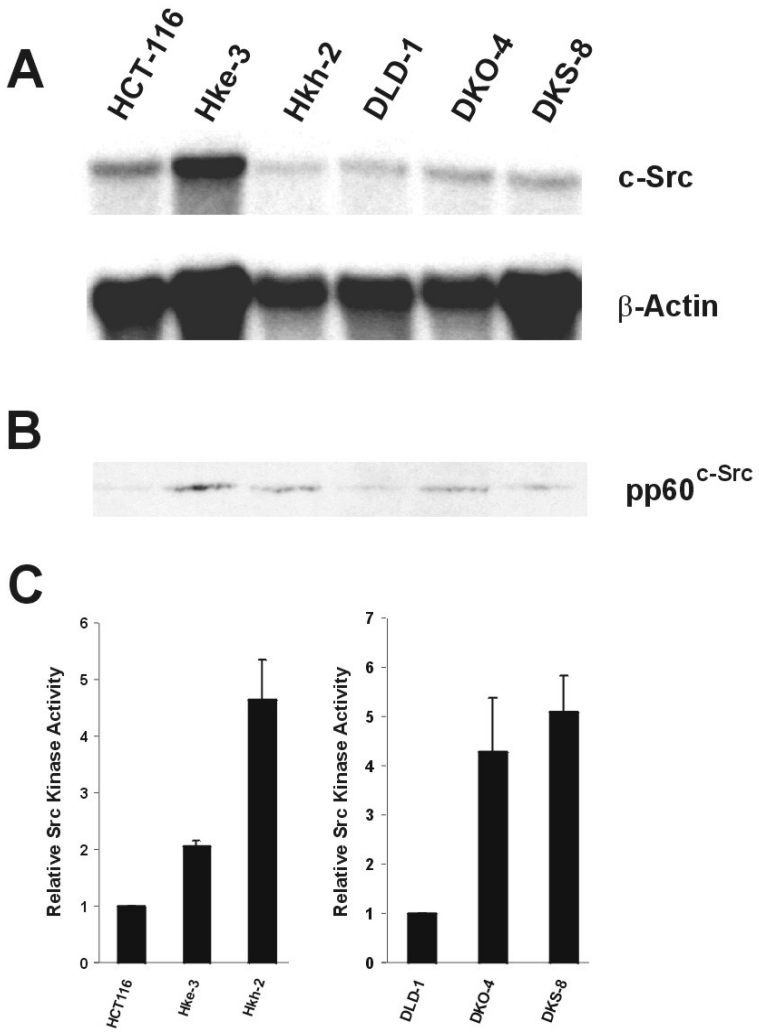

Figure 4.5. c-Src expression and activity in HCCLs with targeted disruption of mutant KRAS. (A) Northern blot of total RNA from semi-confluent cells sequentially probed for c-Src and β-Actin. **(B)** Western blot of total cellular proteins from the same cell lines harvested at the same time as in (A), using a specific anti-pp60$^{c\text{-}Src}$ monoclonal antibody. **(C)** Evaluation of c-Src kinase activity in anti-pp60$^{c\text{-}Src}$ immunoprecipitates using a synthetic peptide (AEEEIYGEFEAKKK) substrate. Bar graphs represent the mean +/- the standard deviation from three replicate experiments.

4.1.7. DISCUSSION

4.1.7.1. SRC Transcriptional Activation in Human Cancer Cell Lines

Activation and/or overexpression of the tyrosine kinase pp60$^{c\text{-}Src}$ has been frequently reported in both human colon cancer cell lines and tumors (Bolen et al., 1987a; Cartwright et al., 1990; Talamonti et al., 1993), However, the precise mechanism of this activation has remained elusive. An activating SRC mutation has been described in a small (12%) subset of highly advanced colon cancers (Irby et al., 1999). This mutation converts glutamine 531 to a stop codon, resulting in a truncated version of c-Src. The truncation at the C-terminal tail prevents phosphorylation of Y530, and hence prevents pp60$^{c\text{-}Src}$ inactivation. Transfection studies have shown this mutation is activating, transforming, tumorigenic, and promotes metastasis. However, the importance of this SRC mutation in the majority of colon cancers is suspect. Numerous follow-up studies have analyzed hundreds of advanced colon cancer specimens, and not one has been able to reproduce the results from the initial report (Daigo et al., 1999; Laghi et al., 2001; Nilbert and Fernebro, 2000). Therefore, SRC mutation is considered a very rare event, and in the vast majority of colon cancers, c-Src is activated through non-mutagenic means.

A subset of the reports describing c-Src tyrosine kinase activation in colon cancer has demonstrated an increase in the specific activity of pp60$^{c\text{-}Src}$. (Bolen et al., 1987a; Bolen et al., 1987b; Cartwright et al., 1989). Early studies attempting to explain this observation focused on the kinase capable of phosphorylating the Y530 residue of pp60$^{c\text{-}Src}$, Csk (Nada et al., 1991). However, no correlation has been observed between Csk expression and/or activity and c-Src activity. In fact, high levels of Csk activity and expression have been observed in colorectal tumors and cell lines with high levels of c-

Src kinase activity (Li et al., 1996). In addition, several proteins capable of enhancing c-Src kinase activity by de-phosphorylating the Y530 residue have been identified, but none have been shown to have elevated expression or activity in colon cancer (Bjorge et al., 2000; Fang et al., 1994; Walter et al., 1999). Therefore, the post-translational mechanisms leading to c-Src kinase activation in colon cancer remain undefined.

One of the more consistent findings in various human tumors and tumor derived cell lines is that increased c-Src activity can be explained by an increase in c-Src protein levels (Biscardi et al., 1999; Iravani et al., 1998; Irby et al., 1997). In this study, these observations have been verified and expanded, and increased c-Src protein levels and resulting kinase activity have been shown to be due to elevated c-Src mRNA expression. Furthermore, transcriptional activation of the SRC gene was demonstrated, which was responsible for the overexpression of c-Src mRNA observed in a subset of HCCLs. Therefore, although controls such as covalent modification exist to finely modulate c-Src kinase activity, coarse controls such as SRC transcriptional activation are an important determinant of overall c-Src expression and activity in HCCLs.

An interesting and unexpected finding from this line of investigation was decreased stability of c-Src mRNA in cell lines that displayed SRC transcriptional activation. These results suggest a negative feedback loop exists that leads to reduced stability of c-Src mRNA when SRC transcription is increased. The mechanism of c-Src mRNA destabilization is currently unknown; however, this observation parallels findings that $pp60^{c\text{-}Src}$ with higher kinase activity is degraded more rapidly by a ubiquitin-dependent mechanism than $pp60^{c\text{-}Src}$ with lower levels of kinase activity (Hakak and Martin, 1999; Harris et al., 1999).

There have also been many reports that have suggested c-Src overexpression and/or activation could play an important role in non-colon cancers. For example, a c-Src specific antisense strategy employed in the SKOv-3 ovarian cancer cell line resulted in diminished anchorage-independent growth and tumor forming ability in a xenograft nude mouse model (Wiener et al., 1999). Similarly, antisense-mediated down-regulation of c-Src expression in NIH3T3 cells engineered to mimic breast cancer by overexpressing the EGFR or an EGFR-HER-2 chimera resulted in reversal of the transformed phenotype of these cells (Karni et al., 1999). Observational studies have also reported increased c-Src expression and/or kinase activity in cancers such as breast, lung, pancreas, and liver (Lutz et al., 1998; Masaki et al., 1998; Mazurenko et al., 1992; Verbeek et al., 1996). Therefore, it is important to note that the c-Src mRNA expression analysis employed in this study detected elevated c-Src mRNA levels in cell lines derived from cancers of the liver and breast. Additional studies will be necessary to determine if SRC transcriptional activation plays a role in determining overall $pp60^{c\text{-}Src}$ activity in these and other cancers.

4.1.7.2. SRC Transcription and Colon Cancer Progression

Colon cancer is the best understood cancer both in terms of staging and documentation of specific genetic lesions. This has allowed for the proposal of stepwise models of colonic tumorigenesis (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). For example, loss of APC function (Kinzler and Vogelstein, 1996; Morin et al., 1997) or mutations in β-catenin that render it unresponsive to APC regulation (Dashwood et al., 1998; Morin et al., 1997; Park et al., 1999) have been identified as

crucial initiating events for colon tumorigenesis. Colon tumor progression is associated with further genetic lesions resulting in the loss of function of additional tumor suppressor genes such as p53, DCC, hMLH1, and hMSH2, and activation of oncogenes such as KRAS (Kinzler and Vogelstein, 1996). KRAS mutations are reported to occur early in colon cancer development, during progression from early adenoma to late adenoma, at a rate of about 50% (Bos et al., 1987; Burmer and Loeb, 1989; Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). These mutations have significant impact on proliferation, transformation, and tumorigenicity (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). Similarly, c-Src overexpression or activation is a frequent early event in colon cancer (Biscardi et al., 1999; Bolen et al., 1987a; Budde et al., 1994; Cartwright et al., 1994; Cartwright et al., 1990; Han et al., 1996; Iravani et al., 1998; Staley et al., 1997; Talamonti et al., 1993; Zhao et al., 1990), and simple overexpression of normal c-Src in mouse fibroblasts is transforming (Lin et al., 1995). Therefore, the observation of a strong inverse correlation between SRC transcriptional activation and the presence of activating KRAS mutations in the HCCLs examined in this study is of potential clinical importance. The possibility is raised that increased SRC transcription and KRAS mutation represent two mutually exclusive and parallel oncogenic pathways activated early in colon tumorigenesis.

The hypothesis of SRC and KRAS mutual exclusion is further supported by the similarities between c-Src and K-Ras activated signaling pathways. For example, inactivation of c-Src or mutant KRAS alleles in HCCLs by antisense technology (HT29) or homologous recombination (HCT-116, DLD-1) results in cells with decreased proliferative rates, focus forming ability, and tumorigenicity (Shirasawa et al., 1993; Staley et al., 1997). These same cells with down-regulated c-Src or disrupted KRAS

also exhibit diminished levels of VEGF, a growth factor closely associated with angiogenesis and tumor growth (Ellis et al., 1998; Shirasawa et al., 1993). Also, c-Src has often been placed upstream of Ras in signal transduction pathways because several studies have shown that v-Src transformation of NIH 3T3 mouse fibroblast cells is dependent on Ras (Rak et al., 1995). However more recent work in rat intestinal epithelial cells and rat fibroblasts has clearly shown that Src and Ras can induce transformation via independent and quite distinct mechanisms (Oldham et al., 1998; Rak et al., 1995). Thus, a de-regulated c-Src or K-Ras can ultimately act on similar or even identical downstream targets and contribute independently to transformation and tumor progression. This theory is supported by the findings from this study that c-Src kinase activity was elevated in HCT-116 and DLD-1 cells that have had the mutant KRAS allele disrupted by homologous recombination. This potentially compensatory increase in c-Src kinase activity did not appear to result directly from SRC transcriptional activation, demonstrating alternative mechanisms can also be activated in HCCLs that result in increased c-Src kinase activity.

4.1.7.3. Scope and Significance

This line of investigation has demonstrated SRC transcriptional activation as a mechanism leading to elevated $pp60^{c\text{-}Src}$ expression and activity in HCCLs. SRC transcriptional activation was only observed in HCCLs with wild type KRAS, suggesting an inverse relationship between c-Src or K-Ras activation. If the observations reported here can be translated into studies with colon and other tumor samples, then this would hold important ramifications for both diagnosis and future targeted therapy of various human cancers.

4.2. MECHANISMS OF SRC TRANSCRIPTIONAL ACTIVATION IN HUMAN CANCER CELL LINES

4.2.1. Differential SRC Promoter Use in Cancer Cell Lines

Studies outlined in the previous chapter showed SRC transcriptional activation is an important determinant of overall c-Src expression and kinase activity levels in a subset of HCCLs. Therefore, an important issue became the mechanisms regulating this transcriptional activation. At the same time the experimentation outlined in the preceding chapter was being carried out, Dr. Keith Bonham and other members of his laboratory had identified and were characterizing the SRC1α promoter (Bonham et al., 2000). The discovery of another SRC promoter, SRC1A, had been reported by Dr. Bonham seven years earlier (Bonham and Fujita, 1993). Interestingly, SRC1α, unlike the Sp-regulated, ubiquitously expressed SRC1A promoter, is regulated by a liver enriched transcription factor, HNF-1α. HNF-1α mediated regulation of SRC1α provides a solid rationale for the tissue-restricted expression of this promoter. For example, SRC1α promoter use is mainly restricted to the same tissues that HNF-1α is expressed in, such as liver, lung, pancreas, kidney, and intestine (Bonham et al., 2000). Interestingly, elevated c-Src activity has been documented in liver cancer (Masaki et al., 1998), and results from Northern blotting demonstrated that c-Src mRNA is overexpressed in the HepG2 hepatocellular carcinoma cell line (Fig. 4.1.). Because of these observations, HepG2 cells were incorporated into the majority of the experiments performed in this thesis. S1 nuclease protection assays, designed to examine relative SRC promoter use in HepG2 cells, have shown that 80-90% of the c-Src transcripts arise from the SRC1α promoter (Bonham et al., 2000). These S1 protections have been

repeated, and verify that c-Src mRNA in HepG2 cells contain a much higher abundance of transcripts with Exon 1α at the extreme 5' terminus than Exon 1A (Fig. 4.6 A). This is in contrast to S1 experiments with HT29 cells, which have shown that c-Src mRNA overexpression in this cell line results from near equal utilization of both SRC promoters (Bonham et al., 2000). Both SRC promoters are also used in SW480 cells, however at much lower levels than HT29 cells, resulting in very weak signals for the two different c-Src mRNA transcripts when assayed via S1 nuclease protections (Bonham et al., 2000). This experiment has been repeated, and confirms both SRC promoters are utilized at very low levels in SW480 cells (Fig. 4.6 B). These findings led to the hypothesis that understanding the mechanisms governing differential promoter usage could lead to an understanding of the mechanisms leading to SRC transcriptional activation in these cell lines.

4.2.2. Transcription Factor Expression Analysis in Cancer Cell Lines

To initially address the differential usage of the two SRC promoters, Western blot analysis was performed to determine the relative expression levels of the HNF-1α, Sp1 and Sp3 transcription factors in various cancer cell lines (Fig. 4.7). At the time these experiments were performed, SPy had not yet been identified as hnRNP K; therefore, expression data for this factor was not obtained. As expected, Sp1 and Sp3 expression was fairly ubiquitous, although expression levels did vary somewhat among cell lines. However, significant differences in HNF-1α expression were observed in these cell lines. HNF-1α expression was highest in the HepG2 hepatocellular carcinoma cell line, intermediate in the SW480, COLO201, WiDr, HT29, COLO205, and SW620 colon

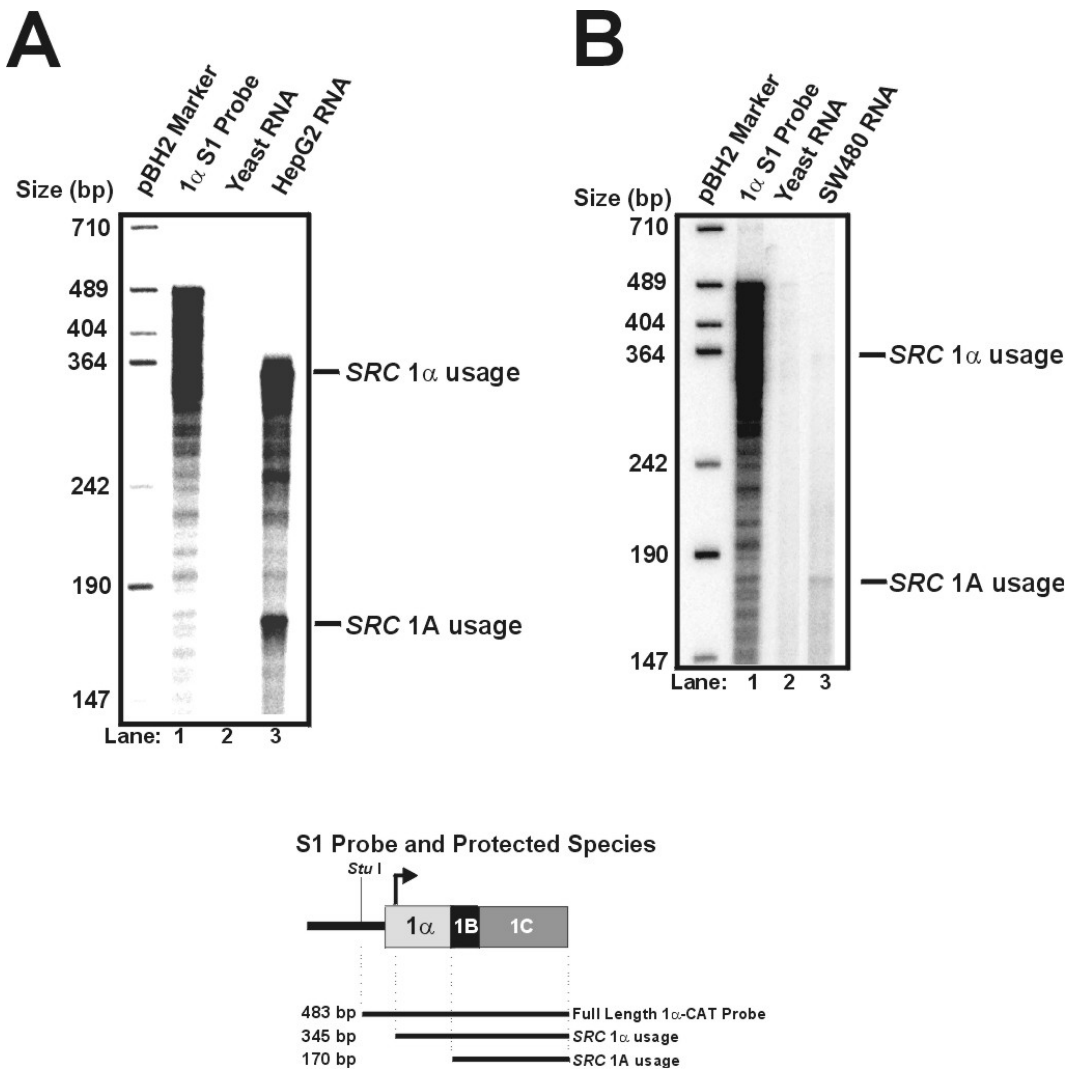

Figure 4.6. Differential Usage of the SRC1α and SRC1A promoters. Total RNA was isolated from HepG2 **(A)** and SW480 **(B)** cells, and hybridized with an S1 probe specific for endogenous 1α transcripts. RNA from yeast was utilized to ensure the specificity of the S1 probe for c-Src mRNA transcripts (A and B, Lane 2). A schematic of the expected protection pattern is shown below. pBH2 refers to fragments from a *Hpa* II pBluescript digest [^{32}P]-labeled via an in-fill reaction with Klenow fragment.

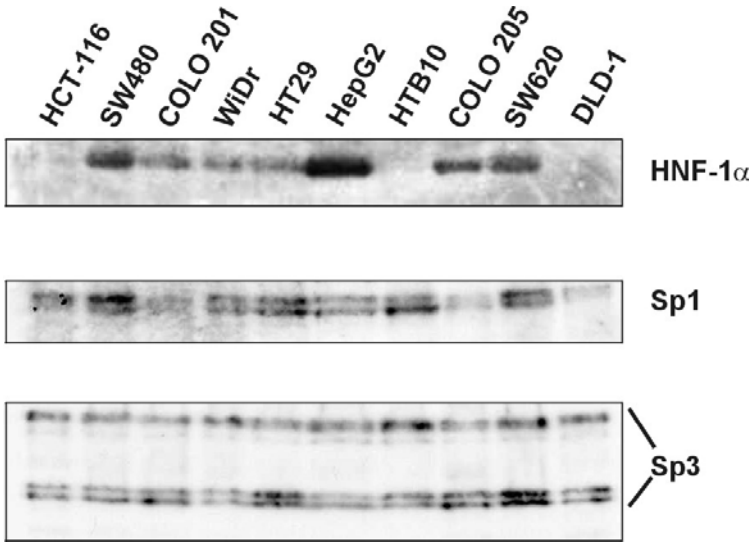

Figure 4.7. HNF-1α, Sp1, and Sp3 expression levels in human cancer cell lines. Western blots were performed using equal amounts of protein from various whole cell lysates of human cancer cell lines. Blots were probed, stripped, and re-probed sequentially using antibodies specific for HNF-1α, Sp1, and Sp3.

carcinoma cell lines, and absent in HCT-116 and DLD-1 colon carcinoma cells as well as the HTB10 neuroblastoma cell line. These results suggested HNF-1α could be an important regulator of SRC promoter usage and expression levels in colon and other cancer cell lines. This possibility led to the development of various models that could explain differential SRC promoter usage. For example, HepG2 cells, which display preferential SRC1α promoter usage, were found to contain high levels of HNF-1α and intermediate levels of Sp1 and Sp3. In this situation, the SRC1α promoter could be strongly transactivated due to high levels of HNF-1α, thus preventing preinitiation complex formation at the SRC1A promoter. This phenomenon, where strong upstream promoter usage inhibits downstream promoter usage, has been termed promoter occlusion (Corbin and Maniatis, 1989; Cullen et al., 1984; Ju and Cullen, 1985; Proudfoot, 1986). Alternatively, in HT29 cells, intermediate levels of HNF-1α, Sp1 and Sp3 expression were observed. Previous S1 analysis has shown the SRC1α and SRC1A promoters are utilized at similar levels in these cells (Bonham et al., 2000). Both promoters could be utilized in this circumstance because less SRC1α promoter usage, due to intermediate HNF-1α levels, would allow for preinitiation complex formation at the SRC1A promoter.

4.2.3 A Strategy for Studying the SRC Promoters in their Natural Linked Context

4.2.3.1. Dual SRC Promoter CAT Reporter Construct

A key observation made early in the course of these studies was that the SRC1A promoter was always stronger in isolation than the SRC1α promoter. As will be demonstrated in subsequent sections, this consistent finding arose from transfection

experiments with SRC1A-CAT and SRC1α-CAT reporter constructs in HepG2, HT29, HCT-116, and SW480 cells. This was a very intriguing observation, because previous S1 analysis had determined that the high levels of c-Src mRNA in HepG2 cells arose from preferential use of the SRC1α promoter, while the high levels of c-Src mRNA in HT29 cells arose from near equal use of both the SRC1α and SRC1A promoters (Bonham et al., 2000). This discrepancy between endogenous SRC promoter use and the strength of SRC promoter CAT constructs in cancer cell lines prompted the design of a CAT-based plasmid construct that would allow the study of both SRC promoters in their natural, physiologically linked context. It was hypothesized that studying the promoters in such a way would circumvent this problematic observation of SRC1A-CAT constructs always being more active than SRC1α-CAT constructs in transfection experiments. The desire was to create a construct that would allow the measurement of overall combined activity of the two SRC promoters as well as quantitative comparison of their relative usage. Such a construct would be a very valuable tool to test models of differential SRC promoter use observed in human cancer cell lines, especially the promoter occlusion model proposed for HepG2 cells. To this end, a series of CAT reporter vectors were generated that contained both SRC promoters and associated exons in their normal genomic architecture, separated by a small intron from Exon 1B, a common non-coding splicing exon for both Exon 1A and Exon 1α (Fig. 4.8 A and B). These SRC Dual Promoter CAT Constructs (SRC DPCATs) differed only in the amount of DNA sequence they harboured upstream from Exon 1α. In addition to being able to drive transcription of the CAT gene, the SRC DPCAT constructs theoretically contained intronic DNA splicing recognition sequences upstream from Exon 1B and downstream

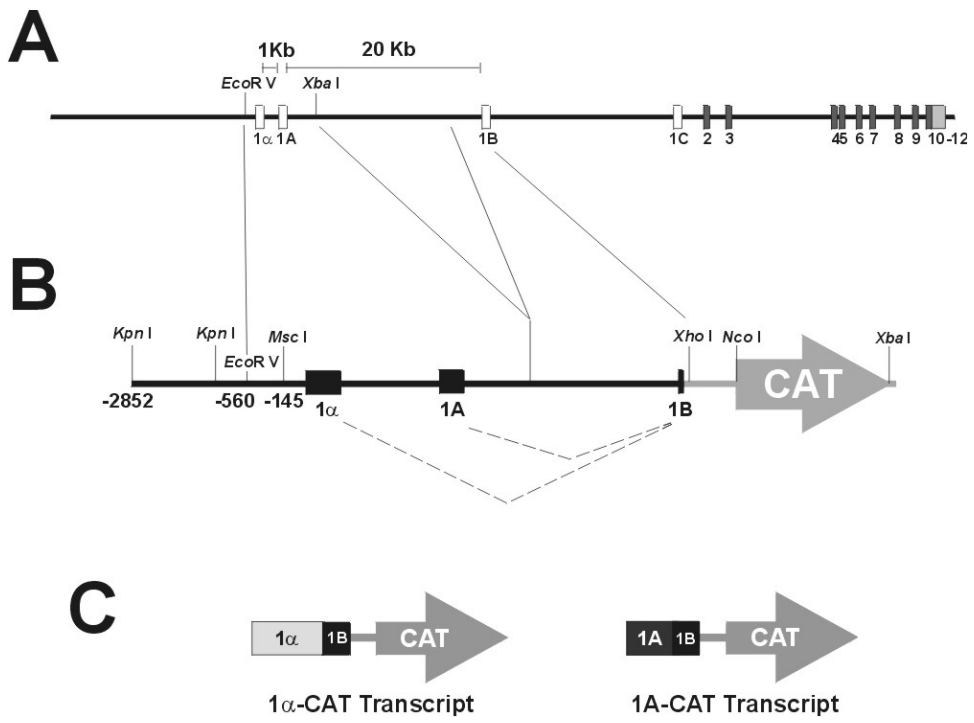

Figure 4.8. Schematic of the SRC DPCAT Strategy. (A,B) The -560 SRC DPCAT construct was generated by inserting a genomic SRC promoter cassette upstream of a genomic DNA fragment containing Exon 1B and upstream sequences. **(B)** Variants of the -560 DPCAT construct, -2852 SRC DPCAT and -145 SRC DPCAT were created by adding or removing genomic fragments upstream from Exon 1α. The extreme 5' boundaries of the -2852, -560, and -145 SRC DPCAT constructs are defined by *Kpn* I, *Eco*R V, and *Msc* I restriction sites, respectively. **(C)** Following transfection into cells, the 1α-CAT transcript would be predicted to arise from usage of the SRC1α promoter, and the 1A-CAT transcript would be predicted to arise from usage of the SRC1A promoter.

from Exons 1α and 1A to allow for selective splicing of Exon 1α or Exon 1A to 1B following transfection into cells (Fig. 4.8 C) (Breathnach et al., 1978; Chu and Sharp, 1981; Lerner et al., 1980; Mount, 1982; Rogers and Wall, 1980; Seif et al., 1979; Treisman et al., 1982; Wieringa et al., 1983).

