ABSTRACT

MOOREFIELD, KRISTOPHER SCOTT. Regulation of Sp2 DNA-binding activity and *trans*-activation (Under the direction of Jonathan M. Horowitz).

Regulated transcription requires the collaboration of a variety of transcription factors, including sequence-specific DNA-binding proteins. The Sp-family of DNA-binding proteins governs the expression of a wide variety of mammalian genes, including housekeeping, cell cycle-regulated, and developmentally regulated genes. A wealth of information has been obtained about the functional and biochemical properties of two Sp-family members, Sp1 and Sp3, whereas studies of other Sp proteins have been limited. The purpose of this research has been to characterize the functional and biochemical properties of Sp2, and to provide insights into mechanisms regulating Sp2 DNA-binding activity and *trans*-activation.

To initiate my analysis of Sp2 I first identified its consensus DNA-binding sequence (5'-GGGCGGGAC-3') using a PCR-based protocol, and determined that Sp2 binds this sequence with high affinity *in vitro* (225 pM). Despite the incorporation of this consensus sequence within the promoter of a well-characterized Sp-dependent gene, I found that Sp2 was a relatively weak activator of transcription compared with Sp1 and Sp3. To begin to define mechanisms limiting Sp2 function, chimeric proteins carrying portions of Sp2 and Sp1 were created and analyzed. These studies demonstrated that Sp2 DNA-binding activity and *trans*-activation are each negatively regulated in mammalian cells.

In mixing experiments I detected an activity in mammalian extracts that abrogates Sp2 DNA-binding activity. I showed further that an 84 kDa mammalian protein bound to the *trans*-activation domain of Sp2, but not that of Sp1 or Sp3. Phosphatase treatment

revealed that Sp2 DNA-binding activity requires phosphorylation, and phosphoamino acid analysis confirmed that Sp2 is phosphorylated *in vivo*. Size-exclusion chromatography indicated that Sp2 is specifically phosphorylated *in vitro* by an activity in fractions carrying high-molecular weight proteins or protein complexes. Indirect immunofluorescence studies indicated that, unlike other Sp proteins, the vast majority of Sp2 localizes to sub-nuclear foci associated with the nuclear matrix. The data reported herein indicates that Sp2 is functionally distinct from other Sp proteins, and provides insight into mechanisms that negatively regulate Sp2-mediated transcription. Taken together, my results suggest that Sp2 may perform a highly specialized role in the regulation of gene expression.

REGULATION OF SP2 DNA-BINDING ACTIVITY AND *trans*-ACTIVATION

by

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APPROVED BY:

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Chair of Advisory Committee

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DEDICATION

I would like to dedicate the efforts presented in this publication to my parents, Kay and Terry, for without their love and support, this journey would not have been possible.

I would also like to dedicate this publication to my sister, Kristen, to whom I have done all that I can to serve as a role model, and to whom I wish nothing but the best out of life.

I would further like to dedicate this work to Brittany, who managed to brighten every day, even at the very end when it was needed most.

And lastly, I would like to dedicate this publication and all that it represents to my friends, for without all of you, life would have been a little less interesting these past years.

THE ROAD NOT TAKEN

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that, the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I dept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

BIOGRAPHY

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List of Abbreviations

DHFR ……Dihydrofolate Reductase

GST……..…Glutathione S-Transferase

PSA…….. Prostate-Specific Antigen

PAS……...Protein A Sepharose

MDR……....Multidrug Resistance

Tk……..... Thymidine Kinase

HIV-1 LTR……...Human Immunodeficiency Virus-1 Long Terminal Repeat

ADH5/FDH…...……..Alcohol Dehydrogenase 5/ Formaldehyde Dehydrogenase Gene

VEGF……...Vascular Endothelial Growth Factor

hTERT……....Human Telomerase Reverse Transcriptase

C/EBP……...CCAAT/ Enhancer Binding Protein

IL-2……....Interleukin-2

HPV-16…….….Human Papillomavirus Virus-16

CBP……...cAMP response element-binding protein (CREB)-binding protein

SV40……Simian Virus 40

PODs……....Promyelocytic Leukemia (PML) Oncogenic Domains

TGF-β……...Transforming Growth Factor-β

EY(G)FP……..Enhanced Yellow (Green) Fluorescent Protein

NMTS……Nuclear Matrix Targeting Signal

TSA…….Trichostatin A

TPA……...Phorbol 12-Myristate 13-Acetate

CHAPTER I
Introduction to the Sp-family of Transcription Factors

1.1 GENERAL INTRODUCTION

The regulation of transcription in mammalian cells is facilitated by the actions of numerous proteins, including components of the basal transcription complex, RNA polymerase II and polymerase-associated cofactors, and sequence-specific DNA-binding proteins. It has been reported that the human genome contains about 25,000 protein-coding genes, of which transcription factors represent the second largest functional category (Nature October, 2004). Acting through *cis*-regulatory promoter elements, sequence-specific DNA-binding proteins govern the spacio-temporal regulation of gene expression and thus many biological events (Chu, et al., Gene, 2005; Dang, journal of biochemistry, 2000). Many transcription factors are classified based on conserved structural motifs within the region responsible for interacting with DNA. The Sp/XKLF family of mammalian transcription factors, comprised of 23 members, shares a highly conserved, carboxy-terminal DNA-binding domain consisting of three zinc-"fingers" of the Cys_2-His_2 class (Bouwman and Philipsen, 2002). The Cys_2-His_2 motif is characterized by conserved cysteines, histidines, and hydrophobic residues stabilizing the three-dimensional structure (Wolfe, et al., 2000). Cys_2-His_2 zinc-"fingers" are the most common DNA-binding motif found in eukaryotic transcription factors and represent the largest family of DNA-binding proteins in the human proteome, about 4% of the human genome (Hoovers et al., 1992; Lander et al., 2001; Tupler et al., 2001). Sp/XKLF proteins are present in species such as C*aenorhabditis elegans*, *Drosophila melanogaster*, and *Danio rerio*, as well as humans and appear to have evolved through multiple gene-duplication events (Ossipova et al., 2002; Schock et al., 1999; Wimmer et al., 1993; Oates et al., 2001).

1.2 ISOLATION AND CLONING OF THE SP-FAMILY

The Sp-subfamily of Sp/XKLF proteins contain 8 members (Sp1-Sp8), of which Sp1 is the founding member and is the best characterized (Dynan and Tjian, 1983). Sp1 (specificity protein 1) was the first mammalian transcription factor cloned based on its selective biding to GC-rich sequences, known as "GC-boxes", within the SV40 early promoter (Dynan and Tijan, 1983; Kadonaga, et al., 1987). Subsequently, Sp1 was shown to stimulate transcription not only of SV40, but also the thymidine kinase (tk) viral promoter (Jones et al., 1985). Further studies revealed the presence of GC-boxes within the promoters of several other cellular and viral genes. Sp1 was originally characterized as responsible for recruiting TATA-binding protein (TBP) and other members of the basal machinery to TATA-less promoters (Pugh and Tijan, 1991; Zenzie Gregory, et al., 1993; Blake et al., 1990; Jolliff et. al., 1991). Soon after, the identification of Sp1-related proteins Sp3 and Sp4 in 1992, however, it became clear that Sp1 is a member of a multigene family (Hagen et al., 1992). Sp3 and Sp4, originally termed SPR-1 and SPR-2 ("Sp1 related protein 1 and 2"), were cloned via their association with a GT-box motif in the rabbit uteroglobin promoter similar to the motif found in the SV40 enhancer region (Hagen et al., 1992). Others cloned Sp3 and yet another Sp1-related protein, Sp2, due to their hybridization to a probe derived from the DNA-binding domain of Sp1. Independently, Sp3 and Sp2 were also found to bind the GT-box motif in the T-cell antigen receptor α promoter (Kingsley and Winoto, 1992). The original cDNA sequence published for Sp3 lacked a consensus translational initiation codon, and it was suggested that Sp3 translation might initiate from a non-AUG start site. This proposal was made, despite the fact that the Sp3 cDNA sequence reported was shorter than transcripts

identified by Northern blotting (Suske, 1999; Kingsley and Winoto, 1992; Hernandez et al., 2002). The high GC-content at the 5' end of the Sp3 gene made isolation of a full-length Sp3 cDNA problematic, however, full-length chicken and mouse Sp3 cDNAs have been isolated that contain a traditional translational initiation codon (Chamboredon et al., 2003; Yajima et al., 1998). A full-length human Sp3 cDNA was subsequently isolated using 5' rapid amplification of cDNA ends and genomic DNA carried by bacterial artificial chromosomes (BACs) (Hernandez et al. (2002) (Moran et al., 2004).

Several years later Sp5, another Sp1-related gene, was isolated from the primitive streak of mouse embryos using a subtractive hybridization approach (Harrison et al. 2000; Treichel et al., 2001). Subsequently, Sp6 was identified by using the sequence of the Sp1 DNA-binding domain as a probe to screen a mouse EST database (Scohy et al. 2000). Cloning and characterization of the human Sp7 gene was accomplished by virtue of its hybridization with the mouse Osterix gene in osteogenic cells (Gao et al., 2004). Finally, human Sp8 was identified by one group based on its homology with the human Sp7 sequence using TblastN software (Milona et al., 2004), and simultaneously by separate group using *in situ* hybridization screening for specific cDNA clones with overlapping pattern of expression with the *gata2* protein (Penberthy et al., 2004). Careful analyses of have been performed on a number of Sp proteins and have implicated them in regulating the expression of a wide variety of mammalian genes, such as housekeeping genes, growth factors, as well as genes responsible for such physiologic processes as cell cycle control, oncogenesis, differentiation, and angiogeneisis (Suske, G., Gene, 1999, 238; Black, A.R., J. Cell physiology, 2001; Phillipsen, S., NAR, 1999; Fandos et al., 1999; Schilling and Farnham, 1995; Black and Azizkhan, 1996).

1.3 CONSERVED STRUCTURAL MOTIFS

Utilizing a panel of deletion mutants, Kadonaga et al. (1988) identified domains of Sp1 based on amino acid content, structural motifs and biochemical function (see Fig. 1). To define functional domains of human Sp1, plasmids expressing deletion mutants of Sp1 were examined in transcriptional assays using reporter constructs containing the SV40 promoter in cotransfections of *Drosophila* cell line (Schneider Line 2; SL2); considered the paradigm for monitoring Sp-family transcription as they lack endogenous Sp homologues yet contain the proper transcriptional machinery necessary to support Sp-mediated transcription (Courey and Tjian, 1988). Functional analyses of mutant Sp1 proteins identified three modular regions termed the *trans*-activation, DNA-binding, and the mutimerizaiton domains (or D domain), proven to be important to transcriptional activation. Sp1, Sp3, and Sp4 contain all three regions, where as Sp2 is absent the B domain, and Sp5-8 are devoid of the entire *trans*-activation domains.

The amino-terminal *trans*-activation domain (Sp1 amino acids 1-619) is responsible for stimulating transcription and consists of three subdomains, termed A, B, and C. Domains A and B each contain a serine- and threonine-rich region ($A^{S/T}$, $B^{S/T}$) composed of 50% serine/threonines (Kadonaga et al. (1988) followed by a C-terminal glutamine-rich region (A^{Q}, B^{Q}) composed of 25% glutamines (Courey and Tijan, Cell, 1988). Transient cotransfection experiments with Sp1 deletion mutants and a reporter construct harboring a GC-rich region derived from the SV40 early promoter indicated that the glutamine-rich portion of each subdomain is required for Sp1 to stimulate transcription. The serine/threonine-rich regions appear to be dispensable for Sp1-mediated transcription (Courey and Tjian, 1988; Santoro et al., 1988). It is worth

mentioning that Sp5, Sp6, Sp7, and Sp8 are devoid of *trans*-activation domain present Sp1-Sp4. The Sp1 C domain is characterized by a highly charged region composed of 12 negatively charged and six positively charged amino acids within a 69-residue segment (Kadonaga et al., 1987). Despite the net negative charge, there appears to be no correlation between charge and transcriptional activity, as a deletion mutant consisting of the C domain and the DNA-binding domain was capable of stimulating transcription 10x compared to the basal activity of transcriptionally inert mutants (Courey and Tijan, 1988).

The extreme amino-terminus of Sp1 (amino acids 1-82) has been proposed to be a putative inhibitory domain (ID) (Murata et al., 1994). Using Gal4-fusion proteins containing portions of the Sp1 *trans*-activation domain, Murata et al. revealed that removal of the amino-terminal 82 amino acid serine/threonine-rich subdomain from Sp1 resulted in a 300 fold increase in relative *trans*-activation potential of the protein. This result was correlated with a reduction in the binding of a 74 kDa protein that bound specifically to the amino-terminus of Sp1 *in vitro*. Recent functional characterization of this domain reveals it to negatively regulate Sp1-mediated *trans*-activation via interactions with co-repressors such as SMRT, NcoR, and BcoR, possibly explaining the role of Sp1 as a repressor under certain circumstances (Lee et al., 2005).

The Sp-family of proteins shares a highly conserved DNA-binding domain (amino acids 620-699) immediately adjacent to the C domain. This region consists of three Cys_2-His_2 zinc-"fingers" required for sequence-specific DNA-binding to GC-rich promoter elements. Sequence alignment and phylogenetic analysis of the DNA-binding domains of Sp-family members indicate that Sp1, Sp3, Sp4, and mSp5 share greater than

90% amino acid conservation, whereas the DNA-binding domains of Sp2, and Sp6 have considerably less sequence similarly (75% and 85%, respectively) (Philipsen and Suske, 1999; Kololl and Crawford, 2002; Kaczynski et al., 2003). Despite sharing a conserved carboxyl-terminal DNA-binding domain, amino acids outside this region are only modestly conserved. Co-crystal structure of zinc-"finger" proteins bound to DNA indicate that each zinc-"finger" protein recognizes and binds to a decameric sequence such that each "finger" interacts with four nucleotides; three amino acids in the α-helix interact with three consecutive nucleotides on the primary DNA strand, whereas one amino acid binds a nucleotide on the complementary strand (Wolf et al., 2000; Pavletich and Pabo, 1991; Fairall et al., 1993). Of those Sp1 amino acids that are predicted to interact with DNA, only one amino acid is divergent among all Sp-family members: in Sp2, a histidine residue within the first "finger" is replaced with a leucine residue.

A high degree of evolutionary conservation not only exists within individual zinc-"fingers", but also in the inter-finger linker regions that connect adjacent "fingers" (Jantz and Berg, 2004). This conserved inter-finger linker sequence appears in virtually all zinc-"finger" proteins encoded by the human genome (Wolf et al., 2000; Lander et al., 2001). In fact, this sequence is so strongly conserved that the DNA sequence encoding the linker has been used to identify new zinc-"finger" proteins by hybridization or by Blast searches using the human genome database (Agata et al., 1998; Hoovers et al., 1992). NMR structural studies indicate that the linker is flexible in solution but becomes rigid upon DNA-binding (Clemens et al., 1994; Wuttke et al., 1997; Bowers et al., 1999; Laity et al., 2000). The strong sequence conservation of the inter-finger linker sequence has led to speculation that evolution has selected against variation within this region

(Choo and Klug, 1993). Some have speculated that this region may serve as a recognition sequence for kinases that regulate DNA-binding activity (Dovat et al., 2002; Jantz and Berg, 2004). Indeed, evidence from the zinc-"finger" protein Ikaros suggests that the single threonine residue in the inter-finger linker sequence could be the target of a mitotic phosphorylation event that leads to exclusion of zinc-"finger" proteins from mitotic chromatin (Dovat et al., 2002; Pandya and Townes, 2002; Jantz and Berg, 2004).

The extreme carboxy-terminus of some members of the Sp-family, termed the D domain (amino acids 700-778), is responsible for protein multimerization and synergistic *trans*-activation (Pascal and Tjian, 1991). This region cannot stimulate transcription alone, however, deletion of this domain reduces Sp1-mediated *trans*-activation by 20% (Courey and Tjian, 1988). The capacity of Sp1, Sp3, and Sp4 to synergistically *trans*-activate promoters proximal or distal to the transcriptional start site relies on its ability to multimerize via the D domain (Courey et al., 1989; Pascal and Tjian, 1991; Mastrangelo et al., 1991). Other Sp-family members are devoid of the D domain and consequently are unable to multimerize. Sp1s ability to multimerize with itself was confirmed using electron microscopy. These crosslinking studies demonstrated that Sp1 was capable of forming tetramers bound to DNA and synergistic activation was accomplished by looping out intervening DNA sequences bringing tetramers bound at disparate sites on DNA into proximity via D domain-mediated homomultimerization (Mastrangelo et al., 1991; Courey et al., 1989; Li et al., 1991).

1.4 AN INTRODUCTION TO SP1, SP3, AND SP4

Sp1, Sp3, and Sp4 are the most well characterized members of the Sp-family of transcription factors. These three proteins share the greatest degree of sequence homology, structural similarities and biochemical properties. Sp1 and Sp3 are ubiquitously expressed in all human, mouse and rat tissues, whereas expression of Sp4 appears to be restricted to the central nervous system (Hagen et al., 1992; Supp et al., 1996). A phylogenetic analysis of the mammalian zinc-"finger" proteins DNA-binding domain indicates that Sp1, Sp3, and Sp4 form a monophyletic group (Kolell and Crawford; 2002). Sp1 is located on human chromosome 12q13, was the first family member cloned (1983), and much of what is known about the biochemical and functional properties of Sp proteins is due to analyses of Sp1. The glutamine-rich regions of domains A and B were identified as the major *trans*-activation domains and, along with an intact zinc-"finger" DNA-binding domain, is required for Sp1-mediated transcriptional activation (Courey and Tijan, Cell, 1988).

Sp3 maps to human chromosome 2q31, and shares 90% amino acid homology with Sp1 within the DNA-binding domain (Hagen et al., 1992). Structurally Sp3 resembles Sp1, containing a *trans*-activation domain consisting of two amino-terminal serine/threonine- and glutamine-rich subdomains, a highly charged C domain and a carboxy-terminal multimerization (D) domain. In addition to structural homology, Sp3 interacts with similar GC-rich promoter elements with comparable affinity to Sp1, and has been shown to regulate the expression of several Sp1-dependent genes (Hagen et al., 1992). Fusion proteins containing segments of the Sp3 *trans*-activation domain attached to the Gal4 DNA-binding domain indicated that, as with Sp1, the glutamine-rich portions

of each A and B domain are responsible for stimulation of transcription (Dennig, et al. 1996). Despite its obvious structural similarity with Sp1, a variety of functional studies reveal that Sp3 also has the capacity to repress transcription (Udvadia et al., 1995; Liang et al., 1996; Ding et al., 1999; Hagen at al., 1994). Reports indicate that Sp3 can repress Sp1-mediated transcriptional activation in both a DNA-binding-dependent and a DNA-binding-independent fashion. In transient co-transfection experiments using *Drosophila* SL2 cells, Sp3 was able to inhibit Sp1-dependent transcription of HIV-1 LTR, and DHFR promoters. Based on the similar DNA-binding site specificities, it was proposed that the repressive effects of Sp3 resulted via a mechanism involving competition with Sp1 for occupancy of GC boxes in promoter regions (Majello, et al., 1994; Hagen et al., 1994; Birnbaum et al. 1995; Dennig et al., 1996, Sjottem et al., 1996). Additionally, Sp3 can also inhibit Sp1/Sp3–dependent transcription in a DNA-binding-independent fashion involving competition for critical constituents of the basal transcription machinery (Kennet et al. 1997). Discussed below, reports have identified a region in the C domain of Sp3 (amino acids 547-559), containing the amino acid triplet KEE, termed the "inhibitor" domain, as responsible for repressing Sp3-mediated transcription (Dennig et al., 1996; Majello et al. 1997). Interestingly, identification of two smaller isoforms (M1 and M2) that arise via internal translational initiation of the Sp3 mRNA as repressor serves to complicate this issue. M1 and M2 have been shown to function as potent repressors of Sp-mediated transcription via a "squelching"-like mechanism, titrating cofactors required for transcription away from Sp proteins (Kennett et al., 1997; Kennet et al., 2002). It is worth mentioning that Sp1 can also inhibit Sp3-mediated *trans*-

activation in a promoter-dependent context, for example the mouse growth hormone L2 (Yu et al., 1999).

Sp4 maps to human chromosome 7p15, encodes a protein which is structurally analogous to Sp1, and a DNA-binding domain which shares 89% homology to the DNA-binding domain of Sp1 (Gaynor et al., 1993; Matera and Ward, 1993; Kalff-Suske et al., 1996). Biochemical analyses indicate that Sp4 binds to a similar GC-rich cognate DNA sequence as Sp1, and functional studies reveal Sp4 to possess *trans*-activation properties, via its glutamine-rich regions, similar to Sp1 (Hagen et al. 1995). However, several reports suggest that Sp4's capacity to stimulate transcription is promoter-specific (Hagen et al., 1994; Ahlgren et al., 1999; Wong et al., 2001). Sp4 is able to activate transcription of the BCAT-1 promoter in transient co-transfections of SL2 cells, but represses the ADH5/FDH gene in an Sp1-dependent manner (Kwon et al., 1999). However, whereas Sp1 can synergistically activate promoters containing multiple binding sites, transactivation by Sp2 only occurs in an additive manner (Hagen et al., 1995).

1.5 FUNCTIONAL ANALYSIS OF THE SP-FAMILY

1.5.1 Target Genes Regulated

The completion of the human genome project in 2001 has facilitated the prediction of "target" genes for many transcription factors based on the localization of cognate DNA-binding sequences within promoters or unannotated regions upstream of genes. Given the widespread distribution of GC-rich sequences within the human genome, "target" gene prediction for Sp-family members has proven to be especially difficult and potentially misleading. Moreover, *in silico* "target" gene prediction for Sp-

family members is unreliable since the DNA-binding preferences of most Sp proteins have yet to be defined. A large number of genes have been designated as Sp1 "target" genes due to their capacity to bind Sp1 *in vitro* and *in vivo* and/or due to increased rates of transcription in transient transfection experiments. Indeed, a literature search of the PubMed database yielded almost 3800 citations for "Sp1-regulated gene expression". Although these citations do not spring from studies of 3800 different "target" genes, it is likely that many genes have been incorrectly assigned as Sp1 "target" genes. It is equally probable that genes assigned as Sp1 "target" genes may also be regulated by one or more additional Sp-family members. Consistent with these notions, and as discussed below, disruption of the Sp1 gene via homologous recombination does not lead to the reduced expression of many genes previously identified as Sp1 "target" genes (Marin et al., 1997).

What evidence provides compelling support for the notion that any given gene is a target of a given Sp-family member? Several techniques have proven to be most informative. Gene inactivation experiments, whether carried out in whole animals (via homologous recombination) or in tissue culture cells (via siRNA and RNAi), have been the most powerful tools to confirm whether a given Sp-family member is required for the regulation of a putative "target" gene. Another invaluable strategy, referred to as chromatin immunoprecipitation (ChIP) experiments, assess whether any given Sp-family member interacts with specific sequences within a candidate "target" gene via direct DNA-binding or through protein-protein contacts (Barski and Frenkel, 2004). Such experiments are most informative when performed in untransfected cells, enabling one to assess the interaction of endogenous Sp proteins with their natural targets. When

combined, gene ablation studies and ChIP experiments provide the best evidence that a gene is a target of any given Sp protein. Using these criteria, only a limited number of genes have been validated as Sp targets. For example, homozygous deletion of Sp1 in mice led to a drastic reduction in the expression level of methyl-CpG binding protein 2 (MeCP2), a protein required for survival of differentiated cells. In these same animals, only a modest decrease in the expression of thymidine kinase (tk) and dihydrofolate reductase (DHFR) was detected despite their long-standing identification as Sp1-dependent genes (Marin et al., 1997; Tate et al., 1996). It is worth pointing out that since more than one Sp-family member may service any given gene, gene ablation experiments may underestimate the numbers of target genes for any given Sp protein. Using the stringent criteria mentioned above, Sp proteins have also been shown to regulate developmental genes such as Wilm's tumor (WT1; Cohen et al., 1997) and vascular endothelial growth factor (VEGF; Pore et al., 2004), as well as cancer related genes including human telomerase reverse transcriptase (hTERT; Wooten and Ogretmen; 2005), and $p27^{Kip1}$ (Huang et al., 2004). Functional analyses of the Sp-family in the context of annotated target genes are discussed below.

1.5.2 Activation/Repression of Transcription

Sp1 and Sp3 have been implicated in the regulation of a wide range of mammalian genes. Despite their ubiquitous expression, their structural and sequence similarity, and their interaction with common cognate DNA-binding sites, it is clear that each plays unique roles in transcriptional regulation (Black et al., 1999). Although it is widely accepted that Sp1 functions as an activator of mammalian gene expression, Sp1

appears to act as a transcriptional repressor for a finite number of genes such as C/EBPα and IL-2 receptor α (Tang et al., 1999; Roman et al., 1990; Shou et al., 1998). In contrast, Sp3 was originally characterized as a transcriptional repressor (Birnbaum et al. 1995; Hagen et al., 1994). More recent functional studies in a variety of insect and mammalian cells, however, indicate that, similar to Sp1, Sp3 and Sp4 predominantly function as transcriptional activators (Udvadia et al., 1995; Liang et al., 1996; Ding et al., 1999; Hagen at al., 1994; Nielsen, 1998). Although, due to similar DNA-binding site preferences, Sp1, Sp3, and Sp4 have also been shown to compete for GC-rich promoter elements in transcription assays. For instance, co-expression of Sp1 and Sp3 in *Drosophila* SL2 reveals that Sp3 inhibited Sp1-mediated transcription (Majello, et al., 1994; Birnbaum et al. 1995; Dennig et al., 1996, Sjottem et al., 1996; Hagen et al., 1994; Hata et al., 1998). Similarly, Sp4 can repress Sp1-mediated transcription of the alcohol dehydorgenase 5 gene (ADH5) via competing for binding to promoter sites (Kwon et al., 1999). It is clear, however, as co-expression of Sp1 and Sp3 occurs in several cell types, that mechanism independent of DNA-binding are be in place to coordinate Sp1- and Sp3-mediated regulation of transcription. For example, several reports show variations in the relative abundance of Sp1 and Sp3 in different cell types and under different cellular conditions results in differential regulation of gene expression (Kwon et al. 1999; Tu et al, 1998; Apt et al., 1996; Krikun ert al., 2000). C2C12 myocyte, as well as human umbilical vein endothelial (HUVEC) cells cultivated under hypoxic conditions resulted in a depletion of Sp3 abundance whereas Sp1 protein levels remained unchanged (Xu et al., 2000; Discher et al., 1998). Likewise, it was demonstrated that hypoxic conditions induced down-regulation of Sp3-mediated repression of β-enolase gene expression. It

was also demonstrated that activation of the epithelial HPV-16 promoter shift from Sp3 to Sp1 concomitant with a change in the Sp1/Sp3 ratio in primary kerotinocytes induced to differentiate *in vitro* upon treatment with calcium (Apt et al., 1996). It is clear that an understanding of the regulation of Sp-dependent transcription will require not only consideration of modifications to the individual Sp proteins, but also a knowledge of the expression patterns and interactions with other members of the Sp-family.

Contrasting reports on the physiologic functions of Sp1 and Sp3 are likely due to promoter context examined or the use of divergent experimental conditions, but also may reflect the structural differences in the two proteins. It is clear that both proteins contain similar potent glutamine-rich activation domains, however, repression domain have also been identified in both Sp1 and Sp3 (Murata et al., 1994; Dennig et al., 1996). The molecular basis for the inhibitory function of Sp1, involving interactions with co-repressors, has been discussed above. An Sp3 inhibitory domain located in the C domain was identified by site-directed mutagenesis (Dennig et al., 1996). In transcriptional studies, this 13 amino acid region was capable of suppressing transcriptional activation when linked to the potent *trans*-activation domain of Sp1, or to a heterologous Gal4-*trans*-acitivation domain (Majello et al. 1997; Dennig et al., 1996). Contained within the inhibitory domain is the KEE amino acid triplet (amino acid 551-553) that has been shown to be both necessary and sufficient to repress Sp3-mediated transcription. In fact, point mutations within this region relieved its inhibitor function, converting Sp3 from transcriptionally inert to a strong activator of transcription (Dennig et al., 1996). The inhibitory function is thought to be mediated through post-translational modifications as the lysine has been shown to be acetylated by the acetyl transferases CBP and p300

(Braun, et al., 2001). Additionally, recent reports demonstrate that Sp3 is sumoylated by Ubc9, a SUMO-1 conjugating enzyme, at lysine 551 (Spengler et al., 2005; Sapetschnig et al., 2002; Ross et al., 2002). Sumoylation is involved in altering protein-protein interactions, subcellular localization, and/or protein stability, and has been correlated to up- or down-regulation of transcriptional activity in several transcription factors including AP-2γ, c-Myb, and p53 (Dohmen, 2004; Bies et al., 2002; Melchior and Hengst, 2002). Mutation of the sumoylation site, however, led only to modest increases in Sp3-mediated transcription in insect and mammalian cells (Spengler et al., 2005). While sumoylated Sp3 has been shown to co-localize within discrete subnuclear regions known as promyelocytic (PML) oncogenic domains (PODs), it is possible that sumoylation plays an important role in regulation of Sp3s activation potential via dictating interactions with transcriptional cofactors known to co-localize to PODs (Spengler et al., 2005).

Despite having similar DNA-binding specificities and co-regulating several common genes, it is suggested that promoter context likely dictates the function of Sp1 or Sp3. For example, Sp3 activates promoters containing a single binding site, whereas promoters containing multiple binding sites are non-responsive to Sp3 (Birnbaum et al., 1995; Dennig et al., 1996). Whether Sp3 activates or represses Sp1-mediated transcription may also be cell-dependent. Reports show that in co-transfections with HERV-H LTR, Sp3 stimulated transcription in NTera2-D1 cells, but acted as a repressor in HeLa and SL2 insect cells (Sjottem et al., 1996). Additionally, subtle differences within the nucleotide sequence of a promoter region may dictate whether Sp1 or Sp3 is responsible for stimulating or repressing transcription. As seen in Table 1, the Sp-family

of proteins differs in their capacity to stably interact with promoter regions of well-defined targets of Sp-mediated transcription, p21, MDR, and DHFR (Kennet et al., 2002). DNA-binding site preference may therefore influence the functional properties of the Sp-family.

The capacity of Sp3 to function as both an activator and a repressor is further complicated by the fact that it encodes for three translational products, a full length Sp3 (110-115 kDa), and two smaller isoforms termed M1 and M2 (70-80 kDa), that arise via internal translational initiation of Sp3 mRNA (Kennet et al., 1997). As translation is initiated within the B domain of Sp3, M1 and M2 are devoid of the A domains present in Sp1, Sp3, and Sp4. In contrast to full length Sp3, functional studies indicate that M1 and M2 serve as potent repressor of Sp1- and Sp3-mediated transcription (Kennet et al., 1997; Kennet et al., 2002). The region required for M2-mediated repression was mapped to the M2 *trans*-activation domain suggesting that M1 and M2 may block Sp-mediated repression via titration of one or more factors required for transcription, possibly via interactions with basal transcription constituents TAF_{II} 70 and TAF_{II} 40 (Kennet et al., 2002). Additionally, M1 and M2 contain the inhibitory KEE domain and are also targets of sumoylation by Ubc9 (Spengler et al., 2005). Of particular interest is the finding that, unlike full length Sp3, mutation of lysine 551 converts M1 from a potent repressor to a strong transcriptional activator, possibly providing insight into regulation of Sp-mediated transcription.

1.5.3 Physiological function of Sp1, Sp3, and Sp4: Knockout studies

Gene ablation studies have proven to be a powerful means to decipher the developmental and physiological properties of individual members of gene families. Given their similar patterns of expression as well as common sequence, structure, and DNA recognition sequences, one might predict that inactivation of a given Sp-family member might not result in a significant loss-of-function. Surprisingly, however, mice nullizygous for Sp1, Sp3, or Sp4 exhibit profound as well as subtle developmental abnormalities. Sp1-deficient mice sustain a broad range of severe developmental abnormalities, eventually succumbing at embryonic day 11 (e11; Marin et al., 1997). Sp1s role in regulating several cellular processes is highlighted by the developmental heterogeneity including many gross morphological defects in structures and tissues, *e.g.*, somites, otic vesicle, limb bud, blood, the developing eye, and heart. In contrast to these severe embryonic defects, Sp1-deficient embryonic stem (ES) cells are viable, proliferate normally, and can contribute to the formation of early chimeric embryos. Sp1-null ES cells injected into wild-type blastocysts are no longer present after day e11. These results indicate that nullizygosity for Sp1 leads to a cell autonomous defect, and that Sp1 is essential for the maintenance of differentiated cells after day 10 of development. Interestingly, the expression of many putative Sp1 "target" genes was shown to be unaffected by the inactivation of Sp1 in ES cells (see above).

In contrast to Sp1 null mice, Sp3- and Sp4-deficient mice survive through birth but have reduced body weight compared to wild type and heterozygous littermates (Bouwman et al., 2000; Supp et al., 1996; Gollner et al., 2001). Sp3 nullizygous animals invariably perish within 10 minutes of birth due to respiratory failure, however; only

minor morphological alterations are observed in the lung. Histological examination of Sp3 nullizygotes revealed pronounced defects in late tooth development that has been related to lack of proper enamel deposition (Bouwman et al., 2000). Two-thirds of nullizygous Sp4 animals die within 4 weeks of birth due to unknown causes. Although Sp4 is largely expressed in the central nervous system tissue of wild-type animals, Sp4-deficient mice do not display obvious neurological abnormalities. On the other hand, $Sp4^{-/-}$ males do not breed despite having fully developed reproductive organs and mature sperm. These results suggest that Sp4 is required for normal male reproductive behavior (Gollner et al., 2001). $Sp5^{-/-}$ does not display an overt phenotype despite expression in wild-type animals throughout early development in the spinal cord, trigeminal ganglia, and somites (Harrison et al., 2000; Treichel et al., 2001). Sp7-deficient mice lack endochondral and intra-membranous bone formation, and osteoblast differentiation is prematurely arrested during embryonic development (Nakashima, et al., 2002; Gao et al., 2004). Mice lacking Sp2, Sp6, or Sp8 function have not as yet been reported.

1.6 REGULATION OF THE SP-FAMILY

Given their conserved DNA-binding domains and co-expression in many, if not most, mammalian cells one might predict that Sp protein functions would be regulated by one or more post-translational mechanisms. Indeed, Sp protein DNA-binding activity and *trans*-activation have been shown to be governed by a variety of post-translational modifications and protein/protein interactions.

1.6.1 Post-translational Modifications

With the cloning of Sp1 and the generation of anti-Sp1 antisera it soon became apparent that Sp1 encodes a family of proteins ranging, in size from 95 to 105 kDa. Given that the apparent molecular weight of each of these proteins is greater than that predicted from its cDNA (80 kDa), it was predicted that Sp1 is subject to one or more post-translational modifications. It has since been reported that the Sp-family is covalently modified by glycosylation (Jackson and Tjian, 1988), phosphorylation (Jackson et al., 1990), acetylation (Dennig et al., 1996), and most recently by sumoylation (discussed above; Spengler et al., 2005; Sapetschnig et al., 2002; Ross et al., 2002). Moreover, post-translational modifications of Sp-family members have been shown to stimulate as well as repress Sp-dependent gene expression.

Phosphorylation

Sp1 was initially identified as a phosphoprotein due to its increased mobility on denaturing polyacrylamide gels following treatment of HeLa cell extracts with potato acid phosphatase (Jackson et al., 1990). Subsequent phosphoamino acid analyses indicated that 95% of phosphorylated residues are phosphoserine, the majority of which are located within the Sp1 *trans*-activation domain. Phosphothreonine residues comprise the remaining 5% of phosphoamino acids. Column chromatography subsequently identified DNA-dependent protein kinase (DNA-PK) to be an Sp1 amino-terminal kinase. Since this initial report, several additional kinases have been identified that utilize Sp1 as a substrate. Sp1 phosphorylation has been shown to be induced upon viral infection and following stimulation of quiescent cells with mitogens (Alroy et al., 1999; Black et al.,

1999; Bonello and Khachigian, 2004; Chun et al., 1998; Kim and DeLuca, 2002). Perhaps the most well characterized example of virally induced phosphorylation of Sp1 is that following SV40 infection (Jackson et al., 1990; Saffer et al., 1990). Upon infection, SV40 "small t" antigen inhibits the enzymatic activity of protein phosphatase 2A (PP2A) leading to the induction of a hyperphosphorylated form of Sp1 (Yang et al., 1991; Garcia et al., 2000). Several growth factors have been shown to stimulate Sp1 phosphorylation. For example, epidermal growth factor (EGF) induces Sp1 phosphorylation via the MEK signal transduction pathway (Chupreta et al., 2000; Milanini et al., 1998; Zheng et al., 2001). Discussed below, phosphorylation of Sp1by cyclinA/cdk2 has also been linked to cell cycle progression (Black et al., 1999; Haidweger et al., 2001; Dovat et al., 2002; Jantz and Berg, 2004). Sp1 phosphorylation can have effects on DNA-binding activity and *trans*-activation. For example, casein kinase II-dependent (CKII) phosphorylation of Sp1 at threonine residues located within the DNA-binding domain inhibits Sp1 binding to its consensus binding site (Armstrong et al., 1997; Leggett et al., 1995). In contrast, other reports have documented instances in which phosphorylation increases Sp1 DNA-binding activity (Rafty and Khachigian, 2001) (Haidweger et al., 2001). For example, phosphorylation of Sp1 by protein kinase C-γ has been shown to stimulate Sp1-mediated expression of platelet-derived growth factor β-chain (PDGF-β) by enhancing Sp1s DNA-binding ability. Taken together, these results indicate that a variety of kinases and signaling pathways can regulate Sp1 DNA-binding activity and *trans*-activation. Presumably, this enables cells to modulate transcription in response to a wide range of stimuli (Chu et al., 2005).

Glycosylation

To date, Sp1 is the only Sp-family member to be shown to be modified by glycosylation. Attachment of O-linked N-acetylglucosamine (O-GlcNAc) moieties to Sp1 appears to impact its *trans*-activation potential, nuclear localization, and interaction with other proteins (Kadoonaga et al., 1988; Jackson and Tjian, 1988; Roos et al., 1997; Han and Kudlow, 1997). Jackson and Tjian (1988) demonstrated that Sp1 carries, on average, eight O-GlcNAc monosaccharides per molecule of protein. Conflicting reports indicate that the attachment of O-GlcNAc residues to Sp1 can increase or decrease Sp1-mediated transcription (Jackson and Tjian, 1988; Roos et al., 1997). Glycosylated Sp1 purified from HeLa cells has been reported to be more competent to stimulate transcription than recombinant Sp1 produced in *E. coli* (Jackson and Tjian, 1988). Additionally, preincubation of purified Sp1 with lectin wheat germ agglutinin (WGA), which binds O-GlcNAc residues, prior to adding the protein to an *in vitro* transcription system, specifically inhibited the *trans*-activation potential of Sp1 in HeLa cells by 4 fold. In contrast, others have reported that glycosylation of the glutamine-rich region of the Sp1 B domain impairs its ability to multimerize, with a consequent reduction in its ability to stimulate transcription (Roos et al., 1997; Yang et al., 2001). O-GlcNAc modification has also been linked to proteolytic degradation of Sp1, as hypoglycosylated Sp1 activates a caspase-mediated pathway leading to cleavage of Sp1 and degradation via a proteosome-dependent mechanism (Han and Kudlow, 1997). Treatment of cells with glucosamine, a metabolic derivative of glucose, or with proteosome inhibitors, such as lactacystin, can prevent Sp1 degradation by the proteosome (Han and Kudlow, 1997; Su et al., 2000). It has been proposed that glycosylation blocks the formation of complexes

between Sp1 and proteins that target it for proteosome-mediated degradation (Roos et al., 1997).

Acetylation and Sumoylation

Sumoylation and acetylation appear to be post-translational modifications that are specific to Sp3, as evidence of sumoylation or acetylation of other Sp proteins has yet to be reported. Both modifications have been implicated as molecular mechanisms responsible for negatively regulating Sp3-mediated transcription (Dennig et al., 1996; Braun et al., 2001; Spengler et al., 2005; Sapetschnig et al., 2002; Ross et al., 2002). Sumoylation, discussed above, occurs at a specific lysine residue (lysine 551) within a so-called "inhibitor domain" not present in other Sp proteins (Dennig et al., 1996). This same lysine residue has also been shown to be the target of acetylation *in vivo* by the acetyltrasnferases CBP and p300 (Braun et al., 2001). Substitution of arginine for lysine 551 results in a substantial increase in Sp3-mediated transcription (Spengler, et al. Dennig et al., 1996; Braun et al., 2001). Acetylation of lysine residues in repressor domains has been reported for C/EBPα and β, p53, and E2F1 (Williams et al., 1995; Angerer et al., 1999; Gu et al., 1997; Martinez-Balbas et al., 2000). Additionally, C/EBPα, and p53 have also been shown to be sumoylated *in vivo* (Melchior and Hengst, 2002). It is currently unclear whether prior acetylation is required for sumoylation of lysine 551 (or vice a versa), nor have cellular conditions been established under which Sp3 is preferentially acetylated or sumoylated (Braun et al., 2001). Although studies examining the sumoylation state of Sp1 or Sp5 have yet to be reported, it has been noted

that putative consensus sites for sumoylation are present in both proteins (Spengler, et al., 2005).

Cell Cycle-Dependent Phosphorylation and Mitotic Exclusion

Cell cycle dependent phosphorylation of Sp1 has been demonstrated at both the G_1-S and G_2-M transitions (Black et al., 1999; Lacroix et al., 2002; Dovat et al., 2002). Serum-stimulation of quiescent fibroblasts results in an increase in the abundance and phosphorylation of Sp1 concomitant with Sp1-dependent activation of the cell cycle regulated dihydrofolate reductase (DHFR) promoter in late G_1 (Black et al., 1999). The kinase responsible for this cell-cycle-dependent induction of Sp1 phosphorylation was later identified as cyclinA/CDK2. CyclinA/cdk2 forms a stable complex with Sp1, and phosphorylates the carboxy-terminus of Sp1 in a cell cycle-dependent fashion (Haidweger et al., 2001).

Phosphorylation of zinc-"finger" proteins at the G_2-M transition has been correlated with the inactivation of DNA-binding activity and exclusion from mitotic chromatin (Raff et al., 1994; Dovat et al., 2002; Jantz and Berg, 2004). It is widely accepted that entry into mitosis is accompanied by the termination of transcription (Prescott and Bender, 1962). It is also known that several transcription factors are inactivated during the G_2-M transition by mechanisms that reduce DNA-binding activity and displace them from chromatin (Segil et al., 1991; Martinez-Balbas et al., 1995; Gottesfeld and Forbes, 1997). Recent reports have indicated that the highly conserved zinc-"finger" inter-finger linker (NH_2-TGEKP-CO_2H) may serve as a recognition sequence for at least one mitotic kinase. It has also been suggested that this mitotic kinase

may be part of a global mechanism to exclude zinc-"finger" proteins from chromatin (Dovat et al., 2002; Jantz and Berg, 2004). Previous studies have indeed showed that the DNA-binding activity of Sp1 is greatly reduced in mitotic extracts (Martinez-Balbas et al., 1995). Phosphopeptide maps of Sp1 in metaphase-arrested 293T cells revealed specific mitosis-associated phosphorylated residues. Moreover, substitution of alanine for threonine within the inter-"finger" linker regions resulted in the disappearance of one mitotic phosphopeptide. These results suggested that at least one of the inter-"finger" linker regions within the Sp1 DNA-binding domain is phosphorylated during mitosis (Dovat et al., 2002). Whether this phosphorylation event is indeed responsible for mitotic chromatin exclusion will be discussed further in Appendix I

Subcellular Localization and Nuclear Matrix Association

Other than localizing within nuclei, little else is known about the distribution of Sp proteins within interphase cells. Hints that Sp proteins can be sequestered within sub-nuclear domains spring from several studies. When co-expressed with promyelocytic (PML) protein, Sp3 isoforms are recruited to sub-nuclear domains termed promyelocytic oncogenic domains (PODs) (Spengler et al., 2005). Although Sp3 does not appear to localize to PODs in the absence of PML over-expression, many sumoylated proteins localize to these nuclear "speckles". Non-sumoylated proteins, such as the retinoblastoma (Rb) protein and Sp1, have also been identified within PODs (Ferbeyre et al., 2000; Vallian et al., 1998). Sp1 and PML have been shown to form protein complexes, and these interactions require the DNA-binding and D domains of Sp1 (Vallian et al., 1998). The formation of Sp1/PML complexes appears to repress Sp1-

mediated transcription by blocking the formation of Sp1/DNA complexes. Whether Sp3-mediated transcription is regulated by interactions within PODs remains to be determined.

van Wijnen et al (1993) have reported that a protein with DNA-binding properties similar to those of Sp-family members is associated with the nuclear matrix. The nuclear matrix is in part an intranuclear macromolecular scaffold composed of A/C- and B-type lamins, additional intermediate filament proteins, as well as other structural proteins and a variety of lamin-associated proteins. The nuclear matrix is also rich in ribonucleoproteins and a constellation of low-abundance proteins, including proteins responsible for chromatin modification, DNA replication and repair, transcription, and RNA splicing (Berezney and Coffey, 1975; McCready et al 1980; Pardoll, et al 1980; Cook, 1991; Reyes et al 1997; review Davie, 1997; Lawrence, et al 1989; Nickerson et at, 1995; Stein et al 2000; Wei et al, 1998; Wei, et al. 1999). Some 398 nuclear matrix-associated proteins have been identified thus far, and amongst these are a variety of sequence-specific DNA-binding proteins, such as steroid hormone receptors, SatB1, ATF, OCT-1, Pit-1, Runx/Cbfa/AML and AP-1 (Mika and Rost, 2005; Simmen et al, 1984; Barrack, 1983; Barrack and Coffey 1980; Rennie et al 1983; DeFranco and Guerro 2000Getzenberg et al, 1991; Getzenberg and Coffey, 1990; Stenoien et al, 2001; Mancini 1999; Merriman, 1995; Stein, 2000; Zeng et al 1997; Zeng 1998). Comparisons of the DNA-binding activity of the nuclear matrix-associated and non-matrix-associated forms of Sp1 indicate that Sp1 retains the capacity to stably interact with DNA despite its association within the nuclear matrix (Bidwell et al., 1993; van Wijnen et al., 1993).

Studies detailing the sub-nuclear localization properties of Sp-family members and their association with the nuclear matrix will be reported in Chapter V.

1.6.2 Protein-Protein Interactions

As for many, if not all, sequence-specific DNA-binding proteins Sp-mediated transcription is regulated via protein/protein interactions (Pugh and Tjian, 1990). Sp proteins have been reported to physically interact with components of the basal transcription machinery, co-activators and co-repressors, chromatin modifying proteins, other sequence-specific DNA-binding proteins, as well as the products of oncogenes and tumor suppressor genes.

Interactions with Components of the Basal Transcription Complex and Co-Activators

Given that the promoters of many GC-box-rich genes lack TATA boxes it has long been suspected that Sp proteins might function, at least in part, by tethering TATA box-binding protein (TBP) and associated factors to DNA. Indeed, Sp1 has been shown to bind *Drosophila* and human TBP *in vitro* and TBP-associated factor 110/130 ($dTAF_{II}110/hTAF_{II}130$) *in vivo* via the glutamine-rich portions of the Sp1 A and B domains (Emili et al., 1994; Hoey et al., 1993). Mutational studies have indicated that interactions of Sp1 with $TAF_{II}110$ are required for *trans*-activation (Gill et al., 1994). At least *in vitro*, Sp-mediated transcription requires TBP, and three members of the TFIID complex: $TAF_{II}110$, $TAF_{II}150$, and $TAF_{II}250$ (Weinzierl et al., 1993; Chen et al., 1994). Interactions of Sp1 and Sp3 with other TATA-binding protein associated proteins have been detected *in vitro* and in yeast "two-hybrid" screens, suggesting that Sp proteins

tether components of the basal transcription complex to DNA via a variety of protein contacts (Dennig et al., 1996; Chiang and Roeder, 1995; Kennett et al., 2002). Although not a component of the basal transcription machinery, a nonomeric protein complex termed CRSP (cofactor required for Sp1) is required along with TFIID proteins for efficient Sp1-mediated transcription *in vitro* (Ryu et al., 1999). Immunodepletion of CRSP from fractionated cell extracts markedly reduces Sp1 transcription *in vitro*.

Interactions with Sequence-Specific DNA-Binding Proteins

As mentioned above, Sp1 can form homomultimeric complexes via its D domain and these multimers facilitate synergistic *trans*-activation. Yet, Sp1 and presumably other Sp-family members, also form higher order complexes with many other sequence-specific DNA-binding proteins resulting in synergistic *trans*-activation of a variety of genes. The first human DNA-binding protein shown to functionally interact with Sp1 was YY1 (Seto et al., 1991). In *Drosophila* SL2 cells, co-expression of Sp1 and YY1 led to the synergistic *trans*-activation of the adenovirus E1A promoter dependent on DNA-binding sites for both proteins (Seto et al., 1993). Subsequently, many similar interactions have been documented. For example, Sp1 associates with AP-2 *in vivo* via the Sp1 D domain, and *trans*-activates the CYP11A1 promoter in an Sp1-dependent manner (Nielsen et al., 1998). Interactions between Sp1, but not Sp3, and Smad proteins 2, 3, and 4 are responsible for TGF-β signaling (Cook and Urrutia, 2000; Inagaki et al, 2001). Complexes between Sp proteins and CREB-binding protein (CBP), a histone acetyltransferase, have also been reported (Naar et al., 1998). CBP was required along with TAFs and SREBP-1a, for synergistic *trans*-activation of the low-density lipoprotein

receptor (LDLR) promoter in *Drosophila* SL2 cells (Naar et al., 1998). Several other interactions between Sp-family members and transcription factor proteins, such as NF-κB (Majello et al, 1994) have been documented, resulting in differential regulation of Sp-mediated transcription, however, an in depth analysis of each is beyond the scope of this review.

Interactions with Co-Repressors

Physical interactions between Sp proteins and other factors can also repress Sp-mediated transcription. The promyelocytic leukemia protein (PML) binds the DNA-binding domain of Sp1 preventing subsequent interactions with DNA (Vallian et al., 1998). More recently, co-repressor proteins such as SMRT, NcoR and BcoR have been shown to co-immunoprecipitate with the Sp1 DNA-binding domain and an amino-terminal portion of the Sp1 *trans*-activation domain (Lee et al., 2005). These interactions with co-repressor proteins are modulated by the Map kinase/Erk kinase (MEK) signaling pathways and serve to negatively regulate Sp1-mediated transcription by recruiting histone deacetylases (HDAC). Sp1, as well as Sp3, have also been shown to be bound directly by histone deacetylases (Doetzlhofer et al., 1999). HDAC-1 was shown to bind the Sp1 DNA-binding domain, and treatment of cells with HDAC inhibitors, such as trichostatin A (TSA), relieves HDAC-1-mediated repression of Sp1 transcription (Karlseder et al., 1996; Doetzlhofer et al., 1999).

Interactions with the Products of Oncogenes and Tumor-Suppressor Genes

Members of the Sp-family are also able to regulate cell cycle progression and cell growth through the formation of complexes with the products of several tumor suppressors and oncogenes. Sp1 has correlated physical interactions with p53 with both alterations in DNA-recognition and binding, and *trans*-activation. Several reports indicate that wild type p53 inhibits Sp1-dependent transcription of insulin-like growth factor-I (IGF-I) by binding Sp1 and preventing it from associating with DNA (Webster et al., 1996; Ohlsson et al., 1998; Lee et al., 2000). This mechanism may be analogous to the granulocyte/macrophage colony-stimulating factor (GM-CSF) induced proliferation of the human erythroleukemia cell line TF-1, where wild type p53, but not mutant p53, is able to inhibit Sp1 DNA-binding activity (Borellini and Glazer, 1993). Interestingly, Sp1 and Sp3 have been reported to bind p53 and synergistically *trans*-activate p53 targets, such as the upregulated mediator of apoptosis (PUMA) and $p21^{Cip1}$, in an Sp1 site-dependent manner (Koutsodontis et al., 2005). This interaction requires the carboxy-terminal portion of p53 and the glutamine-rich regions of the Sp1 *trans*-activation domain.

Functional interactions between Sp-family members and the retinoblastoma (Rb) protein have also been reported (Kim et al., 1992; Udvadia et al., 1993). Rb was initially shown to regulate transcription through retinoblastoma control elements (RCEs) that were themselves bound by Sp1 and Sp3 (Udvadia et al., 1993; Udvadia et al., 1995; Kim et al., 1992; Chen et al., 1994). RCEs were found in several promoter regions, such as the growth-control genes c-fos, c-jun, c-myc, and TGF-β1 (Kim et al., 1991; Robbins et al., 1990). Gal4-Sp1 fusion proteins identified functional interactions between Sp1 and Rb

that required the amino-terminal region of Sp1 (Kim et al., 1992). Co-transfection experiments revealed that Rb "superactivates" Sp1- and Sp3-mediated transcription *in vivo* and that this activity required both amino-terminal and carboxy-terminal portions of Rb (Udvadia et al., 1995). As physical interactions between Sp1 and Rb have been difficult to detect *in vivo*, it is possible that Rb stimulates Sp-mediated transcription by titrating a negative regulator of Sp1 DNA-binding activity (Chen et al., 1994). One potential candidate in this regard is MDM2, a key regulator of p53 function. MDM2 has been shown to inhibit Sp1 DNA-binding activity, thus Rb may relieve MDM2-mediated repression of Sp1 by disrupting MDM2/Sp1 interactions (Johnson-Pais et al., 2001). As mentioned above, maximal Sp1-mediated *trans*-activation requires, among other proteins, $TAF_{II}250$ (Chen et al., 1994). Cyclin D1 has been reported to inhibit Sp1 *trans*-activation either by physically interacting with Sp1 or with $TAF_{II}250$ (Adnane et al., 1999; Opitz and Rustgi, 2000). Since Rb has itself been shown to bind directly to $TAF_{II}250$, some have suggested that Rb may stimulate Sp1-dependent transcription by disrupting cyclin D1/ $TAF_{II}250$ complexes (Shao et al., 1995; Adnane et al., 1999). Regardless of the precise mechanism(s) by which Rb may regulate Sp-mediated transcription, interactions between Sp1 and Rb-related proteins are likely to introduce additional complexities. The Rb protein is itself a member of a protein family that includes two sequence-related proteins, termed p107, and p130. It has been shown that p107, but not p130, binds directly to Sp1 *in vivo* and represses Sp1-dependent transcription (Datta et al., 1995). Despite evidence that co-expression of Rb and Sp1 results in "superactivation" of several mammalian promoters, including the hamster *dhfr* gene, Rb has also been reported to cooperate with p130 in the stable repression of the

hamster *dhfr* promoter. These latter results have led to the suggestion that Rb and Sp1 participate in cell cycle-regulated interactions via a multimeric protein complex that includes HDAC-1 (Noe et al., 1998; Chang et al., 2002).

In addition to interactions with growth regulators, such as Rb and p53, the Sp-family has been implicated in the transcription of cell cycle-regulated genes via direct interactions with members of the E2F family of transcription factors. *In vitro* binding assays have demonstrated that Sp1, Sp2, Sp3, and Sp4 all have the capacity to physically bind E2F-1 (Karlseder et al., 1996; Lin et al., 1996; Rotheneder et al., 1999). Sp1 also forms complexes with E2F-2 and E2F-3, but not E2F-4 nor E2F-5 (Lin et al., 1996). Physical and functional interactions between Sp1 and E2F require the Sp1 carboxy-terminus and the amino-terminus of E2F-1, -2, and –3, a region not present in E2F-4 and –5 (Karlseder et al., 1996). The functional consequence of Sp1/E2F-1 interactions appears to be promoter and cell type specific. Co-expression of E2F-1 and Sp1 in *Drosophila* SL2 cells results in synergistic activation of the DHFR promoter and the DNA polymerase α promoter. Yet, synergistic *trans*-activation was reported for the mouse thymidine kinase (tk) synthetase promoter in mouse 3T6 fibroblasts, but not *Drosophila* SL2 cells (Rotheneder et al., 1999). Experiments in SL2 cells demonstrate further that synergistic *trans*-activation can occur for promoters that contain Sp1- or E2F-1-binding sites via a tethering mechanism that brings both factors into proximity of the basal transcription complex (Lin et al., 1998). Interestingly, Sp1 and E2F-1 physical interactions appear to be cell cycle regulated as co-immunoprecipitation studies reveal that maximal interactions occur in late G_1, concomitant with the expression of the DHFR gene (Lin et al., 1998).

1.7 CONCLUSIONS AND PERSPECTIVES

Sp proteins have been implicated in the regulation of a wide range of mammalian genes, from cell cycle-regulated and growth-related genes to "housekeeping" genes. Despite their structural and sequence homology, it is clear that each also possesses unique transcriptional and regulatory properties. In part, these protein-specific functions are due to isoforms encoded by particular Sp genes, post-translational modifications of Sp proteins, and the interaction of Sp-family members with various regulatory proteins. The functional consequence of these distinctions is perhaps best appreciated by the wide range of phenotypes presented by mice nullizygous for individual Sp-family members. Given their significance as transcriptional regulators, a great deal of effort has been expended in an attempt to understand the biochemical and functional properties of Sp proteins. The vast majority of this effort, however, has focused on Sp1 and Sp3. At the time my research began little or no information was available regarding the biochemical and functional properties of Sp2, nor its role in cell physiology. To address these unknowns, it was my intention to provide a detailed characterization of Sp2 DNA-binding activity and *trans*-activation and define mechanism(s) that regulate these functions. In so doing I hoped to identify Sp2-interacting proteins, and define signal transduction pathways that regulate Sp2 function. In the following chapters I will present detailed biochemical, molecular genetic, and functional evidence indicating that in many respects Sp2 is a unique member of the Sp-family of mammalian transcription factors.