4.2.3.2. SRC DPCAT Splicing Following Transient Transfection

In order to verify the validity of this approach, a number of preliminary transfection experiments were performed with the SRC DPCAT reporters in HepG2 cells (Fig. 4.9). The HepG2 cell line was chosen for these initial experiments due to the observation that these cells can be transfected at a very high efficiency. The CAT levels arising from a 0.54 SRC1A-CAT reporter were higher than for a -145 SRC1α-CAT reporter (Fig. 4.9 A), demonstrating the SRC1A promoter has higher activity in isolation than the SRC1α promoter in HepG2 cells. When CAT expression levels arising from the -145, -560, and -2852 SRC DPCAT reporters were compared, very similar activities were observed. These results demonstrated the amount of genomic sequence contained upstream from Exon 1α had little effect on the overall activity of these constructs. Activity of these SRC DPCAT reporters was very similar to the activity of the SRC1α promoter in isolation (Fig. 4.9 A). These results showed the SRC DPCAT constructs were able to efficiently drive CAT expression in transient transfection experiments.

A combination of RT-PCR and 5' RACE approaches were then utilized to verify correct exon splicing and the generation of predicted transcripts following transfection. First, total RNA was isolated from -560 DPCAT transfected HepG2 cells and subjected to RT-PCR analysis to assess whether Exons 1α and 1A were effectively spliced to

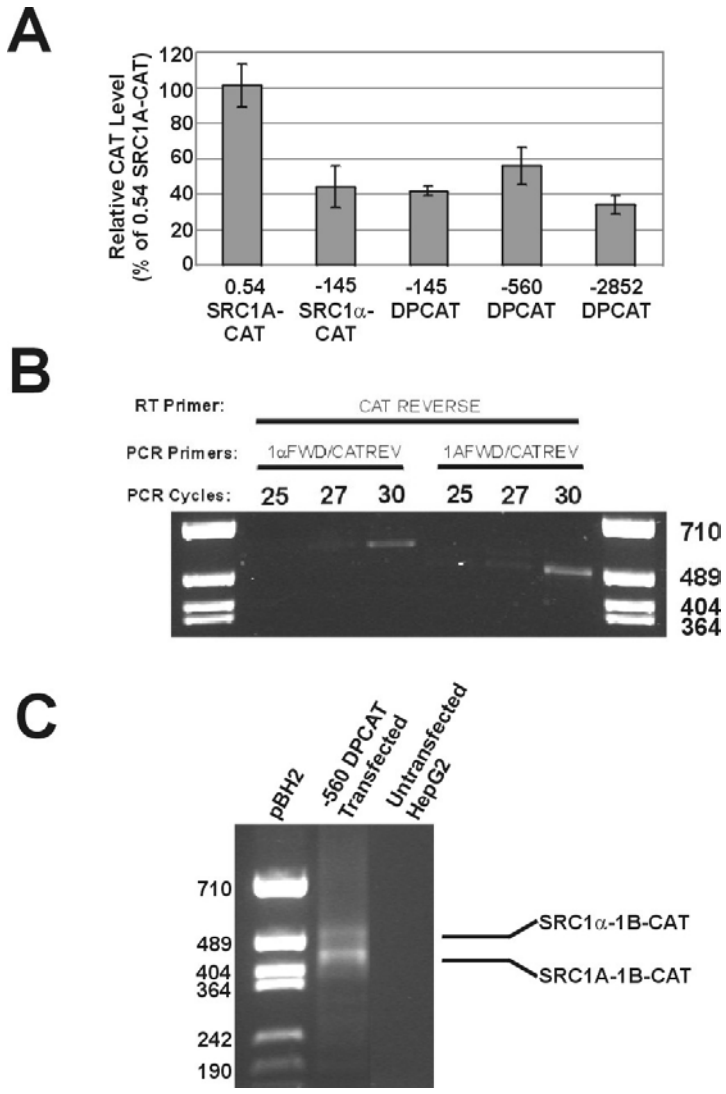

Figure 4.9. Activity and splicing patterns of SRC DPCAT constructs in HepG2 cells. (A) HepG2 cells were transfected with various SRC DPCAT reporters as well as 0.54 SRC1A-CAT and -145 SRC1α-CAT reporters. CAT levels in cell lysates were subsequently determined. Activity of the 0.54 SRC1A-CAT reporter was arbitrarily chosen to represent 100%. Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate. **(B)** Total RNA was isolated from HepG2 cells that were transfected with -560 SRC DPCAT. Reverse transcription was performed using a CAT specific primer. Subsequent PCR was performed using a nested CAT specific reverse primer coupled with Exon 1α or 1A specific forward primers. PCR products were separated on agarose gels. **(C)** Reverse transcription products from (B) were subjected to 5' RACE, and products were separated on agarose gels. Bands were subsequently cloned and sequenced.

Exon 1B. A CAT-anchored primer was utilized for reverse transcription, and subsequent PCR reactions were performed on these reverse transcription products using a nested CAT reverse primer coupled with specific forward primers recognizing Exon 1α or Exon 1A (Fig. 4.9 B). Following electrophoretic separation on agarose gels, bands of the predicted size (508 bp for Exon 1A splicing to Exon 1B, and 585 bp for Exon 1α splicing to Exon 1B) were observed, suggesting that the predicted spliced transcripts were generated following transfections with the SRC DPCAT reporter. To confirm this, these fragments were isolated from the agarose gels and sequenced. Second, to verify that transcripts arising from the SRC DPCAT reporter originated exclusively from the SRC1α or SRC1A promoters, 5' RACE was performed using the reverse transcription reaction generated for RT-PCR experiments. When 5' RACE products were separated on agarose gels, two discrete bands were observed that were exactly the size predicted for transcripts containing Exon 1α or Exon 1A at the extreme 5' terminus (Fig. 4.9 C). To verify this, the two bands were excised from the gel and cloned into pBluescript. Results from DNA sequencing analysis of 5' RACE clones confirmed these two bands represented Exon 1α or Exon 1A properly spliced to Exon 1B-CAT. Taken together, these experiments demonstrated the two SRC promoters were active within the SRC DPCAT construct, and generated pre-mRNA products that properly spliced to form mature mRNAs of a predicted composition following transient transfection.

4.2.3.3. Quantitation of SRC DPCAT Promoter Usage

Due to the demonstration that the predicted mRNA species arose from the SRC DPCAT construct, a S1 nuclease protection strategy was developed to specifically and quantitatively measure their relative abundance following transient transfections. Vectors were engineered that perfectly matched the sequences of 1α-CAT or 1A-CAT transcripts (Fig. 4.10). These vectors were subsequently used in linear amplification reactions to generate [^{32}P]-labeled S1 probes perfectly complementary to these two mRNA species. When the 1α-CAT specific S1 probe was hybridized with RNA from cells that had been transfected with -145 or -560 SRC DPCAT and subjected to digestion with S1 nuclease, three distinct protected species were observed (Fig. 4.10 A, Lanes 4 and 5). The largest, 472 bp band represented the 1α-CAT transcript, the intermediate, 299 bp band represented the 1A-CAT transcript, and the smallest, 217 bp band represented the endogenous c-Src mRNA transcript arising from usage of the SRC1α promoter. The origin of these species was confirmed by the observation that the 1α-CAT S1 probe only protected a 217 bp species in RNA from untransfected HepG2 cells (Fig 4.10 A, Lane 3). Specificity of the 1α-CAT S1 probe was further confirmed by the absence of protected species from yeast RNA (Fig 4.10 A, Lane 2). A higher abundance of 1α-CAT transcripts were generated in HepG2 cells transfected with -145 SRC DPCAT, compared with -560 SRC DPCAT (Fig. 4.10 A, compare Lanes 4 and 5). These findings agree with previous studies that have demonstrated negative regulatory elements present in SRC1α promoter constructs larger than -145 SRC1α-CAT (Bonham et al., 2000). These results also demonstrated a much higher abundance of 1A-CAT transcripts relative to 1α-CAT transcripts arising from the -145 and -560 SRC DPCAT

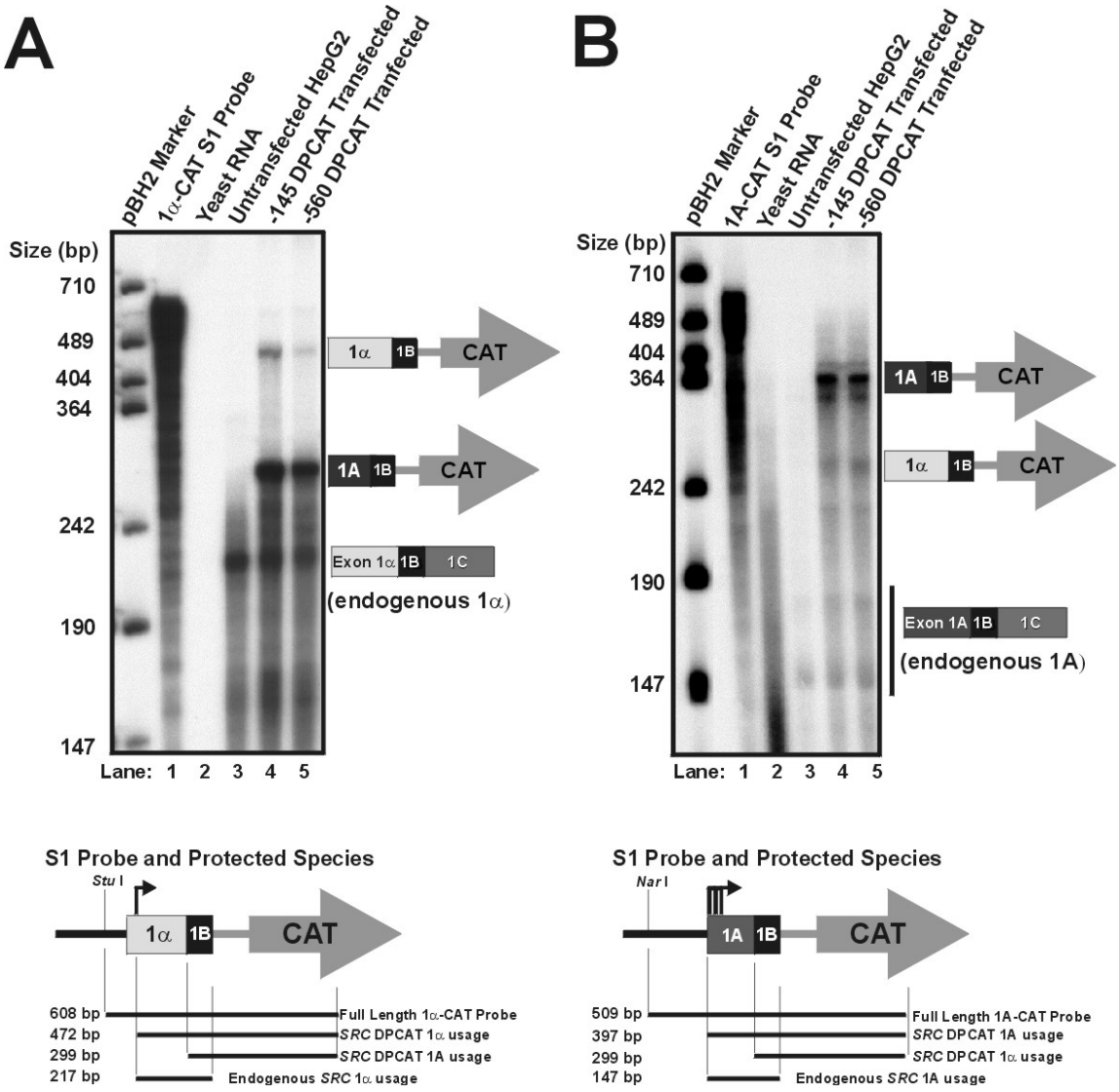

Figure 4.10. Determination of relative promoter usage from the SRC DPCAT reporter following transient transfections. (A) Total RNA was isolated from transfected as well as untransfected HepG2 cells. RNA was hybridized with a 1α-CAT specific S1 probe and then digested with S1 nuclease. Protected fragments were separated on standard DNA sequencing gels. A schematic of the expected protection pattern is shown below. **(B)** Experiments were performed exactly as in (A), using a 1A-CAT specific S1 probe. A schematic of the expected protection is shown below. pBH2 refers to fragments from a *Hpa* II pBluescript digest [^{32}P]-labeled via an in-fill reaction with Klenow fragment

reporters (Fig. 4.10, Lanes 4 and 5). When the reciprocal experiment was performed,and a 1A-CAT specific S1 probe was hybridized with RNA from cells that had been transfected with -145 or -560 SRC DPCAT and subjected to digestion with S1 nuclease, a similar pattern of protected species was observed (Fig. 4.10 B, Lanes 4 and 5). The 397 bp and 299 bp bands represented 1A-CAT and 1α-CAT transcripts, respectively. Bands spanning 147 bp to 180 bp represented endogenous c-Src transcripts arising from multiple transcription start sites within the SRC1A promoter. Quantification of the relative abundance of 1A-CAT and 1α-CAT transcripts determined using the 1A-CAT specific S1 probe correlated very closely with the relative abundance determined using the 1α-CAT specific S1 probe. Taken together, these results demonstrated S1 nuclease protection assays specifically designed to target 1α-CAT and 1A-CAT transcripts represented an excellent approach to measuring relative SRC promoter use following transient transfections with SRC DPCAT constructs.

4.2.4. Search for Enhancer Elements in the SRC Locus

4.2.4.1. Evidence for a SRC Enhancer in Human Cancer Cells

Preliminary SRC DPCAT transfection experiments in HepG2 cells suggested preferential SRC1A promoter use from this construct. Subsequent S1 nuclease protection assays of transfected HepG2 cells have confirmed this initial observation, which is in stark contrast to the preferential SRC1α promoter use seen endogenously in these cells (Fig. 4.11). An S1 probe perfectly complementary to endogenous SRC1α derived transcripts was hybridized with HepG2 RNA, followed by S1 nuclease digestion. The largest, 345 bp protected band representing the endogenous 1α transcript

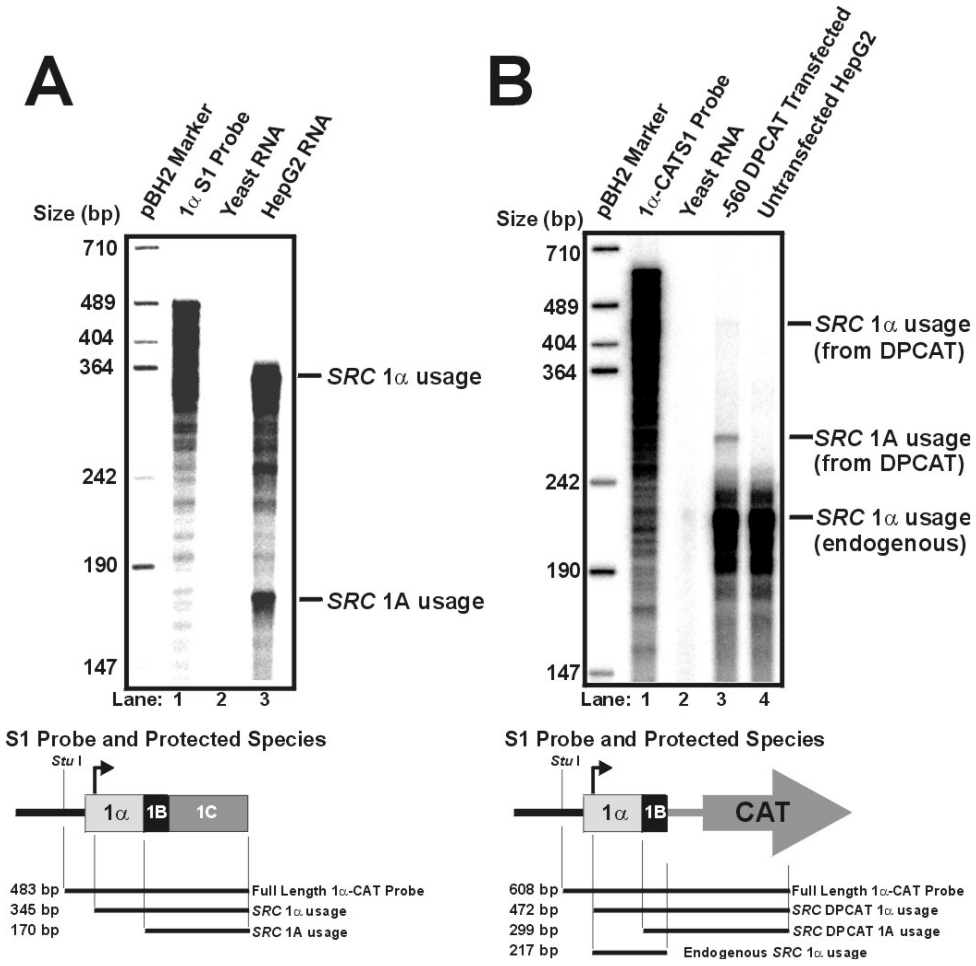

Figure 4.11. Endogenous vs. SRC DPCAT promoter usage in HepG2 cells. (A) Total RNA was isolated from HepG2 cells and hybridized with an S1 probe specific for endogenous 1α transcripts. A schematic of the expected protection pattern is shown below. **(B)** Total RNA was isolated from untransfected or transfected HepG2 cells and hybridized with an S1 probe specific for 1α-CAT transcripts. A schematic of the expected protection pattern is shown below. pBH2 refers to fragments from a *Hpa* II pBluescript digest [^{32}P]-labeled via an in-fill reaction with Klenow fragment.

was observed at a much higher abundance than the smaller, 170 bp protected band representing the endogenous 1A transcript (Fig. 4.11 A). Conversely, 1α-CAT transcripts were in very low abundance compared with 1A-CAT transcripts in HepG2 cells that had been transfected with -560 SRC DPCAT (Figs. 4.10 and 4.11 B). This demonstrated the SRC1A promoter was utilized at a much higher rate than the SRC1α promoter in the SRC DPCAT construct. Therefore, this finding excludes the promoter occlusion model that was developed to explain the preferential SRC1α use in HepG2 cells *in vitro*. In addition, these observations parallel previous transient transfection experiments, which have demonstrated that the SRC1A promoter in isolation is stronger than the SRC1α promoter (Fig. 4.9 A).

To determine if these results extended to HCCLs as well, the same experiments were performed in HT29, SW480, and HCT-116 cells. Indeed, the activity of a SRC1A construct was consistently much higher in isolation than activity of a SRC1α construct in these HCCLs (Fig. 4.12). This difference in activity was even more pronounced than in HepG2 cells (Fig. 4.9 A). In SW480 and HCT-116 cells, the activities of the -145 and -560 DPCAT constructs were comparable to the activity of the SRC1A promoter in isolation (Fig. 4.12 B,C). S1 analysis of SW480 and HCT-116 showed that both of these cell lines display weak endogenous use of both SRC promoters, resulting in the low levels of c-Src mRNA expressed in these cells (Fig. 4.13 A). When S1 assays were utilized to determine promoter use from the -560 DPCAT construct in these cells, near exclusive SRC1A usage was observed (Fig. 4.13 B). Similarly, preferential strong SRC1A usage was observed from the -145 and -560 SRC DPCAT reporters in HT29 cells (Fig. 4.13 C). This contrasts previous studies, which have determined that the

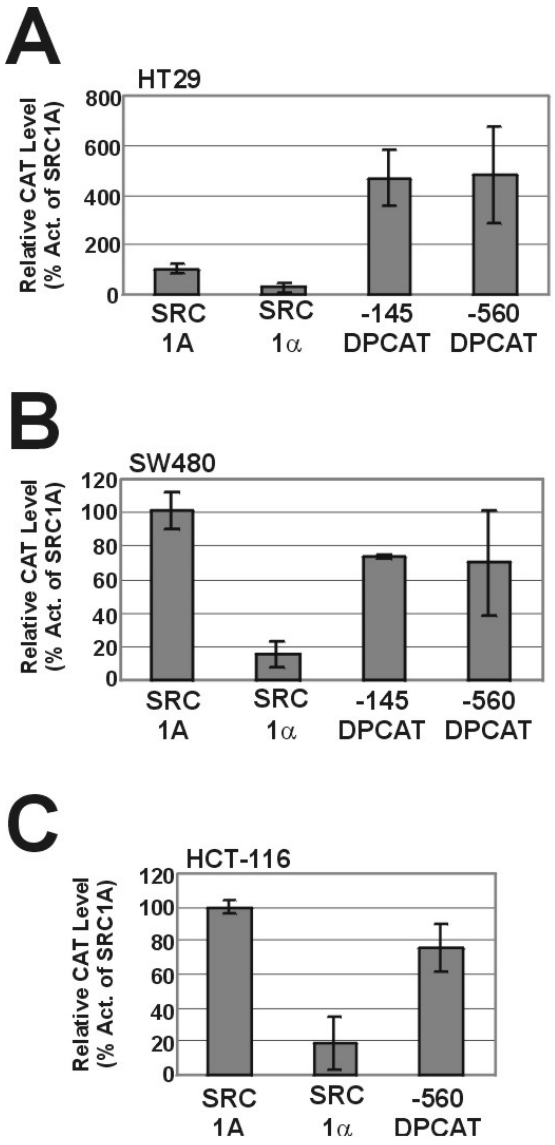

Figure 4.12. Activities of SRC DPCAT constructs relative to isolated SRC promoter-CAT constructs in HCCLs. The indicated reporter constructs were transiently transfected in HT29 (A), SW480 (B), or HCT-116 (C) cell lines. SRC1α refers to the -145 SRC1α-CAT construct. Activity of the 0.54 SRC1A-CAT reporter was arbitrarily chosen to represent 100%. Bar graphs represent the mean +/- the standard deviation from three separate experiments, each performed in duplicate.

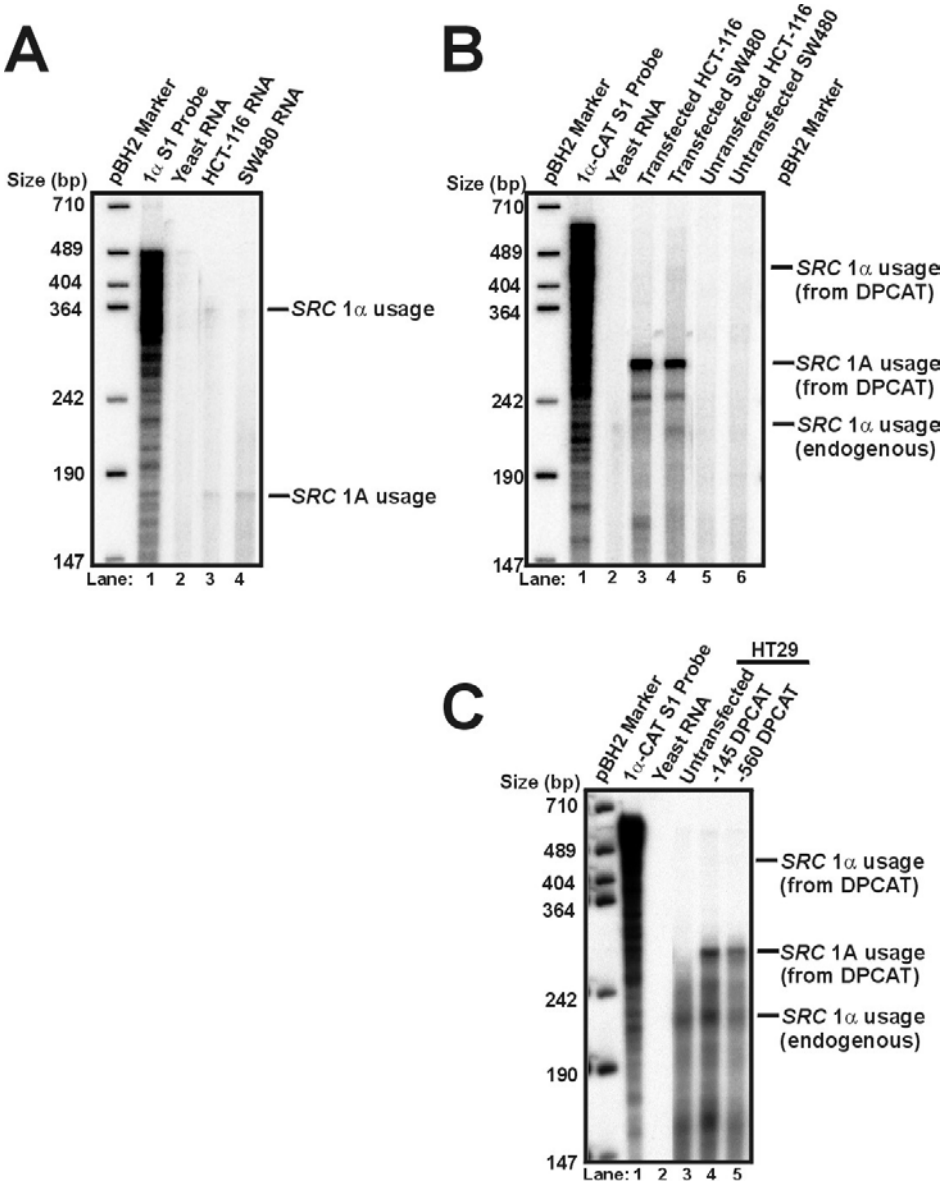

Figure 4.13. Determination of SRC DPCAT promoter use in HCCLs. **(A)** S1 analysis, using an S1 probe complementary to 1α c-Src transcripts, was employed to assess endogenous SRC promoter use in HCT-116 and SW480 cells. An S1 probe complementary to 1α-CAT transcripts was also used in S1 protection assays to determine promoter usage from the SRC DPCAT construct following transient transfections in HCT-116 and SW480 **(B)** and HT29 **(C)** cells.

overexpression of c-Src in these cells results from near equal usage of both SRC promoters. Interestingly, in HT29 cells, the activity of these SRC DPCAT constructs was much higher than the activity of either promoter in isolation, suggesting differential regulation of this construct in HT29 cells than SW480 or HCT-116 (Fig. 4.12 A). Taken together, this investigation has shown the SRC1α promoter displays very weak activity compared with the SRC1A promoter when assayed in isolation. Even in the context of the SRC DPCAT reporter, the SRC1A promoter was observed to be much stronger than the SRC1α promoter, in all cell lines studied. Therefore, there was consistent discrepancy between SRC promoter use in SRC DPCAT constructs, and promoter use seen endogenously in any given cell line. As a result of these observations, the conclusion was made that some element, not present in the SRC DPCAT constructs, was responsible for increasing SRC1α activity in HepG2 cells as well as HCCLs. Strong evidence was therefore provided for the hypothesis that an enhancer element existed and was responsible for elevating SRC1α promoter use relative to SRC1A in human cancer cells. Identification of this putative enhancer element became of immediate interest, because its activity could explain the SRC transcriptional activation observed in human cancer cells.