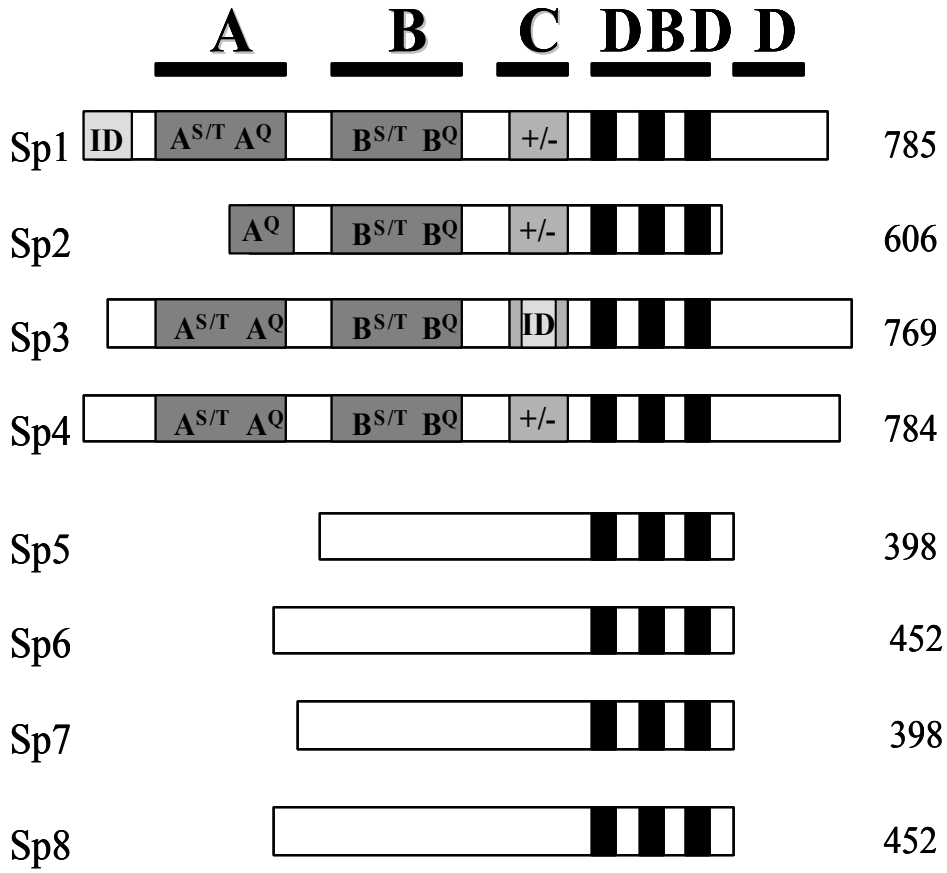

FIGURE 1-Schematic diagram comparing structural motifs of the Sp-family members. The *trans*-activation domain consists of domains A and B, carrying serine- and threonine-rich regions ($A^{S/T}$ and $B^{S/T}$) and glutamine-rich regions (A^{Q} and B^{Q}). The C domain contains highly charged amino acids (+/-). The Cys_2His_2 zinc-"finger" DNA-binding domain (DBD) is indicated with three black bars. The carboxy-terminal D domain (D) is required for multimerization and synergistic activation. The inhibitory domains (ID) are regions functionally determined regions of Sp1 and Sp3.

Table I

Relative binding of recombinant Sp proteins to oligonucleotides carrying Sp-binding sites derived from three Sp-dependent promoters

Protein extracts were prepared from Sf9 cells infected with baculovirus stocks encoding Sp family members, and the volume of each extract required to bind 50% of a radiolabeled oligonucleotide probe derived from the c-*fos* promoter was determined. This volume of cell extract was later employed in protein/DNA-binding assays with oligonucleotides derived from the *DHFR*, *p21*, and *MDR-1* promoters. Binding assays were performed in triplicate and quantified *in situ*. DNA-binding activities for each protein were normalized to the amount of binding activity detected for each protein on the *p21* oligonucleotide.

Sp protein	DHFR				MDR-1	P21
	1	2	3	4		
Sp1	2.2	1.8	1	3.0	2.5	1
Sp2	<0.01	0.8	<0.01	1.4	<0.01	1
Sp3	2.5	1.9	1.2	3.0	1.2	1
M1	1.7	1.2	0.6	2.1	1.5	1
M2	1	1	0.5	1.4	1	1

CHAPTER II
Materials and Methods

2.1 CELL CULTURE

T98G, K562, HCT116, HUT78, Jurkat, SKNBE, DU145, COS-1, HeLa and GoTo cells were obtained from Duke Comprehensive Cancer Center cell culture facility (Duke University Medical Center, Durham, NC). HTLA230 cells were obtained from Dr. Emil Bogenmann (Children's Hospital of Los Angeles, Los Angeles, CA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 50 μg/ml Pipracil. PC12 cells were obtained from Dr. Luis Parada (National Cancer Institute, Frederick, MD) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% calf serum and 50μg/ml Pipracil. Mammalian cells were culture in humidified incubators at 37°C under 5% CO_2. Sf9 cells were obtained from Invitrogen, Inc. (Carlsbad, CA) and *Drosophila* Schneider line-2 (SL2) cells were obtained from Dr. Cheaptip Benyajati (University of Rochester, Rochester, NY). *Drosophila* SL2 cells were cultured in Shields and Sang M3 Medium (Sigma Chemical Co., St. Louis, MO) supplemented with BPYE (0.25% Difco Bacto-peptone, 0.1% Difco TC-yeastolate; Difco Laboratories, Detroit, MI), 10% heat-inactivated fetal calf serum, 1% piperacil, and incubated at 27°C. Sf9 cells were cultured in spinner flasks at 27°C in Grace's complete insect media (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum and 0.1% Pluronic F-68 and 10μg/ml Gentamycin (GIBCO).

2.2 PLASMID CONSTRUCTIONS

2.2.1 Expression Constructs

The *Drosophila* cell expression plasmid pPacSp1 was obtained from Dr. Robert Tijan (University of California Berkeley; Courey and Tijan, 1988). pPacSp3 was generated in our lab by Dr. Sarah Kennet (Kennet, et al. 1997). An Sp2 expression construct, pPacSp2, was generated following a PCR that included a human Sp2 cDNA (provided by Dr. Astar Winoto, University of California, Berkeley; Kingsley and Winoto, 1992) as a template, and the following primers: 5'-GGG GGA TCC GCC ACC ATG GCG GCC GCC GTA ATG AGC-3' and 5'-GGG GGA TCC CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA CAA GTT CTT CGT GAC CAG-3'. The amplified, epitope-tagged cDNA was subsequently cloned at the BamHI sites of pCMV4 to generate the mammalian expression plasmid pCMV4-Sp2 and pBSK to generate pBSKSp2. The plasmids pCMV4-Sp1 and pCMV4-Sp2 were used as substrates to create chimeric Sp1/2 plasmids, and each was cloned into the *Drosophila* expression plasmid pPac using EcoRI and XbaI sites. Each chimera was subcloned into pBSK using EcoRI and XbaI sites and subsequently cloned into the KpnI and XbaI sites of pCMV4. To create Sp1/2 (carrying amino acids 1-619 of Sp1 and 527-613 of Sp2) a PCR reaction was employed using the following primers: Sp1-ABC**:** 5'-CCC CTC GAG GCC ACC ATG GAT GAA ATG ACA GCT GTG GTG-3', and 5'-AAT ATG CTG TTT CTT TTT GCC AGG ATC CC-3'; Sp2-ZnD: 5'-TGC CAC ATC CCC GAC TGT GGC AAG ACG-3' and 5'-CCC GAA TTC TTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA CAA GTT CTT CGT GAC-3'. To generate Sp2/1 (carrying amino acids 1-526 of Sp2 and 620-778 of Sp1) a PCR reaction containing the following primers was used: Sp2

ABCZn: 5'-CCC CTC GAG GCC ACC ATG AGC GAT CCA CAG ACC AGC ATG GCT GCC-3', and 5'-AAC GTG CTT CTT CTT GCC CTG CTC TCC-3'; Sp1-ZnD**:** 5'-TGC CAC ATC CAA GGC TGT GGG AAA GTG TAT GGC-3', and 5'-GGG TCT AGA CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA GAA GCC ATT GCC ACT G-3'. To generate Sp2/1-D (carrying amino acids 1-613 of Sp2, and 700-778 of Sp1) the following primers were used in a PCR reaction: Sp2 ABCZn: 5'-CCC CTC GAG GCC ACC ATG AGC GAT CCA CAG ACC AGC ATG GCT GCC-3', and 5'-GTG GGT CTT GTA ATG CTT GGT GAG GTG GTC-3'; and 5' Sp1 (D)**:** 5'-CAG AAT AAG AAG GGA GGC CCA GG-3', and 5'-GGG TCT AGA CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA GAA GCC ATT GCC ACT G-3'. To create Sp1/2/1 (carrying amino acids 1-619, and 700-778 of Sp1, and 527-613 of Sp2) a PCR reaction was employed using the following primers: Sp1/2: 5'-CCC GGT ACC GCC ACC ATG GAT GAA ATG ACA GCT GTG GTG-3', and 5'-CAA GTT CTT CGT GAC CAG GTG GG-3'; and 5' Sp1 (D): 5'-CAG AAT AAG AAG GGA GGC CCA GG-3', and 5'-GGG TCT AGA CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA GAA GCC ATT GCC ACT G-3'. To create Sp2 (B)/Sp1 (carrying amino acids 196-417 of Sp2 and 620-778 of Sp1) the following oligos were employed in a PCR reaction: Sp2 (B): 5'-CCC GGT ACC GCC ACC AGC GGG GCC AAT GTG GTG AAG CTG ACA GGT GGG GGC-3', and 5'-CCC GGC TGG GGC AAT CTT TGG CAG G-3'; and Sp1-ZnD; 5'-TGC CAC ATC CAA GGC TGT GGG AAA GTG TAT GGC-3', and 5'-GGG TCT AGA CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA GAA GCC ATT GCC ACT G-3'. To generate Sp1 (B)/Sp2 (carrying amino acids 232- 502 of Sp1 and 527-613 of Sp2) the following oligos were employed: Sp1 (B):

5’-CCC GGT ACC GCC ACC CCG GAC GCT AAT AAT GTA CTC TCA GGA CAG ACT CAG-3’, and 5’-AGC AGC TGA GGC AAT GGG TGT GAG AGT GG-3’; and Sp2-ZnD: 5’-TGC CAC ATC CCC GAC TGT GGC AAG ACG-3’, and 5’-CCC GAA TTC TTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA CAA GTT CTT CGT GAC-3’. PGBT9Sp2 with the *trans*-activation domain of Sp2, corresponding to amino acids 1-496, was prepared by PCR using pCMVSp2 as the template and the following primers: 5’-CCC GGA TCC ATG AGC GAT CCA CAG ACC AGC-3’ and 5’-GGG GGA TCC TTA CAA GTT CTT CGT GAC-3’. The amplified product was subsequently cloned at the BamHI sites of pGBT9. pGBT9Sp1 was generated in our lab by Dr. Sarah Kennet (Kennet, et al. 1997). 5’RbAS2 has been described (Sterner, et al., 1998). Glutathione S-transferase fusion expression constructs, pGEX-Sp1, pGEX-Sp3 carrying the *trans*-activation domains of each protein, and pGEX-FSH, encoding a *Schistosome* surface antigen, have been previously described (Murata, et al., 1994; Kennet, et al., 1997). A GST-Sp2 fusion protein carrying the Sp2 *trans*-activation domain (amino acids 1-526) was prepared using the PCR and the following primers: 5’-CCC GGC TCC ATG AGC GAT CCA CAG ACC AGC-3’ and 5’-GGG AGA CCT CTT CTC CCC ATC CTT-3’. Subsequently the amplified Sp2 *trans*-activation domain was cloned into pGEX-2TK at BamHI and BglI sites to create GST-Sp2. GST-Sp2A, GST-Sp2B, and GST-Sp2C were prepared using the PCR and pBSKSp2 as the substrate (Moorefield, et al. 2004). The partial *trans*-activation domain GST fusion proteins carry the following Sp2 amino acids: GST-Sp2A (amino acids 1-195), GST-Sp2B (amino acids 196-417), GST-Sp2C (amino acids 421-528). An Enhanced Yellow Fluorescent Protein (pEYFP-C1) fusion protein containing Sp2 expression construct, pEYFP-C1-

Sp2/flu, was generated following a PCR that included a human Sp2 cDNA as the template and the following primers: 5' GGG GGT ACC GCC ACC ATG AGC GAT CCA CAG ACC AGC ATG GCT GCC-3', and 5'-CCC GGATCC CTA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA CAA GTT CTT CGT GAC C-3'. The amplified, epitope-tagged cDNA was subsequently cloned in frame at the KpnI and BamHI sites of pEYFP-C1 to generate the mammalian expression plasmid pEYFP-C1-Sp2/flu. pEYFP-C1-Sp2/flu deletion mutants were prepared using the pEYFP-C1-Sp2/flu plasmid as the template and Pfx platinum DNA polymerase (Invitrogen) in a PCR reaction. Amino acids carried by each deletion mutant are as follows: ΔA (carrying amino acids 196-619 of Sp2), ΔAB (carrying amino acids 419-619 of Sp2), ΔABC (carrying amino acids 526-619 of Sp2), ΔZnIII (carrying amino acids 1-595 of Sp2), and ΔZnII/ III (carrying amino acids 1-562 of Sp2). Primers employed to create each deletion mutant were as follows: ΔA, 5'-GGG GGG TAC CTG CGA CTG CAG AAT TCG AAG CTT G-3', and 5'-GGG GGG TAC CAG CGG GGC CAA TGT GGT GAA GTT GAC AGG-3'; ΔAB, 5'-GGG GGG TAC CTG CGA CTG CAG AAT TCG AAG CTT G-3', and 5'-GGG GGG TAC CAC CAG CAA AAA GCA CTC AGC TGC AAT TCT C-3'; ΔABC, 5'-GGG GGG TAC CTG CGA CTG CAG AAT TCG AAG CTT G-3', and 5'-GGG GGG TAC CCA GGG CAA GAA GAA GCA CGT TTG CCA CAT C-3'; ΔZnIII, 5'-CCC GCG GCC GCA TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TAG-3', and 5'-GGG GCG GCC GCC TGG GCG CAC TCG AAG CGT TTG TCC CC-3'; ΔZnII/ III, 5'-CCC GCG GCC GCA TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TAG-3', and 5'-GGG GCG GCC GCG ACA AAG GGC CGC TCG CCA GTG TGC-3'. Deletion mutants ΔA, ΔAB, and ΔABC were digested with KpnI and ΔZnIII,

and ΔZnII/III was digested with NotI, and each was re-ligated upon itself. To generate deletion mutant pEYFP-C1-ZnI, containing the first zinc finger of Sp2 corresponding to amino acids 526–561, the pEYFP-C1-Sp2/flu plasmid was used as the template and Pfx platinum DNA polymerase (Invitrogen) in a PCR reaction. To amplify the first Zn-finger region of Sp2, the following primers were employed: 5'-GGG CTC GAG TGC CAC ATC CCC GAC TGT GGC AAG ACG TTC CG-3', and 5'-CCC CCC GGG GAC AAA GGG CCG CTC GCC AGT GTG CAG GCG-3', and to amplify pEYFP-C1, I used primers: 5'-GGG GAC CTC TAG ACT CAG GCC TGA AC-3', and 5'-CCC CCC GGG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TAG-3'. The amplified Zn-finger cDNA was subsequently cloned at the PCR generated XmaI and XhoI sites of pEYFP-C1.

2.2.2 Reporter Constructs

A wild-type hamster DHFR-luciferase reporter construct (DHFR-Lux) has been described (Udvadia, et al., 1995). DHFR-luciferase constructs carrying one or more consensus Sp2-binding sites were prepared as follows. To convert GC-boxes III and IV of the DHFR promoter to consensus Sp2-binding sites, the following oligonucleotide and its complement were employed: 5'-GGG CGG GAC CGG CCT GGT GGG GCG GGA CTC TGA CCT CGT GGG GGC-3'. To convert GC-box II to a consensus Sp2-binding site, the following oligonucleotide and its complement were employed: 5'-TCT GAC CTC GTG GGG GCG GGA CCT CTG ATG -3'. To convert GC-box I to a consensus Sp2-binding site, the following oligonucleotide and its complement were employed: 5'-GGC TTG TTG GGG CGG GAC CTC CGA TTC ACA AG-3'.

A PSA-luciferase reporter construct carrying a 5-kbp portion of the human prostate specific antigen promoter was a generous gift of Dr. Charles J Bieberich (University of Maryland–Baltimore, Baltimore, MD; Chen, et al., 2002). A chloramphenicol acetyltransferase (CAT) reporter gene linked to the adenovirus major late promoter, Δ53MLP-CAT, was obtained from Dr. Adrian R. Black (Roswell Park Cancer Institute, Buffalo, NY; Jensen, et al., 1997)). A CAT reporter gene carrying three multimerized copies of a consensus Sp2-binding site (pSp2-TK-CAT) was generated using the following synthetic oligonucleotide and its complement: 5'-GGG CTC GAG TGG GCG GGA CTA ATG GGC GGG ACT AAT GGG CGG GAC TAA AGA TCT GGG -3'. This duplex was cloned at the XhoI I and BglII II sites of pTK-CAT, which is a reporter gene regulated by a minimal promoter segment derived from the Herpes simplex virus thymidine kinase gene.

2.2.3 Baculovirus Stocks

An Sp2 cDNA carrying an amino terminal V5 epitope tag was generated by the PCR using pPAC-Sp2/flu as the template, Deep Vent DNA polymerase (New England Biolabs), and the following primers: 5'-GGG GCC ACC ATG GGA AAG CCA ATT CCA AAT CCA CTT CTT GGA CTT GAT AGT ACA ATG AGC GAT CCA CAG ACC AGC ATG-3', and 5'-GGG GTT ACA AGT TCT TCG TGA CCA G-3'. The resulting amplified, epitope-tagged cDNA was subcloned into pCR-Blunt$_{II}$-TOPO (Invitrogen) at the XbaI I and Bam HI sites and subsequently sub-cloned into pVL-1392 (Pharmingen, San Diego, CA) to create pVL-1392-Sp2/V5. Recombinant Sp2/V5 was transferred to the viral DNA backbone via homologous recombination using reagents

supplied by a proprietary kit (BaculoGold; Pharmingen). Sf9 cells ($3.0x10^6$) were plated in T-25 flasks with 5 ml Grace's complete media and incubated at 27 °C for 15 min. Sf9 cells were transfected with 0.5 μg of linearized BaculoGold DNA and 2.0 μg of pVL1392-Sp2/V5 according to the manufacturer's protocol and the infection was allowed to proceed for 5 days at 27 °C. Baculovirus stocks were amplified by infecting $2.5x10^6$ Sf9 cells plated in a T-25 flask for 5 days, followed by the infection of $7x10^6$ Sf9 cells plated in a T-75 flask for 5 days, and finally by infecting $7.5x10^7$ Sf9 cells seeded in a spinner flask for 5 days. The resulting Sp2 baculovirus stock was harvested by centrifuging the contents of the infected Sf9 spinner flask at 1000 RPM for 5 min at 4 °C and collecting the supernatant.

2.3 ANTIBODIES

Anti-Sp1 is a polyclonal rabbit antiserum prepared against a GST-fusion protein containing the amino-terminal 603 amino acids of Sp1 (Udvadia et al., 1995). Anti-Sp1 was used at a dilution of 1:1000 for Western blotting. Affinity purified anti-Sp1 (H-225; sc-14027; Santa Cruz Biotechnology) is a rabbit polyclonal antibody against amino acids 121-345 at the amino-terminus of Sp1. Anti-Sp1(H-225) was used at a dilution of 1:1000 for Western blotting. A polyclonal chicken anti-Sp2 antiserum was prepared against a GST-fusion protein containing the amino-terminal 496 amino acids of Sp2 (Aves Labs, Inc., Tigard, OR). Affinity purified anti-Sp2 (K-20; sc-643; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) is a rabbit polyclonal antibody prepared against the amino-terminus of Sp2. Anti-Sp2 (K-20) was used at a dilution of 1:1000 for Western blotting and 1:10 for immunoprecipitations. Anti-Sp3 is a polyclonal rabbit antiserum prepared against a

GST-fusion protein containing the amino-terminal 300 amino acids of Sp3 (Udvadia et al., 1995). Anti-Sp3 was used at a dilution of 1:2000 for Western blotting. Affinity purified anti-V5 (Invitrogen, Inc.) is a mouse monoclonal antibody against the 14 amino acid V5 epitope tag (NH_2-GKPIPNPLLGLDST-CO_2H). Anti-V5 was used at a dilution of 1:1000 for Western blotting. Affinity purified anti-HA (12CA5; Roche, Inc., Indianapolis) is a mouse monoclonal antibody that recognizes a 10 amino acid epitope (NH_2-YPYDVPDYAS-CO_2H) derived from the *Influenza* hemagglutinin protein (HA). Anti-HA was used at a dilution of 1:1000 for Western blotting and 1:10 for immunoprecipitations. Affinity purified anti-HA (Y-11; sc-805, Santa Cruz Biotechnology) is a rabbit polyclonal antibody that recognizes the same HA epitope. Y-11 was used at a dilution of 1:1000 for Western blotting and 1:10 for immunoprecipitations. All antibodies were used at a dilution of 1:20 for the identification of proteins in protein/DNA-binding assays. For indirect immunofluorescence experiments, affinity-purified rabbit (sc-5621, anti-PML (H-238); sc-20682, anti-Lamin B1 (H-90); sc-805, anti-HA (Y-11)) and goat antisera (sc-18555, anti-NuMA (N-20)) were obtained from Santa Cruz Biotechnology, and each was used at a dilution of 1:500. Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 594 goat anti-rabbit, and Alexa Fluor 594 rabbit anti-goat secondary antibodies were obtained from Molecular Probes, Inc. (Eugene, OR). Each was used at a dilution of 1:500 for indirect immunofluorescence experiments.

2.4 OLIGONUCLEOTIDES

2.4.1 Oligonucleotide Probes

Oligonucleotides were obtained from Invitrogen, Inc. Probes were synthesized on automated, proprietary DNA synthesizers using standard cyanoethyl phosphoramidite chemistry, then deprotected and partially purified through proprietary columns. Oligonucleotide probes for standard and quantitative protein/DNA-binding assays were prepared from 60-mers, each carrying a single promoter-derived Sp-binding site flanked by common nucleotide sequence. Oligonucleotides used include the following and their complementary strands (bold lettering indicates Sp-binding sites):

p21: 5'-TCG GTA CCT CGA GTG AAG CTT GA**C CCG CCT CCT** ATG AAT TCG GAT CCG CGG TAA-3'

MDR-1: 5'-TCG GTA CCT CGA GTG AAG CTT GA**C GCC GGG GCG TGG** GCA TGA ATT CGG ATC CGC GGT AA-3'

DHFR 1: 5'-TCG GTA CCT CGA GTG AAG CTT GA**A GGG CGT GGC** ATG AAT TCG GAT CCG CGG TAA-3'

DHFR 2: 5'-TCG GTA CCT CGA GTG AAG CTT GA**G AGG CGG GGC** ATG AAT TCG GAT CCG CGG TAA-3'

DHFR 3: 5'-TCG GTA CCT CGA GTG AAG CTT GA**G AGG CGG AGT** ATG AAT TCG GAT CCG CGG TAA-3'

DHFR 4: 5'-TCG GTA CCT CGA GTG AAG CTT GA**T GGG CGG GGC** ATG AAT TCG GAT CCG CGG TAA-3'

DHFR *: 5'-TCG GTA CCT CGA GTG AAG CTT GA**A GGG CGG GAC** ATG AAT TCG GAT CCG CGG TAA-3'

c-fos: 5'- TCG GTA CCT CGA GTG AAG CTT GA**C CCT TGC GCC ACC** ATG AAT TCG GAT CCG CGG TAA-3'

Annealed, complementary oligonucleotides were labeled with [γ-^{32}P]dATP (ICN, Inc., Costa Mesa, CA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Probes were purified through Sephadex G-50 spin columns.

2.5 *IN VITRO* TRANSCRIPTION/TRANSLATION

In vitro transcribed/translated proteins were generated using the TNT Quick Coupled Transcription/Translation reticulocyte lysate System (TNT; Promega, Inc., Madison, WI) using T7 RNA polymerase. *In vitro* translated proteins were radiolabeled with [^{35}S]-L-methionine (Tran ^{35}S-label; ICN, Inc.; 100μCi/ml).

2.6 LUCIFERASE ASSAYS

One day prior to transfection, *Drosophila* SL2 (7.5×10^5) or DU145 (2.0×10^5) cells were plated in each well of 6 well plates. Transient transfections were performed using SuperFect Transfection Reagent (Quiagen, Inc., Hilden, Germany). Each well of transfected cells received 2.0 μg of total DNA (0.5 μg of expression plasmid, 0.5 μg of *Renilla* Luciferase reporter DNA, 0.5 μg Δ53MLP-CAT reporter DNA, and 0.5 μg of λ^+ DNA) in serum-free media (100 μl), to which SuperFect (10 μl) was then added. Reactions were incubated for 10 min at room temperature, diluted with serum-containing media (600 μl) and added to cells. For luciferase assays, the Dual-Luciferase Reporter Assay System (Promega) was employed to quantify luciferase activity. Two days post-

transfection, cell extracts were prepared from PBS-washed cells following a 15 min incubation in Passive Lysis Buffer (500 µl/well). Debris was cleared by centrifugation at 14,000 rpm for 5 min. Lysates were divided in two, with half quantified for luciferase activity and half for CAT activity. Reactions containing 100 µl of extract and 100 µl of Luciferase Assay Reagent II were mixed and luminescence was detected in a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Results were normalized to Δ53MLP-CAT activity (see below).

2.7 CHLORAMPHENICOL ACETYLTRANSFERASE (CAT) ASSAYS

CAT assays were performed using a liquid scintillation method described previously (Gorman, et al. 1982). Extracts from *Drosophila* SL2 or DU145 cells were prepared two days post-transfection by collecting PBS-washed cells and resuspending them in Passive Lysis Buffer. Cells were transferred to microfuge tubes and freeze/thawed three times in a dry ice-ethanol and 37 °C water baths, debris was removed by centrifugation at 14,000 rpm for 5 min, and CAT assays were performed in scintillation vials. Extracts (25 µl) were added to a cocktail containing 75 µl 0.1 M Tris pH 7.8 and 100 µl of 0.5 µCi/ml ^{3}H-Acetyl CoA (ICN) in 2mg/ml chloramphenicol dissolved in 0.1 M Tris pH 7.8. Reactions were vortexed gently and overlaid with 3 ml Econofluor-2 non-aqueous scintillation fluid (Packard Instrument Company, Inc., Meridian, CT). The abundance of ^{3}H-chloramphenicol in samples was quantified at least three times for 30 sec each over a four hour time course, and the mean counts per minute were used to normalize for plate-to-plate differences in luciferase activity in transient transfection experiments.

2.8 CELL EXTRACTS

2.8.1 Nuclear Extracts

Nuclear extracts were prepared using a method described by Lee, et al. (1988). Cells were rinsed with PBS, scraped from plates and collected by centrifugation at 1000 rpm for 10 min at 4 °C. The volume of the cell pellet was estimated and the pellet was resuspended in an equal volume of hypotonic buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). The cells were incubated on ice for 15 min, then passed through a 25-gauge needle, and nuclei were collected by centrifugation at 3,000 rpm for 5 min at 4°C. The cell pellet volume was again estimated and resuspended in hypertonic buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF) at approximately 1 ml/0.3 ml pellet volume, and nuclei were stirred for 5 min at 4°C. Extracts were subsequently cleared of debris by centrifugation at 14,000 rpm, 5 min at 4°C.

2.8.2 Non-Denatured Whole Cell Protein Extracts

Cells were scraped from tissue culture plates or decanted from spinner flasks and pelleted at 2500 rpm for 5 min at 4°C. Cell pellets were washed with PBS and then resuspended in 200 µl of ice-cold EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 µM Na-orthovanadate, and 10 µg/ml Pepstatin A, PMSF and Leupeptin), and incubated for 60 min at 4 °C with agitation. Extracts were cleared of debris by centrifugation at 14,000 rpm for 30 min at 4°C, and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL) and BSA standards.

2.8.3 Treatment Experiments to Activate Sp2 DNA-binding

2.8.3.1 Ethanol Stress

COS-1 cells plated at 2.5 x 10^6 cells per 100-mm tissue culture dish were treated with DMEM containing 7% ethanol followed by a 20 min incubation at 37 °C under 5% CO_2. Non-denatured whole cell extracts were prepared as described above.

2.8.3.2 Oxidative Stress

COS-1 cells plated at 2.5x10^6 per 100-mm tissue culture dish were treated with media containing 0.7 M H_2O_2 followed by a 15 min incubation at 37 °C under 5% CO_2. Non-denatured whole cell extracts were prepared as described above.

2.8.3.3 Osmotic Stress

COS-1 cells plated 2.5 x 10^6 per 100-mm tissue culture dish were treated with DMEM containing 0.7 M NaCl followed by a 20 min incubation at 37 °C under 5% CO_2. Non-denatured whole cell extracts were prepared as described above.

2.8.3.4 Heat Shock Stress

COS-1 cells plated 2.5 x 10^6 per 100-mm tissue culture dish and cultured in complete DMEM media were subjected to heat shock stress by incubating cells collected by scraping in a 43 °C water bath for 10 min. Non-denatured whole cell extracts were prepared as described above.

2.8.3.5 Neuronal Growth Factor (NGF)

PC12 cells plated 2.5 x 10^6 per 100-mm tissue culture dish and cultured in DMEM supplemented with 10% horse serum and 5% calf serum were treated with 10% horse serum and 5% calf serum were treated with 100 ng/ml NGF (Alomone Labs, Jerusalem,

Israel) in DMEM medium containing 1% horse serum at 37°C for three days prior to making extracts

2.8.3.6 TPA

DU145 cells were plated at 2.5 x 10^6 per 100-mm tissue culture dish and cultured in complete DMEM media with 10 nM TPA (phorbol 12 –myristate 13-acetate; Sigma) in DMSO for 3 hours at 37 °C, nuclear extracts and non-denatured whole cell extracts were prepared as described above.

2.8.3.7 Dibutyryl cAMP

DU145 cells were plated at 2.5 x 10^6 per 100-mm tissue culture dish and cultured in complete DMEM media were treated with 1 mM dibutyryl cAMP (Sigma) for 3 hours at 37 °C. Subsequently, nuclear extracts and non-denatured whole cell extracts were prepared as described above.

2.8.3.8 Calcium ionophore (A23187)

DU145 cells were plated at 2.5 x 10^6 per 100-mm tissue culture dish and incubated with 3 μM of the calcium (Ca^{2+}) ionophore A23187 (Sigma) for 3 hours at 37 °C under 5% CO_2. Nuclear extracts were prepared as described above.

2.9 PROTEIN/DNA-BINDING ASSAYS

2.9.1 Standard Protein/DNA-Binding Assay

Nuclear or non-denatured whole cell extracts (5 μg) prepared from baculovirus-infected Sf9 cells or cells transiently transfected with expression vectors were incubated with poly dI/dC (0.067 μg/μl) in 4 μl binding buffer (10 mM Tris pH 7.5, 50 mM NaCl,

5% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM $ZnCl_2$). Antibodies or competitor oligonucleotides were added to reactions where necessary and each reaction received 10^4 cpm of radiolabeled double-stranded oligonucleotide probe. Binding reactions were incubated for 5 min at room temperature, 4 μl of loading buffer (20% Ficol (Fisher, Inc.) was added to each reaction, and protein/DNA complexes was resolved at 250V for 1 hr on 4% polyacrylamide gels (30% acrylamide, 0.8% bis-acrylamide) that were pre-run for 2 hrs in 0.5 x TBE (0.025 M Tris pH 8.3, 0.025 Boric acid, 0.5 mM EDTA). Gels were transferred to Whatman paper by drying and protein/DNA complexes were visualized by autoradiography.

2.9.2 Nuclear Extract "Mixing" Experiment and Deoxycholate Treatment

Nuclear extracts from T98G, Jurkat and HCT116 mammalian cells and non-denatured whole cell extracts prepared from Sp2 baculovirus-infected Sf9 cells were prepared as described above. Sf9 extracts (5 μg) containing recombinant Sp2 protein were incubated with 1, 2, 4, or 8 μg of mammalian nuclear extracts for 10 min at room temperature. Subsequently, extracts were supplemented with poly dI/dC (0.067 μg/μl), and binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM $ZnCl_2$), and radiolabeled probe (DHFR*; 10^4 cpm). Reactions were incubated for 5 min at room temperature, resulting protein/DNA complexes were resolved on 4% polyacrylamide gels, and visualized by autoradiography.

For treatment with deoxycholate, mammalian nuclear extracts were combined with 5 μg of recombinant Sp2 and incubated with 1% deoxycholate for 20 min on ice. 1.2% NP-40 was added to the reaction prior to the addition of poly dI/dC and binding

buffer. Radiolabeled DHFR* (10^4 cpm) was added and the reaction was incubated for 5 min at room temperature. Resulting protein/DNA complexes were resolved on 4% polyacrylamide gels and visualized by autoradiography as described above.

2.9.3 Phosphatase Treatment

Phosphatase treatment of Sp2 was performed essentially as described by Jurutka, et al., 2002. Whole cell extracts were prepared from Sp2 baculovirus-infected Sf9 cells as described above, and 5 μg of Sf9 extract was incubated with decreasing amounts of Potato Acid Phosphatase (1 – 10^8 units; PAP; Roche) dissolved in PAP reaction buffer (20 mM Tris, pH 7.2, 1 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol) at 25 °C for 15 min in a final volume of 5 μl. Phosphatase activity was neutralized by addition of 100 mM Na_2HPO_4 in a total volume of 10 μl and incubation for an additional 15 min at 25°C. Poly dI/dC, binding buffer, and radiolabeled DHFR* probe (10^4 cpm) were added and reactions were incubated for 5 min at room temperature. Protein/DNA-binding reactions were applied to 4% polyacrylamide gels and processed as described above.

2.9.4 Quantitative Protein/DNA-Binding Assay

Whole cell extracts prepared from Sp2 baculovirus-infected Sf9 cells were incubated as above with a radiolabeled oligonucleotide probe (10^4 cpm) derived from the c-fos promoter (5'-CCC TTG CGC CAC CCC TCT-3'). Resulting protein/DNA complexes were resolved as above, and quantified *in situ* using an InstantImager (Packard, Inc.). Volumes of infected cell extracts that led to half-maximal binding of this

probe were then employed in similar assays performed in triplicate with Sp-binding sites derived from p21, MDR-1 and DHFR promoters and quantified *in situ*.

2.10 WESTERN BLOTTING

PBS-washed cells were pelleted at 1000 rpm for 10 min, resuspended in 200 μl of Western sample buffer (2% SDS, 60 mM Tris pH 6.8, 100 mM DTT), passed through a 22.5 gauge needle, and boiled for 5 min. Debris was cleared by centrifugation at 14,000 rpm for 10 min at 4 °C, and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). Proteins were resolved on denaturing 8% polyacrylamide gels, and transferred to PVDF membranes (Millipore, Inc. Billerica, MA) at 20 V for 20 min using a semidry transfer apparatus (Transblot-SD, BioRad, Inc., Hercules, CA). Membranes were blocked with 5% fat-free dried milk in TBS-T (2.42 g/L Tris pH 7.6, 8 g/L NaCl, supplemented with 1% Tween-20) for 60 min to overnight. Following blocking, membranes were incubated with primary antibodies diluted in TBS-T containing 5% milk for 60 min with gentle rocking. Membranes were then washed four times for 15 min each with TBS-T, and incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min with gentle rocking. Anti-mouse (NA 9310; Amersham Pharmacia Biotech, Inc., Arlington Heights, IL) or anti-rabbit (Promega) secondary antibodies were diluted 1:10,000 or 1:40,000, respectively, in TBS-T containing 5% milk prior to incubation with membranes. Membranes were washed with TBS-T once for 15 mins followed by four subsequent washes for 5 min each. Antigen-antibody complexes were detected using Western Blot Chemiluminescent Detection Reagents (ECL; Amersham).

2.11 IMMUNOPRECIPITATION

Cells were metabolically labeled following pre-incubation for 60 min in methionine-free DMEM supplemented with 2% dialyzed heat-inactivated fetal bovine serum, glutamine, and sodium pyruvate at 37 °C under 5% CO_2. Cells were incubated for an additional 3 hrs in methionine-free DMEM containing 100 μCi/ml [^{35}S]-L-methionine labeled (Tran ^{35}S-label; ICN). Non-denatured whole cell extracts were prepared by incubating PBS-washed cells in ELB$^+$ (0.25 M NaCl, 0.1% NP-40, 50 mM Hepes pH 7.0, 1 mM PMSF, 5 mM EDTA, 0.5 mM DTT; approximately 1 ml per 10 cm plate) for 30 min on ice. Disrupted cells were removed to microcentrifuge tubes, and debris was cleared by centrifugation at 14,000 rpm for 10 min at 4 °C. Antibodies (5μl of affinity purified antibodies and 10μl of polyclonal whole serum) were added to each extract, and reactions were incubated for 60 min at 4 °C with gentle rocking. A 1:1 slurry of Protein A Sepharose (PAS; Zymed, Inc., San Francisco, CA) equilibrated in ELB$^+$ was added at a dilution of 1:50 and incubated for 60 min at 4 °C with gentle rocking. Bead-bound proteins were washed twice with 1 ml ELB$^+$ and three times with ice-cold PBS. Proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer (0.125 M Tris pH 6.8, 2% SDS, 40% glycerol, bromophenol blue, 50 μl/ml β–mercaptoethanol) and were resolved on denaturing polyacrylamide gels. For immunoprecipitation of Tran ^{35}S-label (ICN) mammalian cell extracts or *in vitro* translated proteins, results were visualized by fluorography.

2.12 *IN VITRO* PROTEIN/PROTEIN-BINDING ASSAYS

2.12.1 Expression of GST-Fusion Proteins

BL21 CodonPlus competent bacterial cells (Stratagene, Inc., La Jolla, CA) transformed with GST-fusion expression constructs were cultured in Terrific Broth media (Mediatech, Inc., Herndon, VA) overnight at 37 °C with 250 rpm agitation. Cells were diluted 1:10 with fresh Terrific Broth and cultured until OD=0.5 (approximately 1-1.5 hrs). Protein expression was induced via the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sigma) at a final concentration of 0.2 mM and cells were cultured for an additional three hrs at 37 °C and 250 rpm. Cells were collected by centrifugation at 3000 rpm for 10 min, pellets were resuspended 9 mls in PBS containing 10 μg/ml PMSF, pepstatin A, and leupeptine and 1 mM EDTA and cells were lysed by sonication. Debris was removed by centrifugation at 10,000 for 10 min at 4 °C, and Triton X-100 was added to a final concentration of 1%. The resulting cell pellet was suspended in 1.5% N-lauroylsarcosine (Sarkosyl; Sigma), 25 mM triethanolamine, 1 mM EDTA pH 8.0 and incubated on ice for 10 min. The suspension was centrifuged at 10,000 for 20 min at 4 °C and raised to 1% Triton X-100 and 1 mM $CaCl_2$.

2.12.2 Protein/Protein-Binding Assay

Glutathione-agrose beads (Sigma) were added to cleared bacterial extracts, and the mixture was rocked overnight at 4 °C. Beads were then washed three times with PBS and resuspended in one volume of NETN (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). To quantitate amounts of GST-fusion proteins bound to beads, 25

μl aliquot of bead-bound proteins were boiled in Laemmli sample buffer, eluted proteins were resolved on acrylamide gels in parallel with Bovine Serum Albumin (BSA) standards, and gels were stained with Coomassie Brilliant Blue. *In vitro* protein/protein-binding assays were performed by incubating 5 μg of bead-bound GST-fusion proteins with [^{35}S]-L-methionine labeled (Tran ^{35}S-label; ICN) mammalian cell extracts 1 ml or 10 μl *in vitro* translated proteins in 400 μl NETN for 60 min at 4 °C with gentle rocking. Beads were washed four times with NETN and proteins were eluted by boiling in Laemmli sample buffer. Eluted proteins were resolved on denaturing acrylamide gels and visualized by fluorography.

2.13 DNA SEQUENCING

Double-stranded DNA sequencing was performed using Sequenase version 2.0 T7 DNA polymerase (USB, Cleveland, OH). Plasmid DNAs (5μg) were denatured by incubation at 37 °C for 30 min in 20 μl dH_2O plus 2 μl (2 M NaOH, 2 mM EDTA). Reactions were neutralized and DNAs were precipitated with 2.2 μl 3 M NaOAc and 65 μl absolute ethanol. Annealing reactions were performed by resuspending denatured DNAs in 1.5 μl dH_2O, 1 μl Sequenase buffer, and 200 ng of primer, and incubating at 37 °C for 30 min. Annealed DNAs was extended by adding 0.5 μl 0.1 M DTT, 1.0 μl labeling mix (diluted 1:5 in dH_2O), 1.0 μl Sequenase DNA polymerase (diluted 1:8 in Sequenase dilution buffer), and 0.5 μl (5 μCi) [α-^{35}S]dATP (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA) and incubation at room temperature for 5 min. To terminate extension reactions, 1.75 μl of the extension reaction was added to 1.3 μl each of four dideoxy termination mixtures. Termination reactions were incubated at 37

°C for 5 min, and then stopped by the addition of 2 µl FDE (90% deionized formamide, 20 mM EDTA, bromophenol blue (0.2%), xylene cyanol (0.2%). Sequencing reactions were boiled for 2 min, resolved on acrylamide gels at 70W for 2 hrs in 0.5 x TBE (5.45 g/L Tris (pH 8.0) 2.78 g/L Boric acid, and 0.51 g/L EDTA. Resulting sequencing reaction were visualized by autoradiography.

2.14 SITE DIRECTED MUTAGENESIS

The QuickChange Site-Directed Mutatagenesis system (Stratagene) was employed for PCR-mediated site-directed mutatagenesis. A wild-type hamster DHFR-luciferase reporter construct (DHFR-Lux) was employed as a template with complementary oligonucleotides listed in section 2.4.2. The PCR cycles were performed in a 50 µl reaction containing 50 ng of circularized template, 125 ng of each oligonucleotide, 500 µM dNTPs, and 2.5 U *PfuTurbo* DNA polymerase (Stratagene). PCR reaction conditions were 95 °C for 30 sec, 55 °C for 1 min and 68 °C for 12 min (2 minutes/kb of plasmid) for 18 successive rounds of PCR amplification. The entire 50 µl reaction was digested with DpnI (10 U; New England Biolabs) to remove methylated, non-mutated template plasmid, and 1 µl of DpnI I-digested amplified DNA was used to transform competent DH5α E. coli. Resulting bacterial colonies were screened by DNA sequencing (section 2.13). Successive rounds of mutagenesis were employed to generate DHFR reporter constructs with multiple consensus Sp2-binding sites.

2.15 CELL CYCLE ANALYSIS

T98G human glioblastoma cells (2.5×10^5) were plated in 60-mm tissue culture dishes containing DMEM supplemented with heat-inactivated FBS, and 50 μg/ml Pipracil, glutamine, and sodium pyruvate at 37 °C under 5% CO_2. Cells were incubated in media containing 0.2% fetal calf serum for 72 hrs to arrest growth, and then stimulated to re-enter the cell cycle by the addition of media containing 20% fetal calf serum. Whole cell or nuclear extracts were prepared at 0, 8, 12, 20, 24, 28, and 32 hrs post-serum stimulation.

2.16 YEAST "TWO-HYBRID" SCREEN

The Matchmaker yeast "two-hybrid" system (Clontech Laboratories, Palo Alto, CA) was employed for this genetic screen. A cDNA fragment encoding the *trans*-activation domain of Sp2, corresponding to subdomains A, B and C (amino acids 1-496), was sub-cloned into the TRP1+ GAL4 DNA-binding domain vector pGAD9 creating pGBR9-Sp2 (ABC). Yeast strain Y190 (*his3 leu2 trp1*), carrying Gal4-dependant lacZ and HIS3 genes, was cultured to OD_{600}=0.5, pelleted at 1000 rpm for 5 min at room temperature, resuspended in 1:10 volume of dH_2O, and pelleted again. Yeast cells were made competent by resuspending in 1.5 ml of sterile TE/LiAc (10 mM Tris pH 7.5, 1 mM EDTA, 100 mM LiAc), and 0.1 ml competent cells were transformed with 0.1 μg of pGBT9-Sp2 (ABC) DNA and 0.1 mg of herring testes DNA in 600 μl (40% PEG + TE/LiAc). The cell/DNA mixture was vortexed and incubated at 30 °C for 30 min, and cells were subsequently incubated at 42 °C for 15 min following the addition of 10%

DMSO. Yeast transformants were pulse-centrifuged, resuspended in 0.5 ml sterile TE (10 mM Tris pH 7.5, 1 mM EDTA) and plated on SD-selection agar plates lacking tryptophan (6.7 g/L yeast nitrogen base (Difco), 20 g/L agar, 100 ml/L amino acid solution (-TRP), 40 m/L 50% dextrose, pH 5.8) supplemented with 25 mM 3-AT (3-amino-1,2,4-triazole; Sigma). A culture derived from a single Trp+His+ transformant was subsequently transformed with a cDNA library derived from HeLa cells fused to the Gal4 *trans*-activation domain in LEU2+ plasmid pGAD424. $Leu^{+}Trp^{+}His^{+}$ transformants capable of growth on agar plates containing 25 mM 3-AT were screened *in situ* using β-galactosidase colony-lift and liquid culture assays. Yeast cells were transferred to Whatman #5 filter paper and permeabilized by freezing in liquid nitrogen and thawing at room temperature. Filters carrying disrupted yeast colonies were placed on sheets of filter paper that had been presoaked in Z buffer/X-gal solution (100 ml Z buffer [16.1 g/L $Na_2HPO_4 \bullet 7H_2O$, 5.5 g/L $Na_2HPO_4 \bullet H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4 \bullet 7 H_2O$, pH 7.0], 1.67 ml X-gal [20 mg/ml 5-bromo,4-chloro,3-indolyl, β-D-galactoside (β-gal; Sigma) in N,N-dimethylformamide (Sigma)], 0.27 ml β-mercaptoethanol) and incubated at 30 °C 30 min to overnight. Filters were monitored for the appearance of blue colonies. Following three rounds of *in situ* screening, positive colonies were subsequently screened with liquid culture assay using ONPG (o-nitorphenyl β-D-galactopyranoside; Sigma) as substrate. For liquid assays, $Leu^{+}Trp^{+}His^{+}lacZ^{+}$ colonies grown on agar plates containing 25 mM 3-AT were cultured in SD media ($Leu^{-}Trp^{-}His^{-}$) overnight at 30 °C shaking at 250 rpm. The following day, 2 ml of cultured yeast were added to 8 ml of YPD media (20 g/L Dicfo peptone, 10 g/L yeast extract and 2% dextrose) and cultured at 30 °C until growth OD_{600}=0.5. Cells were pelleted and resuspended in 300 μl Z buffer, and

permeabilized by freezing in liquid nitrogen and thawing at 37 °C three times. Subsequent to adding 700 µl Z buffer + β-mercaptoethanol, 160µl of ONPG in Z buffer was added to the reaction and the culture was incubated at 30 °C until reactions appeared yellow, about 45 min. Reactions were treated with 0.4 ml of 1 M Na_2CO_3, cells were pelleted, and OD_{420} values were obtained as an indication of the strength of protein/protein interactions. To establish whether protein/protein interactions were specific, matings were performed with Y187 cells transformed with one of the following control plasmids: pGBT9Sp1 (Sp1 *trans*-activation domain), pGBT9Sp3 (Sp3 *trans*-activation domain), 5'RbpAS2 (Rb amino-terminus), pLAMC (Lamin A), pVA3 (p53), pCL1 (full length, wild type GAL4), pTD-1 (SV40 large-T antigen). Candidate library DNAs were recovered and purified form yeast cells that tested negative for interactions with 5'RbpAS2, pLAMC and pVA3. To prepare plasmid DNAs, cells were pelleted and then resuspended in approximately 50 µl supernatant. Cells were disrupted by addition of 0.2 ml (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), 0.2 ml phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 ml glass beads followed by vortexing for 2 min. After centrifugation at 14,000 rpm, the aqueous phase was removed and DNA was recovered by ethanol precipitation. To rescue pGAD424 DNA, competent KC8 bacterial cells (carrying hisB, leuB and trpC mutations) were transformed with plasmid DNA, and plated on M9 minimal agar plates (1 ml 1M thiamine-HCL, 100 ml/L amino acid solution (-LEU), 40 mg/L proline, 20 g/L agar, and 50 mg/L ampicillin). All resulting colonies were amplified and cDNA inserts sequenced.

2.17 DERIVATION OF CONSENSUS Sp-2-BINDING SITE ("CASTing")

Sf9 cells cultured in a 100 ml spinner flask at 27 °C were infected with an Sp2 baculovirus stock, and non-denatured whole-cell extracts were prepared 48 hrs later. Infected-cell extracts (300 μl) were incubated with 50 μl of mouse monoclonal α-V5 (Invitrogen) antibody with of 500 μl ESB buffer (20 mM Hepes pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 10% glycerol, 1 mM PMSF, 3 mg/ml BSA, 2% Ficol, and 25 μg/ml salmon testes DNA) for 60 min at 4 °C with gentle agitation. To precipitate antibody-antigen complexes, a 1:1 slurry of Protein A Sepharose (PAS; Zymed) equilibrated in ESB was added at a dilution of 1:20 and incubated for 60 min at 4 °C with gentle rocking. Following three washes with ESB, bead-bound proteins were incubated with 300 μl of ESB containing a double-stranded 60-mer carrying a 16-nucleotide central region of random sequence (ED1; 5'-TCG GTA CCT CGA GTG AAG CTT GAN NNN NNN NNN NNN NNN ATG AAT TCG GAT CCG CGG TAA-3' and its complementary strand) for 30 min at room temperature with gentle rocking. Following three washes with ESB, protein-bound DNAs were amplified by the PCR using 200 ng ED3 (5'-TCG GTA CCT CGA GTG AAG CTT GA-3') and 200 ng ED4 (5'-TTA CCG CGG ATC CCA CGA ATT CAT-3') as primers in a 50 μl PCR reaction containing 200 μM dCTP, 500 μM dNTP (- dCTP), 10 μg BSA, 1 mM $MgSO_4$, and 5 μl [α-^{32}P]dCTP (3000 μCi/mmol; ICN) and 10 U DeepVent polymerase (NEB). Reaction parameters were: 15 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. Radiolabeled amplified DNAs were purified as described above using Sephadex G-50 spin columns. Sp2-infected Sf9 cell extracts were once again precipitated with anti-HA

antibody, and following washes with ESB, amplified DNAs from the first round of immunoprecipitation/PCR were added to precipitates as detailed above. Protein/DNA mixtures were precipitated with PAS, washed, and subjected to another round of PCR. Following eight successive rounds of immunoprecipitation and DNA amplification, resulting DNAs were cloned into pCR-Blunt II-TOPO (Invitrogen) vector and 21 independent clones were subjected to dideoxy-sequencing as described above.

2.18 EQUILIBRIUM DISSOCIATION CONSTANT (K_d) DETERMINATION

Calculations of K_d values were performed by incubating dilutions of non-denatured cell extracts prepared from Sp2 baculovirus-infected Sf9 cells with 200 pmol of radiolabeled DHFR* oligonucleotide until a volume of cell extract was identified that complexes with 50% of the input probe (5 μl of Sp2 extract). The abundance of free probe and Sp2 protein-DNA complexes was quantified *in situ* using an InstantImager (Packard). To ensure that Sp2-binding sites were limiting under these conditions, additional protein/DNA-binding assays were performed with five-fold increasing and decreasing amounts of radiolabeled probe. An excess of infected cell extract was then assayed with 200 pmol of DHFR* radiolabeled probe and increasing amounts of unlabeled homologous competitor DNA. The concentration of unlabeled oligonucleotide that led to 50% occupancy of the radiolabeled probe was determined by quantification *in situ,* and this concentration was divided in half to determine the amount active Sp2 in diluted protein extracts. This value was then divided by the original extract dilution factor to derive the equilibrium dissociation constant (225 pmol for Sp2).

2.19 NUCLEAR MATRIX FRACTIONATION

Nuclear matrix fractionations were performed essentially as described by Zaidi, et al., 2001. COS-1 cells (2.5×10^6) were plated in 100-mm tissue culture dishes containing DMEM supplemented with heat-inactivated FBS and 50 μg/ml Pipracil at 37 °C under 5% CO_2. The following day, cells were transfected with 10 μg of pCMV-Sp1/flu, pCMV-Sp2/flu, pCMV-Sp3/flu, or pCMV-Sp4/flu using SuperFect transfection reagent (Quiagen) as described above, and incubated at 37 °C under 5% CO_2. For subcellular fractionation, cells were collected in ice-cold PBS containing 1x Complete™ protease inhibitors (Roche). Cell pellets were resuspended in 300 μl CSK buffer (100 mM NaCl, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X-100) containing 0.3 M sucrose, 1.6 mM ribonucleoside-vanadyl complex (New England Biolabs), and 1.2 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride; Fisher Scientific, Suwanee, GA) for 10 min on ice. Nuclei were collected by centrifugation and supernatants containing soluble proteins were stored at -80 °C. Nuclei were subsequently extracted with 300 μl Digestion Buffer (50 mM NaCl, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X-100) containing 400 units per ml RNase-free DNase I (Roche) for 30 min at room temperature. To terminate digestion reactions, 250 mM NH_4SO_4 was added to nuclei and incubated for 5 min at room temperature. Extracted nuclei were centrifuged to separate soluble nuclear proteins (supernatant; designated the chromatin fraction) and insoluble nuclear matrix (pellet; designated the nuclear matrix fraction). Nuclear matrix fractions were resuspended in 300 μl of Western sample buffer. Equal volumes of each fraction were resuspended in Laemmli sample buffer and resolved on denaturing 8% polyacrylamide gels.

2.20 INDIRECT IMMUNOFLUORESCENCE

2.20.1 Indirect Immunofluorescence and Time Lapse Confocal Microscopy

For confocal microscopy, 2-4 x 10^4 COS-1 cells were plated in 35 mm glass-bottom dishes (MatTek Corp., Ashland, MA) and cultured overnight at 37 °C in DMEM supplemented with heat-inactivated FBS, and 50 μg/ml Pipracil at 37 °C under 5% CO_2. The following day cells were transfected with 3 μg of pEYFP-Sp2 using SuperFect transfection reagent (Qiagen) as described above, and cultured for 24 hours at 37 °C under 5% CO_2. Plates were transferred to a humidified microscope chamber (INC-2000 Incubator System; 20/20 Technologies, Wilmington, NC) heated to 37 °C, and supplemented with 5% CO_2. Nuclei were imaged using a Nikon Eclipse TE-2000E microscope configured with a 40x oil immersion lens (1.4 N.A.), YFP BP HYQ filter cube with a 535 nm bandpass filter, and C1 confocal workstation administered by EZ-C1 2.30 confocal laser scanning software. EYFP-positive cells were identified via widefield epifluorescence and subsequently scanned into 35 Z-sections with an argon ion laser at 488 nm every 10 min for 18 hrs. Volume rendering software was applied to generate a composite image of all 35 Z-sections at each time point and then combined to generate a time-lapse video of the collected data as well as a series of static images each representing data collected each hour of observation.

2.20.2 *In Situ* Nuclear Matrix Preparation and Analysis

Cells were analyzed *in situ* by direct and indirect immunofluorescence following the solubilization of nuclei and preparation of nuclear matrices essentially as described by Javed, et al 2000. COS-1 cells (2 X 10^5) were plated on sterile glass coverslips in each

well of 6-well plates and cultured at 37 °C overnight in DMEM culture media supplemented with heat-inactivated FBS, and 50 μg/ml Pipracil at 37°C under 5% CO_2. The following day, some wells were transfected with expression vectors carrying GFP-fusion constructs (EYFP-Sp1, EYFP-Sp2 or EGFP-Sp3; 2 μg per well) using SuperFect transfection reagent (Qiagen) as described above, and cultured for 48 hours at 37 °C under 5% CO_2. Plates were placed on ice, washed twice with ice cold PBS, and cells were solubilized in CSK buffer. Following removal of CSK buffer chromatin was digested at 30° C for 60 min via incubation of nuclei in Digestion Buffer. Following removal of Digestion Buffer, nuclei were incubated for 10 min on ice in Stop Solution (Digestion Buffer containing 250 mM NH_4SO_4). Nuclei were subsequently fixed with 2% paraformaldehyde at room temperature for 15 min, washed with PBS, and stained with DAPI (4',6-diamidino-2-phenylindole) in 0.5% Triton X-100/PBS. Coverslips were washed with PBS, then dH_2O, and mounted using Vectashield mounting media (Vector Labs, Burlingame, CA). Fluorescence was imaged using a Nikon TE-200 inverted epifluorescence microscope equipped with appropriate optics and filter blocks at a magnification of 100x under oil immersion. Results were recorded with a digital camera (SPOT, Jr.: Diagnostics Instruments, Inc., Sterling Heights, MI) and proprietary software using the manufacturer's instructions.

2.21 COLUMN FILTRATION CHROMATOGRAPHY

Non-denatured whole cell extracts prepared from twenty 100 mm tissue culture plates of COS-1 cells, cultured at 37 °C in complete DMEM culture media under 5% CO_2, were prepared in 10 ml of EBC buffer as described above. To fractionate whole

cell lysates, a HiPrep Sephacryl S-300 High Resolution 26/60 chromatography column (26/60; Amersham) with a bed volume of 320 ml containing 47 μm particles of a cross-linked copolymer of allyl dextran and N,N-methylenebisacrylamide was employed. This resin is designed for the separation of proteins in the range of 1,500-10 kDa. The column was calibrated using 3.9 ml of a 1:2 dilution of distilled water and Start Buffer (0.05 M Na_2HPO4, 0.15 M NaCl, pH 7.2) and the Molecular Weight Marker Kit for Gel Filtration Chromatography (Sigma; includes molecular weight marker proteins of 669, 443, 150, 66, and 29 kDa). Prior to applying cell lysates, the column was pre-run at 21 psi of pressure in running buffer (50 mM Tris, 100 mM NaCl, 0.02% Sodium Azide) at a flow rate of 1 ml/min for 60 min. The entire 10 ml volume of lysate was applied to the column, ran at 21 psi at a flow rate of 1 ml/min, and eluted proteins were collected in 3 ml aliquots every 3 minutes for an additional 105 minutes using the Biologic LP Chromatography system (BioRad). Elutions were immediately stored on ice. Collected fractions were concentrated using Centricon YM-30 centrifugal filter devices (Millipore). One ml of each fraction was applied to the centricon filter and centrifuged at 5000 rpm for 60 min at 4 °C. To recover filter-bound proteins, the centricon filter device was inverted and proteins were by centrifugation at 1000 rpm for 2 min at 4 °C. Concentrated, fractionated proteins were assayed for Sp2 by Western blotting using anti-Sp2 (K-20; Santa Cruz) as described above. Using the fusion protein GST-Sp2 as substrate, 10 μl of bead-bound GST-Sp2 recovered as described above, was incubated overnight with the remaining 2 ml of each fraction and analyzed for kinase activity the following day using the *in vitro* kinase assay described below.