4.2.4.2. Search for Nuclease Hypersensitive Sites in the SRC Locus

There are many examples of genes that require liver- or intestine-specific enhancer elements to achieve specific and full activation in these tissues (Bisaha et al., 1995; Shachter et al., 1993; Shelley and Baralle, 1987; Vergnes et al., 1997). Such elements, therefore, could also exist within the SRC locus. As shown in Fig. 4.14 A, the SRC

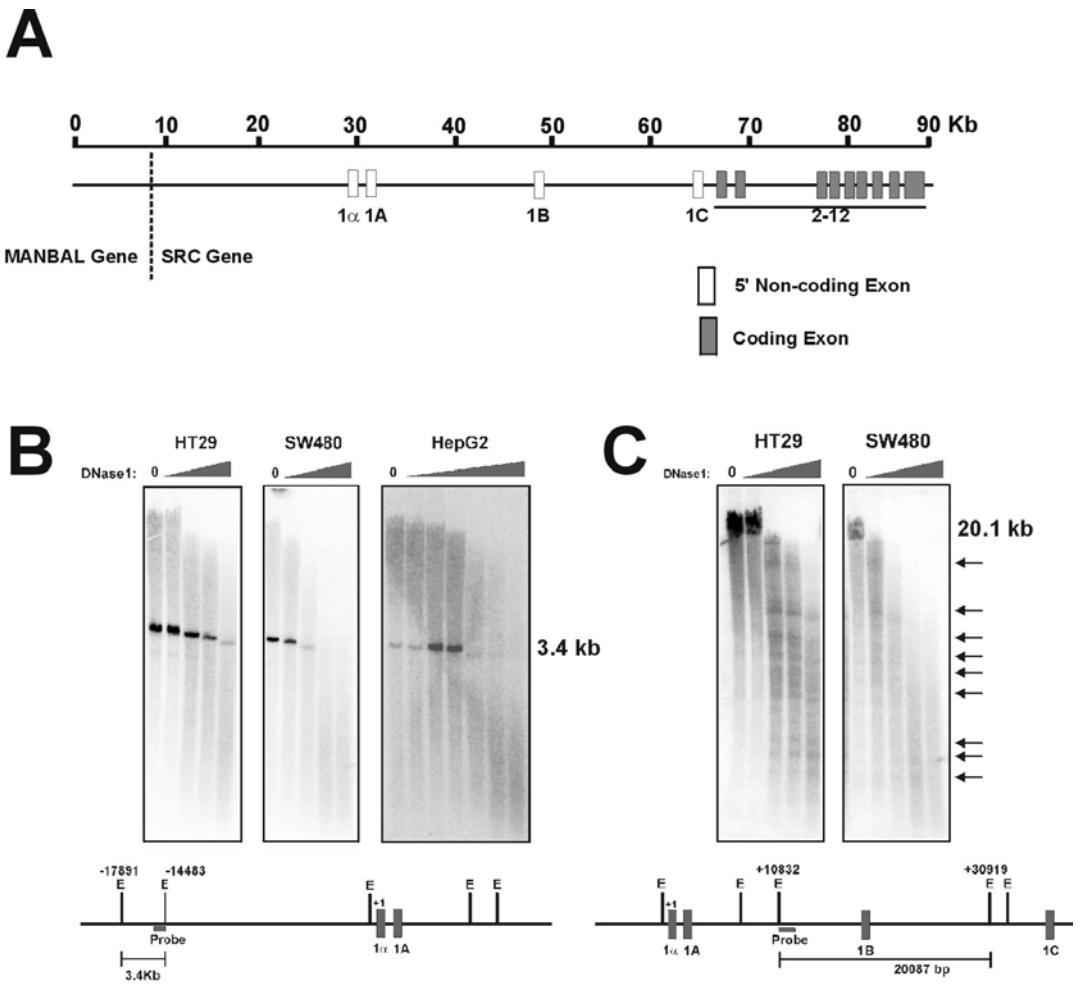

Figure 4.14. SRC enhancer search in human cancer cells. (A) Schematic of the human SRC gene on chromosome 20q12. The neighboring MANBAL gene, predicted by gene search algorithms (NCBI) to exist upstream of Exon 1α, is shown. **(B)** Nuclei from HT29, SW480, or HepG2 cell lines were digested with increasing concentrations of DNase I. Genomic DNA was subsequently purified and subjected to digestion with *EcoR* V. Southern blot analysis was employed, and DH sites were detected with a labeled DNA probe specifically targeted to the 3' end of an *EcoR* V fragment immediately upstream from the SRC promoters. **(C)** Nuclei were prepared and genomic DNA was digested as in (B), and DH sites were detected with a labeled probe specifically targeted to the 5' end of an *EcoR* V fragment encompassing Exon 1B. DH sites are identified by black arrows. Maps of the relative locations of the *EcoR* V fragments detected in this experiment are shown at the bottom of each figure.

locus is quite large, with 20 kb introns separating the 5' non-coding exons (Bonham and Fujita, 1993). In addition, 20 kb of non-coding genomic DNA is contained upstream of Exon 1α and downstream of the hypothetical MANBAL gene (Lander et al., 2001 and references therein). As a result of these observations, the enhancer proposed to elevate SRC1α activity in human cancer cells was hypothesized to exist within this 60 kb region of genomic DNA, which spanned 20 kb upstream of Exon 1α to Exon 1C (Fig. 4.14 A). Although the putative SRC enhancer could also exist outside this relatively small search area, this 60 kb region at the 5' end of the SRC gene was selected as the primary focus. A strategy of searching for DNaseI hypersensitive (DH) sites was therefore developed to identify putative enhancer elements within this search area. DH sites often represent functional regions of genomic DNA, and identification of these regions has allowed for the isolation of enhancer elements within other genes (Bisaha et al., 1995; Grandien et al., 1993; Pullner et al., 1996; Shachter et al., 1993; Vaulont et al., 1989). Combinations of overlapping restriction fragments encompassing the entire 60 kb region were assessed for their DH site composition in HepG2, HT29, SW480, and HepG2 cells. The typical DH patterns observed within these restriction fragments are illustrated in Fig 4.14. For example, a 3.4 kb *EcoR* V restriction fragment upstream of the SRC promoter region did not display any obvious DH sites in any of the cell lines examined when detected with a probe targeted to its 3' terminus (Fig. 4.14 B). Conversely, when a larger 20 kb fragment located downstream of the SRC promoters was assessed, many weak DH sites were observed in HT29 and SW480 cells (Fig. 4.14 C). This approach, which resulted in the generation of dozens of Southern blots, did not reveal any obvious, strong DH sites in HT29, SW480, or HCT-116 cells that might represent candidate enhancer

elements. However, a single intense DNaseI hypersensitive site, specific to HepG2 cells was discovered using this approach, and was denoted DH3 (Fig. 4.15). This site was contained within an *Eco*R V fragment, immediately upstream of Exon 1α, and mapped to a multitude of potential transcription factor binding motifs as determined by the TFSearch transcription factor binding site search algorithm. Two common DH sites, termed DH1 and DH2, were also observed within this restriction fragment. Additional restriction enzyme combinations were employed to more precisely map this interesting DH3 site, and confirmed its location between -4400 and -4000, relative to the transcription start site in the SRC1α promoter.

4.2.4.3. Cloning and Characterization of a Putative HepG2 Specific SRC Enhancer

Due to the marked discrepancy between endogenous SRC promoter use and promoter use observed from SRC DPCAT constructs in HepG2 cells, an immediate priority was to clone and characterize the genomic DNA fragment representing the DH3 site. It was hypothesized that this element could be an enhancer that was responsible for the preferential, strong SRC1α promoter usage seen in HepG2 cells. The region encompassing this putative HepG2 specific enhancer element was contained within existing SRC genomic cosmid clones (Bonham and Fujita, 1993), allowing this element to be readily isolated. Therefore, various reporter constructs harboring this putative enhancer were generated. When analyzed in transient transfection experiments, this element displayed a very modest ability to increase the activity of the SRC1α promoter (Fig. 4.16 A). For example, the presence of the fragment representing the HepG2 DH3 site (-6882 SRC1α-CAT) increased the activity of a -2852 SRC1α-CAT reporter

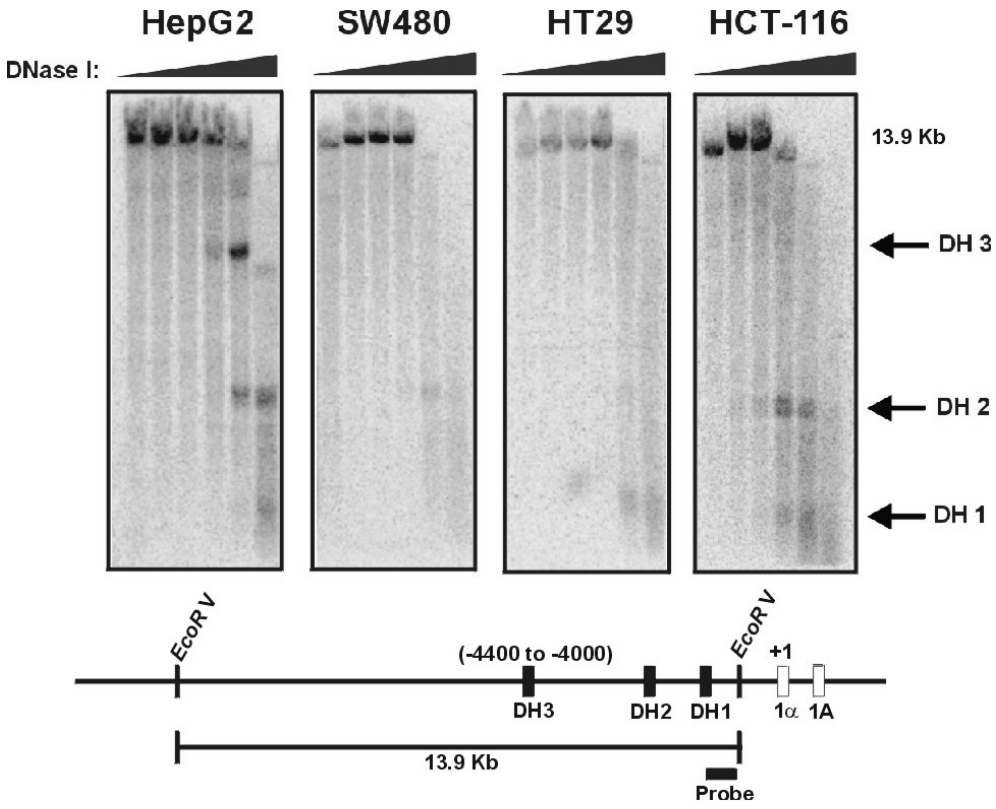

Figure 4.15. A HepG2 specific DH site. Nuclei from HepG2, SW480, HT29, or HCT-116 cell lines were digested with increasing concentrations of DNaseI. Genomic DNA was subsequently purified and subjected to digestion with *EcoR* V. Southern blot analysis was employed, and DH sites were detected with a labeled DNA probe specifically targeted to the 3' end of an *EcoR* V fragment immediately upstream from the SRC promoters. A map of the relative locations of the DH sites in this experiment is shown at the bottom of the figure.

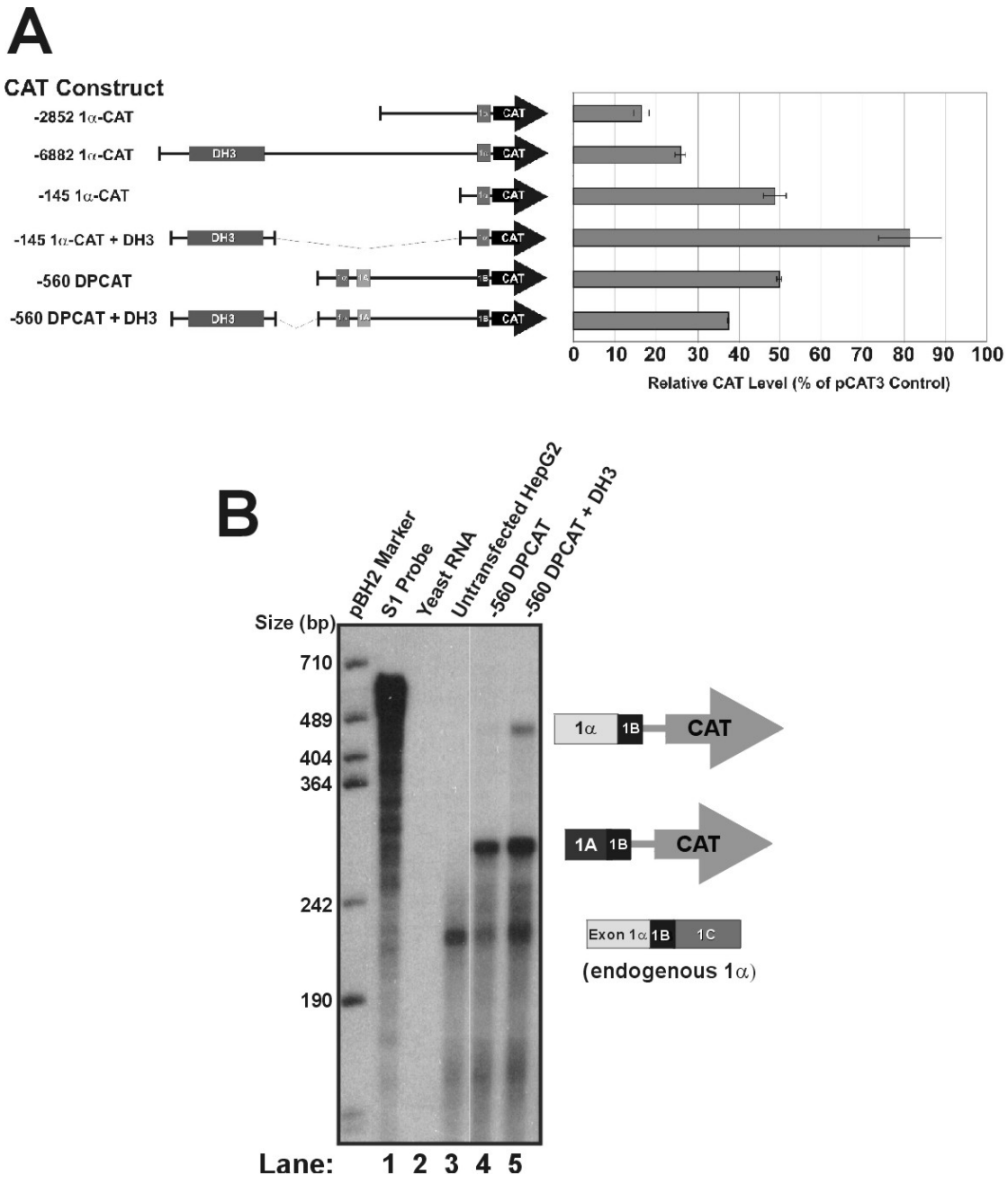

Figure 4.16. Characterization of the putative HepG2 specific SRC enhancer. (A) The genomic fragment representing the HepG2 DH3 site was cloned upstream of the promoter(s) in various SRC reporter constructs shown at left. Activities of these constructs were subsequently determined in transient HepG2 transfections. CAT levels are shown relative to a viral promoter driven pCAT3 control vector. Bar graphs represent the mean +/- the standard deviation from three separate experiments, each performed in duplicate. **(B)** RNA from HepG2 cells transfected with -560 SRC DPCAT or -560 SRC DPCAT containing the putative enhancer was hybridized with an S1 probe specifically targeted to 1α-CAT transcripts; probe:target duplexes were subsequently digested with S1 nuclease and separated on sequencing gels.

construct approximately 80 to 90%. This fragment mediated a similar level of increased activity in a -145 SRC1α-CAT reporter. When analyzed in the context of the -560 SRC DPCAT construct, a slight decrease in CAT levels was observed. In order to specifically assess the effect of this genomic DNA fragment on SRC1α promoter activity in the -560 SRC DPCAT reporter, S1 protection analysis was employed. In the construct harboring the HepG2 DH3 site, a slightly higher signal for SRC1α promoter use was observed relative to SRC1A promoter use compared with -560 SRC DPCAT alone (Fig. 4.16 B, compare lanes 4 and 5). However, SRC1A was still the predominant promoter used to drive CAT expression from this construct. In conclusion, these observations suggested the HepG2 DH3 site contained detectable, yet very weak activating potential towards the SRC1α promoter in HepG2 cells.

4.2.5. Differential Activity of the SRC DPCAT Reporter in HT29 Cells

4.2.5.1. SRC DPCAT Activity and Promoter Use in HCCLs

An interesting observation noted during SRC DPCAT transfection experiments in various human cancer cell lines was that the SRC DPCAT constructs displayed a much higher activity than either SRC promoter in isolation in HT29 cells (Fig. 4.12 A). This finding contrasted the observation that the activity of SRC DPCAT constructs was comparable to the isolated SRC promoters in all other cell lines examined (Figs. 4.9 A, 4.12 B and C). Therefore, the combined activities of the individual SRC promoters could not account for the activity observed from the SRC DPCAT constructs in HT29 cells. Previous S1 protection assays demonstrated that SRC1A is the predominant promoter used from the SRC DPCAT constructs in these cell lines (Fig. 4.13 C). These

findings suggested the SRC1A promoter in these reporter constructs was somehow activated, exclusively in HT29 cells.

4.2.5.2. The SRC1α HNF Site Activates the SRC1A Promoter in HT29 Cells

To address the mechanism of SRC1A promoter activation in the SRC DPCAT construct in HT29 cells, various deletion and mutant DPCAT constructs were generated (Fig. 4.17 A) and their activities assessed following transient transfections in SW480, HCT-116, and HT29 cells (Fig. 4.17 B-D). Deletion of the SRC1A promoter from the -560 DPCAT reporter severely impaired CAT expression in all three cell lines. These findings corroborated the results from S1 protection assays (Fig. 4.13), which demonstrated the SRC1A promoter was primarily responsible for generating the CAT levels observed from the SRC DPCAT in HCCLs. The remaining activity in this Δ1A DPCAT construct was very similar to -145 SRC1α-CAT, confirming weak residual activity of the SRC1α promoter was responsible for the low CAT expression levels detected. Surprisingly, deletion of the SRC1α promoter from the -560 SRC DPCAT construct also severely impaired SRC DPCAT activity in HT29 cells (Fig. 4.17 D). This deletion reduced SRC DPCAT activity to identical levels observed from the 0.54 SRC1A-CAT construct. This same deletion, however, did not have any detectable effect on CAT expression levels in SW480 or HCT-116 cells (Fig. 4.17 B,C). In these cell lines, rather, this Δ1α DPCAT construct displayed an activity very similar to 0.54 SRC1A-CAT, verifying S1 protection findings that the SRC1α promoter contributed little to the overall CAT levels generated from SRC DPCAT constructs in these cell lines. Most significantly, however, these findings implicated that an element within the

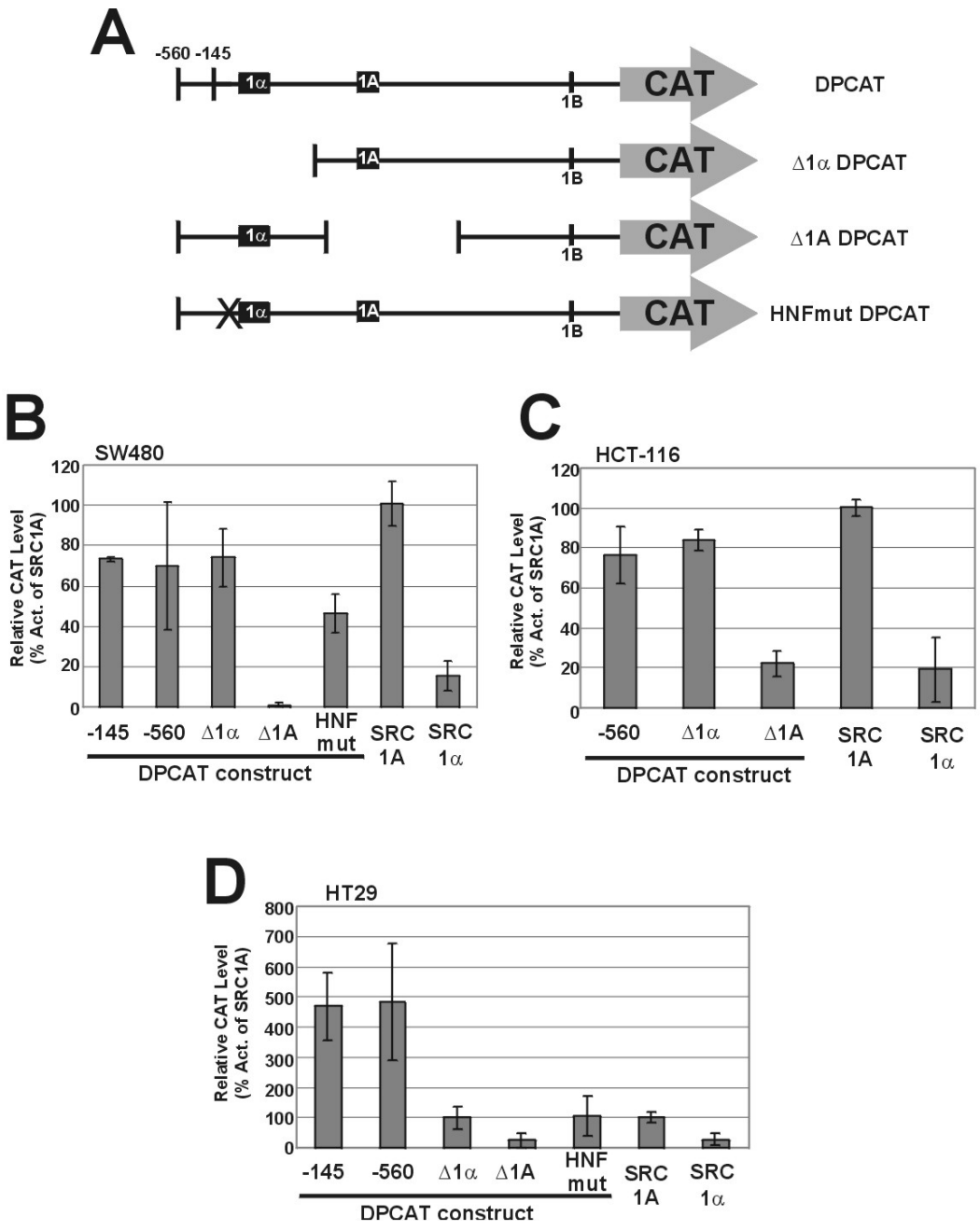

Figure 4.17. Activity of deletion and point mutant SRC DPCAT constructs. (A) Schematics of the constructs utilized in this study. Various DPCAT reporters and mutants derived from -560 DPCAT were transiently transfected in SW480 **(B)**, HCT-116 **(C)**, and HT29 **(D)** cells. Activities of the 0.54 SRC1A-CAT and -145 SRC1α-CAT constructs are included; all CAT levels are shown relative to 0.54 SRC1A-CAT, which was arbitrarily chosen as 100%. Bar graphs represent the mean +/- the standard deviation from two to three separate experiments, each performed in duplicate.

deleted SRC1α fragment was activating the SRC1A promoter in the SRC DPCAT constructs, specifically in HT29 cells. A hypothesis was subsequently developed that the SrcHNF binding site in the SRC1α promoter could be responsible for the observed SRC1A specific activation in HT29 cells. Therefore, a -560 DPCAT variant harboring point mutations in the SRC1α HNF binding site was generated, and its activity determined in HT29 and SW480 cells (Fig 4.17 B, D). Previous studies have demonstrated such a mutation has a drastic effect
on SRC1α promoter activity as well as HNF-1α binding in HepG2 cells (Bonham et al., 2000). In this study, mutation of the SrcHNF site in the -560 DPCAT construct had a nearly identical effect as deletion of the entire SRC1α promoter in HT29 cells, reducing activity approximately 5-fold. In SW480 cells, conversely, the SrcHNF mutation reduced -560 DPCAT activity by only 25%. These results clearly demonstrate the SrcHNF site in the SRC DPCAT construct increases the activity of the SRC1A promoter, only in HT29 cells.