2.22 *IN VITRO* KINASE ASSAYS

COS-1 cells (2.5 x 10^6) were plated in 100-mm tissue culture dishes containing DMEM supplemented with heat-inactivated FBS, and 50 μg/ml Pipracil at 37 °C under 5% CO_2. The following day, cells were transfected with 10 μg of pCMV4-Sp2/flu pCMV4-Sp1/flu or HA-tagged Sp1/Sp2 chimeras using SuperFect transfection reagent (Quiagen) as described above, and incubated at 37 °C under 5% CO_2 for 48 hrs. PBS-washed cells were solubilized in 1 ml ELB^+ (0.25 M NaCl, 0.1% NP-40, 50 mM Hepes pH 7.0, 1 mM PMSF, 5 mM EDTA, 0.5 mM DTT) by incubating at 4 °C for 30 min with gentle rocking. To immunoprecipitate epitope tagged proteins, 10 μg of anti-HA (12CA5; Roche) antibody was added to cell extracts and incubated for 60 min at 4 °C with gentle rocking. To precipitate antigen-antibody complexes, a 1:50 dilution of PAS (Zymed) equilibrated in RIPA was added and incubated for 30 min at 4 °C with gentle rocking. Following three washes with 500 μl RIPA, bead-bound proteins were washed twice in kinase buffer (15 mM Hepes pH 8.0, 2 mM DTT, 2 mM $MgCl_2$, 50 μM dATP). *In vitro* kinase reactions were performed by incubating washed beads in 30 μl of kinase buffer containing 20 μCi/ml [γ-^{32}P]dATP (10 mCi/ml; ICN) for 30 min at 30 °C. Proteins were eluted from the beads by boiling kinase reactions for 10 min in Laemmli sample buffer, resolved on denaturing acrylamide gels, and results were visualized by autoradiography.

2.23 PHOPHOAMINO ACID ANALYSIS

Phosphoamino acid analysis was performed essentially as described by Cooper, et al., 1983 using the Hunter Thin Layer Peptide Mapping Electrophoresis System (HTLE-

7002; C.B.S. Scientific Company, Inc., Del Mar, CA). COS-1 cells ($2.5x10^6$) were plated in triplicate in 100 mm tissue culture plates containing complete DMEM at 37 °C under 5% CO_2. The following day, cells were transfected with 10 μg of HA-tagged expression constructs encoding full length Sp1, Sp2 or Sp3 using SuperFect transfection reagent (Quiagen) and incubated at 37 °C under 5% CO_2 for 48 hrs. Transfected cells were pre-incubated for 1 hr in 10 ml Phosphate-Free Media (0.2g/L $CaCl_2$, 0.4 g/L KCl, 0.2 g/L $MgSO_4$, 6.4 g/L $NaHCO_3$, 4.5g/L d-glucose, 2x MEM Amino Acids (Invitrogen), 4x MEM Vitamins (Invitrogen), 1 ml/L 0.01% $Fe(NO_3)_3 \bullet 9H_2O$, 0.15% Phenol Red Solution (Invitrogen), pH 6.8), containing 2% dialyzed fetal calf serum, glutamine (Invitrogen), and sodium pyruvate (Invitrogen) at 37 °C under 5% CO_2. Cells were incubated for an additional 3 hrs in 2 ml Phosphate-Free Media containing 2 mCi ^{32}P-orthophosphate (10 mCi/ml; ICN) at 37 °C under 5% CO_2. Non-denatured whole cell extracts were prepared from PBS-washed cells solubilized in 1 ml RIPA (300 mM NaCl, 1 mM Na_4VO_3, 2 mM NaF) for 1 hr at 4 °C with gentle rocking. Subsequently, cells were passed through a 26g needle and debris pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C. Extracts were incubated with anti-HA (12CA5; Roche) antibody for 1 hr at 4 °C with gentle rocking. To precipitate the antibody-antigen complex, a 1:1 slurry of PAS (Zymed) equilibrated in RIPA was added at a dilution of 1:20 and incubated for 60 min at 4 °C with gentle rocking. Following three washes with RIPA, bead-bound radiolabeled proteins were boiled in 25 μl Laemmli sample buffer, resolved on 8% SDS-polyacrylamide gels and transferred to a PVDF membrane (Millipore) using a semi-dry apparatus as described above. The moist membrane was wrapped in Saran Wrap, and radiolabeled proteins were detected following exposure to Hyperfilm (Amersham)

overnight at room temperature. A membrane slice containing a band of interest was excised using exposed film as a guide and subsequently incubated in 100% methanol for 1 min followed by two rinses in 500 µl dH_2O. Proteins were hydrolyzed by incubation in 100 µl 6 M HCl in boiling water for 1 hr. Following centrifugation for 2 min at 14,000 rpm at room temperature, the liquid hydrolysate was lyophilized for 3 hrs in an Automated Environmental SpeedVac containing a VaporNet hood (ThermoSavant, Inc., Holbrook NY), and the amount of radioactivity determined by the Cherenkov method using a scintillation counter (Packard Instrument Company). The hydrosylate was resuspended in 5 µl of pH 1.9 buffer (25 ml/L 88% formic acid, 78 ml/L glacial acetic acid, 897 ml/L dH_2O) containing nonradioactive phosphoamino acid standards (1 µl at1.0 mg/ml phosphoserine, phosphotyrosine and phosphothreonine) and spotted 1 µl at a time on one origin of a 20 cm x 20 cm x 100µm glass-backed cellulose thin-layer chromatography plate. First-dimension electrophoresis was performed in pH 1.9 buffer at 1000 V for 25 min using the HTLE apparatus. The TLE plate was air dried for 20 min, rotated 90°C counterclockwise, and placed back into the HTLE apparatus. Electrophoresis in the second-dimension was performed at 1300 V for 16 min in pH 3.5 buffer (50 ml/L glacial acetic acid, 5 ml/L pyridine, 945 ml/L dH_2O). TLE plates were incubated for 10 min at 65 °C, and unlabeled phosphoamino acids standards were visualized by spraying with 0.25% ninhydrin dissolved in acetone and incubation for an additional 15 min at 65°C. ^{32}P-labeled phosphoamino acids were visualized by autoradiography.

CHAPTER III

Sp2 DNA-Binding Activity and *trans*-Activation Are Negatively Regulated in Mammalian Cells

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The Sp-family of sequence-specific DNA-binding proteins governs the expression of a wide variety of mammalian genes. Transcriptional regulation by Sp proteins begins with their binding to GC-rich promoter elements via a highly conserved Cys_2-His_2 DNA-binding domain located at the carboxy-terminus of each family member. As indicated in Chapter I, detailed characterizations of the biochemical and functional properties of Sp-family members have focused primarily on Sp1, and to a lesser degree, Sp3. At the time I began my research project, Sp2 and Sp4 had been cloned but little information was available regarding their functional attributes, regulation, and role in cell physiology. For Sp2, protein/DNA-binding assays using *in vitro* translated proteins indicated that Sp2 bound only modestly to sequences bound more avidly by other Sp-family members. Little or nothing was known about the relative capacity of Sp2 to regulate gene expression. In this first chapter I detail the DNA-binding and transcriptional properties of Sp2 in comparison with Sp1 and Sp3, and in so doing define one or more mechanisms that appear to negatively regulate Sp2 function in mammalian cells.

3.1 SP2 IS A RELATIVELY WEAK *TRANS*-ACTIVATOR OF THE HAMSTER DHFR PROMOTER *IN VIVO*

To begin to analyze Sp2 function, I compared its capacity to stimulate the transcription of DHFR, a well characterized target of Sp-mediated transcription, with that of Sp1 and Sp3 in *Drosophila* SL2 cells (Udvadia et al., 1995; Kennett et al., 1997; Swick et al., 1989). SL2 cells are a convenient milieu for such studies, since they lack transcription factors closely related to Sp family members although they support Sp-mediated transcription. A full-length, human Sp2 cDNA was subcloned in an insect cell

expression vector (pPac), and this construct or analogous constructions carrying Sp1 (pPacSp1/flu) or Sp3 (pPacSp3/flu) cDNAs were transiently co-transfected with a DHFR-luciferase reporter gene (DHFR-Lux) (Kennett et al., 2002). Four Sp-regulated elements (termed DHFR1–DHFR4) have previously been identified and characterized within the 170-base pair portion of the DHFR promoter carried by DHFR-Lux. As I have previously reported, Sp1 and Sp3 stimulated DHFR transcription between 130- and 300-fold over the range of input DNA concentrations examined (Fig. 2*A*) (Udvadia et al., 1995; Kennett et al., 1997). In contrast, Sp2-directed transcription was significantly weaker, ranging between 4- and 20-fold over baseline DHFR-Lux activity. This relatively modest level of *trans*-activation was obtained despite the inclusion of up to a 64-fold molar excess of Sp2 DNA compared with Sp1 or Sp3. Western blotting with an anti-Sp2 polyclonal antiserum indicated that Sp2 was expressed abundantly in transfected cells and at levels comparable with that of other Sp proteins expressed in this setting (Fig. 2*B*, *Lane 2*, and data not shown). I conclude from these titration experiments that Sp2 is a relatively weak *trans*-activator of DHFR transcription in SL2 cells.

3.2 SP2 BINDS RELATIVELY WEAKLY TO SP-REGULATED PROMOTER ELEMENTS WITHIN THE DHFR PROMOTER

Given that the DNA-binding domain of Sp2 is only 75% identical to that of Sp1 and Sp3, I speculated that this significant structural difference might account for Sp2's relatively modest stimulation of DHFR transcription; *i.e.* I reasoned that amino acid differences within the DNA-binding domain of Sp2 may induce it to bind fewer Sp-regulated elements within the DHFR promoter and/or bind them less efficiently than Sp1

and Sp3. To test this hypothesis, I prepared a recombinant baculovirus stock that expresses full-length human Sp2 protein in infected insect cells and compared its DNA-binding activity with that of Sp1 and Sp3 as I have recently reported (Kennett et al., 2002). To facilitate detection of this recombinant protein, I incorporated a 14-amino acid V5 epitope tag at the Sp2 amino terminus. Consistent with previous reports, infection of Sf9 cells with this baculovirus stock led to the synthesis of an 80-kDa protein that is detected with polyclonal antibodies directed against Sp2 (Fig. 3*A*, *Lane 2*) and a monoclonal anti-V5 antibody (Fig. 3*A*, *Lane 4*) (Kingsley and Winoto, 1992). As expected, a protein of similar molecular weight was not detected with these antibodies in uninfected cell extracts (Fig. 3*A*, *Lanes 1* and *3*). As is also shown in Fig. 3*A*, Western blots with anti-epitope tag antibodies showed that comparable amounts of each Sp protein were synthesized in infected Sf9 cells (*Lanes 5–7*). To compare the DNA-binding capacities of Sp1, Sp2, and Sp3 two experiments were performed. First, Sf9 cells were infected with Sp1, Sp2, or Sp3 baculovirus stocks, cell extracts were prepared, and extracts were incubated with each of four radiolabeled oligonucleotides carrying Sp-binding sites from the DHFR promoter. Each radiolabeled probe carried 10 DHFR-derived nucleotides flanked by common nucleotide sequences. Following incubation, resulting Sp protein-DNA complexes were resolved in parallel in non-denaturing acrylamide gels and prepared for autoradiography. As shown in Fig. 3*B*, Sp1 and Sp3 bound each DHFR-derived Sp-binding site with comparable efficiency. In contrast, Sp2 bound DHFR2 and DHFR4 weakly, and binding of Sp2 to DHFR1 and DHFR3 was negligible. To more accurately compare the efficiency with which Sp2 bound each Sp-binding site, a series of quantitative protein/DNA-binding assays were performed using

the aforementioned DHFR-derived oligonucleotides as well as oligonucleotides carrying Sp-binding sites from the human *p21* (site I) and *MDR-1* promoters. Analogous experiments comparing the relative capacities of Sp1 and Sp3 to interact with each of these six Sp-binding sites have recently been reported (Kennett, et al., 2002). To compare results for Sp2 with previous studies of Sp1 and Sp3, I normalized the amount of Sp2 DNA-binding activity included in these experiments using a radiolabeled Sp-binding site derived from the mouse c-*fos* promoter (Udvadia et al., 1992). Next, to gauge the relative capacity of Sp2 to bind Sp-binding sites from the DHFR, *p21*, and *MDR-1* promoters, a volume of Sp2 protein extract that bound 50% of the c-*fos* probe was employed in protein/DNA-binding assays with these additional oligonucleotides. Each protein/DNA-binding assay was performed in triplicate and quantitated *in situ*. Consistent with results presented in Fig. 3*B*, Sp2 bound appreciably to only two of four DHFR-derived Sp-binding sites (DHFR2 and DHFR4). In addition, Sp2 bound a *p21*-derived Sp-binding site but not a site derived from *MDR-1* (Table I). Little difference was noted in the relative capacity of Sp2 to bind DHFR2, DHFR4, and p21. This latter result is in marked contrast with results for Sp1 and Sp3, since I have recently reported that Sp1 and Sp3 bind each of these six Sp-binding sites, and their capacity to bind varies over a 3-fold range *in vitro* (Kennett et al., 2002). I conclude from these comparative DNA-binding studies that Sp2 binds DHFR-derived Sp-binding sites relatively weakly *in vitro* and that this may at least partially account for the modest stimulation of DHFR transcription by Sp2.

3.3 DERIVATION OF A CONSENSUS SP2-BINDING SEQUENCE AND CHARACTERIZATION OF SP2 DNA-BINDING ACTIVITY *IN VITRO*

One plausible explanation for the modest stimulation of DHFR transcription by Sp2 is its relatively weak capacity to associate with two of the four Sp-regulated elements within this promoter. To determine whether this is the only property of Sp2 limiting *trans*-activation, I reasoned that I needed to first identify high affinity Sp2-binding sites and analyze Sp2-mediated *trans*-activation in the context of a promoter carrying such sites. Thus, I employed a PCR-mediated protocol termed "CASTing" or SELEX that takes advantage of the capacity of a DNA-binding protein to select preferred DNA sequences from a pool of oligonucleotides carrying a partially degenerate sequence. Based on the crystallographic structure of Zif268, a related zinc finger protein bound to DNA and a handful of consensus DNA-binding sites derived for other zinc-finger proteins, I predicted that Sp2 should bind with high affinity to a GC-rich nonameric sequence (Swirnoff et al., 1995; Shi and Berg, 1995; Pavletich and Pabo, 1991). To derive a consensus Sp2-binding site, extracts prepared from Sp2 baculovirus-infected cells were incubated with a double-stranded 60-mer carrying a 16-nucleotide central region of random sequence, a monoclonal α-V5 antibody was added to precipitate protein-DNA complexes, and precipitated DNAs were amplified using the PCR. Following eight successive rounds of immunoprecipitation and DNA amplification, resulting DNAs were subcloned, and 21 independent clones were sequenced and compared. As shown in Fig. 4*A*, a nonameric consensus Sp2-binding sequence (5'-GGGCGGGAC-3') was developed following alignment of the recovered DNA sequences. Little nucleotide variability was noted within the nonamer. Three nucleotide positions

(*numbered 10, 13*, and *16* in Fig. 4*A*) were invariant in all 21 clones, and five additional nucleotide positions were conserved in 86–90% of sequenced clones. The first nucleotide position of the nonamer was the most variable position noted, nearly one-third of sequenced clones substituting adenine for guanine at this position. Nucleotides flanking the consensus nonamer, especially the three nucleotides immediately 3' of the consensus-binding site, exhibited a high degree of conservation as well, suggesting that nucleotides neighboring the nonamer may influence Sp2/DNA interactions. Consistent with the validity of the consensus Sp2 sequence I derived, naturally occurring Sp-binding sites that are most closely related to the consensus Sp2 sequence, such as DHFR2 and DHFR4, are bound by Sp2 *in vitro* (Fig. 4*B*). In contrast, Sp-binding sites such as DHFR1, DHFR3, and MDR1 that exhibit the greatest divergence from the Sp2 consensus sequence show little or no capacity to stably interact with Sp2 *in vitro*.

To confirm that the derived consensus Sp2-binding sequence indeed represents a high affinity Sp2-binding site, two additional experiments were performed. First a competition experiment was developed in which recombinant Sp2 protein was incubated with a radiolabeled probe carrying the consensus Sp2-binding site and challenged with increasing concentrations of unlabeled homologous and heterologous competitor DNAs. For this experiment, I compared the capacity of Sp2 to bind DHFR1 (5'-GGGCGTGGC-3'), a sequence that Sp2 binds poorly with a derivative of DHFR1 (DHFR1*; 5'-GGGCG**G**G**A**C-3') in which two nucleotides were altered such that a consensus Sp2-binding site was created. Thus, a radiolabeled DHFR1* oligonucleotide was incubated with Sp2 alone or with increasing concentrations of unlabeled DHFR1* or DHFR1 oligonucleotides as competitors. As shown in Fig. 4*C*, a 50-fold molar excess of DHFR1*

was more than sufficient to eliminate specific protein-DNA complexes (indicated by an *arrowhead*), whereas a 800-fold molar excess of DHFR1 was incapable of abolishing Sp2-DHFR1* complexes. Additional competition experiments quantified *in situ* confirmed that the affinity of Sp2 for these two oligonucleotides differs by at least 16–20-fold (data not shown). Second, I employed a standard protocol to derive an equilibrium dissociation constant (K_d) for baculovirus-produced Sp2 and its consensus DNA-binding sequence (DHFR1*). For comparative purposes, I attempted to determine a K_d for Sp2 and DHFR1; however, due to the extremely weak association of Sp2 with this oligonucleotide, I were unable to occupy more than 30% of input radiolabeled DNA with Sp2. Hence, an accurate calculation of the K_d of Sp2 for DHFR1 was not possible. In contrast, I had no such difficulty with DHFR1* and calculated a K_d for Sp2 and this sequence of 225 pM (data not shown). This value is similar to K_d values reported for zinc finger transcription factors, such as Sp1, Zif268, Egr-3, Krox-20, and NGFI-C, bound to their consensus sites (125–530 pM) (Swirnotff et al., 1995; Letovsky and Dynan, 1989). To quantify the extent to which the DNA-binding domains of Sp1, Sp2, and Sp3 specify differing affinities for DNA, I compared the affinity of Sp2 to bind its consensus sequence with that of Sp1 and Sp3. Sp1 and Sp3 bound the Sp2 consensus DNA-binding sequence with significantly less affinity. The K_d values of Sp1 and Sp3 are 3-fold (700 pM) and 40-fold higher (8.9 nM), respectively, than that of Sp2 for its consensus-binding site (data not shown). I conclude from binding site selection and competition studies as well as kinetic experiments that Sp2 binds its consensus DNA sequence with high affinity and that Sp1 and Sp3 bind this sequence significantly less tenaciously.

3.4 SP2 IS A RELATIVELY WEAK *TRANS*-ACTIVATOR OF A DHFR PROMOTER MODIFIED TO CARRY ONE OR MORE CONSENSUS SP2-BINDING SITES

The data presented thus far indicate that Sp2 is a relatively weak *trans*-activator *in vivo*. However, these data were obtained using a wild-type DHFR promoter whose Sp-regulated promoter elements are bound relatively poorly by Sp2 *in vitro*. To determine the absolute capacity of Sp2 to stimulate transcription, I reasoned that I had to analyze Sp2 activity using a promoter carrying high affinity Sp2-binding sites. Given that Sp2 target genes have yet to be identified, I concluded that insertion of consensus Sp2-binding sites within an endogenous promoter at well-characterized positions of regulation by Sp family members would provide a reasonable setting for an analysis of Sp2-mediated transcription. Consequently, I employed oligonucleotide mutagenesis to convert one or more Sp-regulated promoter elements within the DHFR promoter to consensus Sp2-binding sites (Fig. 5*A*). The transcriptional activity of each of these mutated DHFR promoters was then analyzed in transient transfection assays with or without the addition of an Sp2 expression vector. For comparative purposes, analogous expression vectors carrying Sp1 or Sp3 cDNAs were analyzed in parallel. As might be predicted, incorporation of Sp2-binding sites within the DHFR promoter did not influence basal levels of transcription in SL2 cells (data not shown). In contrast, the inclusion of a single consensus Sp2-binding site within the DHFR promoter led to a 2–3-fold increase in Sp2-mediated transcription, depending on the site of insertion (Fig. 5*B*). Although the inclusion of consensus Sp2-binding sites resulted in a significant increase in DHFR transcription, absolute levels of *trans*-activation by Sp2 were still 10–20-fold less than that of Sp1 or Sp3. Interestingly, Sp1- and Sp3-directed transcription was influenced less

uniformly by the insertion of Sp2-binding sites within the DHFR promoter. Inclusion of an Sp2-binding site within DHFR2 increased Sp1-mediated transcription 3-fold, whereas transcription of other single-site mutants was essentially unchanged by Sp1. Inclusion of an Sp2-binding site within DHFR3 or DHFR4 increased Sp3-directed transcription 2-fold, whereas transcription of other mutants was essentially unchanged by Sp3. To extend these results, I analyzed a series of DHFR-derivatives that carry two or more Sp2-binding sites at positions of regulation by Sp family members. As shown in Fig. 5*C*, inclusion of consensus Sp2-binding sites at four sites, three sites, or two sites of transcriptional regulation resulted in little or no additional expression compared with that elicited by Sp1, Sp2, or Sp3 and DHFR reporter genes carrying single Sp2-binding sites. I conclude from these results that incorporation of single consensus Sp2-binding sites within the DHFR promoter led to a modest increase in Sp2-mediated transcription, and this level of *trans*-activation was not significantly increased in promoters carrying up to four consensus Sp2-binding sites. Moreover, despite the analysis of reporter genes carrying one or more consensus Sp2-binding sites, Sp2-directed transcription remains weak relative to Sp1 and Sp3.

3.5 SP2 IS A RELATIVELY WEAK *TRANS*-ACTIVATOR OF THE PSA PROMOTER IN PROSTATE EPITHELIAL CELLS

To extend our studies of Sp2-mediated transcription, I wished to analyze its relative capacity to stimulate transcription in mammalian cells. I also wished to determine whether the relatively weak capacity of Sp2 to stimulate transcription might be ascribed to its association with active histone deacetylases. For these studies, I focused on the *trans*-activation of the PSA promoter in DU145 human prostate epithelial cells. PSA has

previously been reported to be regulated by androgens, NF-κB, Nkx3.1, p53, and PDEF, a member of the Ets family of transcription factors, as well as other transcriptional regulators, and I have shown that PSA is stimulated by a subset of Sp family members in prostatic epithelia (Chen et al., 2002; Chen and Sawyers, 2002; Huang et al., 1999; Gurova et al., 2002). DU145 cells were transiently transfected with a PSA-luciferase reporter gene alone or together with expression vectors encoding Sp1 or Sp2. To account for plate-to-plate variations in transfection efficiency, results were normalized to a chloramphenicol acetyltransferase reporter gene regulated by the adenovirus major late promoter (Δ53MLP-CAT). To determine the influence of associated histone deacetylase activity on induced PSA transcription, a subset of transfected cells were treated with 100 nM trichostatin A (TSA) 24 h following transfection. Extracts were prepared for analysis following an additional 24 h of incubation. As shown in Fig. 6*A*, transient expression of Sp1 in DU145 cells induced PSA transcription more than 20-fold, whereas little or no change in PSA expression was noted following co-expression with Sp2. Treatment of transfected cells with TSA resulted in an increase in the absolute levels of basal and induced transcription; however, PSA expression remained only marginally induced by Sp2 (Fig. 6*A*). In contrast, co-expression of Sp1 led to a nearly 250-fold induction of transcription relative to basal levels of PSA expression in the absence of TSA treatment. I conclude from these results that Sp2 is a relatively weak *trans*-activator in mammalian cells and that this modest level of activity is not due to association with active histone deacetylases.

Given the large relative difference in Sp1- and Sp2-mediated PSA transcription, I reasoned that analyses of chimeric molecules might identify portions of Sp2 that limit its

capacity to stimulate transcription. Thus, a series of chimeras were generated via the PCR, and each was analyzed for its stability, subcellular localization, and stimulation of transcription *in vivo*. In addition, the capacities of each chimera to bind DNA *in vitro* were also determined. As shown schematically in Fig. 6*B*, each chimera carries the DNA-binding and/or *trans*-activation domains of Sp2 as well as appropriate corresponding portions of Sp1. Surprisingly, despite the substitution of the Sp1 *trans*-activation (*e.g.* Sp1/2 and Sp1/2/1) or DNA-binding (*e.g.* Sp2/1) domains for the corresponding domains of Sp2, each chimeric construction induced PSA transcription to levels akin to that of wild-type Sp2. The addition of the Sp1 D domain, a region thought to play an important role in multimerization of Sp proteins, also led to levels of PSA transcription equivalent to that of wild-type Sp2 (Fig. 5*B*, Sp2/1D). To confirm these results, I prepared an additional reporter construct that carries multimerized consensus Sp2-binding sites and a basal herpes simplex virus thymidine kinase promoter (pSp2-TK-CAT) and determined the relative capacity of Sp1, Sp2, and each chimera to stimulate expression of this artificial gene in DU145 cells. Results from these assays were entirely consistent with data obtained with PSA (data not shown).

To determine whether the unexpected transcription results obtained with PSA and pSp2-TK-CAT reporter genes might be ascribed to the instability of chimeric proteins or their improper subcellular localization, Western blotting and indirect immunofluorescence were employed to examine transiently transfected cells. Western blotting of COS-1 cells transfected with Sp1/Sp2 chimeras indicated that each is stably expressed *in vivo* at levels comparable with those of Sp1 and Sp2 (Fig. 6*C*). I presume that post-translational modification of ectopically expressed parental and chimeric Sp

proteins gives rise to the various isoforms detected in this assay. Indirect immunofluorescence analyses using an antibody (anti-HA) that binds an epitope tag at the carboxyl terminus of Sp1, Sp2, and each chimeric protein showed that each is excluded from nucleoli yet otherwise diffusely distributed within the nuclei of COS-1 cells (Fig. 6*E*). Although each parental and chimeric protein was abundantly expressed and karyophilic, I wished to determine whether these ectopically expressed proteins were competent to bind DNA specifically. As such, an *in vitro* protein/DNA-binding assay was employed using a radiolabeled probe carrying a consensus Sp2-binding site (DHFR1*). Interestingly, although expressed abundantly in transiently transfected cells, only a subset of parental and chimeric proteins was capable of forming protein-DNA complexes *in vitro*. Whole cell extracts prepared from cells transfected with Sp1, Sp1/2, and Sp1/2/1 expression vectors gave rise to significant amounts of protein-DNA complexes (Fig. 6*D*). In marked contrast, extracts prepared from cells transfected with Sp2, Sp2/1D, or Sp2/1 led to negligible amounts of protein-DNA complexes. Based on studies with chimeric Sp1/Sp2 proteins, I conclude that Sp2 and chimeric proteins carrying the Sp2 *trans*-activation domain give rise to little or no DNA-binding activity when expressed in mammalian cells. As will be discussed below, this conclusion is entirely consistent with the observation that little or no endogenous Sp2 DNA-binding activity can be detected in many human and mouse cell lines. In addition, I conclude that the Sp2 DNA-binding domain can also limit *trans*-activation as constructions carrying it, as well as portions of the Sp1 *trans*-activation and multimerization domains (*i.e.* Sp1/2 and Sp1/2/1), exhibit minimal capacity to stimulate transcription *in vivo*. It is worth noting that these latter

chimeras exhibit little capacity to stimulate transcription despite readily forming protein-DNA complexes *in vitro*.

3.6 SP2 IS EXPRESSED IN MANY, IF NOT ALL, HUMAN AND MOUSE CELL LINES, YET LITTLE OR NO SP2 DNA-BINDING ACTIVITY IS APPARENT IN EXTRACTS PREPARED FROM THESE CELLS

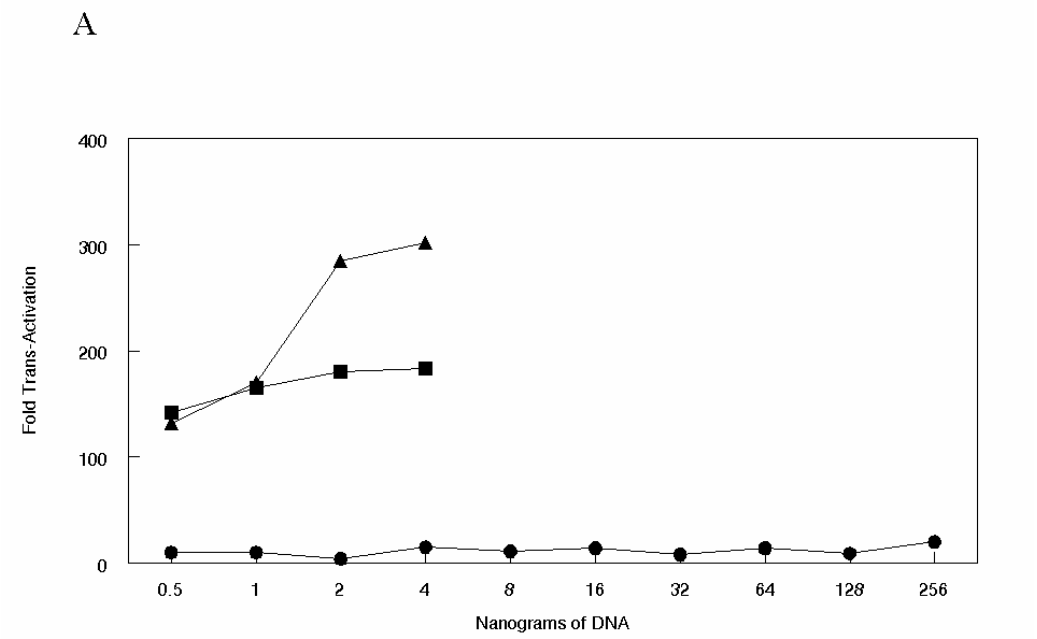
Close inspection of the protein/DNA-binding assays shown in Fig. 6*D* revealed that extracts prepared from mock-transfected COS-1 cells (*Lane 1*) exhibited little Sp2-DNA-binding activity. To explore this observation further, denatured and non-denatured whole cell extracts were prepared from mock-transfected and Sp2-transfected COS-1 cells and examined for Sp2 protein expression and Sp2 DNA-binding activity. As shown in Fig. 7*A*, anti-Sp2 antiserum detects a protein of ~80 kDa in COS-1 (*Lane 1*) cells that migrates slightly faster than recombinant baculovirus-produced Sp2 (rSp2). Transient transfection of COS-1 cells with an Sp2 expression vector leads to the synthesis of a novel protein bound by anti-Sp2 antiserum that migrates with slightly decreased mobility, relative to endogenous Sp2, due to the addition of an epitope tag (*Lane 2*). Next, non-denatured extracts prepared from mock-transfected and Sp2-transfected COS-1 cells were examined for Sp2 DNA-binding activity using a radiolabeled DHFR1* probe. To detect Sp2-DNA complexes, I employed a commercially available anti-Sp2 peptide antibody derived from the amino terminus of Sp2 in "supershift" experiments. I also prepared an Sp2 antiserum directed against the entirety of the Sp2 *trans*-activation domain and compared the capacity of this antiserum to detect Sp2-DNA complexes with that of preimmune serum. As shown in Fig. 7*B*, neither anti-Sp2 antisera detected Sp2-DNA complexes in mock-transfected cells (compare *Lane 1* with *Lanes 3* and *4*). Moreover,

each immune serum detected only a modest amount of Sp2 DNA-binding activity in extracts prepared from cells transfected with Sp2 (compare *Lane 5* with *Lanes 7* and *8*). Preimmune anti-Sp2 antiserum did not react with Sp2-DNA complexes (*Lane 6*).