4.2.5.3. HNF-1 Represses SRC DPCAT Activity in HCCLs

The results from these studies strongly suggested that HNF-1α, through binding the SrcHNF site in the SRC1α promoter, was activating the SRC1A promoter in HT29 cells. It was therefore investigated whether co-expression of HNF-1α could further elevate SRC1A activity in the -560 SRC DPCAT reporter in these cell lines (Fig. 4.18). Surprisingly, however, exactly the opposite result was observed; HNF-1α or HNF-1β co-expression effectively inhibited activity of the -560 SRC DPCAT construct in HT29 cells (Fig. 4.18 A). A -560 SRC DPCAT construct harboring mutations in the SrcHNF

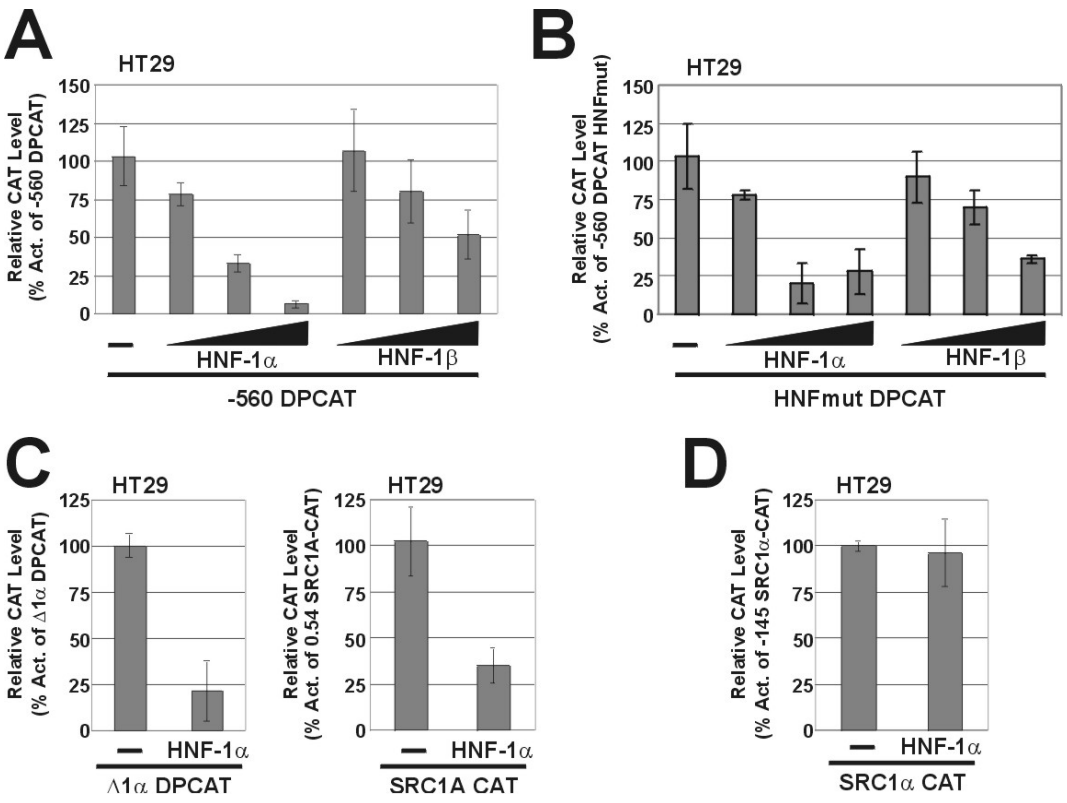

Figure 4.18. Effect of HNF-1α expression on activities of SRC promoter constructs in HT29 cells. (A) The -560 DPCAT reporter was co-transfected with increasing amounts of HNF-1α or HNF-1β expression vector. **(B)** The HNFmut DPCAT reporter was transfected as in (A). **(C)** The Δ1α DPCAT or 0.54 SRC1A-CAT reporters were co-transfected with the highest amount of HNF-1α from (A). **(D)** The -145 SRC1α-CAT reporter was co-transfected with the highest amount of HNF-1α from (A). Bar graphs represent the mean +/- the standard deviation from three separate experiments, each performed in duplicate.

binding site was also significantly repressed by both HNF-1α and HNF-1β co-expression (Fig. 4.18 B). Interestingly, this effect appeared to be due to direct inhibition of SRC1A, because this promoter in isolation was also significantly repressed by HNF-1α expression (Fig. 4.18 C). Most significantly, however, and contrary to previous observations (Bonham et al., 2000), HNF-1α was not able to transactivate the SRC1α promoter in HT29 cells (Fig. 4.18 D). To corroborate these observations, transfection experiments were repeated in SW480 cells (Fig. 4.19). A very similar effect of HNF-1α or HNF-1β co-expression on SRC DPCAT (Fig. 4.19 A), HNFmut DPCAT (Fig. 4.19 B), or SRC1A-CAT (Fig. 4.19 C) was observed in these cells compared with HT29. However, as has previously been reported (Bonham et al., 2000), and in contrast to HT29 cells in this thesis study, HNF-1α co-expression was able to effectively activate the SRC1α promoter approximately 6-fold in SW480 cells (Fig. 4.19 D). Therefore, HNF-1α appeared to be the factor responsible for SRC1α activity in SW480 cells. However, these results also suggested a factor other than HNF-1α was responsible for activating SRC1α transcription and increasing SRC1A activity in HT29 cells.

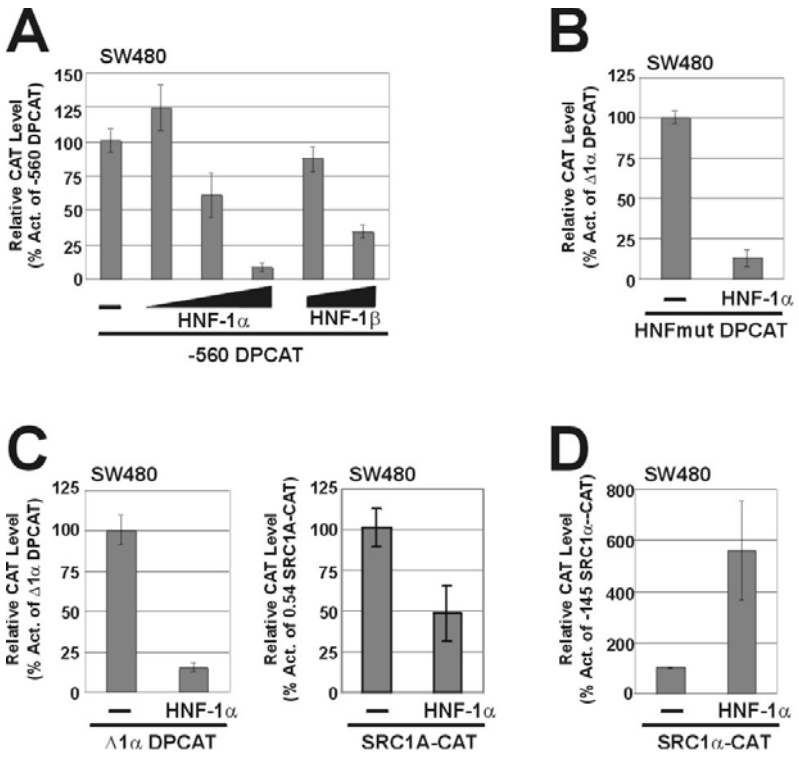

Figure 4.19. Effect of HNF-1α expression on activities of SRC promoter constructs in SW480 cells. **(A)** The -560 DPCAT reporter was co-transfected with increasing amounts of HNF-1α or HNF-1β expression vector. **(B)** The HNFmut DPCAT reporter was co-transfected with the highest amount of HNF-1α from (A). **(C)** The Δ1α DPCAT or 0.54 SRC1A-CAT reporters were co-transfected with the highest amount of HNF-1α from (A). **(D)** The -145 SRC1α-CAT reporter was co-transfected with the highest amount of HNF-1α from (A). Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate.

4.2.6. DISCUSSION

4.2.6.1. Evidence for a SRC Enhancer in Human Cancer Cells

This study detailed the construction of a SRC dual promoter reporter vector that was intended to alleviate the problematic finding that the relative strengths of the SRC promoters in isolation did not match the relative usage of these promoters seen endogenously. Specifically, the SRC1α promoter was consistently much weaker in isolation than the SRC1A promoter as assessed by transient HepG2, HT29, SW480, and HCT-116 transfections. However, when SRC DPCAT transfections were performed in these same cell lines, it was determined that promoter use was still consistently biased for SRC1A activity, despite the SRC promoters being assayed in their natural, physiologically linked context. These findings strongly suggested an enhancer was responsible for SRC1α specific up-regulation in various cancer cell lines. Because of these observations, a strategy of searching for DH sites in the SRC locus was developed, and led to the identification of a strong, HepG2 specific DH site situated approximately 4 kb upstream of Exon 1α. This approach, however, was unsuccessful in identifying any DH sites specific to HT29 or other colon cancer cells. Numerous weak, apparently ubiquitous, DH sites were observed in all the cell lines studied, but none presented with the strength and restricted nature of the HepG2 DH3 site.

When the HepG2 DH3 element was cloned, it displayed a very weak ability to activate the SRC1α promoter, both in isolation, and in the context of the SRC DPCAT reporter. These results suggested that this element at -4000 to -4400 was not responsible for the full SRC transcriptional activation from the SRC1α promoter in HepG2 cells. However, the activity of this putative enhancer element was assessed in transient HepG2

transfection experiments, where the reporter plasmid remained episomal. Therefore, any significant activity of this element that relies upon eliciting changes in chromatin structure would not be observed using this approach. The best example of this type of enhancer action is the locus control region (LCR), which was first described for the human β-globin gene cluster (Grosveld et al., 1987). Other genes have also been identified that contain LCRs, but the β-globin LCR has been the best characterized, primarily due to its compact, linked nature. LCRs are defined as elements that often map to DH sites, and enhance the expression of linked genes to physiological levels in a tissue specific and copy number dependent manner following integration into chromatin (Li et al., 2002). Numerous arrays of binding sites for ubiquitous and tissue restricted transcription factors are located within these LCRs. The fact that levels of reporter gene expression are copy number dependent when linked to LCR elements in stable transfection experiments supports the view that LCR activity creates open chromatin (Forrester et al., 1990). Additional investigation of the properties of the SRC DH3 site in the context of stably integrated reporter constructs is therefore necessary to fully exclude this DH site as a functional enhancer, and potential LCR in HepG2 cells.

In addition to the DH3 site mapped 4 kb upstream of Exon 1α, numerous ubiquitous, weak DH sites were observed throughout the SRC locus in HepG2 cells. It is very likely that one or many of these sites could be required, perhaps in conjunction with DH3, for strong SRC1α activity in transient or stable HepG2 transfection experiments. This possibility is supported by studies of the human apoA-I/apoCIII/apoA-IV gene cluster, which have identified a myriad of discrete, highly dispersed elements within the locus that play a combined role in directing the tissue

specificity and overall levels of expression of the apolipoprotein family members (Zannis et al., 2001). However, it is also quite likely that the putative element(s) responsible for elevating SRC1α activity in HepG2 cells, as well as HCCLs, reside outside of the narrow search area focused on in this study. For example, DH site composition was not assessed within or downstream from the SRC coding region in this study. Therefore, it will be important for future studies to expand the search for putative enhancer elements by assessing DH site composition outside of the 60 kb search area encompassing the SRC promoter region.

4.2.6.2. Binding of Factors to the SrcHNF site in the SRC1α Promoter

An interesting, but very unexpected finding arising from DPCAT transfection experiments in HCCLs was that the SrcHNF site activates the SRC1A promoter, exclusively in HT29 cells. Even more striking was the observation that HNF-1α binding to this site did not appear responsible for this phenomenon. Rather, HNF-1 co-expression inhibited the activity of the SRC DPCAT reporter. This repression appeared to be due to a non-specific squelching phenomenon, because a -560 DPCAT construct with a mutant SrcHNF site, and the SRC1A promoter in isolation, were also significantly inhibited by HNF-1 expression. Squelching is a term used to describe the paradoxical repression of an episomal reporter gene by a strong transactivator (Natesan et al., 1997). It is believed to result from titration of limiting, essential general transcription factors or co-activators away from the template being analyzed. Therefore, it is likely the repression observed in these experiments was the result of HNF-1 sequestering factors essential for full SRC1A activity.

A previous report has determined that the SrcHNF site is absolutely essential for SRC1α promoter activity in HepG2 cells. For example, mutation of this site nearly abolishes all SRC1α activity in this cell line (Bonham et al., 2000). Further analysis with HepG2 nuclear extracts demonstrated the primary factor that interacts with this site is HNF-1α (Bonham et al., 2000). Also, co-transfection experiments in HT29 cells showed that HNF-1α, but not HNF-1β, is able to transactivate the SRC1α promoter (Bonham et al., 2000). Consistent with these observations, experiments described in this thesis showed that HNF-1α was able to transactivate an isolated SRC1α promoter construct in SW480 transfections. However, in contrast to previous observations, studies in this thesis demonstrated that HNF-1α was not capable of SRC1α transactivation in HT29 cells. The best explanation for this lack of HNF-1α mediated SRC1α transactivation in HT29 cells is that another factor binds to and activates the SrcHNF site. This possibility led to a re-evaluation of data in Dr. Bonham's laboratory, which do indeed hint at this possibility that another factor exists, and can compete with HNF-1α for binding to the SrcHNF site. For example, in EMSA experiments with HepG2 nuclear extracts and a [^{32}P]-labeled probe representing the SrcHNF site, the major complex observed contained HNF-1α (Bonham et al., 2000). Significantly, however, competition experiments employing an unlabeled competitor representing a consensus HNF binding site disrupted this HNF-1α binding, but also enriched for binding of a smaller, unknown factor to the SrcHNF probe (Bonham et al., 2000). This observation is even more evident in a similar HepG2 EMSA experiment previously performed by Dr. Bonham, which utilized even higher levels of competitor oligonucleotides (Fig. 4.20). In this experiment, competitors representing the SrcHNF

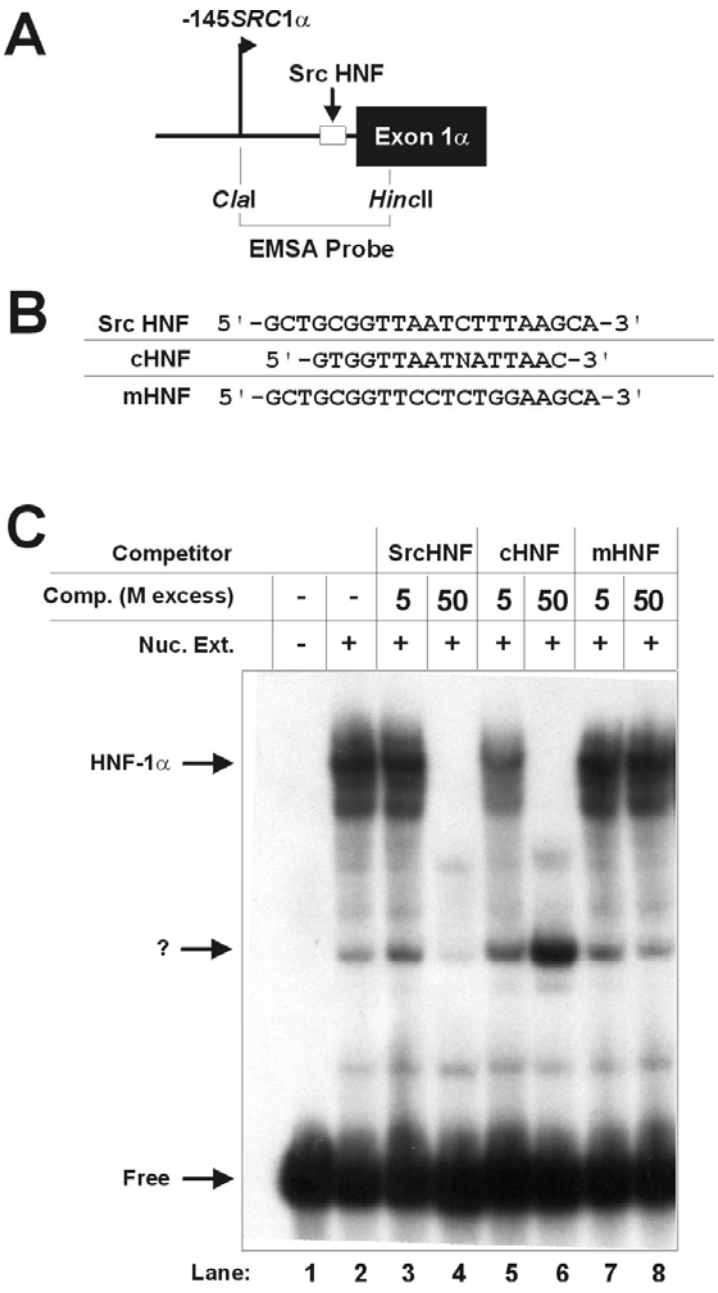

Figure 4.20. An unknown factor binds the SrcHNF site. (A) Origin of the probe used for these EMSA experiments. **(B)** Competitor oligonucleotides. **(C)** HepG2 nuclear extracts were incubated with a labeled SRC1α promoter restriction fragment. When present, competitor oligonucleotides were added at a 5 or 50 fold molar excess. The major complex, previously identified as containing HNF-1α, is shown. A unknown factor that binds the probe is denoted by a question mark.

or a consensus HNF site, but not a mutant HNF site, were able to effectively block the binding of HNF-1α to a labeled SRC1α promoter restriction fragment. However, with a 50 M excess of consensus HNF competitor oligonucleotide present, enrichment for binding of a smaller, unknown factor to the labeled SRC1α probe was very evident (Fig. 4.20, Lane 6). These results therefore suggest that the major factor binding the SrcHNF site in HepG2 cells is HNF-1α, but another factor also exists in these cells that is able to compete with HNF-1α for SrcHNF binding. This factor likely binds the SrcHNF site in HCCLs also, because HCT-116 cells don't express detectable levels of HNF-1α (Fig. 4.7), yet still display SRC1α activity endogenously (Fig. 4.13) as well as following transient transfections (Figs. 4.12 and 4.17). If a similar factor that competes for SrcHNF binding exists in HT29 cells, and is the main factor that interacts with this binding site, then this would strongly implicate it in SRC1α transactivation. Unfortunately, the findings shown here represent the very last sets of experiments performed for this thesis study. Therefore, adequate experimental pursuit of this very interesting factor could not be carried out. However, the current hypothesis is that preferential binding of this factor to the SrcHNF site is responsible for SRC1α activity in isolation, as well as the high SRC1A activity seen in HT29 cells following SRC DPCAT transfection experiments. Therefore, identification and characterization of this factor will be very important in order to understand the mechanism of communication between the SrcHNF site in the SRC1α promoter and the SRC1A promoter.

4.2.6.3. Significance of SrcHNF mediated SRC1A Activation

Initial experiments with the SRC DPCAT constructs in various cancer cell lines led to the conclusion that one or more enhancer elements were needed to elevate SRC1α activity in the context of these reporters. It was also hypothesized that this element(s) could be responsible for the SRC transcriptional activation observed in HT29 and HepG2 cells. Therefore, it could be argued that the observation of SrcHNF mediated SRC1A activation within the SRC DPCAT reporters in HT29 cells is irrelevant, because the putative element(s) necessary for full SRC1α activity was absent from these constructs. However, S1 nuclease protection assays previously performed with T47D breast cancer cells showed this cell line to express high levels of c-Src mRNA, nearly exclusively from the SRC1A promoter (Bonham et al., 2000). Therefore, the possibility can be raised that the SrcHNF site could play a role in the elevated SRC1A activity seen in these breast cancer cells. This theory could be addressed by repeating the experiments detailed in this study in the T47D breast cancer cell line.

Perhaps the best illustration of the potential relevance of SrcHNF mediated SRC1A activation comes from comparison of the SRC promoter regions in humans and mice. The SRC1A promoter, and its associated exon, Exon 1A, are highly conserved between these two species, with an interesting finding being that the TC tracts are shorter in mouse than human (Fig. 4.21). Similarly, the regions surrounding human Exon 1α are highly conserved between human and mouse, with a near identical match for the SrcHNF site. However, sequences representing Exon 1α are not apparent in the mouse SRC locus. In addition, no potential splice recognition junctions downstream from the SrcHNF are identifiable. These findings therefore suggest a fundamental

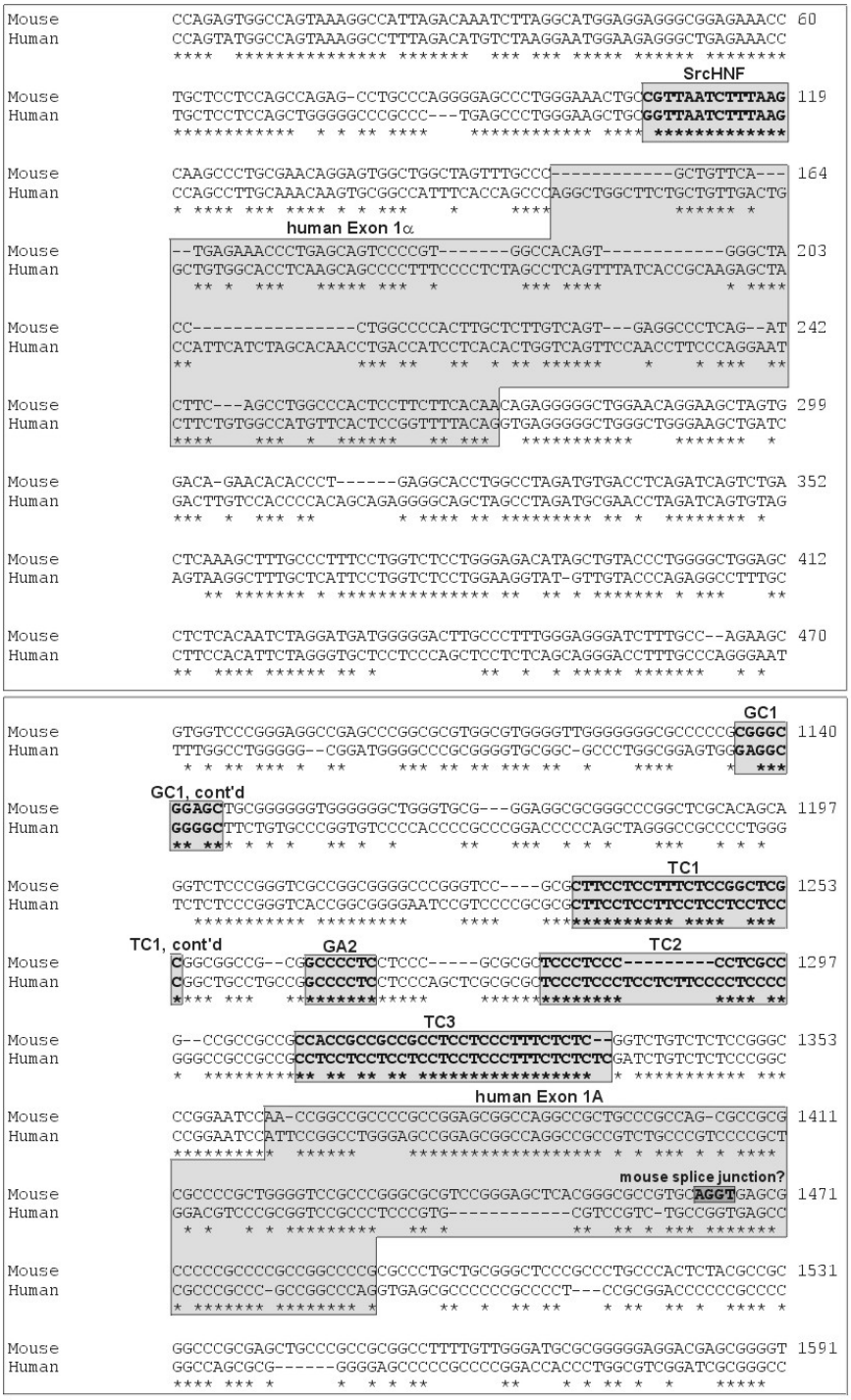

Figure 4.21. Sequence comparison of the human and mouse SRC promoter loci. Functional elements in the human SRC1α and SRC1A promoters are highlighted in grey and labeled. Stars below the aligned sequences denote perfect homology. Numbering at right refers to mouse genomic sequence. Alignments were generated using the ClustalW DNA sequence alignment algorithm.

difference between mouse and human SRC transcriptional regulation is that the human genome harbors Exon 1α while the mouse genome does not. These conclusions are supported by observations that 5' RACE experiments with mouse tissues have identified c-Src mRNA transcripts that contain Exon 1A equivalents, but no Exon 1α equivalents (K. Bonham, personal communication). Interestingly, however, not only is the SrcHNF site highly conserved between mouse and human, its location relative to the SRC1A promoter is also very similar. The possibility arises then that this putative murine SrcHNF site acts as an activator element for the SRC1A promoter, very much like what was observed with SRC DPCAT transfections in HT29 cells. Various experiments are necessary to determine whether this site binds HNF-1α and/or other factor(s) in mice, and what functional role this binding could play in mouse SRC1A transcription.

4.2.6.4. Models of Differential Promoter Use and Transcriptional Activation

The studies outlined in this chapter were unsuccessful in identifying enhancer elements responsible for elevating SRC1α activity, and potentially activating SRC transcription in HepG2 and HT29 cells. In all cell lines studied, CAT expression from the SRC DPCAT reporter originated from the SRC1A promoter. However, S1 analysis determined that HepG2 cells display strong, preferential SRC1α usage, and HT29 cells display strong SRC1α and SRC1A usage endogenously (Bonham et al., 2000). The signals for c-Src mRNA transcripts containing Exon 1α and Exon 1A were very weak in SW480 cells, but previous experiments agree with the findings from this thesis study that both promoters are utilized very weakly, but nearly equally, in these cells (Bonham et al., 2000). Therefore, it is important that testable models of enhancer action be

designed that can account for all of these observations. Indeed, models for activity of these putative enhancer elements can be derived from the findings presented in this chapter. For example, it is possible that separate HepG2 and HCCL enhancers exist, due to the observation of very different patterns of SRC promoter use seen between these different cell lines. Precedent has been made for this possibility by studies of tissue restricted expression patterns of the apolipoprotein gene cluster. For example, in the case of the apoA-I gene, transgenic approaches led to the identification of two enhancers responsible for liver or intestine specific expression. The region for liver restricted expression was located directly upstream of the transcription start site (Shachter et al., 1993), whereas the region for intestine restricted expression was localized to 9 kb upstream of the apoA-I transcription start site, within the apoC-III gene promoter (Bisaha et al., 1995).

Based on the studies presented, a HepG2 specific enhancer element would be hypothesized to exclusively and strongly activate the SRC1α promoter. As discussed in previous sections, the DH3 element identified in HepG2 cells could account in part or in whole for this enhancer activity. The model proposed for HepG2 specific SRC enhancer activity involves multiple ubiquitous and liver specific trans-acting factors binding to their cognate cis-acting elements, which in turn are able to communicate directly or indirectly with HNF-1α and/or the transcriptional apparatus at the SRC1α promoter (Fig. 4.22 A). HNF-1α has previously been characterized as a transcription factor with very weak transactivation potential; rather, this factor serves as a nucleation point for recruitment of various co-activator molecules that synergistically enhance transactivation (Soutoglou et al., 2000). Therefore, this model predicts a multi-protein

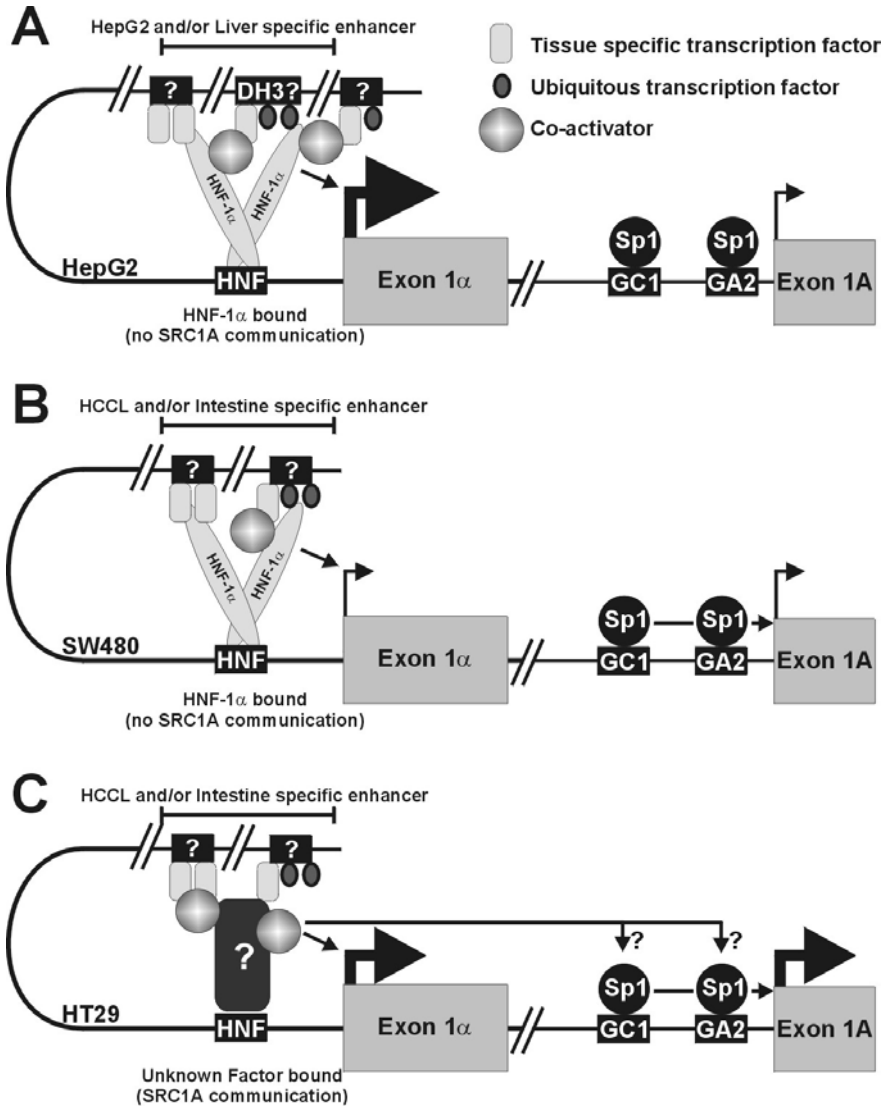

Figure 4.22. Models for differential SRC promoter use and transcriptional activity in human cancer cells. (A) DNA elements that bind tissue specific and ubiquitous transcription factors synergize with HNF-1α and co-activator molecules to elicit very strong SRC1α promoter use in HepG2 cells. **(B)** DNA elements that bind tissue specific and ubiquitous transcription factors cooperate with HNF-1α and co-activator molecules to elicit increased SRC1α promoter use in SW480 cells. **(C)** When a factor other than HNF-1 is bound to the SrcHNF site in HT29 cells, the HCCL specific enhancer complex is much stronger, and mediates very strong activation of both the SRC1α and SRC1A promoters. These models depict DNA elements as existing upstream of the SRC promoters, but they could exist anywhere within the SRC locus. Thickness of the bent arrows represents relative levels of transcriptional activity arising from either SRC promoter.

complex, consisting of DNA-protein and protein-protein interactions, would exist between the SRC1α promoter and the putative enhancer element(s) that would allow for very strong transcriptional activity from the SRC1α promoter. SRC1A promoter activity would be expected to remain at the same levels observed in SRC1A-CAT constructs or in the context of the SRC DPCAT reporters. The end result of enhancer presence in the SRC DPCAT reporter would be the elevation of SRC1α activity to the same level seen endogenously, relative to the SRC1A promoter.

The interplay between factors that bind the SrcHNF site and a putative HCCL specific enhancer form the basis of models explaining differential SRC transcriptional activity and equal use of both promoters in HCCLs. In all HCCLs, regardless of their relative SRC transcription levels, activity of this putative enhancer would elevate the activity of the SRC1α promoter. For example, in SW480 cells, this HCCL specific enhancer element could bind various tissue specific and ubiquitous transcription factors. Because HNF-1α is the major factor interacting with the SRC1α promoter HNF site in these cells, factors binding the HCCL specific enhancer would interact with HNF-1α. This would result in the generation of a weak, SRC1α specific enhancer complex that boosts SRC1α activity to the same levels seen from the SRC1A promoter in isolation or in the context of SRC DPCAT reporters in SW480 cells (Fig. 4.22 B). Conversely, this study has raised the possibility that a novel factor can also bind to the SrcHNF site. This factor, therefore, is hypothesized to be responsible in part for the activation of the SRC1A promoter seen in SRC DPCAT reporter constructs in HT29 cells. This model suggests that communication between the SrcHNF site and the SRC1A promoter takes place through GC1 and/or GA2, the two Sp-family binding sites in the SRC1A

promoter. However, the mechanism of this communication requires experimental investigation. Interaction between factors that bind to the putative HCCL specific enhancer and a novel SrcHNF-bound factor would result in the generation of a very strong enhancer complex (Fig. 4.22 C). Therefore, these models suggest the strength of the HCCL specific enhancer is mediated by whether HNF-1α or other factors are bound to the SrcHNF site. Because the SrcHNF site is "wired" to the SRC1A promoter in HT29 cells, the strong activity of this enhancer would be distributed over both the SRC1α and SRC1A promoters, resulting in the high SRC transcriptional activity observed endogenously. Identification of the proposed HepG2 and/or HCCL specific enhancers will be a key step in testing these models and understanding the mechanisms of SRC transcriptional activation in these human cancer cell lines.

4.2.6.5. Significance of Dual SRC Promoters and Differentially Spliced Exons

The presence of two SRC promoters does not appear to be conserved, and might represent an important difference in the regulation of SRC transcription between humans and other species. Therefore, this implies that two SRC promoters could uniquely exist in humans to allow for increased diversity of c-Src expression in different tissues and/or developmental stages. This is highlighted by the observation that while c-Src expression appears to be ubiquitous in nearly all normal human tissues, c-Src transcripts containing Exon 1α are more restricted to tissues such as stomach, kidney, pancreas, and fetal lung (Bonham et al., 2000). Lower levels of SRC1α-derived transcripts are seen in colon, liver, prostate, fetal kidney, and fetal liver (Bonham et al., 2000). These findings therefore suggest the fairly ubiquitous expression of c- Src in normal tissues is due to

transcription from the SRC1A promoter, since it is regulated by the ubiquitous Sp-family of transcription factors (Philipsen and Suske, 1999), and has a typical make-up of the housekeeping class of promoters (Bonham and Fujita, 1993). SRC transcriptional up-regulation in specific tissues during differentiation, development, or transformation, however, is likely due to increased usage of the SRC1α or even both the SRC1α and SRC1A promoters. This could arise through activation of enhancer elements, or promoter cross-talk mediated by differential binding of factors to the SrcHNF site. These possibilities highlight the importance of future experiments designed to identify and characterize enhancer elements and SrcHNF binding factors in various cell types.

Differential SRC promoter use also results in two c-Src mRNA species that differ in their extreme 5' non-coding terminus. These two different transcripts, however, are identical in their protein coding capacity. Therefore, the possibility exists for further diversity in regulation of human c-Src expression by differential stability or translation potential of these two transcripts. Studies detailed in section 4.1.4 examined c-Src mRNA half-life in HCCLs, and suggested differential c-Src mRNA stability in these cells was not the case. However, there is a strong possibility that the two c-Src mRNA species differ in their translation efficiency. For example, Exon 1α harbors an ATG codon, whereas Exon 1A does not (Bonham and Fujita, 1993; Bonham et al., 2000). Therefore, there is potential for premature engagement of ribosomes at this ATG codon during translation initiation. This phenomenon is common for oncogenes and growth factors, which, like c-Src transcripts, contain relatively long 5' non-coding regions with potential open-reading frames (Willis, 1999). Also, models have been developed that propose the affinity of ribosomal RNA for different mRNAs could also play a role in

regulating translational initiation (Mauro and Edelman, 2002). Clearly, however, these theories must be addressed experimentally before any definitive conclusions can be made regarding differential translation efficiency between 1A or 1α containing c-Src mRNAs.

4.2.6.6. Scope and Significance

This line of investigation described a novel dual promoter reporter system to study the SRC1α and SRC1A promoters in their natural, physiologically linked context in human cancer cell lines. Various experiments with this dual promoter reporter strongly suggested that SRC transcriptional activation in human cancer cell lines results from activation of an enhancer element(s). In addition, these studies identified that the SRC1α and SRC1A promoters are able to communicate in HT29 cells, potentially through a novel factor that binds the SrcHNF site in the SRC1α promoter. Identification of putative enhancer elements, in addition to purifying factors that interact with the SrcHNF site in HT29 nuclear extracts will form the basis for future detailed understanding of the mechanisms of SRC transcriptional activation in colon and other cancer cell lines. This knowledge could lead to the development of therapeutic compounds targeting specific processes that regulate SRC transcription in human cancer. Such strategies would be an important addition to the large arsenal of compounds that have already been developed to target various functions of the c-Src protein (Sawyer et al., 2001).

4.3. Repression of SRC Transcription by Histone Deacetylase Inhibitors

4.3.1. HDIs Directly Repress SRC Transcription

The previous section detailed findings that suggested an enhancer(s) mediates SRC transcriptional activation in colon and liver cancer cell lines. However, the possibility that both SRC promoters were actively repressed in cell lines such as SW480 and SW620 cells was also examined concurrently. The observation that these cell lines expressed very low levels of c-Src mRNA despite expressing levels of HNF-1α, Sp1, and Sp3 similar to HepG2 and HT29 cells strengthened this theory (see Section 4.2.2). The two most well studied mechanisms responsible for such repression are direct hypermethylation of CpG residues, or hypoacetylation of core histones at promoters. These modifications are generally believed to cooperate and elicit a silent, closed chromatin conformation (Richards and Elgin, 2002). Promoters that are contained within CpG islands, such as SRC1A, are especially susceptible to these forms of epigenetic regulation (Antequera et al., 1990; Esteller, 2002). To address the possibility of promoter hypermethylation, SW480 and SW620 cells were treated with the drug 5'-azacytidine, a methylation inhibitor. Following drug treatment, an increase in c-Src mRNA expression was not observed, suggesting promoter CpG hypermethylation was not responsible for the low levels of c-Src expression observed in SW480 or SW620 cells (data not shown). To address the possibility that histone hypoacetylation was responsible for closed, inactive chromatin at the SRC promoter, SW620 cells were treated with the drug Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor (HDI). Surprisingly, a rapid decrease in c-Src mRNA expression was observed following treatment with TSA (Fig. 4.23). Of particular relevance, HDIs have

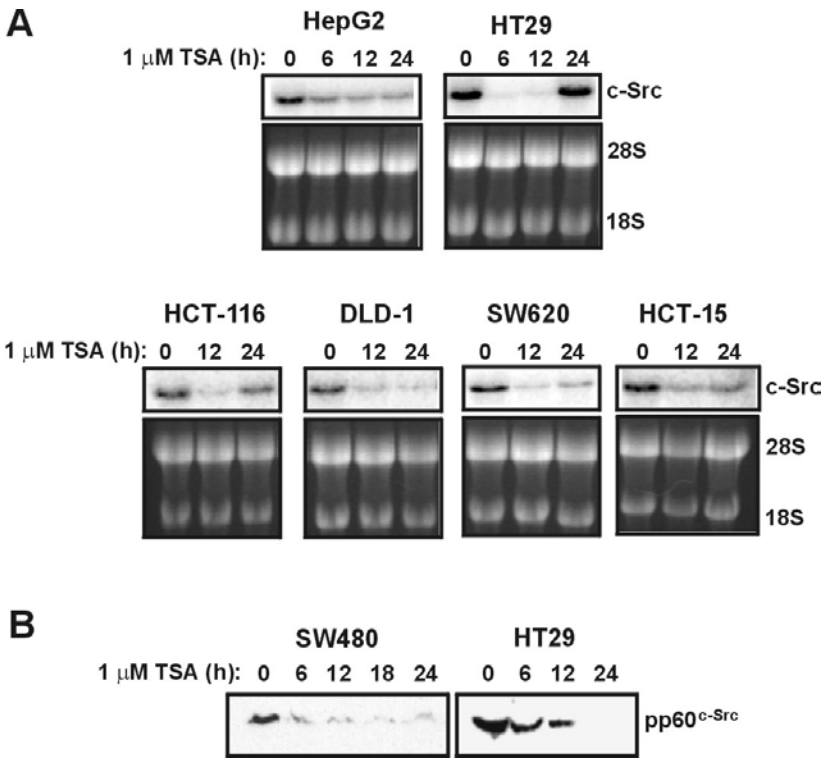

Figure 4.23. Effect of TSA on c-Src mRNA and protein expression in human cancer cell lines. (A) Total RNA was isolated from the indicated cancer cell lines following varying periods of exposure to 1 μM TSA and analyzed by Northern blot with a c-Src specific probe. Equal RNA loading is demonstrated by ethidium bromide stained gels. Northern blots shown are representative of several replicate experiments. **(B)** Total cellular proteins were isolated from SW480 or HT29 cells following various periods of exposure to 1 μM TSA, and analyzed by Western blot for $pp60^{c\text{-}Src}$ expression. Much longer exposures to imaging screens were necessary to obtain signals for c-Src expression in HCT-116, DLD-1, SW620, and HCT-15 cells (2 days) compared with HepG2 and HT29 cells (5 hours).

chemotherapeutic and chemopreventive action towards a wide range of human tumors and tumor derived cell lines (Suzuki et al., 2000; Vigushin et al., 2001). Thus, the anti-neoplastic properties of various HDIs are currently being investigated in clinical trials (Marks et al., 2001a). Interestingly, a very similar degree of c-Src inhibition in SW620 cells was observed following treatment with another HDI, the short chain fatty acid butyrate (Kostyniuk et al., 2002). Butyrate is a natural component of the human large intestine, and is created by anaerobic bacterial fermentation of dietary fibre (Topping and Clifton, 2001). This observation has prompted the theory that butyrate production could in part explain the preventative action of high-fibre diets towards colon tumors (Reddy, 1995; Trock et al., 1990). Indeed, this idea has been supported by the demonstration that butyrate can protect from carcinogen-induced colon tumorigenesis in a rat model system (D'Argenio et al., 1996). Significantly, and has been discussed in previous sections, c-Src has been strongly implicated in the development and progression of various human cancers, especially colon cancer. These observations led to the hypothesis that the decrease in c-Src mRNA expression elicited by HDI treatment may explain in part these agents' anti-cancer activity. A major interest therefore became elucidating the mechanism of c-Src repression by HDIs.

The effect of HDIs on c-Src expression was examined in various additional cancer cell lines (Fig. 4.23). Regardless of whether cell lines displayed high levels of c-Src mRNA (HT29, HepG2), or low levels of c-Src mRNA (HCT-116, DLD-1, SW620, HCT-15), a rapid and effective decrease in c-Src expression was observed following treatment with 1 μM TSA (Fig. 4.23 A). A concomitant decrease in c-Src protein expression was also observed following TSA treatment (Fig. 4.23 B). The response to

TSA at the mRNA level was observed to be transient in most cell lines studied, and was attributed to a short cellular half-life of this agent. Again, nearly identical results were observed when these same cell lines were treated with butyrate (Kostyniuk et al., 2002). By treating HT29 cells with a combination of cyclohexamide and butyrate, it was demonstrated that the decrease in c-Src mRNA expression was direct, and did not require new protein synthesis (Kostyniuk et al., 2002). Therefore, HDIs directly inhibited c-Src mRNA and protein expression in various human cancer cell lines.

To determine if the repressive effect of HDIs on c-Src expression was at the level of transcription, the 0.38 SRC1A-CAT and -145 SRC1α-CAT reporter plasmids were assessed for their response to these agents in transient transfection experiments in HepG2 and SW480 cells (Fig. 4.24). Following 24 hours of treatment with either 1 mM TSA or 5 mM butyrate, CAT levels from both promoter constructs were diminished 80 to 90 % relative to untreated controls. Therefore, TSA and butyrate were exerting a very similar level of repression on the transcriptional activity of either SRC promoter. These results suggested that repression of the SRC promoters was primarily responsible for the decreased c-Src expression observed upon HDI treatment. In large part, however, the mechanism of gene repression elicited by these agents has not been described.

4.3.2. Effect of HDIs on Factors bound to the SRC Promoters

One possibility that could explain SRC repression by HDIs could be that these agents inhibit binding of factors to the SRC promoters. Therefore, to determine whether HDI treatment caused decreased binding of HNF-1α, Sp1, or Sp3 to their cognate cis-acting DNA elements, EMSAs were performed using nuclear extracts from HepG2 cells

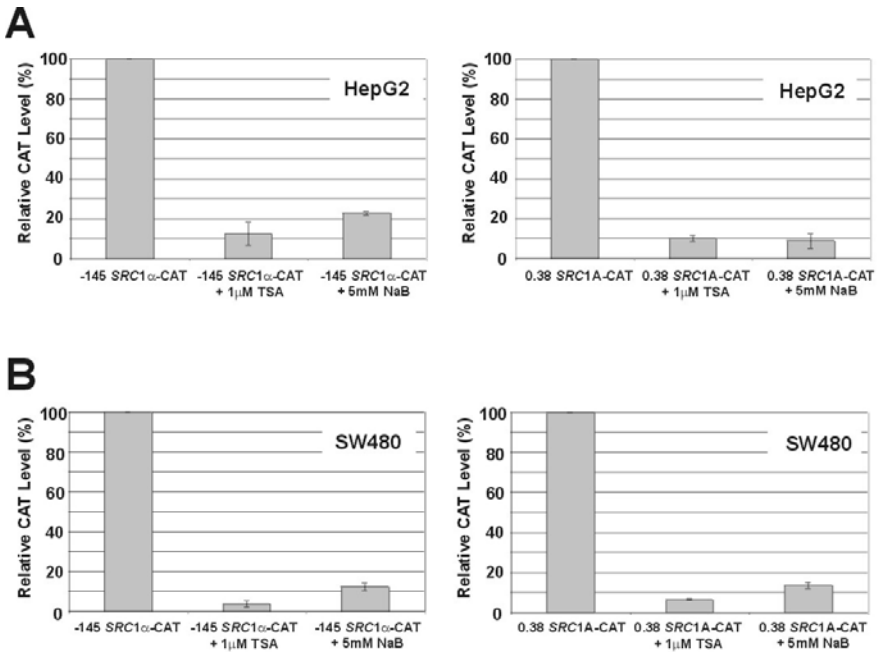

Figure 4.24. Effect of HDIs on activities of the SRC promoters. HepG2 **(A)** and SW480 **(B)** cells were transfected with SRC1α-CAT and SRC1A-CAT reporter plasmids, and treated with 5 mM sodium butyrate (NaB) or 1 μM TSA for 24 h. CAT expression levels are shown relative to untreated cells. Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate.

treated with TSA for various time points (Fig. 4.25). Large promoter restriction fragments encompassing the SRC1α or SRC1A promoters were used as probes in these experiments (Fig. 4.25 A). As shown in Fig. 4.25 B, when a *Cla* I/*Hinc* II fragment representing the SRC1α promoter was incubated with untreated or treated HepG2 nuclear extracts, two major complexes were observed, which have been previously identified as containing HNF-1α (Bonham et al., 2000). Treatment with TSA for up to 12 hours did not result in an observable change in HNF-1α binding to the SRC1α promoter. When SRC1A promoter fragments encompassing the GC1 and GA2 Sp-family binding sites were utilized as EMSA probes, major species previously characterized as Sp1 or Sp3 complexes were observed (Fig. 4.25 B and C). Similar to the results observed for the SRC1α promoter, there was no observable change in Sp1 or Sp3 binding to these SRC1A promoter fragments. These results therefore demonstrated the repression of activities of the SRC promoters was not due to any obvious changes in binding of transcription factors to their respective cis-acting elements *in vitro*.