To determine whether the lack of endogenous Sp2 DNA-binding activity in COS-1 cells is common in other mammalian cell lines, denatured and non-denatured extracts were prepared from 14 human and mouse cell lines and examined similarly. Although all mammalian cell lines examined by Western blotting expressed Sp2 to varying degrees, none yielded evidence of Sp2 DNA-binding activity using a radiolabeled consensus Sp2-binding site probe and anti-Sp2 antiserum (Fig. 7, *C* and *D*, and data not shown). Given this surprising result, I reasoned that Sp2 DNA-binding activity might be restricted to primary cells (*i.e.*, cells that have not been adapted to *in vitro* cell culture). To explore this possibility, non-denatured whole cell extracts were prepared from mouse primary prostate tissue and examined for Sp2 DNA-binding activity (Fig. 8). As seen in Fig. 8, a single prominent protein/DNA complex was detected in extracts prepared from mouse prostate tissue. This complex did not co-migrate with a protein/DNA complex generated by recombinant Sp2 protein prepared in baculovirus-infected Sf9 cells (dark arrowhead), but instead co-migrated with a protein/DNA complex generated by recombinant Sp3 protein. Indeed, Sp3 was shown to be a major constituent of this prostate-derived protein/DNA complex as inclusion of an antiserum against the Sp3 *trans*-activation domain in protein/DNA-binding reactions completely blocked its formation (compare *Lane* 5 with *Lane* 6). In contrast, inclusion of an anti-Sp2 antiserum in parallel reactions did not affect the abundance of this protein/DNA complex. Taking these prostate results together with results from cell lines, I conclude that Sp2 DNA-binding activity is

undetectable in nuclear and whole cell extracts prepared from many, if not all, mammalian cells. This stands in marked contrast to other analyzed Sp-family members, such as Sp1 and Sp3.

In this chapter I have provided several pieces of evidence in support of the notion that Sp2 is a functionally distinct member of the Sp-family. First, I identified a consensus Sp2 DNA-binding sequence and noted that it is considerably different from that derived previously for Sp1. I showed that Sp2 binds this sequence quite avidly and to a greater extent than Sp1 or Sp3. Second, I showed that Sp2 stimulates transcription only modestly even when assayed in the context of an Sp-dependent promoter engineered with high-affinity Sp2 consensus binding sites. Third, I showed that unlike other Sp-family members Sp2 DNA-binding activity is undetectable in extracts prepared from primary tissue and cell lines. Finally, using a series of Sp1/Sp2 chimeras I showed that the Sp2 *trans*-activation and DNA-binding domains are independently negatively regulated in mammalian cells. In the following two chapters I will present a number of studies and results that speak to possible mechanisms regulating Sp2 function.



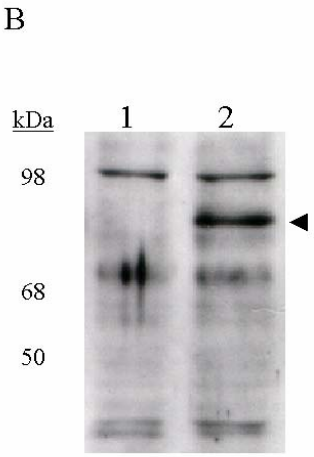


FIGURE 2-Fold *trans*-activation of the DHFR promoter by Sp1, Sp2, and Sp3 and expression of Sp2 in *Drosophila* SL2 cells. *A*, relative *trans*-activation of DHFR promoter by Sp family members. *Drosophila* SL2 cells were transiently transfected with a DHFR-luciferase reporter construct (DHFR-Lux) alone or with increasing quantities of

pPacSp1/flu, pPacSp2/flu, or pPacSp3/flu. Luciferase activity was quantified 48 h later and normalized to protein abundance. Levels of *trans*-activation obtained from two or three independent plates of transfected cells were averaged ± S.D., and -fold activation of the DHFR promoter was determined relative to that of DHFR alone (set equal to 1.0). Levels of *trans*-activation are indicated by *boxes* (Sp1), *triangles* (Sp3), and *circles* (Sp2). *B*, Western blot of human Sp2 protein expressed in *Drosophila* SL2 cells. Denatured cell extracts were prepared from mock-transfected cells (*Lane 1*) and cells transiently transfected with pPacSp2/flu (*Lane 2*). Cell extracts were resolved through an acrylamide gel, transferred to nitrocellulose, and incubated with a polyclonal anti-Sp2 antibody. A *filled arrowhead* indicates that Sp2 and molecular weight markers are indicated on the *left*.

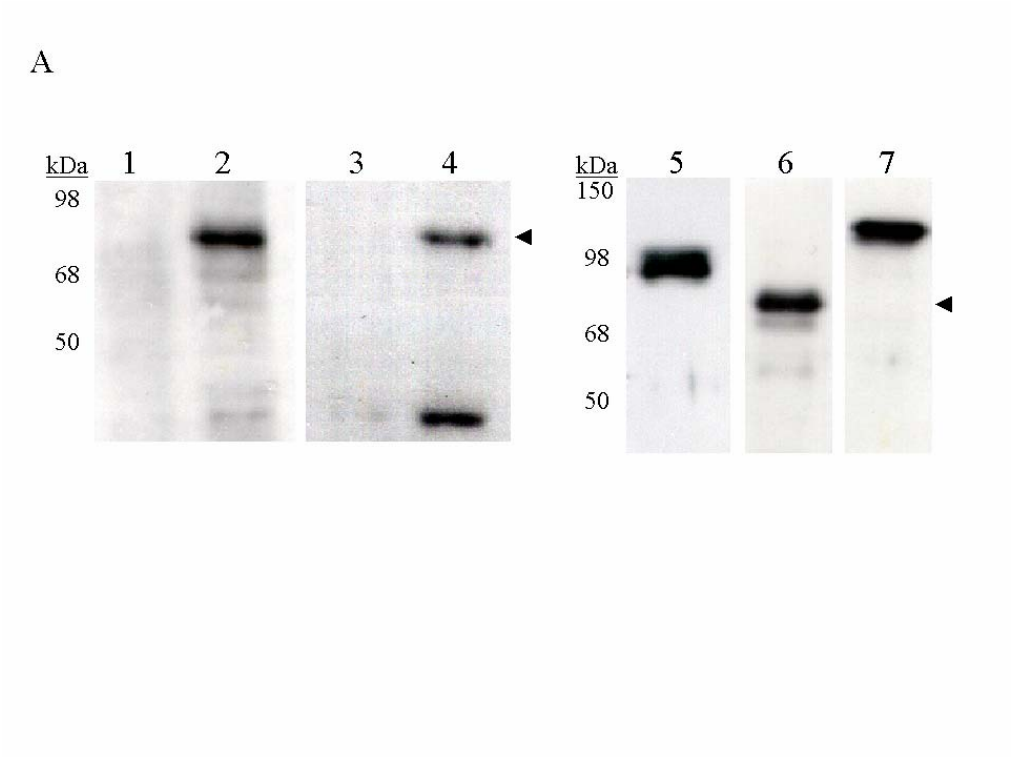

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FIGURE 3-Western blotting of Sp proteins and characterization of protein-DNA complexes in extracts prepared from Sf9 cells infected with recombinant baculoviruses. *A*, Western blots of whole cell extracts probed with anti-Sp2, anti-HA, and anti-V5 antibodies. Equivalent amounts of cell extracts were prepared from uninfected Sf9 cells (*Lanes 1* and *3*), cells infected with a recombinant Sp2 baculovirus stock that carries a V5 epitope tag (*Lanes 2, 4*, and *6*), and cells infected with recombinant Sp1 or Sp3 baculoviruses (*Lanes 5* and *7*, respectively). Extracts were resolved through acrylamide gels and transferred to nitrocellulose, and filters were incubated with anti-Sp2 antiserum (*Lanes 1* and *2*), a monoclonal anti-V5 antibody (*Lanes 3, 4*, and *6*), or an anti-HA monoclonal antibody (*Lanes 5* and *7*). A *closed arrowhead* indicates Sp2, and molecular weight markers are indicated on the *left*. *B*, protein/DNA-binding assay with recombinant Sp1, Sp2, and Sp3 proteins and four Sp-binding sites derived from the DHFR promoter. Nuclear extracts prepared from uninfected Sf9 cells (Sf9) or cells infected with Sp1, Sp2, or Sp3 baculovirus stocks were incubated with one of four radiolabeled DHFR-derived probes (indicated at the *bottom*) and resolved on non-denaturing acrylamide gels. Extracts prepared from Sp1- and Sp3-infected cells were diluted 20- and 50-fold, respectively prior to incubation with radiolabeled probes, whereas extracts prepared from uninfected and Sp2-infected cells were employed undiluted. The *arrows* indicate protein-DNA complexes generated by recombinant Sp2 protein. The *asterisks* indicate irrelevant insect cell-derived protein-DNA complexes.

A

	58 %	71 %	90 %	100 %	86 %	86 %	100 %	90 %	86 %	100 %	79 %	71 %
Consensus	T	**G**	**G**	**G**	**C**	**G**	**G**	**G**	**A**	**C**	T	A
DHFR2	G	A	G	G	C	G	G	G	G	C		
DHFR4	T	G	G	G	C	G	G	G	G	C		
p21		A	G	G	A	G	G	C	G	G	G	
c-fos	G	G	G	G	T	G	G	C	G	C	A	
DHFR1	A	G	G	G	C	G	T	G	G	C		
DHFR3		G	A	G	G	C	G	G	A	G	T	
MDR1		C	G	C	C	G	G	G	G	C	G	T

B

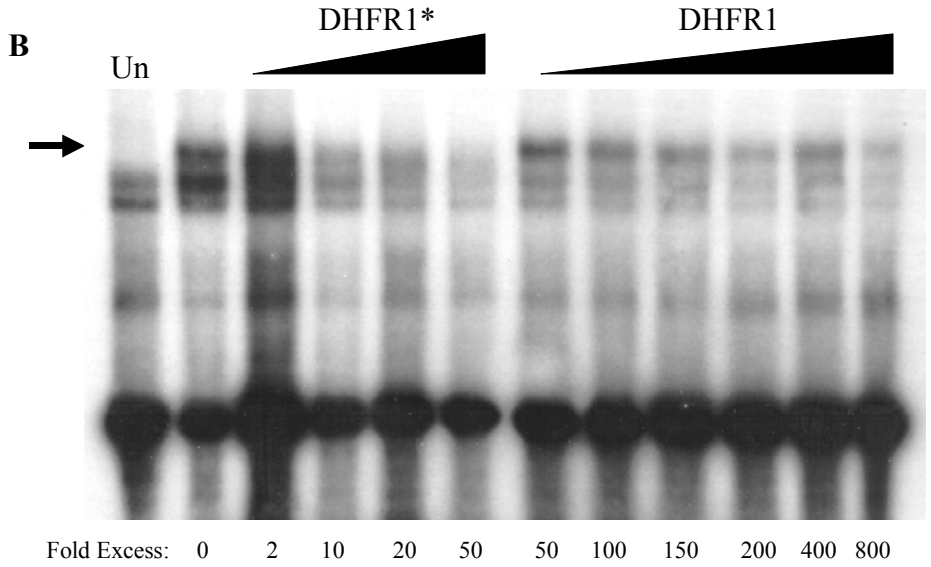
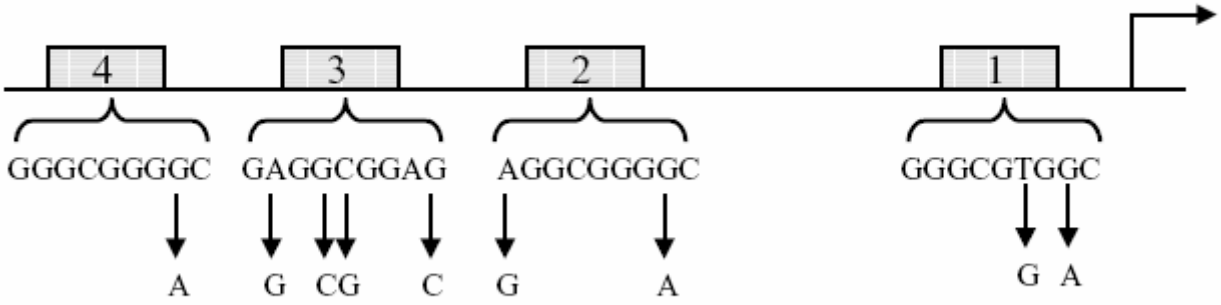


FIGURE 4-Sequence alignment of Sp2-binding sites recovered in "CASTing" experiments, derivation of an Sp2 consensus sequence, and confirmation of this sequence via oligonucleotide competition. *A*, alignment of Sp2-binding sites. Following successive rounds of immunoprecipitation and PCR amplification, DNAs were cloned

and sequenced, and Sp2-binding sites were aligned with respect to each other. The *shaded boxes* indicate nucleotides that define the consensus Sp2-binding sequence. The most frequently recovered residues at each position are indicated, and the percentages of independent clones that carry these nucleotides are noted. *Boldface letters* indicate the Sp2 consensus sequence. *B*, alignment of Sp2 consensus sequence with Sp-binding sites stably bound by Sp2 (DHFR2, DHFR4, p21, and c-*fos*) and Sp-binding sites bound poorly or not bound by Sp2 (DHFR1, DHFR3, and MDR1). Nucleotides shared by the Sp2 consensus sequence and Sp-binding sites derived from the DHFR, *p21, MDR-1*, and c-*fos* promoters are indicated by *shaded boxes. C*, oligonucleotide competition experiment. Nuclear extracts prepared from uninfected Sf9 cells (Sf9) or Sf9 cells infected with an Sp2 baculovirus stock were incubated with a radiolabeled oligonucleotide carrying a consensus Sp2-binding site, DHFR1* (5'-GGGCG**G**G**A**C-3), and challenged with increasing concentrations of unlabeled DHFR1* or a related oligonucleotide, DHFR1 (5'-GGGCG**T**G**G**C-3'), derived from the DHFR promoter. An *arrowhead* indicates Sp2 protein-DNA complexes, and -fold excesses of unlabeled oligonucleotides are indicated at the bottom

A


4
3
2
1
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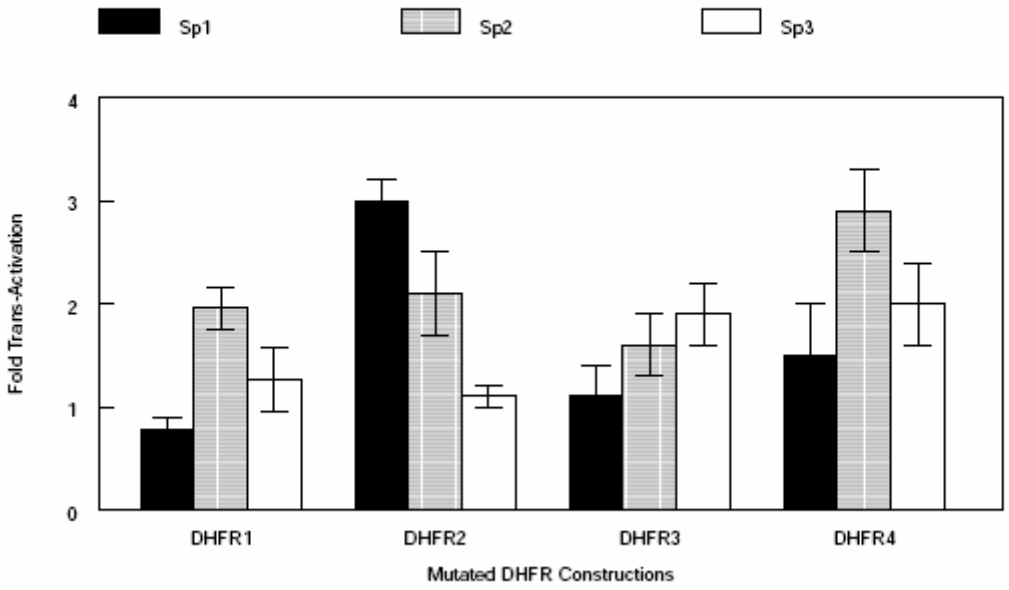
GAGGCGGAG
AGGCGGGGC
GGGCGTGGC
A
G
CG
C
G
A
G
A

B


Sp1
Sp2
Sp3
Fold Trans-Activation
0
1
2
3
4
DHFR1
DHFR2
DHFR3
DHFR4
Mutated DHFR Constructions

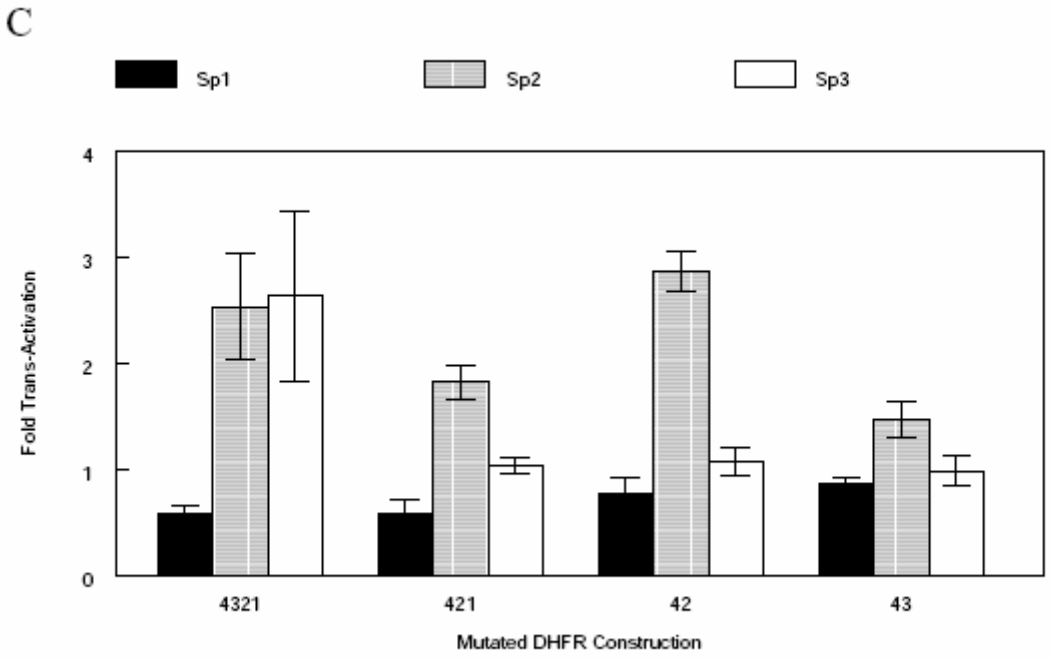
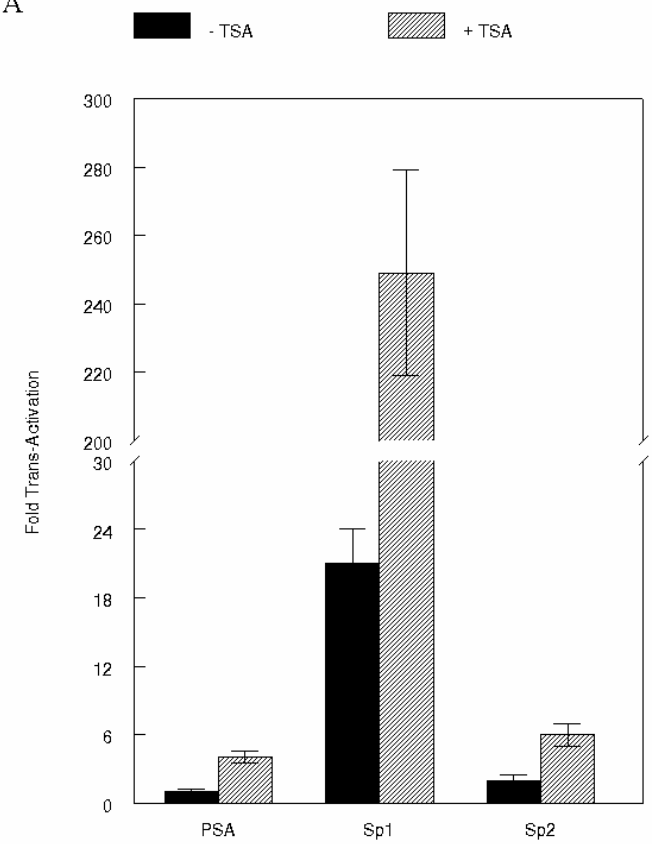
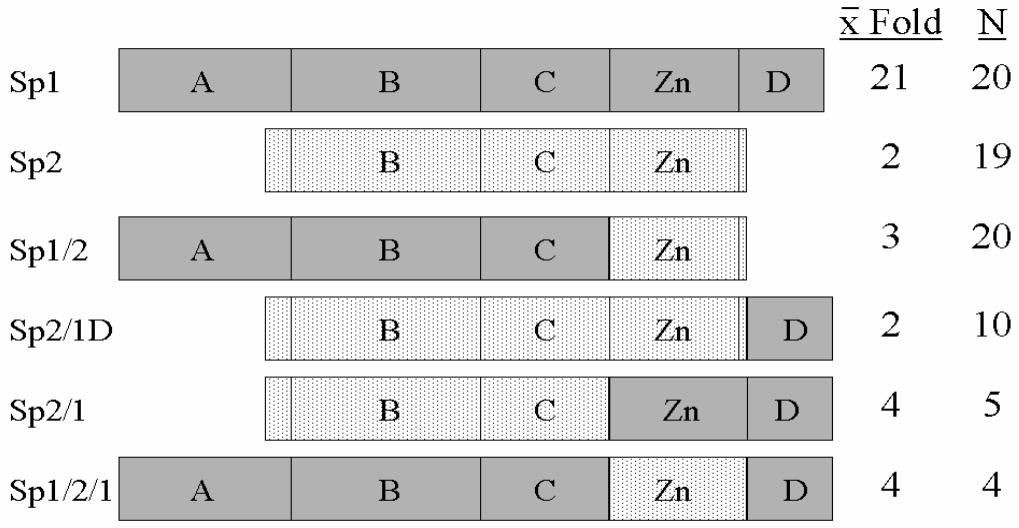


FIGURE 5-Comparison of Sp1-, Sp2-, and Sp3-mediated *trans*-activation of the wild-type DHFR promoter and derivatives carrying one or more consensus Sp2-binding sites. *A*, schematic diagram illustrating the sequences of four Sp-binding sites within the hamster DHFR promoter and nucleotide substitutions introduced to convert each binding site to a consensus Sp2-binding site. The *numbering* of each site is as reported previously (29). *B*, -fold *trans*-activation of wild-type and mutated DHFR promoters in SL2 cells. DHFR-luciferase reporter genes carrying single consensus Sp2-binding sites within DHFR1, DHFR2, DHFR3, or DHFR4 were assayed in transient co-transfection experiments in *Drosophila* SL2 cells with Sp1, Sp2, and Sp3 expression vectors. Illustrated is the mean -fold activation ± S.D. of each construction by each *trans*-activator relative to their activation of a wild-type DHFR construct in parallel transfections. A minimum of six independent plates of cells was examined for each construction and each *trans*-activator. *C*, -fold *trans*-activation of wild-type and mutated DHFR promoters in SL2 cells. DHFR-luciferase reporter genes carrying multiple consensus Sp2-binding sites were analyzed precisely as outlined in *B*. Indicated is the mean -fold activation ± S.D. of four DHFR-derived promoters carrying consensus Sp2-binding sites at all positions (denoted 4321), three positions (denoted 421), or two positions (denoted 42 and 43) of Sp regulation.

A


- TSA
+ TSA
Fold Trans-Activation
300
280
260
240
220
200
30
24
18
12
6
0
PSA
Sp1
Sp2

B


x̄ Fold
N
Sp1
A
B
C
Zn
D
21
20
Sp2
B
C
Zn
2
19
Sp1/2
A
B
C
Zn
3
20
Sp2/1D
B
C
Zn
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B
C
Zn
D
4
4

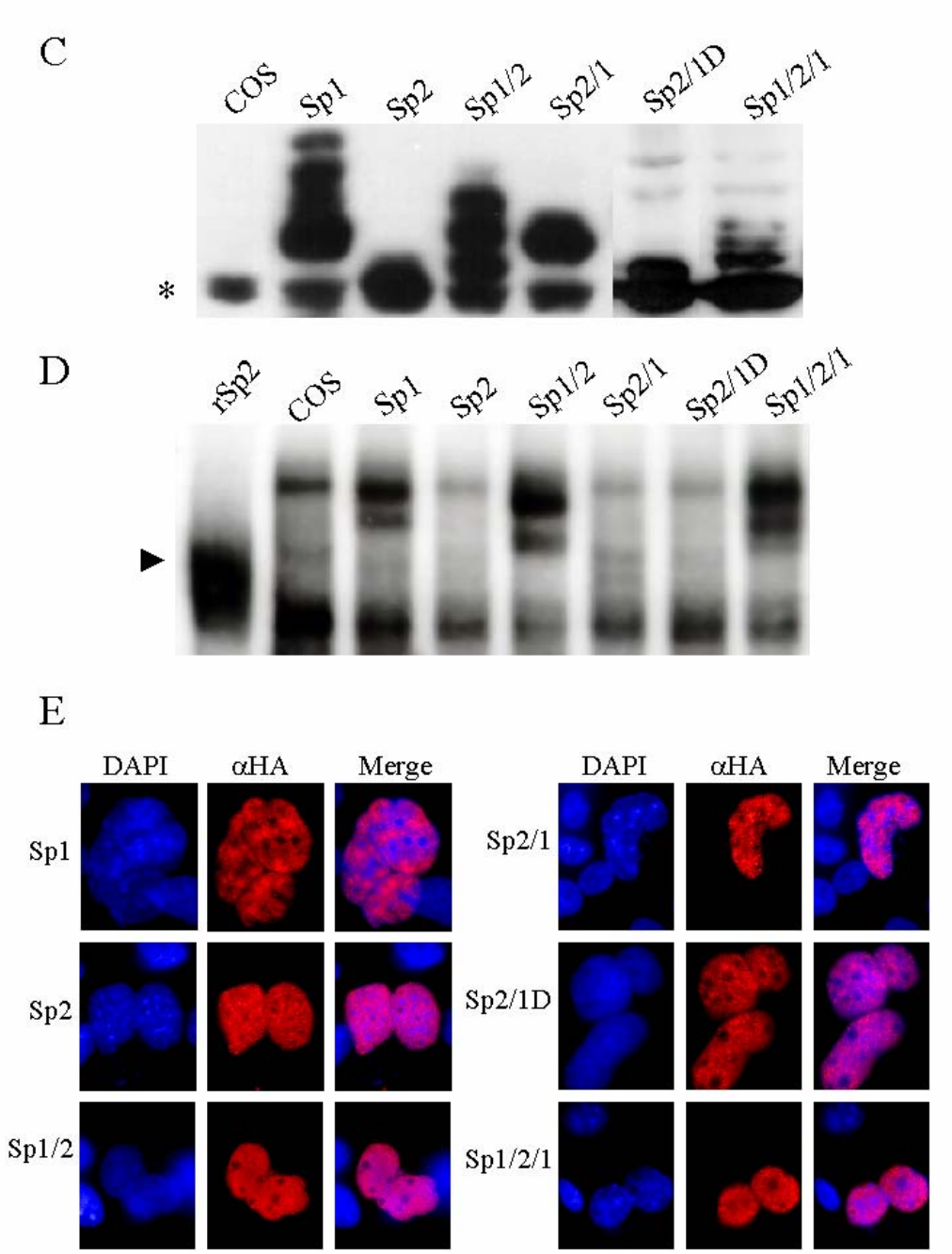


FIGURE 6-*trans*-Activation of the PSA promoter in human prostate-derived epithelia and characterization of chimeric Sp1/Sp2 constructs. *A*, transient co-transfection of DU145 cells. A human PSA-luciferase reporter gene was transfected alone or in conjunction with Sp1 or Sp2 expression vectors in human DU145 cells. Half of the plates of transfected cells were treated with 100 nM TSA 24 h after transfection. To normalize for plate-to-plate differences in transfection efficiency, all cells also received a

CAT reporter gene derived from the adenovirus major late promoter (Δ53MLP-CAT). Extracts were prepared for analysis following another 24 h of incubation, and luciferase results were normalized to resulting CAT activities. Shown are levels of mean -fold *trans*-activation ± S.D. from a minimum of six independent plates of cells for each *trans*-activator and treatment analyzed. Basal levels of PSA transcription in the absence of TSA were set equal to 1. *B*, schematic diagram of Sp1, Sp2, and chimeric Sp1/Sp2 proteins. Illustrated are portions of the *trans*-activation domains of Sp1 and Sp2 (denoted by A, B, and C) as well as the DNA-binding domains of Sp1 and Sp2 (*Zn*) and the multimerization domain of Sp1 (denoted by D). Protein names are listed on the *left*, and levels of mean -fold *trans*-activation of the PSA promoter in DU145 cells (*Fold*) are indicated on the *right* as well as the number of transfected plates of cells (*N*) analyzed. *C*, Western blot of parental and chimeric proteins. Denatured extracts were prepared from mock-transfected COS-1 cells (*COS*) and cells transfected with Sp1, Sp2, or chimeric Sp1/Sp2 proteins. Transfected constructs are indicated at the *top* of each *Lane*. Extracts were resolved on acrylamide gels, transferred to nitrocellulose, and incubated with an anti-HA monoclonal antibody. An *asterisk* indicates an irrelevant background band. *D*, protein/DNA-binding assay. Non-denatured extracts were prepared from mock-transfected COS-1 cells and cells transfected with Sp1, Sp2, or chimeric Sp1/Sp2 proteins. COS-1 extracts and baculovirus-expressed recombinant Sp2 protein (rSp2) were incubated with a radiolabeled DHFR1* probe, and protein-DNA complexes were resolved on a non-denaturing acrylamide gel. *Lanes* are labeled as in *C*. A *closed arrowhead* indicates the position of Sp2-DNA complexes. *E*, indirect immunofluorescence. COS-1 cells transfected with Sp1, Sp2, or chimeric Sp1/Sp2 proteins were incubated with an anti-HA monoclonal antibody and then stained with an Alexa Fluor 594 goat anti-mouse secondary antibody and DAPI. Columns of DAPI-stained nuclei (*DAPI*) and antibody-stained nuclei (*HA*) are presented as well as merged images (*Merge*) for each transfected cDNA.