HDIs have been shown to effect changes in chromatin structure, which results from altered histone acetylation and deacetylation dynamics (Marks et al., 2000). Therefore, the effect of TSA treatment on the acetylation status of histone H3 at the SRC promoters was determined using a chromatin immunoprecipitation (ChIP) approach. Hyperacetylation of promoter bound histone H3 at lysines 9 and 14 is highly correlated with active transcription, due to a proposed role in aiding TFIID recruitment (Agalioti et al., 2002). Therefore, any change or paradoxical decrease in the acetylation of histone H3 elicited by HDIs would be expected to influence SRC transcriptional activity. An antibody specific for histone H3 acetylated at Lys 9 and Lys 14 was employed for

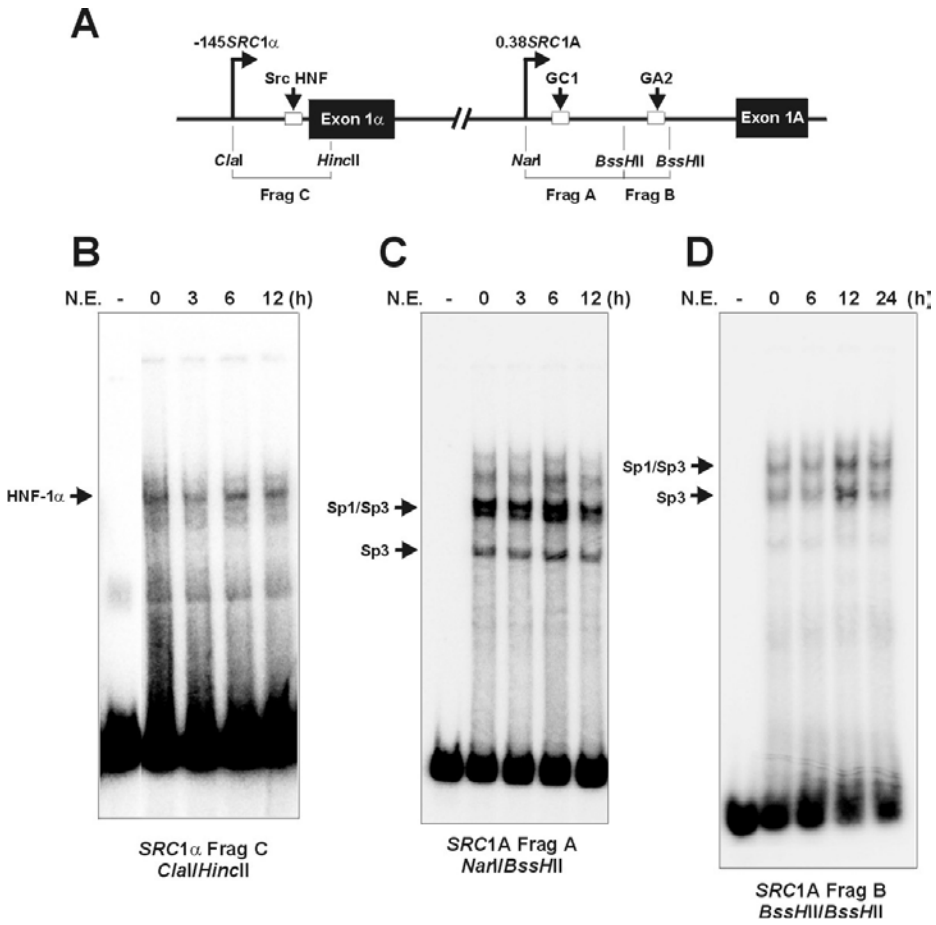

Figure 4.25. Effect of HDIs on binding of factors to the SRC1α and SRC1A promoters. (A) Schematic of the origin of probes used for EMSA analysis. **(B-D)** Nuclear extractions were performed on HepG2 cells exposed to 1 μM TSA for varying periods of time. EMSA analysis was subsequently performed by incubating these nuclear extracts with a probe spanning the SRC1α promoter **(B)**, or probes spanning the SRC1A promoter **(C,D)**. Complexes that have previously been identified as containing the transcription factors Sp1, Sp3, and HNF-1α are labeled.

immunoprecipitation of cross-linked protein-DNA complexes from treated and untreated HT29 (Fig. 4.26 A) and SW480 (Fig. 4.26 B) cells. Following detection via PCR using primers specific for the SRC1α or SRC1A promoter, acetylated histone H3 was found to be associated with both promoters in untreated SW480 and HT29 cells. In addition, there was no observable change in this pattern of acetylated H3 association in cells that had been treated with TSA for up to 6 hours. Therefore, these results suggest HDIs were not affecting the acetylation pattern of histone H3 at either SRC promoter. Combined with EMSA experiments, no change in the factors associated with either SRC promoter was observed following treatment with HDIs.

4.3.3. Search for Distinct 5' HDI Response Elements in the SRC Promoters

Previous studies with the WAF1 promoter have implicated Sp-family binding sites in transcriptional activation following HDI treatment (Huang et al., 2000; Nakano et al., 1997). Therefore, the role of the SRC1A GC1 and GA2 elements in HDI-mediated repression was assessed. In most circumstances, assessing the roles of Sp1 and/or Sp3 in mammalian transcription is difficult via co-transfection experiments due to the high endogenous levels of these transcription factors. This potential pitfall prompted an alternative approach, using an engineered SRC1A promoter containing GAL4 recognition sites, in co-transfection experiments with GAL4-Sp-family fusions. An additional benefit of this approach is it allows for a separate analysis of the effect of Sp1 or Sp3 on SRC1A transcription. To this end, the GC1 and GA2 sites were replaced in a 0.38 SRC1A promoter reporter construct with binding sites for the GAL4 yeast transcription factor (Fig. 4.27 A), and transfections in HepG2 cells performed (Fig. 4.27

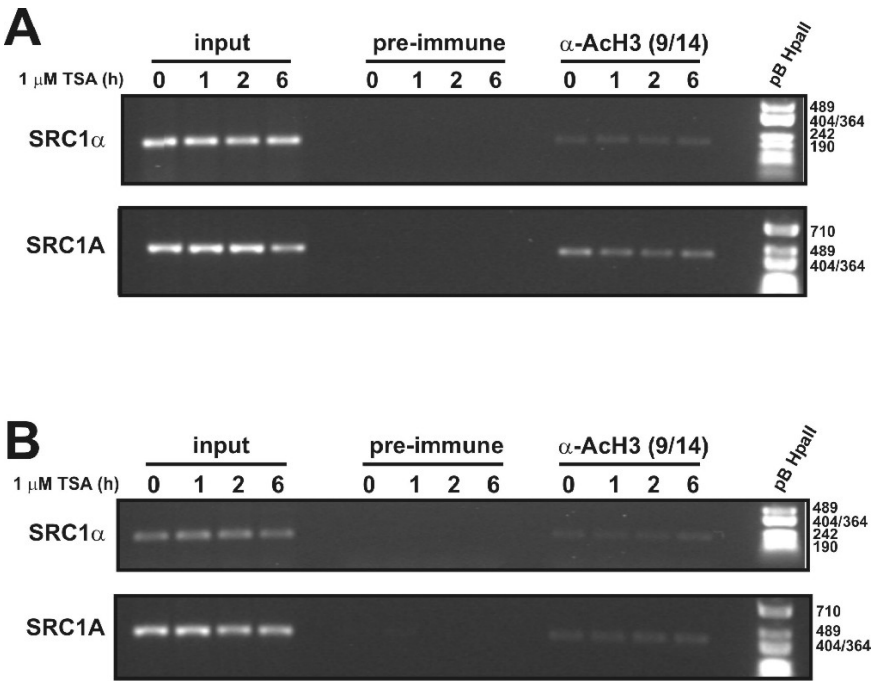

Figure 4.26. Effect of HDIs on acetylation of histone H3 at the SRC promoters. ChIP assays were performed on HT29 **(A)** and SW480 **(B)** cells that had been treated with 1 μM TSA for various periods of time. Following cross-linking of proteins bound to DNA, immunoprecipitations were performed on cell lysates with purified pre-immune rabbit IgG, or a monoclonal antibody specific for histone H3 acetylated at lysines 9 and 14. Following reversal of crosslinks, immunoprecipitates were subjected to PCR with primers specific for the SRC1α or SRC1A promoters.

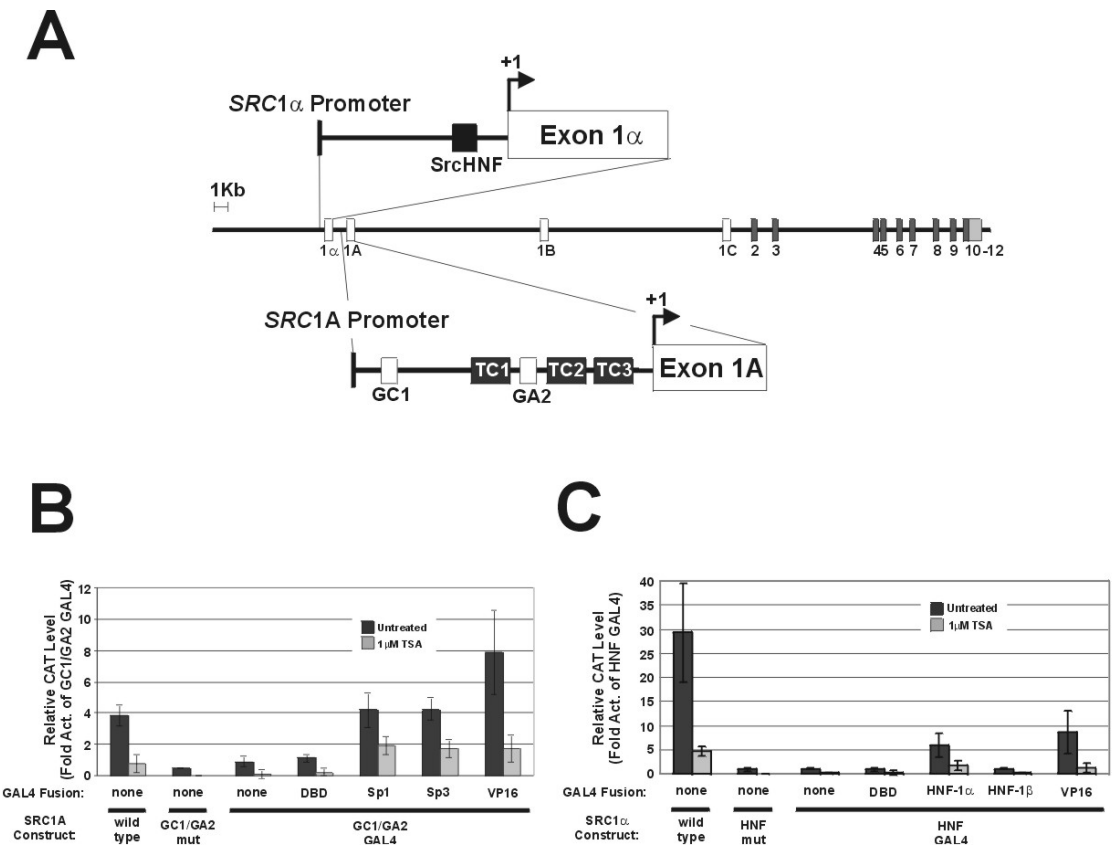

Figure 4.27. Functional necessity of the Sp- and HNF-binding sites for HDI-mediated SRC transcriptional repression. (A) Schematic of the SRC locus. The GC1 and GA2 sites in a SRC1A promoter construct, and the HNF site in a SRC1α promoter construct were employed in this study. **(B)** The *SRC*1AΔGC1/GA2-GAL4 promoter was transactivated with various GAL4-transcription factor fusions, and the response to 24 hr treatment with 1 μM TSA assessed in HepG2 cells. **(C)** The SRC1αΔHNF-GAL4 promoter was transactivated with various GAL4-transcription factor fusions, and the response to treatment with 1 μM TSA assessed in HepG2 cells. Bar graphs represent the mean +/- the standard deviation from four separate experiments, each performed in duplicate.

B). GAL4 replacement impaired SRC1A activity to a similar degree as mutation of these sites. Following co-transfection with GAL4-Sp1 or GAL4-Sp3 fusions, but not the GAL4 DNA binding domain (DBD) alone, SRC1A activity was restored to levels of the wild-type promoter. This represented the first evidence that Sp3, like Sp1, could activate the SRC1A promoter in mammalian cells. When these transfected cells were exposed to TSA, SRC1A activity was consistently repressed regardless of whether the promoter construct was analyzed alone, or if an Sp1 or Sp3 GAL4 fusion was employed for co-transfection. Strikingly, when the SRC1A GC1/GA2-GAL4 promoter was transactivated by GAL4 fused to the strong viral VP16 activator, repression was still observed following TSA treatment. These findings suggested the repressive effects of TSA were not specific for Sp1 or Sp3 binding to GC1 or GA2. Therefore, TSA was not inhibiting SRC1A activity through the Sp-family binding sites. A similar approach was taken to analyze the HNF-1α binding site in the SRC1α promoter (Fig. 4.27 A and C). As shown in Figure 4.27 C, a GAL4-HNF-1α fusion, but not a GAL4-HNF-1β fusion or the GAL4 DBD alone, was able to moderately transactivate the SRC1α HNF-GAL4 promoter. However, regardless of whether the SRC1α HNF-GAL4 construct was transactivated by GAL4-HNF-1α or GAL4-VP16, it was still significantly repressed by TSA. These findings suggested the SrcHNF site in the SRC1α promoter was not responsible for mediating the repressive effect of HDIs.

Next, it was determined if element(s) other than GC1 or GA2 were responsible for mediating the inhibitory effects of TSA on the SRC1A promoter. Obvious candidate elements were the SPy/hnRNP K binding sites, TC1, TC2, and TC3. A number of 5' and TC1, TC2, and TC3 SRC1A internal promoter deletions based on the 0.38 SRC1A-CAT

promoter construct have been previously analyzed in transfection experiments (Bonham and Fujita, 1993; Ritchie et al., 2000). In this thesis study, deletion of TC1 had a pronounced effect on SRC1A promoter activity in HepG2 cells, diminishing CAT levels approximately 60% (Fig. 4.28). However, regardless of the internal deletion generated, these constructs were still all further repressed following treatment with TSA. A 0.2 SRC1A-CAT reporter was subsequently created, which had the entire SRC1A promoter 5' to the TC3 element deleted (Fig. 4.28). Despite the very low activity of this truncated SRC1A reporter construct, repression was still observed following treatment with TSA. In summary, a distinct 5' TSA response element, which would block the repressive effects of TSA when deleted, was not observed in either SRC promoter. These findings therefore implicate SRC core promoter elements in mediating the repressive effects of TSA.

4.3.4. Search for Distinct HDI Response Elements in the SRC Core Promoters

4.3.4.1. SRC Core Promoter Architecture

The major transcription initiation site in the SRC1α promoter maps to a CCA(+1)GGCT motif 39 bp downstream from the SrcHNF site in HepG2 and HT29 cells (Fig. 4.29 A) (Bonham et al., 2000). Alternatively, previous analysis of the SRC1A promoter determined that transcription was initiated from multiple weak start sites (Bonham and Fujita, 1993). However, because these observations were generated in cells other than those used in this study, S1 nuclease protection analysis was employed to determine the major SRC1A transcription start sites in HepG2, HT29, and SW480 cells (Fig. 4.29 B). Three major sites of transcription initiation were mapped to

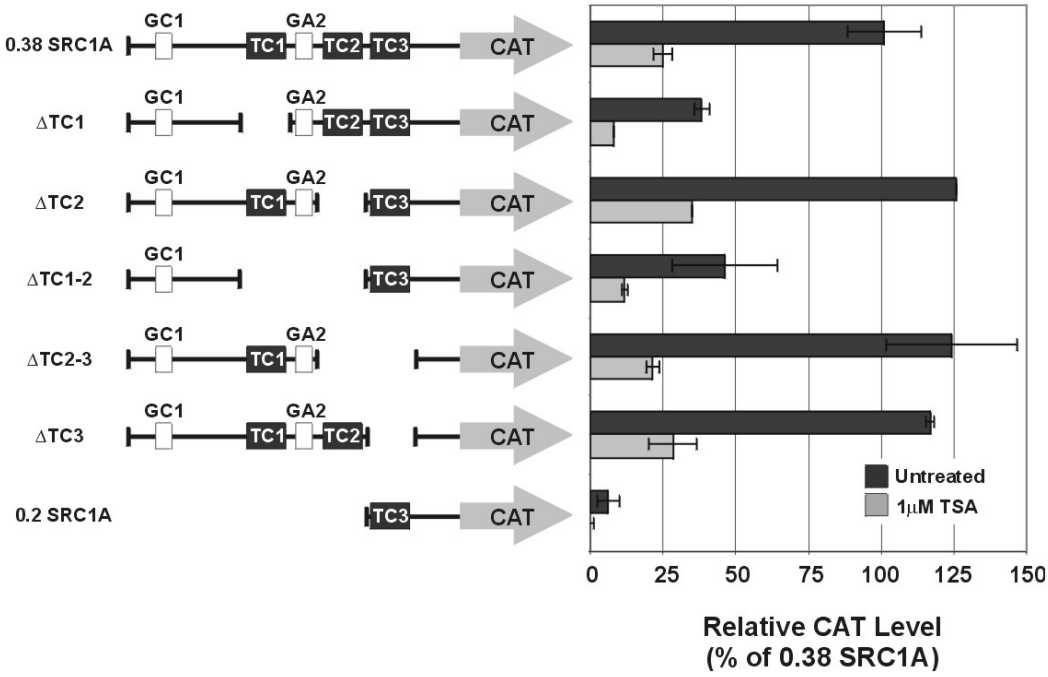

Figure. 4.28. Search for a 5' TSA response element. Various SRC1A constructs harboring deletions in upstream activation sequences were evaluated for their response to 1 μM TSA in HepG2 cells. Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate.

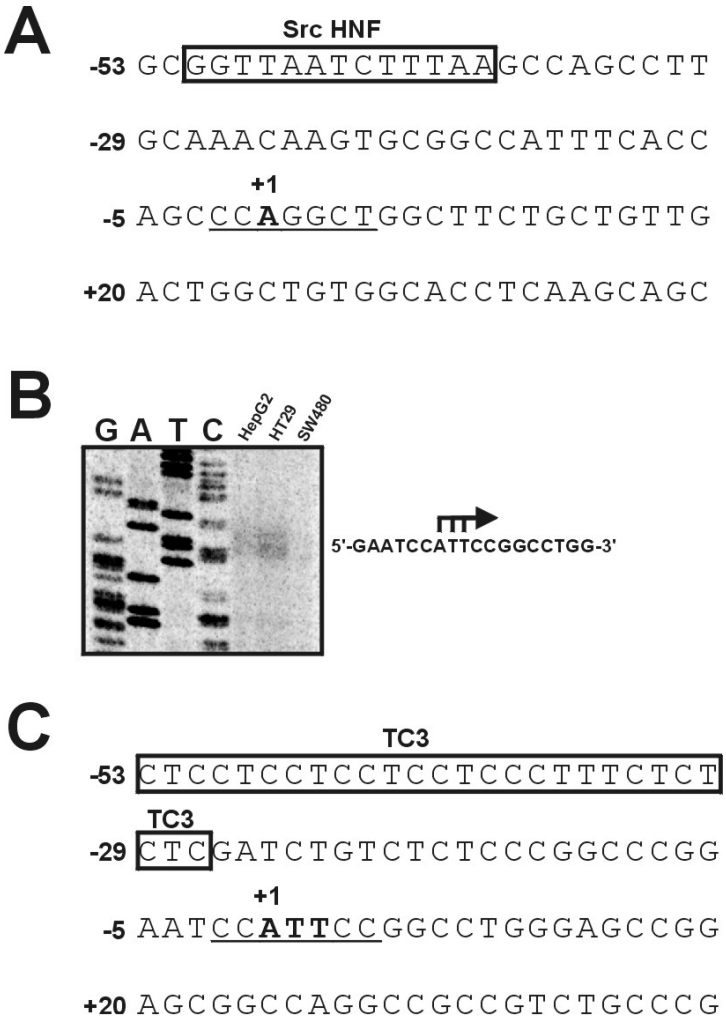

Figure 4.29. Inr elements in the SRC core promoters. (A) Sequence of the SRC1α core promoter regions surrounding the SRC1α transcription start site. The Inr element is underlined. **(B)** S1 protection analysis was performed to determine the initiation sites for transcription in the SRC1A promoter in HepG2, HT29, and SW480 cells. The residues mapped to the extreme 5' termini of exon 1A-containing transcripts are denoted with bent black arrows on the right. **(C)** Sequence of the SRC1A core promoter regions surrounding the SRC1A transcription start site. The Inr element is underlined.

a core CCA(+1)TTC in these cells, 27 bp downstream from the TC3 tract in the SRC1A promoter (Fig. 4.29 C). This SRC1A transcription start site core perfectly matched the Inr consensus sequence of YYA(+1)NTYY (Smale et al., 1998). Furthermore, the SRC1α transcription start site core loosely matched this consensus motif. These findings suggested that both SRC promoters were TATA-less, but Inr driven. It was therefore hypothesized that this commonality in SRC core promoter architecture might explain why both of these very different promoters were repressed by HDIs.

4.3.4.2. Effect of SRC1A and SRC1α 3' Deletions on TSA Mediated Repression

The role the SRC core promoter elements played in SRC1A and SRC1α transcription, as well as their response to HDIs were addressed by analyzing a series of SRC promoter-CAT constructs harboring deletions in the core promoter regions (Figs. 4.30 A and C). As shown in Fig. 4.30 B, 3' deletion of the SRC1A promoter to the *Sac*II site and up to +13 had a much more pronounced effect in SW480 cells compared to HepG2 cells. Deletions that eliminated the Inr element from the SRC1A promoter (Δ-26, Δ-60) nearly abolished all detectable transcriptional activity in both these cell lines, thus supporting the hypothesis that the SRC1A promoter was Inr driven. However, despite these observations, core promoter deletions were unable to block transcriptional repression in response to TSA treatment. Deletions in the SRC1α core promoter had a very similar effect to deletions in the SRC1A core promoter, and exclusion of the Inr element (Δ-20) severely compromised promoter activity (Fig. 4.30 D). Again, regardless of the effect the core promoter deletions had on transcriptional activity, further repression was still consistently observed following treatment with TSA. These

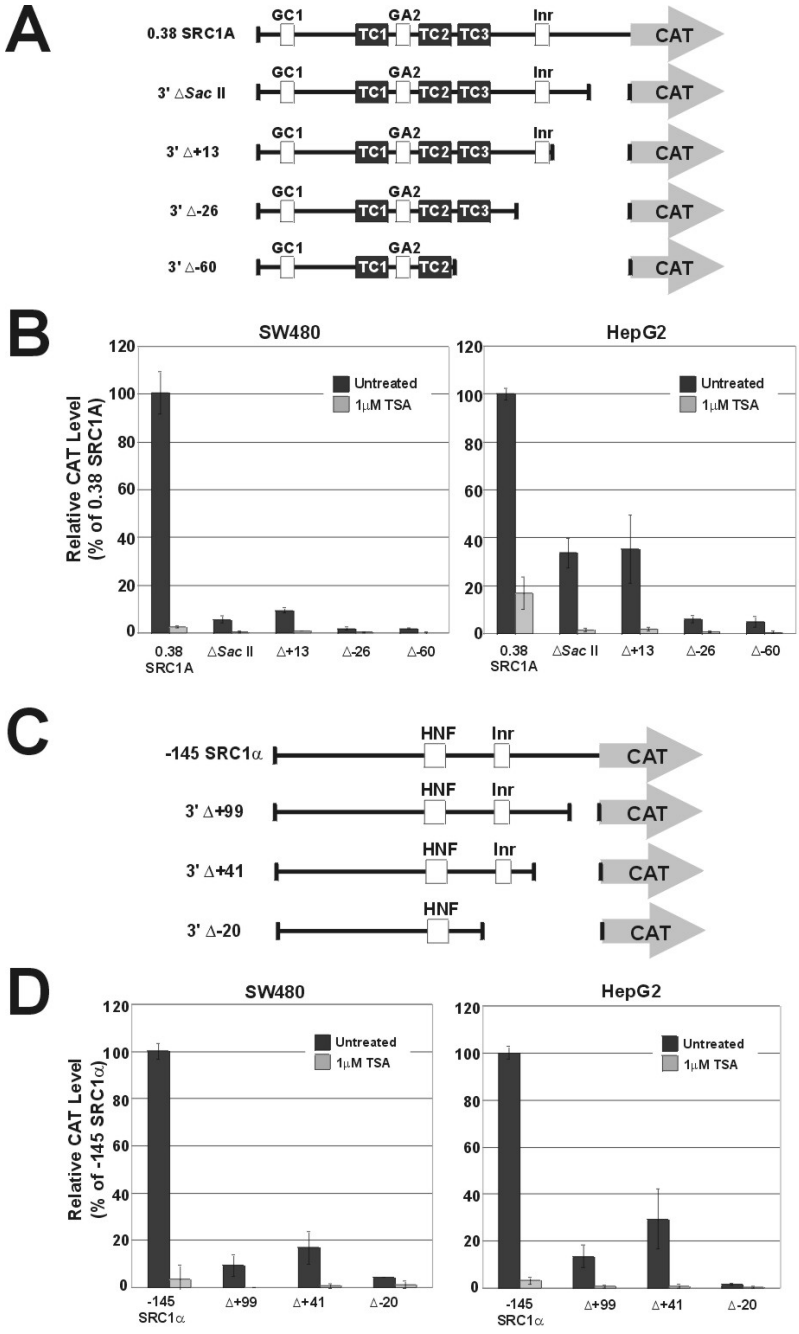

Figure 4.30. Effect of SRC core promoter deletions on TSA mediated repression. **(A)** SRC1A 3' deletion constructs. **(B)** SRC1A 3' deletion constructs were transfected in HepG2 and SW480 cells and analyzed for their response to 1 μM TSA. **(C)** SRC1α 3' deletion constructs. **(D)** SRC1α 3' deletion constructs were transfected in HepG2 and SW480 cells and analyzed for their response to 1 μM TSA. Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate.

data suggested a distinct core promoter element in either SRC promoter was not responsible for mediating the effects of HDIs. The SRC1A promoter was systematically deleted in its entirety in this study (Figs. 4.28 and 4.30 A,B), and no single deletion resulted in a significant change in its response to TSA. These 5' and 3' deletion approaches, therefore, were unable to identify any distinct element that was responsible for HDI mediated SRC transcriptional repression. This suggested some other common, inherent property of the SRC promoters was responsible for their repression by HDIs.

4.3.5. SRC1A and SRC1α Core Promoter Characterization

4.3.5.1. Binding of TAF1 to the SRC Core Promoters

Although discrete TSA response elements were not identified in either SRC promoter, the commonality in their core architecture still provided an attractive rationale for their inhibition by HDIs. A major inherent property of many promoters with Inr elements is their dependence on the TFIID component, TAF1, for full activity. Significantly, TAF1 possesses AT activity, which could potentially play a role in HDI-mediated SRC repression. Therefore, to address whether the SRC core promoters could bind TAF1, EMSAs were performed with a recombinant TAF1-TAF2 heterodimer. These experiments were designed by the candidate, and subsequently performed by collaborators Dr. Edith Wang and Traci Hilton at the University of Washington. The sequences of the double stranded oligonucleotides utilized as labeled EMSA probes and unlabeled competitors are shown in Fig. 4.31 A. As shown in Fig. 4.31 B and C, both the wild-type SRC1α and SRC1A core promoters were able to bind recombinant TAF1-TAF2. This binding was effectively competed away with an excess of unlabeled probe

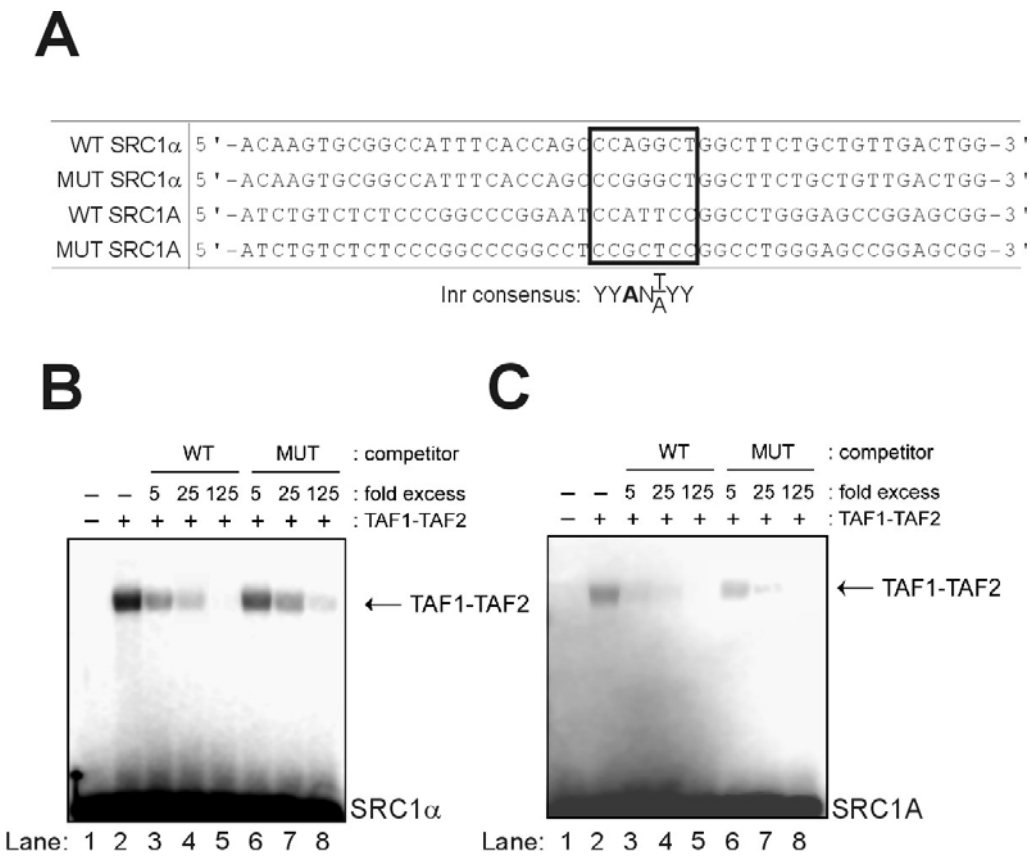

Figure 4.31. TAF1 binding to the SRC core promoters. (A) Oligonucleotides used in EMSA experiments. EMSAs were performed using a recombinant TAF1-TAF2 heterodimer and [^{32}P]-labeled probes representing the SRC1α **(B)** or SRC1A **(C)** core promoters. Competitions were performed using excess unlabeled wild type probe (lanes 3-5), or unlabeled duplexes harboring mutations in the Inr cores (lanes 6-8).

(Figs 4.31 B and C, lanes 3-5). However, unlabeled double stranded oligonucleotides representing Inr mutant forms of the SRC1α or SRC1A core promoters were less efficient competitors for TAF1-TAF2 binding (Figs. 4.31 B and C, compare lanes 6-8 to lanes 3-5). These studies showed the SRC1α and SRC1A core promoters bind a TAF1-TAF2 heterodimer *in vitro*, and that the Inr core plays a role in this binding.

4.3.5.2. TAF1 Dependence of the SRC Core Promoters

Given the SRC1α and SRC1A core promoters contain Inr elements and bind TAF1-TAF2, it was important to determine if SRC transcriptional activity was dependent on functional TAF1. A useful tool for studying TAF1 function is the BHK-21 (baby hamster kidney) derived tsBN462 cell line, which harbors a G690D mutation in the TAF1 protein (Hayashida et al., 1994). These cells grow normally at 33^{o}C, but undergo G1/S arrest when shifted to the restrictive temperature of 39^{o}C. At the permissive temperature, G690D TAF1 retains wild-type function; however, at 39^{o}C, G690D TAF1 AT (Dunphy et al., 2000) as well as Inr binding (Hilton and Wang, 2003) activities are compromised. A shift of these cells from 33^{o}C to 39^{o}C results in the activation of genes encoding p21 and p27, and reduction in the expression of cyclins D1 and A (Rushton et al., 1997; Sekiguchi et al., 1996; Suzuki-Yagawa et al., 1997). These transcriptional responses are generally believed to exert the halt in cell cycle progression. Indeed, cell cycle arrest at 39^{o}C in ts13 cells, which are similar to tsBN462 cells, can be rescued by expression of wild-type TAF1, suggesting disruption of TAF1 activity is directly responsible for this temperature sensitive phenotype (Wang and Tjian, 1994).

Transient transfections were therefore performed with SRC1α and SRC1A CAT reporters in tsBN462, as well as parental BHK-21 cells. Following a shift in growth temperature from 33°C to 39°C, the activity of the SRC1A promoter was decreased in tsBN462 but not BHK-21 cells (Fig. 4.32 A). This decrease in SRC1A activity at 39°C in tsBN462 cells was partially rescued by co-expression of wild-type TAF1 (Fig 4.32 B). Interestingly, however, the SRC1A promoter was no longer repressed by TSA or butyrate at the restrictive temperature in tsBN462 cells (Fig. 4.32 A). Conversely, this promoter construct was still repressed by HDIs at 39°C in the parental BHK-21 cell line. This was the first time repression of either SRC promoter was not observed in response to HDIs. These data therefore suggested the SRC1A promoter was TAF1 dependent, and compromised TAF1 activity blocked the repressive effects of HDIs on this promoter. Similar experiments could not be performed with the SRC1α promoter because it was found to have lower activity at 39°C compared to 33°C in both tsBN462 and BHK-21 cells (Fig. 4.32 C). As a result, the decrease in SRC1α activity following a shift from 33°C to 39°C in tsBN462 cells was not rescued by wild-type TAF1 co-expression (Fig. 4.32 D).

Previous studies have shown that the G690D TAF1 mutation compromises both AT activity and core promoter Inr binding at the restrictive temperature (Dunphy et al., 2000; Hilton and Wang, 2003). Therefore, to address if the decrease in SRC promoter activity following shift to the restrictive temperature in tsBN462 cells was due to reduced TAF1 core promoter binding, binding reactions were carried out using the SRC1A and SRC1α core promoters with wild-type or G690D mutant TAF1-TAF2 heterodimers at 25°C or 37°C. These temperatures have previously been shown to be

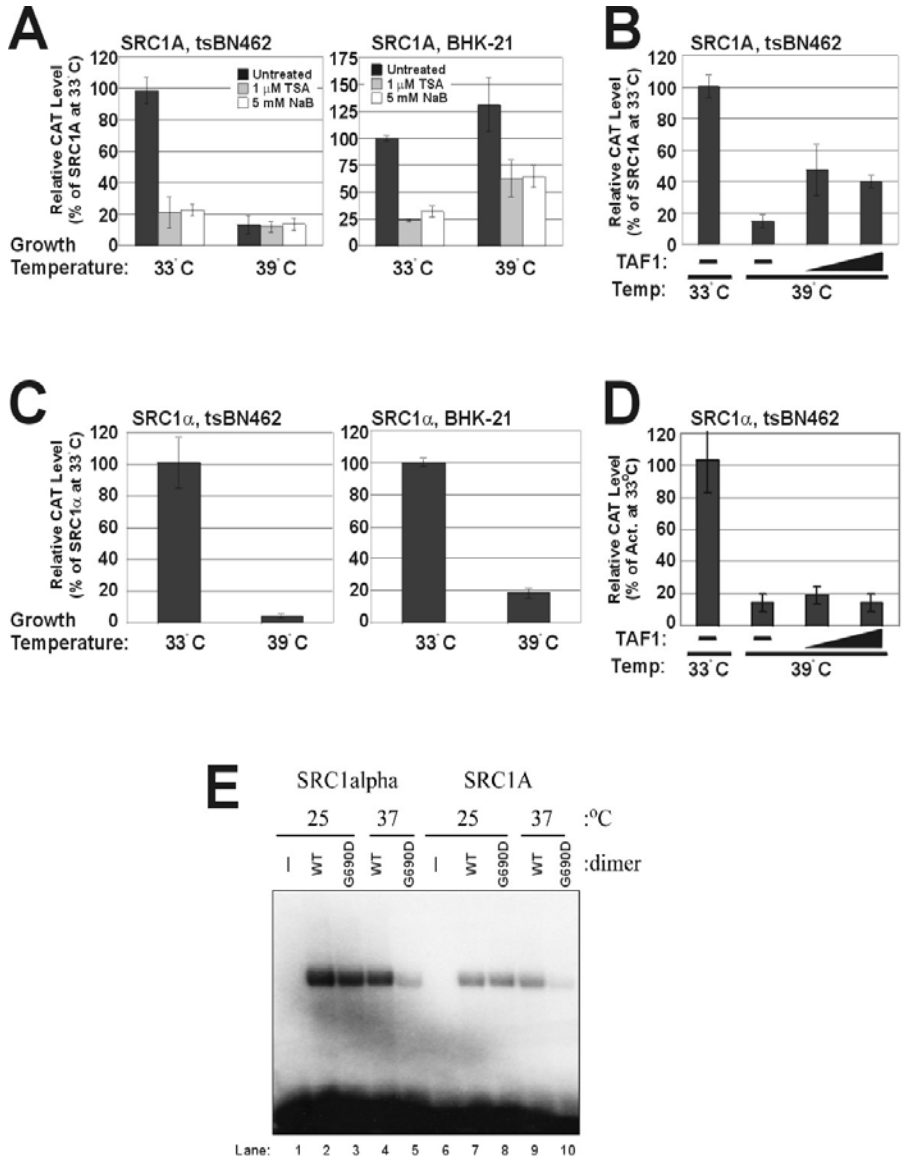

Figure 4.32. TAF1 dependence of the SRC promoters. (A) tsBN462 and BHK-21 cells were transfected with 0.38 SRC1A-CAT and grown at 33°C for 36 hours. Following treatment with 1 μM TSA or 5 mM sodium butyrate, transfected cells were grown at 33°C (permissive) or 39°C (restrictive) for 18 hours. CAT levels were subsequently determined. **(B)** tsBN462 cells were transfected with 0.38 SRC1A-CAT with or without CMV-hTAF(II)250. Following 36 hours of growth at 33°C, cells were shifted to 39°C, and grown for an additional 18 hours. CAT levels were subsequently determined. **(C)** tsBN462 and BHK-21 cells were transfected with -145 SRC1α-CAT and grown at 33°C for 36 hours. Cells were then shifted to 39°C, or maintained at 33°C for an additional 18 hours. CAT levels were subsequently determined. **(D)** tsBN462 cells were transfected with -145 SRC1α-CAT, as described in (B). Bar graphs represent the mean +/- the standard deviation from three separate experiments, each performed in duplicate. **(E)** EMSAs were performed at 25°C or 37°C with a wild type or G690D mutant TAF1 in a TAF1-TAF2 heterodimer and [^{32}P]-labeled probes representing the SRC1α or SRC1A core promoters.

permissive and restrictive, respectively, for *in vitro* transcription reactions with ts13 nuclear extracts (Wang and Tjian, 1994). As with previous TAF1 EMSA analysis, these experiments were designed by the candidate, and performed by Dr. Edith Wang and Traci Hilton at the University of Washington. Strong binding of TAF1-TAF2, containing either wild type or G690D forms of TAF1, to the SRC1α and SRC1A core promoters was observed at 25°C (Fig. 4.32 E). However, binding of the TAF1-TAF2 dimer containing G690D TAF1 to both SRC core promoters was compromised at 37°C. Conversely, the TAF1-TAF2 dimer containing wild type TAF1 did not display a decrease in binding to the SRC core promoters at this temperature. These data suggest the decrease in SRC promoter activity at the restrictive temperature in tsBN462 cells is due to G690D TAF1 inability to bind the core promoter in addition to the well-documented loss of AT activity.

In summary, these sets of experiments clearly showed the SRC1A promoter is TAF1 dependent. Results for the SRC1α promoter were less definitive due to the observation of a decrease in activity following a shift from 33°C to 39°C in both tsBN462 and BHK-21 cells. However, the ability of TAF1 to bind the SRC1α core promoter strongly suggested that this promoter was also TAF1 dependent. This was supported by the observation that binding of the G690D TAF1 mutant to the SRC1α core promoter was compromised at the restrictive temperature of 37°C.

4.3.6. Analysis of SRC:WAF1 Chimeric Promoters

4.3.6.1. Response of SRC:WAF1 Promoter Chimeras to TSA

The previous section detailed findings that suggested the SRC core promoters were both Inr driven and TAF1 dependent, and implicated a role for TAF1 in the repression of the SRC promoters by HDIs. An important question therefore was whether the TAF1 dependence of the SRC promoters was responsible for their repression in response to HDIs. Interestingly, previous studies with mammalian cells have shown that both core promoter elements and upstream activation sequences from a TAF1 dependent promoter can independently confer TAF1 dependence on a normally TAF1 independent promoter (Wang et al., 1997). It was therefore asked whether upstream activation sequences and core promoter elements from the SRC promoters could independently confer HDI mediated repression on a TAF1 independent, heterologous promoter normally activated by HDIs. To this end, a series of chimeric SRC:WAF1 promoter CAT reporters were generated, and analyzed for their response to HDIs following transfection in HT29 and SW480 colon cancer cells (Fig. 4.33). Previous studies have shown the WAF1 promoter contains a consensus TATA box (Fig. 4.33 A), and a crucial Sp-family binding element, Sp1-3, which mediates its activation following HDI treatment (Huang et al., 2000; Nakano et al., 1997). In agreement with these studies, two WAF1 promoter CAT constructs, -210 WAF1-CAT and -101 WAF1-CAT, were significantly induced following TSA treatment in transfected HT29 cells (Fig. 4.33 B). Conversely, in SW480 cells, constitutively strong WAF1 promoter activity was observed, which was not induced following TSA treatment (Fig. 4.33 B). However, when the WAF1 core promoter was replaced in the -210 WAF1 or -101 WAF1 constructs with either the SRC1A (Fig. 4.33 B) or SRC1α (Fig. 4.33 C) core

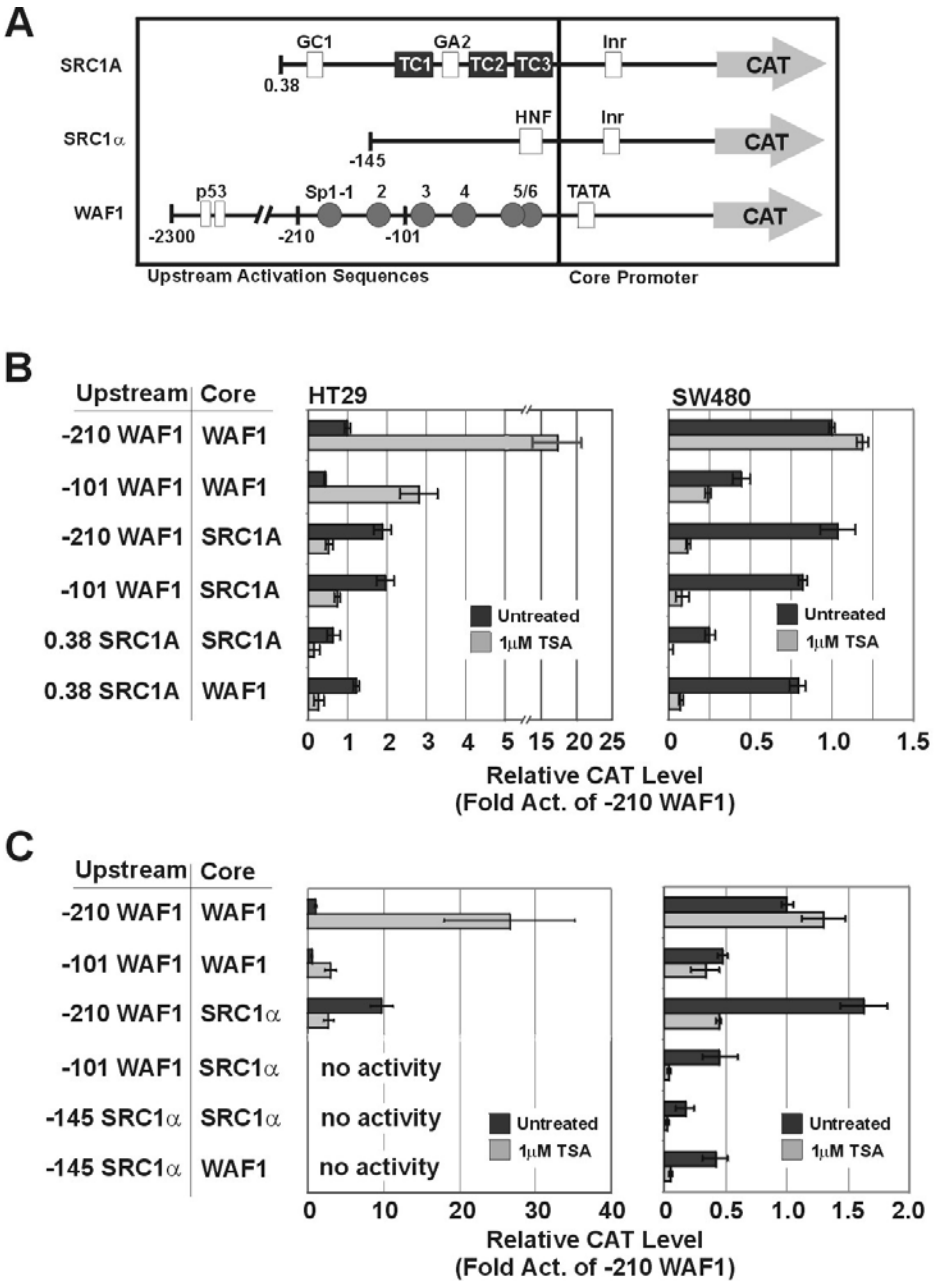

Figure 4.33. Response of SRC:WAF1 promoter chimeras to TSA. (A) Schematic of the SRC1A, SRC1α, and WAF1 promoter constructs utilized to generate chimeras. **(B)** Wild type SRC1A and WAF1 promoter constructs depicted in (A), as well as various promoter chimeras, were assessed for their response to 1 μM TSA following transfection in HT29 and SW480 cells. **(C)** Wild type SRC1α and WAF1 promoter constructs depicted in (A), as well as various promoter chimeras, were assessed for their response to 1 μM TSA following transfection in HT29 and SW480 cells. Bar graphs represent the mean +/- the standard deviation from three separate experiments, each performed in duplicate.

promoters, repression was observed following TSA treatment in both HT29 and SW480 cells. With these chimeras, special care was taken to ensure the Inr elements from the SRC core promoters were positioned the same distance away from the Sp1-6 site as the major site of transcription initiation observed with the wild type WAF1 promoter A similar repression was observed upon TSA treatment when the WAF1 upstream activation sequences were replaced in these constructs with SRC1A (Fig. 4.33 B) or SRC1α (Fig. 4.33 C) upstream activation sequences. With these chimeras, spacing was selected such that the WAF1 TATA element was positioned the exact same distance from the TC3 or HNF sites as the Inr elements in the SRC1A and SRC1α core promoters, respectively. In these sets of experiments, it was observed that the -145 SRC1α, -101 WAF1:SRC1α, and -145 SRC1α:WAF1 reporters had extremely low activities in HT29 cells. Taken together, these results demonstrated that core promoter elements and upstream activation sequences from both the SRC1A and SRC1α promoters could independently confer HDI-mediated repression on the heterologous WAF1 promoter.

4.3.6.2. TAF1 Dependence of SRC:WAF1 Promoter Chimeras

Upstream activation sequences and core promoter elements from the SRC promoters were independently able to confer HDI mediated repression on the WAF1 promoter. To examine whether the SRC upstream activation sequences and core promoter elements were also able to independently confer TAF1 dependence on the WAF1 promoter, the activities of the chimeric SRC:WAF1 and WAF1:SRC constructs were analyzed in tsBN462 cells (Fig. 4.34). It was found that all WAF1 constructs had

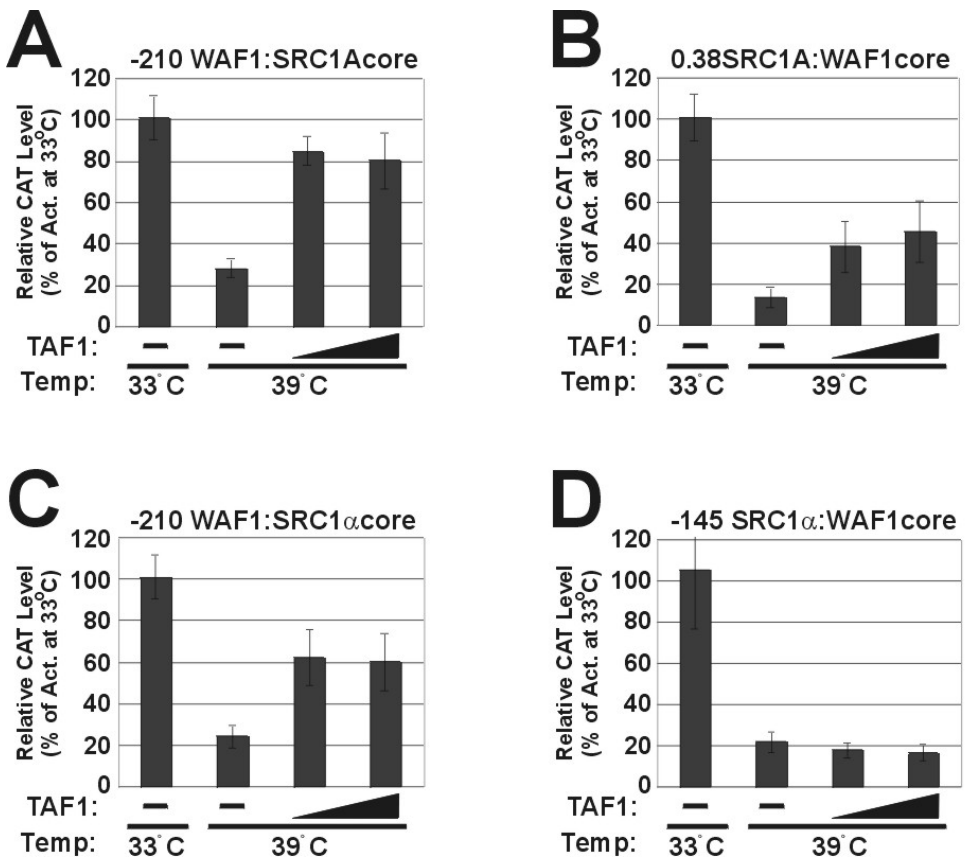

Figure 4.34. TAF1 dependence of SRC:WAF promoter chimeras. (A) A chimeric -210 WAF1:SRC1Acore construct was transfected alone or in combination with increasing amounts of a wild type TAF1 expression vector into tsBN462 cells. Cells were grown at 33°C for 36 h, then maintained at 33°C or shifted to 39°C for an additional 18 h. CAT levels were subsequently determined. **(B)** A chimeric 0.38 SRC1A:WAF1core construct was analyzed as described in (A). **(C)** A chimeric -210 WAF1:SRC1αcore construct was analyzed as described in (A). **(D)** A chimeric -145 SRC1α:WAF1core construct was analyzed as described in (A). Bar graphs represent the mean +/- the standard deviation from two separate experiments, each performed in duplicate.

extremely high activity in tsBN462 and BHK-21 cells at both 33°C and 39°C (data not shown). However, previous studies using ts13 cells stably expressing a WAF1-CAT construct showed that WAF1 transcription is normally induced approximately 2-fold following a shift from 33°C to 39°C in these cells (Rushton et al., 1997). In contrast, a strong decrease in promoter activity was observed for both the -210 WAF1:SRC1A and 0.38 SRC1A:WAF1 chimeras following a shift from 33°C to 39°C in tsBN462 cells (Figs. 4.34 A and B). The decrease in activity of these chimeras at 39°C was rescued by co-expression of wild-type TAF1. These findings confirmed the SRC1A core promoter elements and upstream activation sequences independently conferred TAF1 dependence on the WAF1 promoter. Similarly, the -210 WAF1:SRC1α chimera showed diminished activity following a shift from 33°C to 39°C (Fig 4.34 C). However, in contrast to previous results with the *SRC*1α promoter alone (Fig 4.32 D), the activity of the -210 WAF1:SRC1α chimera was partially rescued by co-expression of wild type TAF1 at 39°C. Rescue of the transcriptional block at 39°C by TAF1 was not observed for the -145 SRC1α:WAF1 chimera (Fig. 4.34 D). These results therefore suggested the SRC1α core is indeed TAF1 dependent, and that the unusual temperature sensitive property described for this promoter in BHK-21 cells is mediated by upstream activation sequences. Furthermore, similar to the results attained for the SRC1A promoter, the TAF1 dependence of the SRC1α core promoter was conferred on the heterologous WAF1 promoter.