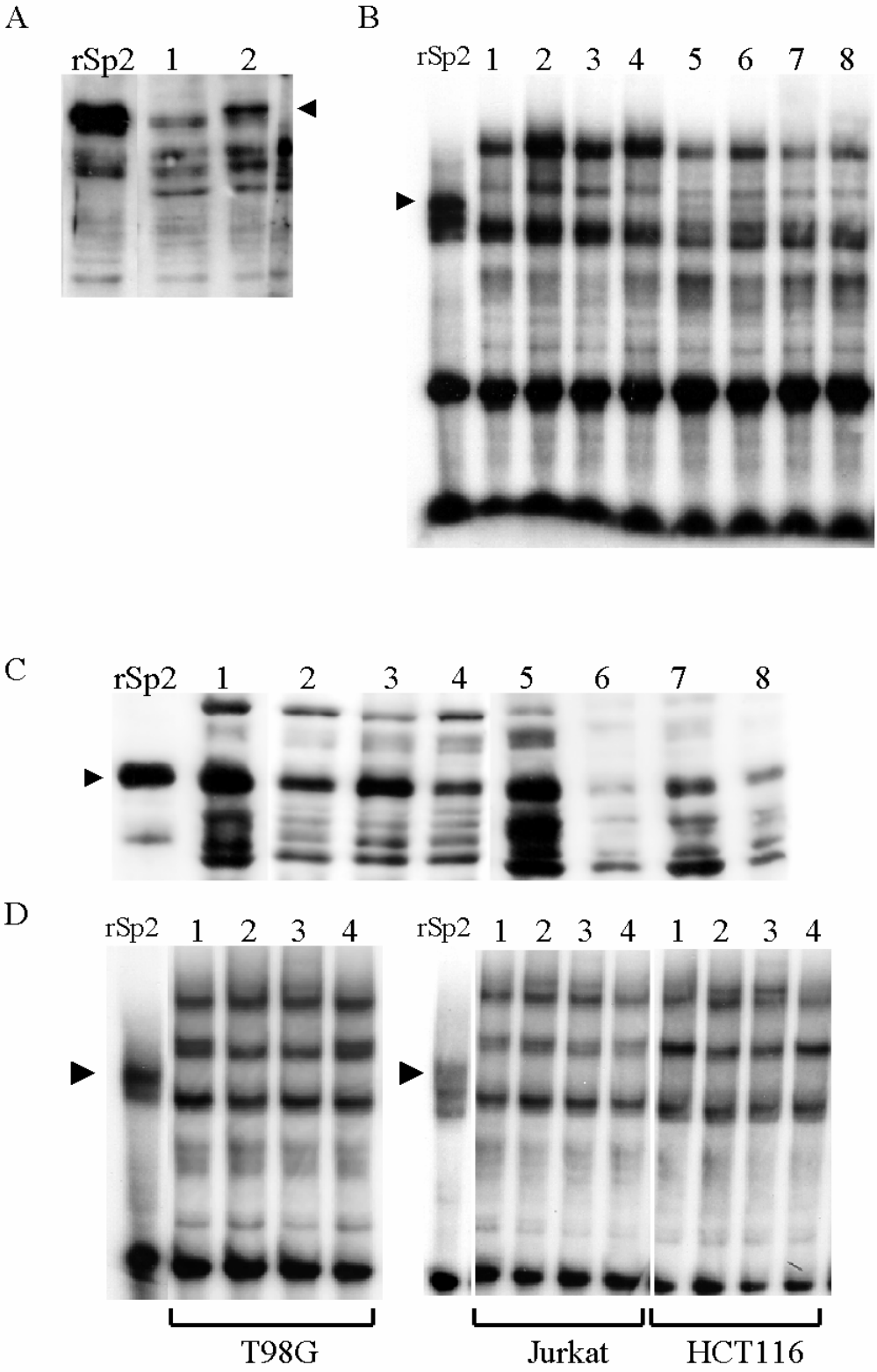

A
rSp2 1 2
B
rSp2 1 2 3 4 5 6 7 8
C
rSp2 1 2 3 4 5 6 7 8
D
rSp2 1 2 3 4
rSp2 1 2 3 4 1 2 3 4
T98G
Jurkat
HCT116

FIGURE 7-Analysis of Sp2 abundance and function in transfected and mock-transfected COS-1 cells as well as mouse and human cell lines. *A*, Western blot. Denatured whole cell extracts were prepared from mock-transfected COS-1 cells (*Lane 1*), COS-1 cells transfected with an Sp2 expression vector (*Lane 2*), and Sf9 cells infected with an Sp2 baculovirus stock (*rSp2*). Extracts were resolved on an acrylamide gel, transferred to nitrocellulose, and probed with an anti-Sp2 peptide antiserum. A *closed arrowhead* indicates exogenous Sp2 expressed in COS-1 cells. *B*, protein/DNA-binding assay. Non-denatured whole cell extracts were prepared from mock-transfected COS-1 cells (*Lanes 1–4*), COS-1 cells transfected with an Sp2 expression vector (*Lanes 5–8*), and Sf9 cells infected with an Sp2 baculovirus stock (*rSp2*). Extracts were incubated with a radiolabeled DHFR1* probe and resolved through an acrylamide gel. To detect Sp2-DNA complexes, anti-Sp2 peptide antiserum (K-20; *Lanes 4* and *8*) or anti-Sp2 *trans*-activation domain antiserum (*Lanes 3* and *7*) was added to binding assays and compared with reactions receiving preimmune antiserum (*Lanes 2* and *6*). A *closed arrowhead* indicates protein-DNA complexes formed by exogenous Sp2. *C*, Western blot. Denatured whole cell extracts were prepared from human and mouse cell lines, resolved through acrylamide gels in parallel with extracts prepared from Sp2 baculovirus-infected Sf9 cells (*rSp2*), transferred to nitrocellulose, and challenged with an anti-Sp2 peptide antiserum (K-20). *Lane 1*, GoTo; *Lane 2*, HTLA230; *Lane 3*, SKNBE(2); *Lane 4*, T98G; *Lane 5*, K562; *Lane 6*, HCT116; *Lane 7*, HUT78; *Lane 8*, Jurkat. A *closed arrowhead* indicates Sp2 expressed in infected Sf9 cells. *D*, protein/DNA-binding assay. Non-denatured whole cell extracts were prepared from T98G cells, Jurkat cells, and HCT116 cells and analyzed as in *B* in parallel with extracts from Sp2 baculovirus-infected Sf9 cells (*rSp2*). Preimmune antiserum was added to reactions in *Lanes 2*, anti-Sp2 *trans*-activation domain antiserum was added to reactions in *Lanes 3*, and anti-Sp2 peptide antiserum (K-20) was added to *Lanes 4*. A *closed arrowhead* indicates protein-DNA complexes formed by baculovirus-expressed Sp2.

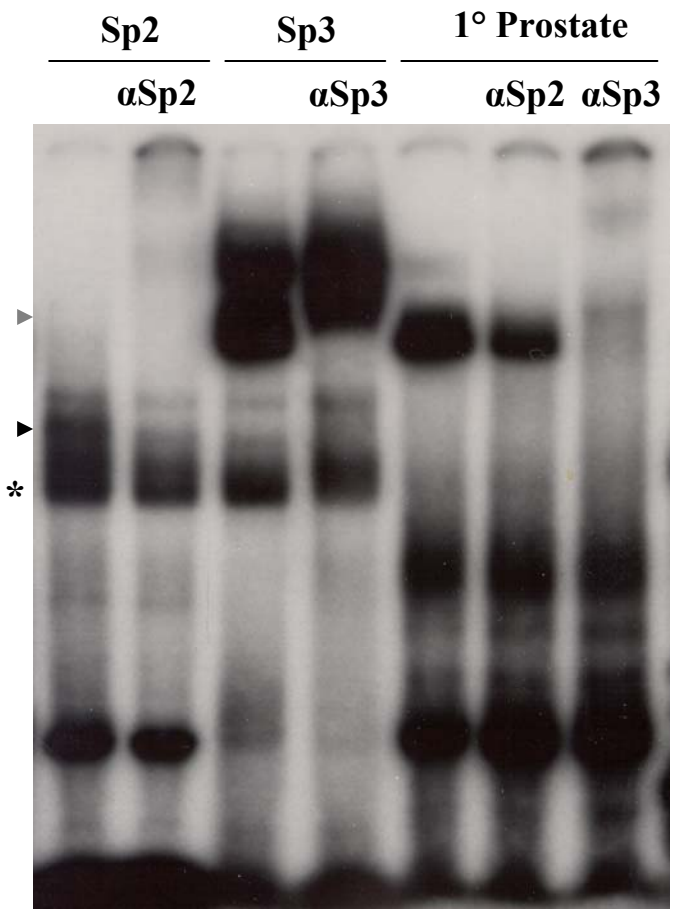


FIGURE 8- Characterization of protein/DNA complexes in extracts prepared from primary mouse prostate tissue. Protein/DNA complexes formed by recombinant Sp2 and Sp3 proteins prepared from baculovirus-infected Sf9 cells were compared with complexes generated by whole cell extracts prepared from mouse prostate tissue. Non-denatured extracts were incubated with a radiolabeled DHFR1* probe, and protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel. The d*ark arrowhead* indicates the position of the Sp2/DNA complexes. The *light arrowhead* indicates the position of the Sp3/DNA complexes. Protein/DNA complexes were identified via the addition of antisera prepared against the amino-terminus of Sp2 (αSp2; K-20) as well as antisera prepared against the entire *trans*-activation domain of Sp3 (αSp3). The *asterisk* indicates an irrelevant insect cell-derived protein/DNA complex.

CHAPTER IV

Insight into the Mechanisms Regulating Sp2 DNA-Binding and *trans*-Activation

(a portion of this chapter appeared in K. Scott Moorefield, Sarah J. Fry, and Jonathan M. Horowitz, Sp2 DNA-Binding Activity and *trans*-Activation are Negatively Regulated in Mammalian Cells, *J. Biol. Chem.*. 279:13911-13924, 2004)

The results I have reported thus far indicate that Sp2 is expressed in a variety of human and mouse cell lines, yet Sp2 DNA-binding activity is undetectable in extracts prepared from these cells. Similar results were noted in extracts prepared from mouse prostate tissue, suggesting that the absence of Sp2 DNA-binding activity in cell extracts may be a universal finding. Consistent with the notion that Sp2 DNA-binding activity may be negatively regulated in mammalian cells, only modest amounts of Sp2 protein/DNA were detected when Sp2 was ectopically expressed. In addition to these findings, I also reported that Sp2-mediated transcription is negatively regulated by a TSA-independent mechanism. I confirmed and extended these results by analyzing an array of chimeric proteins in which functionally analogous regions of Sp2 were exchanged with those of Sp1. These studies with Sp1/Sp2 chimeras proved that the Sp2 DNA-binding and the *trans*-activation domains are each negatively regulated in mammalian cells as each was capable of dominantly-interfering with Sp1 DNA-binding activity and Sp1-mediated *trans*-activation. As I have detailed in Chapter I, DNA-binding and *trans*-activation by Sp1 and Sp3 have been shown to be influenced by post-translational modifications and/or protein/protein interactions and I reasoned that similar mechanisms may regulate Sp2 (Jackson and Tjian, 1988; Dennig et al., 1996; Spengler et al., 2005; Sapetschnig et al., 2002; Ross et al., 2002; Pugh and Tjian, 1990). In an effort to explore these possibilities, I began a series of biochemical experiments designed to identify and characterize cellular mechanisms that negatively regulate Sp2.

4.1 AN ACTIVITY IN MAMMALIAN CELL EXTRACTS CAN INHIBIT THE ASSOCIATION OF SP2 WITH DNA

Given that 1) Sp2 appears to be widely expressed in a fashion that precludes DNA-binding activity and 2) inclusion of the Sp2 *trans*-activation domain in chimeric Sp1/Sp2 proteins appears to diminish DNA-binding activity in transfected cells, I hypothesized that one or more cellular proteins that target the Sp2 *trans*-activation domain may negatively regulate the capacity of Sp2 to bind DNA. To test this hypothesis, I performed a series of mixing experiments in which increasing amounts of mammalian cell extracts were incubated with recombinant Sp2 protein prepared in baculovirus-infected Sf9 cells. Sp2 DNA-binding activity was subsequently measured using a radiolabeled consensus Sp2 DNA-binding site probe. Recombinant Sp2 protein bound the radiolabeled probe efficiently; however, this level of DNA-binding activity was rapidly quenched following the addition of increasing amounts of nuclear extracts from K562 cells (Fig. 7*A*). Identical results were obtained in similar mixing experiments that included nuclear extracts prepared from Jurkat, T98G, and HTC116 cells (data not shown). To determine whether the loss of Sp2 DNA-binding activity was due to its degradation, parallel mixing reactions were examined by Western blotting using a monoclonal antibody that binds an epitope tag included at the amino terminus of recombinant Sp2 protein (anti-V5; Fig. 7*B*). As shown in Fig. 7*B*, recombinant Sp2 protein was not degraded following incubation with mammalian cell extracts. In sum, I conclude from these mixing results that Sp2 DNA-binding activity is negatively regulated by one or more proteins in mammalian cells. Moreover, this regulatory mechanism can be reconstituted *in vitro* using recombinant Sp2 protein produced in insect cells as substrate.

4.2 AN 84-kDa MAMMALIAN PROTEIN BINDS THE SP2, BUT NOT THE SP1 OR SP3, *TRANS*-ACTIVATION DOMAIN VIA THE SP2 B DOMAIN

Since Sp1/Sp2 chimeras implicate the Sp2 *trans*-activation domain as playing a role in the regulation of Sp2 DNA-binding activity and mixing experiments indicate that one or more proteins in mammalian cells regulate the association of Sp2 with DNA, I initiated a series of *in vitro* studies to identify proteins that specifically bind the Sp2 *trans*-activation domain. As a first step, the *trans*-activation domains of Sp1, Sp2, and Sp3 were amplified via the PCR and inserted "in frame" downstream of GST in a bacterial expression vector. The synthesis of GST-Sp fusion proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to bacterial cultures, and recombinant proteins were collected on glutathione-agarose beads. An irrelevant GST-fusion protein, GST-FSH, was also prepared as a negative control for these studies. Equivalent amounts of each GST-fusion protein (as shown by staining with Coomassie Brilliant Blue and Western blotting with an anti-GST antiserum; data not shown) were subsequently incubated with extracts prepared from mammalian cells that were metabolically labeled with [^{35}S]-methionine, loosely adherent proteins were removed by washing, and bead-bound proteins were collected by boiling in SDS and resolved on polyacrylamide gels. As shown in Fig. 8*A*, each GST-Sp fusion protein bound a protein of ca. 74 kDa (p74; indicated by an *open arrowhead*) in extracts prepared from HCT116, T98G, HTLA230, and SL2 cells that was not recovered with GST-FSH. Perhaps of greater interest, however, these protein/protein-binding assays also revealed a novel Sp2-specific protein of 84 kDa (p84; indicated by a *closed arrowhead*) in each mammalian, but not insect, cell extract. An additional Sp2-specific binding protein of ca. 100 kDa was noted sporadically in HCT116 cell extracts but not other mammalian extracts

analyzed (Fig. 7*A*, *asterisk*). To define regions of the Sp2 *trans*-activation domain required for interaction with Sp2-binding proteins, three additional GST-fusion proteins were prepared that carry either the Sp2 A, B, or C domains. Once again, equivalent amounts of each of these GST-Sp2 fusion proteins were challenged with radiolabeled mammalian cell extracts, and recovered proteins were compared with those collected using a GST-fusion protein prepared from the entirety of the Sp2 *trans*-activation domain. Although the abundance of radiolabeled proteins was diminished compared with protein/protein-binding assays using the entire Sp2 *trans*-activation domain, these studies revealed that at least one binding site for the Sp2-specific binding protein (p84) as well as the pan-Sp-binding protein (p74) is carried by the Sp2 B domain (Fig. 8*B*). I conclude from *in vitro* protein/protein-binding assays that the Sp2 B domain interacts specifically with at least two cellular proteins, one of which (p84) binds to the *trans*-activation domain of Sp2 and not to the *trans*-activation domains of two closely related Sp family members, Sp1 and Sp3.

4.3 YEAST "TWO-HYBRID" SCREEN FOR NOVEL CELLULAR PROTEINS BOUND SPECIFICALLY TO SP2

In an effort to identify cellular proteins that bind specifically to the *trans*-activation domain of Sp2, I conducted a genetic screen in yeast using a cDNA library derived from HeLa cells. The *trans*-activation domain of Sp2, encompassing the A, B and C subdomains (amino acids 1-496), was amplified via the PCR and cloned "in frame" with the Gal4 DNA-binding domain in a yeast expression vector. A yeast strain (Y190), that is auxotrophic for tryptophan, leucine, and histidine synthesis, was successively transformed with (1) a Gal4 DNA-binding domain-Sp2 *trans*-activation domain fusion construct that carries the TRP1 gene, serving as the "bait", and (2) a

human cDNA library fused to the Gal4 *trans*-activation domain that carries the LEU1 gene. This yeast strain carries GAL4-regulated histidine (HIS1) and β-Galactosidase genes to facilitate identification of interacting clones. Transformants capable of growth on $trp^{-}leu^{-}his^{-}$ agar plates containing 25 mM 3-AT were subsequently screened for *in situ* β-Gal activity using a colony-lift filter assay. Following three rounds of selection and screening to eliminate false positives, mating experiments were performed with $Leu^{+}Trp^{+}His^{+}lacZ^{+}$ Y190 transformants carrying candidate cDNAs and a yeast strain (Y187) carrying the Gal4-DNA-binding domain and the Sp2 *trans*-activation domain. To ensure that protein/protein interactions in resulting diploid cells were specific, analogous mating experiments were performed with Y187 cells transformed with plasmids carrying the Gal4-DNA-binding domain fused with either p53, or lamin C as negative controls. Plasmids carrying candidate cDNAs were recovered from diploid cells exhibiting interactions with the *trans*-activation domain of Sp2, but not p53 or lamin C. In total, 8000 $Leu^{+}Trp^{+}His^{+}lacZ^{+}$ candidate yeast colonies were screened representing 2.6x coverage of the human genome. Of these, I recovered only one plasmid that interacted specifically with Sp2, and sequence analysis identified this cDNA as encoding interferon-γ inducible protein 30. Subsequent sequence analysis revealed this to be a false positive due to the fact that the sequence was out of frame with the GAL4 *trans*-activation domain, so the interferon inducible cDNA was being read in the wrong frame. Although disappointing, this result is not uncommon as similar results were obtained in a yeast "two-hybrid" screen using Sp1 (J.M. Horowitz, personal communication).

4.4 ACTIVATION OF SP2 DNA-BINDING ACTIVITY IN MAMMALIAN CELLS BY TREATMENT EXPERIMENTS

Thus far I have reported that one or more proteins in mammalian cells inhibit Sp2 DNA-binding activity. Assuming that this must be a regulated process, I hypothesized that one or more signaling pathway may govern the abundance of Sp2 DNA-binding activity. To define such signal transduction pathways, I began a series of experiments in which I treated mammalian cells with effectors of well-characterized signaling cascades (see Appendix II for a list of cellular treatments). Non-denatured whole cell extracts were prepared from treated cells and examined in protein/DNA-binding assays using a radiolabeled DHFR1* probe. As shown in Fig. 11, I was unable to detect Sp2 DNA-binding activity in DU145 cells treated with either TPA (phorbol 12 –myristate 13-acetate) or dibutyryl cAMP. Although treatment of DU145 cells with TPA and dibutyryl cAMP resulted in a novel protein/DNA complex in Fig. 11, *Lane*s 1 and 5, anti-Sp2 antisera revealed these bands must not be Sp2-related. Similarly, I saw no Sp2/DNA-binding activity despite the treatment strategy employed (data not shown). I conclude from this relatively brief survey that mechanisms inhibiting Sp2 DNA-binding activity in mammalian cells are likely to not rely on the signaling pathways examined in these experiments.

4.5 ANALYSIS OF THE EXPRESSION, MODIFICATION STATE, AND DNA-BINDING ACTIVITY OF SP2 AS A FUNCTION OF CELL CYCLE PROGRESSION

To determine whether the abundance and/or modification state of Sp2 fluctuates as a function of cell cycle progression, a series of experiments were performed using human T98G glioblastoma cells. The proliferation of these cells is readily arrested via

serum starvation and these cells uniformly re-enter the cell cycle upon serum addition. T98G cells were arrested by serum withdrawal for 72 hrs, stimulated with serum, and cultured for a further 24 hrs. Non-denatured whole cell extracts were prepared from growth-arrested cells and following serum addition, and examined for Sp2 protein expression and DNA-binding activity. As shown in Fig, 12A, anti-Sp2 antiserum detected a protein of ca. 80 kDa in all T98G extracts that co-migrates with recombinant baculovirus-derived Sp2 (rSp2). A somewhat diminished signal was noted at 20 hrs post-serum addition, however this is very likely a technical artifact due to incomplete protein transfer to the PVDF membrane. It is also worth noting from the results depicted in Fig. 12A that there is little or no obvious change in the mobility of Sp2 as a function of cell cycle progression. It is well established that for many proteins, including Sp-family members, fluctuations in post-translational modifications will have significant observable affects on protein mobility in polyacrylamide gels. The data in Fig. 12A would appear to indicate that if such cell cycle-dependent modifications occur they have little or no impact on the apparent molecular weight of Sp2. I next asked whether Sp2 DNA-binding activity fluctuates as a function of cell cycle progression. Cell extracts prepared as above were examined for Sp2 DNA-binding activity in protein/DNA-binding assays using a radiolabeled DHFR1* probe (data not shown). Consistent with results from asynchronous *in vitro* cell populations and primary tissue, protein/DNA-binding assays detected little or no Sp2 DNA-binding activity in any cell cycle phase. To confirm that T98G cells re-entered the cell cycle uniformly, a parallel set of cells were prepared for flow cytometry by staining with propidium iodide, and cells were sorted and quantified by FACS analysis. The histograms in Fig. 12,B indicate that the cells examined were

synchronized, and progressed through the cell cycle as expected. Given these results I conclude that the abundance and DNA-binding activity of Sp2 does not fluctuate as a function of cell cycle progression.

4.6 SP2, BUT NOT SP1, APPEARS TO REQUIRE PHOSPHORYLATION FOR DNA-BINDING ACTIVITY *IN VITRO*

The data presented thus far indicate that Sp2 DNA-binding activity is negatively regulated in mammalian cells by an as yet unknown mechanism. Mixing experiments had indicated that one or more proteins in mammalian cell extracts inhibit the DNA-binding activity of Sp2 produced in insect cells, and I considered two possible mechanisms that might account for this result: complex formation by an inhibitor and post-translational modification of Sp2. Protein/protein-binding assays had identified a candidate protein, p84, which might block DNA-binding activity via complex formation, yet I wished to determine whether the loss of Sp2 DNA-binding activity is accompanied by changes in its post-translational modification state. I focused my initial studies on phosphorylation as this modification has been shown to regulate the DNA-binding activity of Sp1 and Sp3. To begin, I asked whether Sp2 DNA-binding activity requires phosphorylation. I utilized non-denatured whole cell extracts prepared from Sf9 cells infected with Sp2 or Sp1 baculovirus stocks, and incubated these extracts with potato acid phosphatase (PAP). Protein/DNA complexes formed by untreated Sf9 extracts were compared to those resulting from extracts incubated with varying amounts of potato acid phosphatase (Fig. 13). Sp2/DNA complexes were identified with the addition of an anti-Sp2 antibody (Fig. 13A). As indicated in Fig. 13A, Sp2 DNA-binding rapidly diminished as increasing amounts of PAP were added to Sf9 extracts. Concentrations of

PAP greater than 10^{-8} units were sufficient to abolish all Sp2 DNA-binding activity. In contrast to these results, PAP addition had little or no effect on the formation of Sp1/DNA complexes (Fig. 13C). To ensure that the loss of Sp2/DNA-binding activity was not due to a proteolytic event, experiments incubating recombinant Sp2 with analogous amounts of PAP were examined by Western blotting using a polyclonal rabbit anti-Sp2 antibody (K-20; Fig. 13B). As shown in Fig. 13B, recombinant Sp2 remained intact following incubation with PAP. I conclude from these experiments that recombinant Sp2, but not Sp1, requires phosphorylation for DNA-binding activity *in vitro*.

The above dephosphorylation studies suggested that, at least in insect cells, Sp2 is a phosphoprotein. To determine whether this is also true in mammalian cells, I metabolically labeled COS-1 cells with ^{32}P-orthophosphate and immunoprecipitated endogenous Sp2 protein with an anti-Sp2 antiserum. Indeed, these studies indicated that, akin to Sp1 and Sp3, Sp2 is phosphorylated in mammalian cells (data not shown). Since Sp2 DNA-binding activity depends on phosphorylation and it is phosphorylated in both insect and mammalian cells, I reasoned that Sp2 might be phosphorylated differently in both cell types. That is, I speculated that phosphorylation of Sp2 at cell type-specific residues might account for DNA-binding activity in insect cells but not mammalian cells. To approach this issue I began a series of experiments to first determine the phosphoamino acid content of Sp2 in mammalian cells. Non-denatured cell extracts were prepared from Sp2-transfected COS-1 cells that were metabolically labeled with ^{32}P-orthophosphate. Sp2 was precipitated via incubation with an anti-Sp2 antibody (K-20), and partially hydrolyzed by boiling 6 M hydrochloric acid. Resulting hydrolysates were

resolved by two-dimensional thin layer electrophoresis (TLE) on glass plates coated with cellulose in parallel with unlabeled phosphoamino acids. As shown in Fig. 14, the vast majority of Sp2 produced in transfected mammalian in cells is phosphorylated on serine residues. Indeed, I quantitated the abundance of each phosphoamino acid by scraping TLE plates and confirmed that 95% of Sp2 phosphorylation occurs on serine residues. Taken together, these results indicate that phosphorylation is one mechanism regulating Sp2 DNA-binding activity *in vitro*, and that Sp2 is a physiologic target of phosphorylation *in vivo*.

4.7 ONE OR MORE KINASES DETECTED IN A MOLECULAR WEIGHT PROTEIN COMPLEX CAN PHOSPORYLATE SP2 *IN VITRO*.

To characterize further the role that phosphorylation may play in regulating Sp2 DNA-binding activity, I wished to determine whether there might be many Sp2 kinases or whether Sp2 phosphorylation was likely to be restricted to a small subset of the kinases expressed in mammalian cells. To this end, I developed an Sp2 *in vitro* kinase assay that utilized recombinant human Sp2 as substrate and mammalian cell extracts that had been fractionated through a size-exclusion column. Sp2 coding sequences were amplified via the PCR and inserted "in frame" downstream of a GST-fusion bacterial expression vector. Recombinant GST-Sp2 synthesis was induced by the addition of IPTG to bacterial cultures, and the fusion protein was collected on glutathione-agarose beads. For comparison, bacterial cultures expressing GST alone were induced and GST protein was also collected on glutathione-agarose beads. Non-denatured whole cell extracts were prepared from COS-1 cells and subjected to size-exclusion chromatography

to fractionate proteins based on molecular weight. Each eluted fraction was subsequently incubated with equivalent amounts of GST or GST-Sp2 proteins (quantified by staining with Coomassie Brilliant Blue, data not shown), and *in vitro* kinase assays were performed following incubation with [γ-^{32}P]dATP. Reaction products were resolved on denaturing acrylamide gels and phosphorylated proteins were identified by autoradiography. As shown in Fig. 15A, only eluted fractions carrying high molecular weight proteins or protein complexes (ca. 445-600 kDa) led to phosphorylation of GST-Sp2 (indicated by a closed arrowhead). In contrast, fractionated extracts incubated with GST alone did not lead to the radiolabeling of a protein of similar molecular weight. (105 kDa; compare Fig.15 A and B, *Lane* 2). Instead, an irrelevant 60 kDa protein was detected in all fractions incubated with GST alone as well as a number of smaller phosphorylated proteins. The identity of the radiolabeled proteins in GST-only *Lane*s is unclear, but they presumably represent bacterial proteins that were not collected on GST-Sp2 beads. Thus it would appear that at least one Sp2 kinase can be detected using this *in vitro* kinase assay and it is either a very large protein or it is found in a high molecular weight protein complex.