4.3.7. DISCUSSION

4.3.7.1. HDIs as Anti-Cancer Agents and Modulators of Gene Expression

HDIs have been described as exciting agents with impressive anti-cancer potential (Vigushin and Coombes, 2002). Indeed, these agents effectively target the transformed phenotype of various tumor cell lines *in vivo* and *in vitro* by inhibiting proliferation and inducing differentiation and/or apoptosis (Marks et al., 2000). The anti-cancer properties of HDIs have been hypothesized to result from the highly selective changes in gene expression that ensue following treatment. For example, the most well-described cellular response to treatment with these agents is the p53 independent activation of the potent cell cycle inhibitor, p21/WAF1 (Huang et al., 2000; Nakano et al., 1997). Because of these previous findings, agents from different classes of HDIs are currently being analyzed for their anti-tumor effectiveness in various phases of human clinical trials (Vigushin and Coombes, 2002).

Given the potential clinical importance of these agents, it is surprising that very few studies have reported their mechanisms of gene expression modulation. The X-ray crystal structures of TSA and suberoylanilide hydroxamic acid (SAHA) in complex with a Zn^{2+} dependent HDAC enzyme have been solved (Finnin et al., 1999). These structures suggest the mechanism of HDAC inhibition by these agents is through active site binding, and sequestration of the Zn^{2+} cofactor. Proposed rationales for subsequent activation of genes following this HDAC inhibition involve unbalanced activity of histone AT (HAT) enzymes, leading to hyperacetylation of histones at promoters. Indeed, treatment with HDIs *in vivo* has been shown to lead to accumulation of hyperacetylated nuclear histone proteins in both tumor and normal tissues (Warrell et al., 1998). Such an overall increase in the acetylation of histones at promoters is widely

believed to result in an open and relaxed chromatin conformation. Therefore, these agents have been described as exerting their effects at the level of chromatin structure (Kouzarides, 2000). This model is supported by the observation that many co-activator proteins, including GCN5, p300/CBP, P/CAF, SRC-1 and ACTR possess intrinsic HAT activity, while co-repressor complexes are associated with HDAC enzymes (Marks et al., 2001b; Vigushin and Coombes, 2002). However, this theory offers very little explanation for the mechanism of gene repression by HDIs, and global gene expression studies have shown just as many genes are repressed as are activated by these agents (Mariadason et al., 2000). In addition, such a global phenomenon as a general opening in chromatin structure could not account for the observation that the expression of a very select subset of genes is altered in response to HDIs (Mariadason et al., 2000; Van Lint et al., 1996). Indeed, the most detailed studies into the mechanism of gene activation by these agents have involved the WAF1 promoter, and these concluded that specific Sp-family binding sites were essential for transcriptional induction (Huang et al., 2000; Nakano et al., 1997). Preliminary analysis of molecular pathways leading to activation or repression of genes in response to butyrate have suggested phosphorylation cascades co-operate with acetylation events to elicit these cellular effects. For example, investigation into the activation of histone $H1^{o}$ expression or repression of c-myc gene expression by butyrate demonstrated an okadaic acid sensitive Ser/Thr phosphatase pathway plays a role in the transcriptional response to this HDI (Cuisset et al., 1997). In addition, Ser/Thr kinase inhibition blocks the induction of choline acetyltransferase gene expression, and protein kinase C inhibition blocks the induction of WAF1 in response to butyrate (Espinos et al., 1999; Han et al., 2001). Therefore, it is probably more accurate to describe HDIs as affecting acetylation-mediated signal transduction cascades, as well

as altering chromatin structure at promoters. This is supported by the observation that transcriptional regulators such as p53 (Gu and Roeder, 1997), Sp3 (Braun et al., 2001), EKLF (Zhang and Bieker, 1998), GATA-1 (Boyes et al., 1998), TFIIE, and TFIIF (Imhof et al., 1997) are acetylated by AT enzymes.

4.3.7.2. HDIs and SRC Repression

This study has determined that HDIs were able to effectively inhibit c-Src mRNA and protein expression in a wide variety of human cancer cell lines. This inhibition of expression was the result of direct SRC transcriptional repression. Given the observation that SRC is transcriptionally activated in human colon cancer cell lines, and HDIs block this activation, it is hypothesized that SRC is an important cellular target of these agents. One HDI used in these experiments, sodium butyrate, is particularly relevant to this theory because it is a breakdown product of dietary fibre, resulting from bacterial fermentation in the colonic lumen (Topping and Clifton, 2001). Theories that suggest butyrate is in part responsible for the anti-cancer effect of a high fibre diet are strengthened, considering its ability to repress SRC transcription. For example, inhibition of c-Src expression using antisense approaches in HT29 or a breast cancer cell culture model resulted in diminished parameters of transformation, including slower growth, reduced tumor-forming ability in nude mouse xenografts, and increased apoptosis (Karni et al., 1999; Staley et al., 1997). Therefore, the reduction in c-Src expression elicited by HDI treatment would be expected to have a similar negative effect on cell proliferation and viability as these antisense approaches. Experiments designed to address this hypothesis will be important to determine the significance of SRC repression by these agents.

4.3.7.3. Core Promoter Architecture, TAF1 Dependence, and HDI Mediated SRC Repression

As a result of the potential clinical importance of HDIs, and the general lack of information regarding the mechanisms of gene repression by these agents, this study focused on addressing how SRC repression was elicited by HDIs. GAL4 replacement strategies and promoter deletion analysis was employed, but a single element responsible for mediating repression of either the SRC1A or SRC1α promoters by HDIs was not implicated. However, it was noted following deletion analysis of the SRC core promoters that elimination of very small fragments in the 3' end of SRC promoter constructs drastically impaired SRC transcriptional activity (Fig. 4.30). These results therefore suggest the SRC core promoters are just as important for determining overall SRC expression in human cancer cell lines as upstream activation sequences. Further investigation is therefore called for to understand how sequences in the SRC core promoter regions regulate SRC transcription, and potentially contribute to activation in human colon cancer cell lines. Functional analysis of the SRC1A and SRC1α core promoters demonstrated that they were similar in that they were both Inr-driven and absolutely dependent on the TFIID component, TAF1, for full activity. However, a preliminary examination of the core sequences downstream from the Inr elements in the SRC promoters has not revealed any obvious homology or similarity that might implicate functional elements in these regions.

TAF1 has been characterized as a core promoter selectivity factor that binds the Inr element and responds to upstream activation sequences (Shen et al., 1998). Initial TAF1 studies in yeast, using chimeric promoters, demonstrated TAF1 dependence is dictated

solely by the core promoter (Shen and Green, 1997). However, a similar approach involving a mammalian system determined that TAF1 dependence could be conferred on a heterologous, TAF1 independent promoter by both upstream activation sequences and core promoter elements (Wang et al., 1997). These previous studies formed the basis for the experimental design of this study, which determined that TAF1 dependence could be conferred on the heterologous, TAF1 independent, WAF1 promoter by SRC promoter upstream activation sequences or core promoter elements. Furthermore, this study also demonstrated that HDI mediated repression was conferred upon the WAF1 promoter by SRC upstream activation sequences or core promoter elements. These observations implicated a potential functional link between TAF1 dependence and HDI mediated gene repression that was strengthened by the observation that the repressive effects of HDIs on the SRC1A promoter were completely blocked in tsBN462 cells grown at the restrictive temperature of 39^{o}C. Although the activity of the SRC1A promoter was already low at 39^{o}C, this finding was very significant because this was the first time a complete absence of SRC1A repression was demonstrated following HDI treatment. Unexpectedly, a similar approach with the SRC1α promoter showed it to have lower activity at 39^{o}C compared to 33^{o}C in both tsBN462 and BHK-21 cells. It was determined this temperature sensitive change in activity was actually due to an increase in SRC1α promoter activity at 33^{o}C compared with 37^{o}C. This unusual "cold-induced" property of the SRC1α promoter was verified in HT29 colon cancer cells as well (data not shown). The significance and mechanism of SRC1α induction at lower temperatures in these mammalian cell lines is currently not known.

A single study has previously addressed the mechanism of gene repression by butyrate, using the cyclin D1 promoter as a model (Lallemand et al., 1996). This report identified an 11 bp butyrate response element that could confer weak butyrate-mediated repression when placed upstream of a thymidine kinase (TK) promoter. However, the repression mediated by the cyclin D1 butyrate response element was only 2-fold, and the basal activities of the promoter constructs in untreated cells were not included in the report (Lallemand et al., 1996). In this thesis, it was observed that the basal activities of reporter constructs in untreated cells could significantly affect the fold induction or repression mediated by HDIs. Therefore, it was deemed prudent to take a qualitative approach and catalogue responses to HDIs as activated, repressed, or unaffected. Failure to do so could have inaccurately implicated discrete SRC promoter elements in mediating repression by HDIs. A need for such caution is supported by the observation that elements in the WAF1 promoter that have been concluded as important for induction in response to HDIs are those that impair promoter activity most significantly when mutated and analyzed in transfection experiments (Huang et al., 2000; Nakano et al., 1997).

4.3.7.4. Models for HDI Mediated SRC Repression

Two models have been developed to explain the mechanism of SRC gene repression by HDIs (Fig. 4.35). These models both propose that TAF1 serves a vital core promoter recognition and TFIID recruitment function at the SRC promoters. In tsBN462 cells shifted to the restrictive temperature, these vital TAF1 functions are abrogated. This study has shown this could be due to decreased TAF1 binding to the core promoters, compromised AT activity, or a combination of both of these effects. In

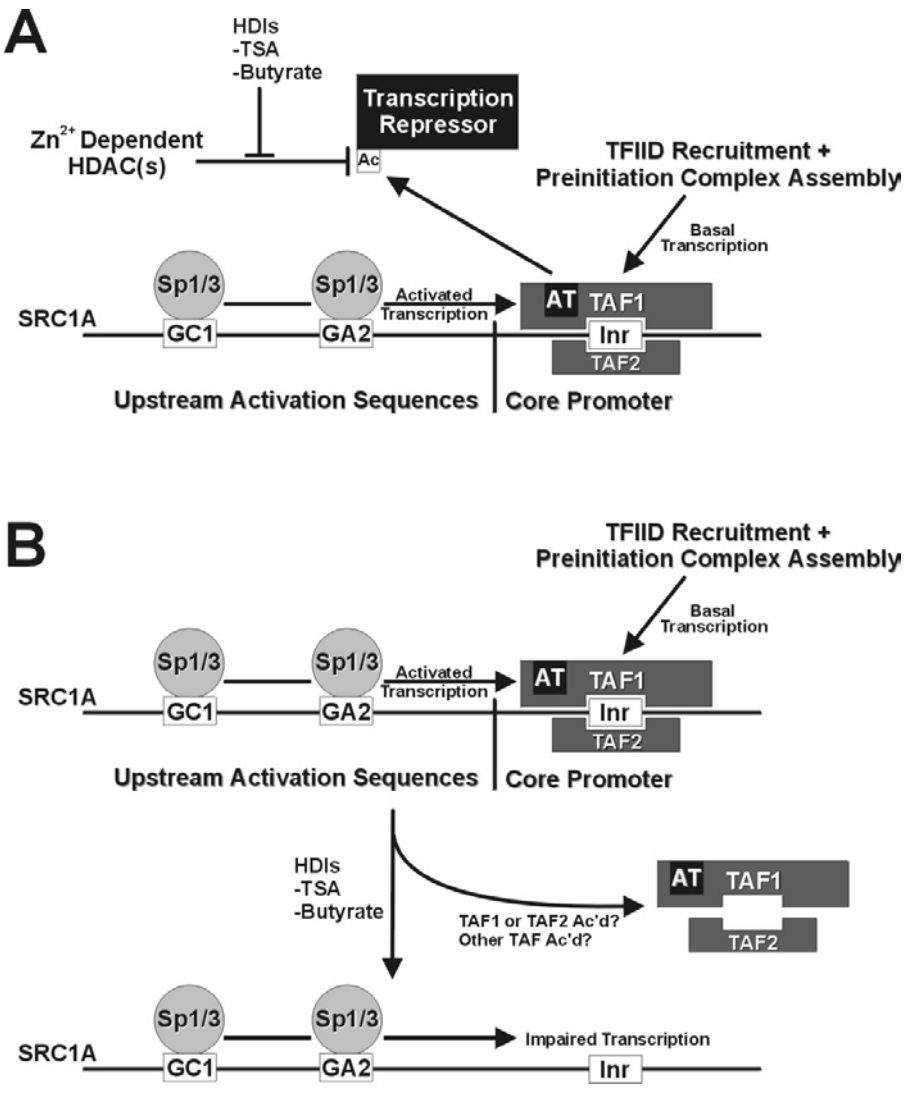

Figure 4.35. Models for HDI-mediated SRC repression. (A) HDIs prevent TAF1 from acetylating a SRC transcriptional repressor. **(B)** HDIs block TAF1 from binding the SRC core promoters.

the first model (Fig. 4.35 A), an unidentified factor is acetylated at the SRC promoters by TAF1, which can subsequently elicit direct transcriptional repression. Normally, acetylation of this putative factor is balanced by one or more of the Zn^{2+} dependent HDAC enzymes, thus preventing its negative influence on SRC transcription. Treatment with HDIs, however, would shift the balance towards hyperacetylation of this factor, resulting in the SRC transcriptional repression observed in various cell lines. In tsBN462 cells, the compromised G690D TAF1 AT activity and/or core promoter recognition prevents TAF1 from acetylating this factor, thus explaining the block of HDI mediated SRC repression in tsBN462 cells at 39^{o}C. In the second model (Fig. 4.35 B), HDIs prevent TAF1 binding to the SRC core promoters, thus accounting for SRC transcriptional repression. This could be due to direct TAF1 acetylation, or acetylation of another component of the basal transcriptional apparatus essential for TAF1 core promoter binding. This model suggests that the block in HDI mediated SRC repression in tsBN462 cells at 39^{o}C is due to the fact that G690D TAF1 core promoter binding has already been abolished at the restrictive temperature.

These models also provide potential explanation for the response of SRC:WAF1 and WAF1:SRC promoter chimeras to HDIs. For chimeras containing SRC core promoter elements and WAF1 upstream activation sequences, the Inr elements in the SRC core promoters could dictate TAF1 dependence. Repression following HDI treatment could then result from one of the two models proposed in Figure 4.35. For chimeras containing WAF1 core promoter elements and SRC upstream activation sequences, TAF1 dependence could be dictated from undefined elements in the SRC upstream activation sequences. In this situation, TAF1 probably is not responsible for TFIID recruitment, because the TATA element in the WAF1 core promoter would bind

TBP. Therefore, the model depicted in Figure 4.35 A would best explain the repression of these chimeras by HDIs. Likely, however, these models represent two extremes, and the precise mechanism of repression of the SRC promoters, as well as SRC:WAF1 chimeras, is through a combination of these two theories.

In light of these models, it is important to note the TAF1 AT substrates that mediate TAF1 dependence in tsBN462 cells have not yet been identified. TAF1 has very weak activity towards histone proteins (Wassarman and Sauer, 2001), but has been shown to acetylate TFIIEβ and the RAP74 subunit of TFIIF *in vitro* (Imhof et al., 1997). The effect of these modifications on the function of these general transcription factors is not known. In order to clarify the role of TAF1 AT activity in core promoter recognition and the repressive effects of HDIs, critical acetylated TAF1 substrates will have to be identified specifically in the context of the SRC promoters.

4.3.7.5. Other TAF1 Dependent Genes Repressed by HDIs

This study has described the first potential mechanistic link between promoter TAF1 dependence and repression by HDIs. The importance of this relationship was provided by studies with SRC:WAF1 chimeras, which demonstrated that conferring TAF1 dependence on the WAF1 promoter also made it repressible in response to TSA treatment. Interestingly, the cyclin D1 and cyclin A promoters, much like the *SRC*1α and *SRC*1A promoters, are also Inr driven, significantly inhibited in tsBN462 cells following a shift to the restrictive temperature, and repressed in response to HDI treatment (Iacomino et al., 2001; Suzuki et al., 2000; Suzuki-Yagawa et al., 1997; Wang et al., 1997). Conversely, promoters of genes that are activated in response to HDI

treatment, such as WAF1, C-FOS, and CMV, contain TATA elements and are slightly induced or unaffected in tsBN462 cells following a shift to the restrictive temperature (Archer et al., 1998a; Rushton et al., 1997; Wang et al., 1997). Interestingly, previous work that showed TAF1 dependence could be dictated independently by upstream activation sequences and core promoter elements utilized chimeras generated between the TAF1 dependent cyclin A promoter and the TAF1 independent FOS promoter (Wang et al., 1997). Therefore, it would be of great interest to expand the findings reported here and determine if these cyclin A:FOS promoter chimeras would also be repressed by HDI treatment. Taken together, these observations suggest there could be a more general, potentially functional, link between Inr driven, TAF1 dependent promoters and HDI mediated repression. Indeed, treatment of various cells with HDIs elicits a similar response of G1/S arrest and/or apoptosis as does the shift of tsBN462 or ts13 cells from 33^{o}C to 39^{o}C. To better understand this relationship, it will be important to dissect the complicated array of elements that dictate both TAF1 dependence and HDI mediated repression in the upstream activation sequences and core promoter elements of Inr driven genes.

4.3.7.6. Scope and Significance

HDIs are anti-neoplastic agents that inhibit proliferation and induce differentiation and/or apoptosis of cancer cells in culture and animal models. The ability of these agents to re-program gene expression plays an important role in their chemotherapeutic action. The transcriptional effects induced by these agents include the induction of growth inhibitory genes such as WAF1, and repression of growth promoting genes such as c-Src and cyclins D and A1. Despite these well-defined cellular responses, the

knowledge surrounding the precise mechanisms of action of these agents on transcription is limited. This study has provided evidence for a potential functional link between the SRC promoters' shared TAF1 dependence, and their repression following HDI treatment. Interestingly, cyclins D and A1 are also TAF1 dependent genes, suggesting the conclusions derived for the SRC promoters could be more general, and provide fundamental information about the gene repression elicited by these important agents.

5. CONCLUSIONS AND FUTURE STUDIES

Activation of the 60 kDa c-Src non-receptor tyrosine kinase is a frequent finding in cancers of the colon, breast, liver, lung, and pancreas. Many previous reports have concluded this activation is accounted for by overexpression of c-Src protein (Biscardi et al., 1999). This overexpression is deemed important for the cancerous phenotype of various tumor cells because antisense strategies preventing c-Src expression result in diminished proliferation, anchorage independent growth, tumor forming ability, and viability (Ellis et al., 1998; Karni et al., 1999; Staley et al., 1997; Wiener et al., 1999; Windham et al., 2002). In this study, c-Src activation was determined to arise at the level of transcription in a subset of HCCLs. These findings therefore suggest that SRC transcriptional activation could play an important role in the fully transformed phenotype of colon and other cancer cell lines. Important future studies will complement these findings, and assess the contribution of SRC transcription to the expression and activity of c-Src in a wide variety of cancer cell lines where c-Src activation has been documented.

SRC transcription is controlled by two disparate promoters separated by approximately 1 kb. The ubiquitously expressed SRC1A promoter is absolutely dependent on the Sp-family of transcription factors for full activity, but is also regulated in part by SPy/hnRNP-K (Ritchie et al., 2000; Ritchie et al., 2003). The SRC1α

promoter, alternatively, is more tissue restricted in its pattern of expression and is absolutely dependent on a single HNF site, which has been shown to bind and respond to HNF-1α (Bonham et al., 2000). In cancer cell lines, both promoters are utilized, albeit at very different ratios. For example, HT29 and HepG2 cells both display very high c-Src expression levels; however, HepG2 cells display preferential SRC1α promoter usage while HT29 cells display nearly equal usage from both of these promoters. The SW480 and HCT-116 cell lines, conversely, display very low levels of c-Src mRNA expression, which arises equally from both SRC promoters. Despite these observations of differential SRC promoter use and transcriptional activity endogenously, transient transfection experiments with SRC promoter reporters consistently showed SRC1A to be a stronger promoter than SRC1α in isolation. Because of these observations, it became of immediate interest to determine the mechanisms regulating differential SRC promoter usage and transcriptional activation. This study detailed the development of a SRC DPCAT reporter system specifically designed to allow measurement of overall CAT activity, as well as relative SRC promoter usage following transient transfection in various human cancer cell lines.

When the SRC DPCAT reporters were assayed, consistently stronger SRC1A activity relative to SRC1α was still observed in all cell lines examined, regardless of their relative endogenous SRC promoter use. A working hypothesis has therefore been developed that one or more enhancer element exist, and are likely important for the transcriptional activation observed in HepG2 and HT29 cells, respectively. These elements are also proposed to play a general role in elevating SRC1α transcription in HepG2, HT29, SW480, and HCT-116 cells. A strategy of searching for and testing the

function of DNaseI hypersensitive sites within the SRC locus was employed to identify potential functional elements that could represent this putative enhancer activity. Although this approach was unsuccessful, a discrete element was identified in HepG2 cells that displayed a very weak ability to activate the SRC1α promoter. Because this element did not account for the full relative SRC1α activity seen endogenously in HepG2 cells, it was deemed likely that a combination of elements might be required to constitute the enhancer activity that this study has suggested to exist. Therefore, it will be important in the future to broaden the search for DNaseI hypersensitive sites in the SRC locus. In addition, enhancer trap experiments could be employed in an effort to systematically test for potential enhancer activity of overlapping 1 to 2 kb DNA fragments derived from the SRC locus. Such an approach, however, would require dozens, or even hundreds, of SRC1α reporter constructs to be developed and tested in transient or stable transfection experiments. Nevertheless, identification and characterization of this potential enhancer will be essential in determining the mechanism of SRC transcriptional activation in colon and other cancer cell lines.

Transfection experiments with SRC DPCAT reporters in HT29 cells showed that the HNF site within the SRC1α promoter acted as a positive regulator of SRC1A promoter activity. However, co-transfection experiments with HNF-1α and a SRC1α-CAT reporter construct suggested that HNF-1α was unable to transactivate the SRC1α promoter in HT29 cells. These observations have led to the proposal that an additional factor can bind to the HNF site in HT29 cells, and plays a role in the SRC1A activation observed in the SRC DPCAT reporter. Results form the Bonham laboratory, showing the ability of an oligonucleotide probe to compete specifically for HNF-1 binding to the

SrcHNF site as well as to enrich for binding of a smaller, unknown factor in HepG2 nuclear extracts, strengthened this theory. Unfortunately, results described in this section were generated from the last sets of experiments performed in pursuit of this thesis study. Therefore, it is very necessary for future experiments to gather more evidence that this factor exists, and then determine its identify and precise role in regulating SRC1α transcription, as well as SRC1A transactivation. This could be accomplished first by using binding assays with various SrcHNF oligonucleotide probes to identify the critical sequences in this site that mediate binding of this unknown factor. This knowledge could then be employed in an affinity-based approach to preferentially purify this putative unknown factor from HT29 and other cellular extracts.

Because of the strong link between c-Src activation and transformation, a significant arsenal of anti-Src compounds has been developed. These include natural products, synthetic peptides, peptidomimetics, and small molecules that target the SH2, SH3, or kinase domain (Sawyer et al., 2001). All of these potential therapeutic agents are intended to block various cellular c-Src functions. This study has demonstrated that HDIs are another groups of compounds that have anti-Src activity, and exert their effects in part by directly blocking SRC transcription in various cancer cell lines. The significance of these findings are highlighted by many reports describing potent anti-cancer activity of HDIs towards transformed cells in culture and animal models (Vigushin and Coombes, 2002). Specifically, HDIs inhibit proliferation, and promote differentiation and/or apoptosis of a wide variety of cancer cells (Marks et al., 2000). Interestingly, these are a few of the many cellular processes for which c-Src function is necessary (Biscardi et al., 1999). It will therefore be important for future experiments to

determine the precise contribution of decreased c-Src expression to the anti-cancer activities of HDIs. This could be accomplished by a stable transfection approach in HT29 cells, where a promoter that is not repressed by HDIs controls overexpression of a c-Src transgene. Such a model system would allow investigators to directly assess the effects of HDIs on these cancer cells when c-Src inhibition is prevented, and obtain clues to the essential c-Src signaling pathways that are shut down by HDIs.

Investigation into the mechanism of SRC transcriptional repression by HDIs has also revealed the importance of the SRC1A and SRC1α core promoters in regulating SRC transcription. These studies defined both SRC core promoters as lacking TATA motifs, but containing functional Inr elements. Therefore, despite the apparent differences between the SRC1A and SRC1α promoters, they shared a dependence on the TFIID component, TAF1, for full activity. Of particular interest, it appears that this commonality is the primary reason why both of these disparate promoters are repressed following HDI treatment. In addition, further important core promoter elements were hinted at by the observation that very small deletions from the 3' ends of either SRC promoter had significant negative effects on overall transcriptional activity. Therefore, narrowing in on these elements will be important in the future, and could be initiated by introducing smaller deletions, followed by specific mutagenesis strategies to identify precise residues important for the function of these elements. Identification and/or purification of factors binding to or interacting with these elements would be the goal of this line of investigation.

Ideally, the findings from these studies will all converge on a set of experiments involving a series of SRC DPCAT reporters designed to probe the interactions between

putative upstream (or downstream) enhancer element(s), previously defined transcription factor binding sites, and core promoter elements in the SRC locus. Interactions between these DNA elements and the multitude of protein factors they recruit represent the "wiring" of the entire SRC promoter locus. Understanding the interplay between factors in this complex is the ultimate, long-term goal for which this thesis has provided groundwork. Introduction of mutations within the SRC DPCAT reporter, which would disrupt specific wiring circuits, or co-transfection with various factors deemed important for full activity, which will enhance specific wiring circuits, will likely prove crucial to a full understanding of the mechanisms of SRC transcriptional activation in human cancer cell lines. Once the interactions between these DNA elements and protein factors are fully understood, the precise mechanism of disruption of this complex following HDI treatment could be elucidated. Employment of other technologies, such as mass spectrometry, could be used to enhance this set of proposed experiments, and play a key role in identifying all the individual proteins that constitute a complete and active SRC transcription complex.

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