With the detection of this high molecular weight kinase activity, I wondered whether it might indeed be of physiological relevance. For example, I wished to know whether the kinase or kinases in this cell fraction could activate Sp2 DNA-binding activity. To this end I incubated GST-Sp2 with column fraction 2 and unlabeled dATP, and looked for affects on Sp2 DNA-binding activity in protein/DNA-binding assays using a radiolabeled DHFR1* probe. Although this experiment needs to be repeated

several more times before I am convinced, preliminary indications are that incubation of GST-Sp2 with fraction 2 and unlabeled dATP increased Sp2 DNA-binding activity (data not shown).

4.8 SP2 IS DETECTED IN A HIGH-MOLECULAR WEIGHT PROTEIN COMPLEX IN MAMMALIAN CELLS.

Given that I detected one Sp2-specific binding protein (p84) in mammalian cell extracts, I wished to determine whether Sp2 might indeed be a part of a larger protein complex *in vivo*. With the characterization of an Sp2-specific kinase in high-molecular weight cell fractions I speculated that perhaps p84 was a kinase and that p84/Sp2 complexes were components of a larger macromolecular complex. To address this possibility, size-fractionated COS-1 extracts were also examined for Sp2 by Western blotting with an Sp2 antibody directed against the amino-terminus of Sp2 (K-20). Interestingly, Sp2 was detected in a wide range of column fractions spanning proteins of approximately 60-600 kDa in size (Fig. 15C). Of particular interest is the fact that a significant portion of Sp2 was detected in high-molecular weight proteins (ca. 445-600 kDa; fraction 2). This is precisely the same column fraction in which one or more Sp2-specific kinases were detected (Fig. 15A). Significant amounts of Sp2 were also detected in fractions carrying proteins of 274-85 kDa (fractions 4 and 5) as well as lesser amounts in other fractions (fractions 3 and 6). Given these results it would appear that Sp2 is indeed a component of one or more larger protein complexes *in vivo*, and that at least one Sp2 protein complex elutes in a column fraction coincident with at least one Sp2-specific kinase.

This chapter details some of my efforts to define mechanisms involved in the negative regulation of Sp2 DNA-binding activity and *trans*-activation. I report that one or more cellular proteins is (are) capable of eliminating Sp2 DNA-binding activity in mixing experiments, and that the Sp2 B domain is bound by an Sp2-specific protein of 84 kDa. Additionally, I have demonstrated that phosphorylation is required for Sp2 DNA-binding activity, and I have characterized at least one mammalian kinase that appears to phosphorylate Sp2 specifically. This kinase(s) was detected in a column fraction that carries high-molecular weight proteins or protein complexes, and Sp2 can be detected in this same column fraction. Additional experiments will be required to determine if p84 is a kinase and whether Sp2 is a component of a high-molecular weight complex that includes Sp2-kinase activity. In the following chapter, I will extend my analysis of Sp2 to its subcellular localization in comparison with other members of the Sp2-family.

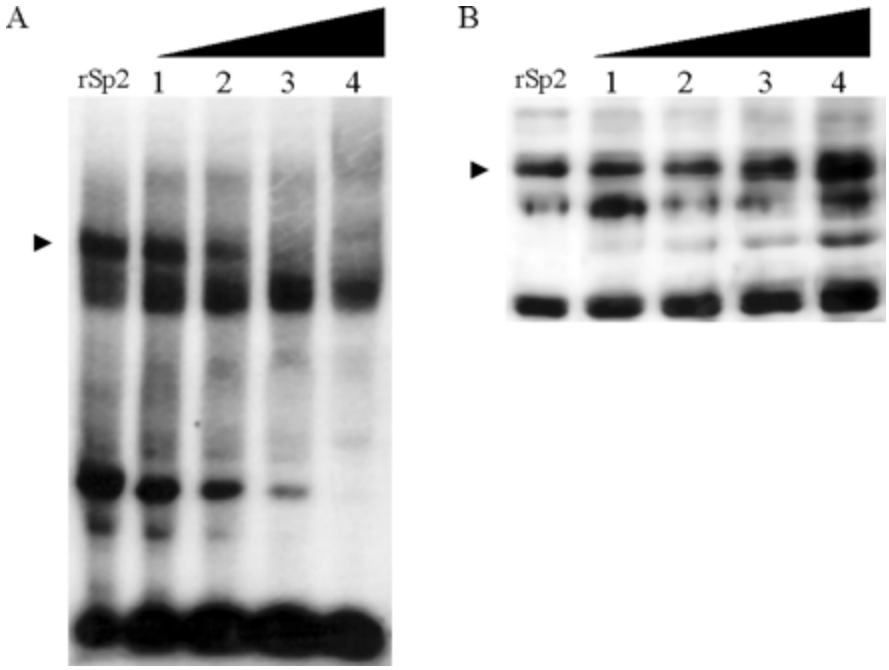


FIGURE 9-Protein/DNA-binding assay and Western blot of mixed extracts. *A*, protein/DNA-binding assay. Recombinant baculovirus-expressed human Sp2 protein was incubated at room temperature for 20 min with a radiolabeled DHFR1* probe or with probe and increasing amounts of nuclear extracts prepared from human K562 cells. Protein-DNA complexes were resolved on a non-denaturing acrylamide gel and prepared for autoradiography. A *closed arrowhead* indicates Sp2-DNA complexes. Protein-DNA complexes formed following incubation of probe with recombinant Sp2 (*rSp2*) protein alone are indicated in the *leftmost Lane. Lane 1*, rSp2 plus 1 µl of K562 extract; *Lane 2*, rSp2 plus 2 µl of K562 extract; *Lane 3*, rSp2 plus 4 µl of K562 extract; *Lane 4*, rSp2 plus 8 µl of K562 extract. *B*, Western blot of mixed extracts. A parallel set of mixed extracts prepared as in *A* were boiled in SDS, resolved through an acrylamide gel, transferred to nitrocellulose, and incubated with an anti-V5 monoclonal antibody. A closed arrowhead indicates recombinant Sp2. *Lanes* are *numbered* as in *A*.

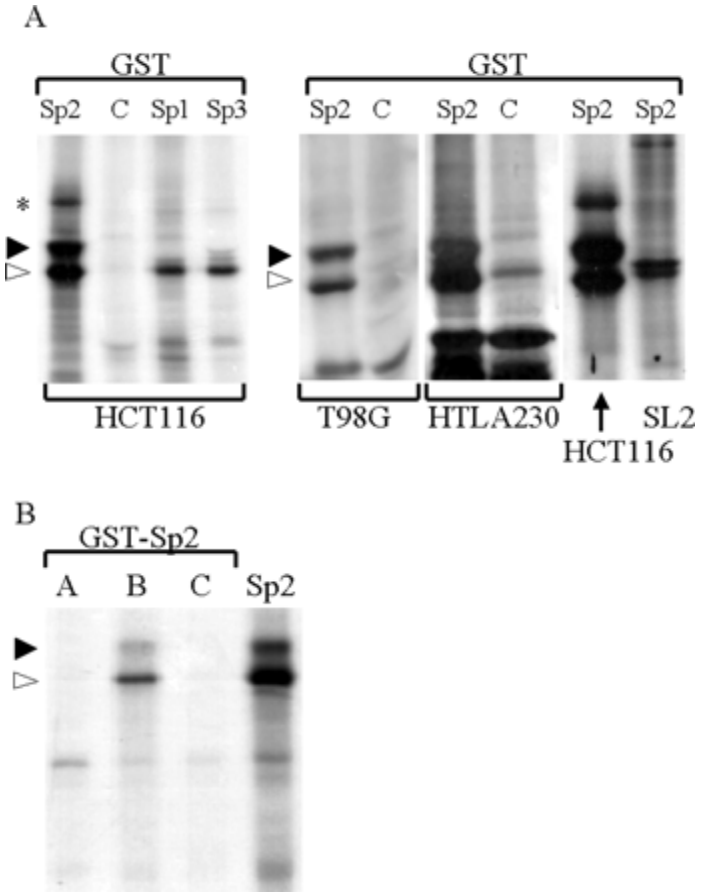


FIGURE 10-Protein/protein binding assays. *A*, protein/protein binding assays with GST fusion proteins prepared from the *trans*-activation domains of Sp1, Sp2, and Sp3. GST fusion proteins were induced in bacteria, bound to glutathione-agarose beads, and incubated with [^{35}S]methionine-labeled extracts prepared from the indicated mammalian (HCT116, T98G, and HTLA230) or insect (SL2) cell lines, and bead-bound proteins were resolved on acrylamide gels. GST-Sp1, -Sp2, and -Sp3 fusion proteins are indicated at the *top* of each *Lane*, as is GST-FSH, a negative control protein (denoted by *C*). A *closed arrowhead* indicates an Sp2-specific binding protein of ~84 kDa (referred to throughout as p84). An *open arrowhead* indicates a pan-Sp-binding protein of ~74 kDa (p74). *B*, protein/protein binding assays with GST fusion proteins prepared from portions of the Sp2 *trans*-activation domain. GST fusion proteins prepared with the entirety of the Sp2 *trans*-activation domain (*Sp2*) or discrete portions (*A, B*, or *C*) were analyzed as in *A* with radiolabeled extracts prepared from HCT116 cells. The *arrowheads* indicate proteins as in *A.*

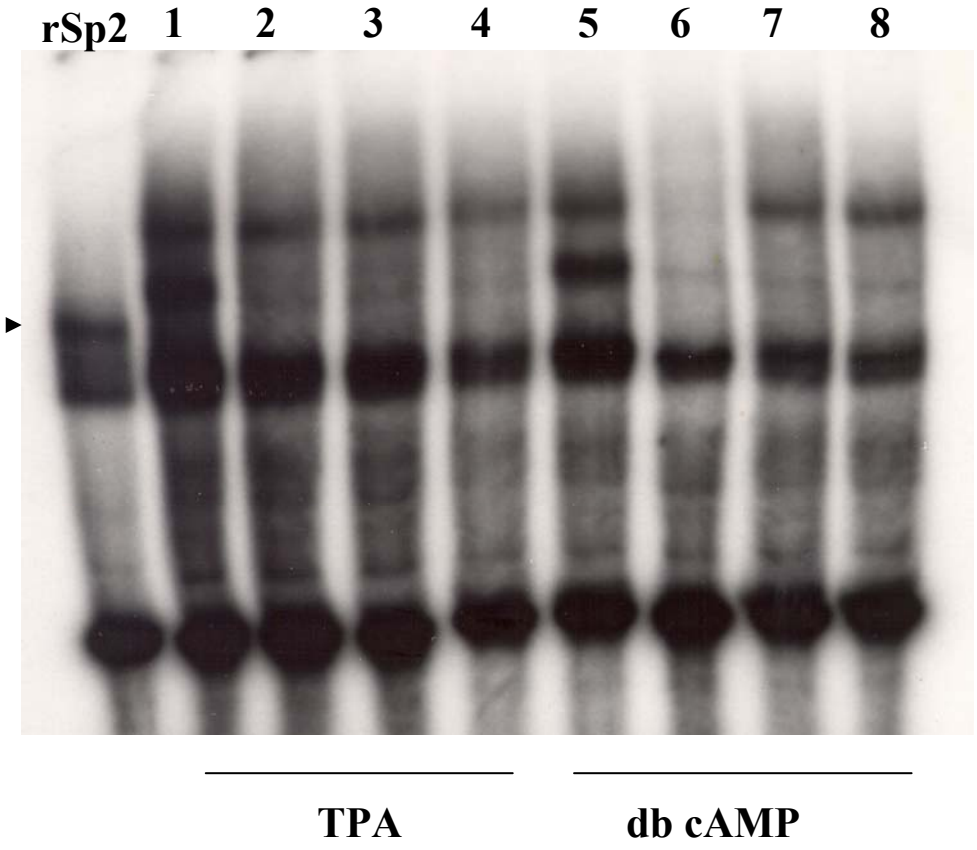
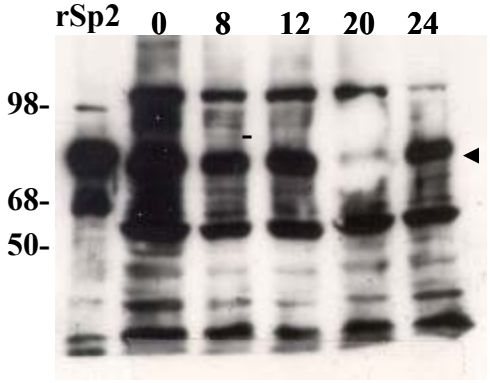
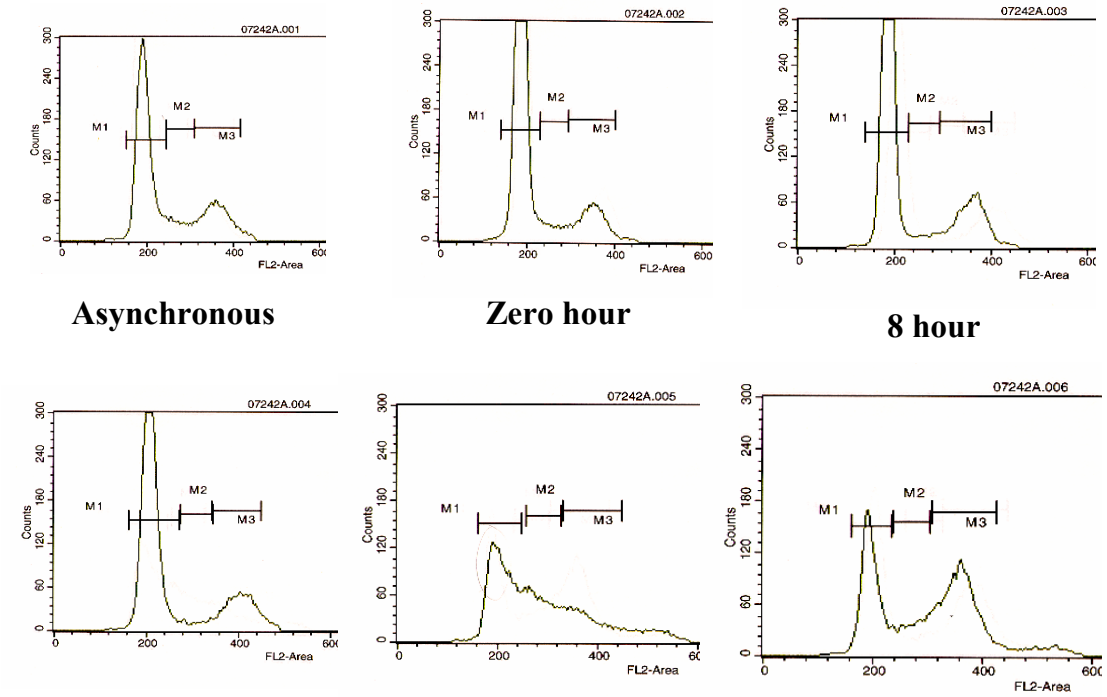


FIGURE 11- Analysis of Sp2 DNA-binding activity in DU145 cells following treatment with TPA or dbcAMP. Non-denatured whole cell extracts were prepared from DU145 cells transfected with a human Sp2 expression vector and following treatment with either phorbol 12 –myristate 13-acetate (TPA; *Lanes 1-4*) or dibutyryl cAMP (db cAMP; *Lanes 5-8*). DU145 extracts or baculovirus-expressed recombinant Sp2 (rSp2) were incubated with a radiolabeled DHFR1* probe, and protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel. Protein/DNA complexes were challenged with chicken pre-immune serum (*Lanes 2 and 6*), anti-Sp2 chicken antiserum prepared against a GST-fusion protein carrying the Sp2 *trans*-activation domain (*Lanes 3 and 7*), or rabbit antiserum raised against the amino-terminus of Sp2 (αSp2; K-20; *Lanes 4 and 8*). The *closed arrowhead* indicates the position of Sp2/DNA complexes.

A


rSp2
0
8
12
20
24
98-
68-
50-

B


07242A.001
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M1
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M3
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FL2-Area
Asynchronous
Zero hour
8 hour
12 hour
20 hour
24 hour

FIGURE 12- Cell cycle analysis of endogenous Sp2 expression over a 24-hour time period. A, Western blot. Human T98G glioblastoma cells were growth-arrested by serum starvation, stimulated to re-enter the cell cycle via the addition of serum, and denatured whole cell extracts were prepared at the indicated time points. Baculovirus-expressed recombinant Sp2 (rSp2) and whole cell extracts were resolved on an acrylamide gel, transferred to nitrocellulose, and incubated with and anti-Sp2 (K-20) antiserum. A *closed arrowhead* indicates endogenous Sp2 protein. B, FACS analysis. Parallel plates of T98G cells were growth arrested and stimulated with serum as in *A*, and prepared for flow cytometry by staining with propidium iodide. Cells were subsequently sorted into cell-cycle compartments according to their DNA content. Histograms represent number of T98G cells in G_1- (M1), S- (M2), or G_2/M (M3)-phase of the cell cycle in a total population of synchronized cells.

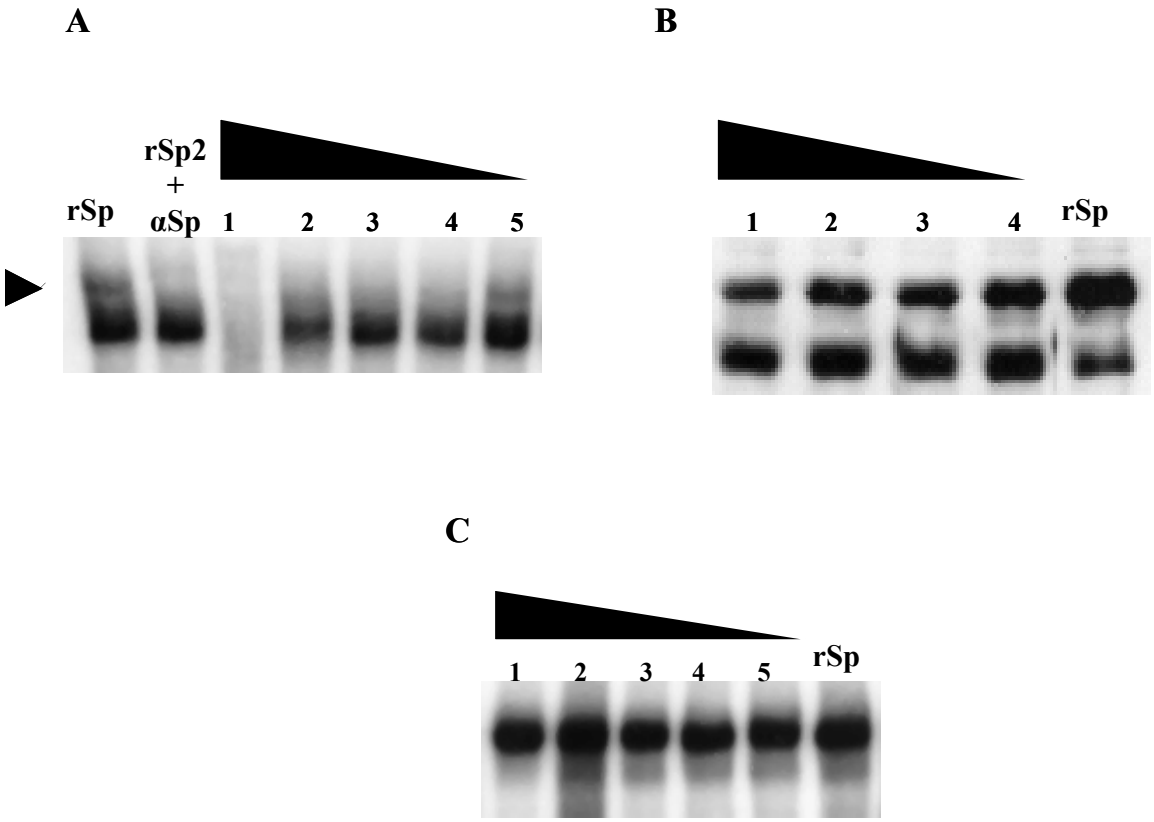


FIGURE 13-Recombinant Sp2, but not Sp1, appears to require phosphorylation for DNA-binding activity. A, Protein/DNA-binding assay of baculovirus-infected Sf9 cell extracts and extracts treated with Potato Acid Phosphatase (PAP). Sf9 cells were infected with an Sp2 baculovirus stock, and non-denatured extracts were prepared. Prior to incubation with a radiolabeled DHFR1* probe, extracts were challenged with varying quantities of phosphatase. Resulting protein/DNA complexes were resolved on non-denaturing acrylamide gels and prepared for autoradiography. A *filled arrowhead* indicates the position of Sp2/DNA complexes identified by the addition of anti-Sp2 antisera (K-20). B, Western blot of Sf9 extracts employed in *A*. Sf9 extracts employed in *A* were boiled in SDS, resolved on an acrylamide gel, transferred to nitrocellulose and incubated with an anti-Sp2 antibody (K-20). Units of PAP added for *A*, *B and C*: *Lane 1*, 1.0. *Lane 2*, 10^{-2}. *Lane 3*, 10^{-4}. *Lane 4*, 10^{-6}. *Lane 5*, 10^{-8}. Untreated lanes are indicated with a -. C, Protein/DNA-binding assay of baculovirus-infected Sf9 cell extracts and extracts treated with PAP. Sf9 cells were infected with an Sp1 baculovirus stock, and treated as in *A*.

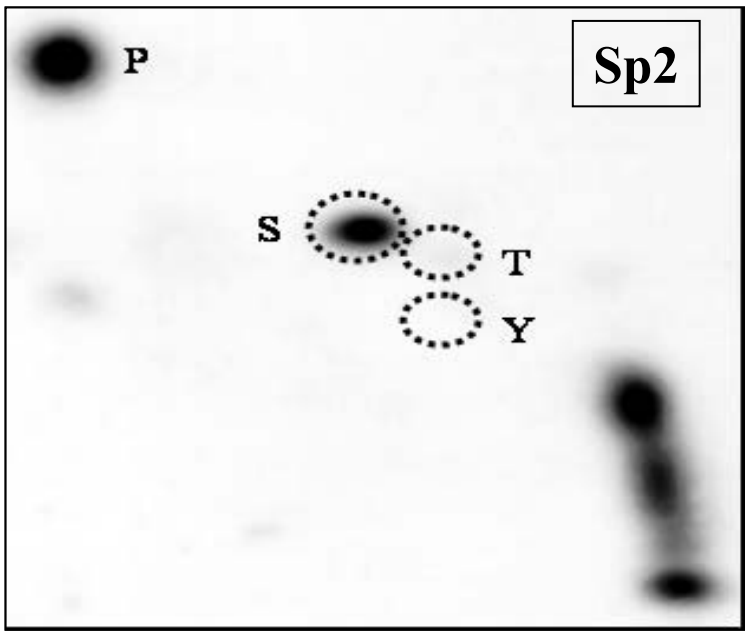


FIGURE 14-Phosphoamino acid analysis of Sp2. COS-1 cells transfected with a human Sp2 expression vector were metabolically labeled with ^{32}P-orthophosphate, non-denatured cell extracts were prepared, and Sp2 was precipitated via incubation with an antibody prepared against the amino-terminus of Sp2 (K-20). Immunoprecipitates were partially hydrolyzed by boiling in 6M hydrochloric acid, resulting phosphoamino acids were resolved by two-dimensional thin layer electrophoresis on glass plates coated with cellulose, and prepared for autoradiography. The high mobility signal (P) results form unincorporated ^{32}P-orthophosphate. The signals at the origin (lower right corner) result from partially hydrolyzed peptides. Unlabeled phosphoamino acid standards were resolved simultaneously to facilitate the identification of radiolabeled phosphoamino acids. S, phosphoserine, T, phosphothreonine, Y, phosphotyrosine.

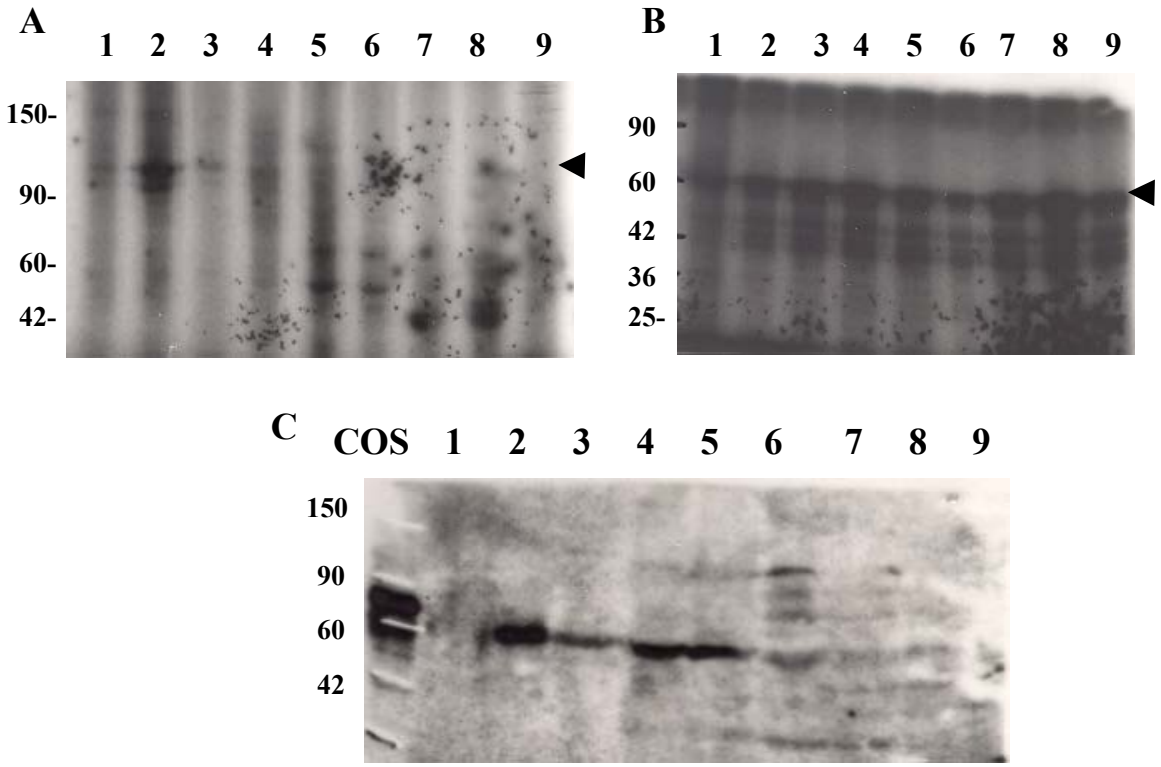


FIGURE 15-*In vitro* kinase assay and Western blotting analysis of endogenous Sp2 in column chromatography elutions. Non-denatured whole cell protein extracts prepared from COS-1 cells were subjected to column chromatography. A, *in vitro* kinase assay. A GST fusion protein prepared from full length Sp2 (GST-Sp2) was induced in bacteria, bound to glutathione-agrose beads, and incubated with fractionated COS-1 whole cell extracts. Using GST-Sp2 as substrate for proteins separated by column chromatography, *in vitro* kinase assays were performed in the presence of [γ-^{32}P]dATP. Bead-bound protein complexes were resolved on an 8% denaturing acrylamide gels and phosphorylated GST-Sp2 was identified by autoradiography. A *dark arrowhead* indicates the position of phosphorylated GST-Sp2. B *in vitro* kinase assay with GST prepared as in A, and resolved on a 12% denaturing acrylamide gel and prepared for autoradiography. A *dark arrowhead* indicates non-specific protein phosphorylation in extracts derived from GST. C. Western blotting of endogenous Sp2. Following fractionation, non-denatured whole cell COS-1 extracts were concentrated, resolved through acrylamide gels in parallel with extracts prepared from COS-1 cells not separated by column chromatography, transferred to PVDF membranes, and challenged with an anti-Sp2 antiserum (K-20). An *arrowhead* represents endogenously expressed Sp2. Lanes represent fraction number corresponding to molecular weights of eluted proteins: *Lane* 1, 700-600 kDa; *Lane* 2, 559-445 kDa; *Lane* 3, 444-275 kDa; *Lane* 4, 274-150 kDa; *Lane* 5, 149-85 kDa; *Lane* 6, 84-60 kDa; *Lane* 7, 59-40 kDa; *Lane* 8, 39-30 kDa; *Lane* 9, 29-5 kDa.

CHAPTER V
Sp2 Localizes to Stable, Sub-Nuclear Foci Associated with the Nuclear Matrix

(a portion of this chapter will appear in K. Scott Moorefield, Teresa D. Nichols, Christopher Cathcart, Steven O. Simmons, and Jonathan M. Horowitz, Sp2 Localizes to Stable, Sub-Nuclear Foci Associated with the Nuclear Matrix, to be submitted 2005)

Results presented in Chapter III indicate that Sp2 is a functionally distinct member of the Sp-family of mammalian transcription factors. Akin to Sp1 and Sp3, Sp2 is a ubiquitously expressed nuclear phosphoprotein. Yet my studies have shown that, unlike other Sp1 and Sp3, Sp2 DNA-binding activity is undetectable in extracts prepared from many human and mouse cell lines, as well as mouse prostate tissue. My studies have also shown that Sp2 is a weak activator of transcription when compared to Sp1 and Sp3. Using a molecular genetic approach, I showed further that the Sp2 DNA-binding and *trans*-activation domains are independently negatively regulated in mammalian cells. Taken together these results appear to indicate that Sp2-mediated gene expression is tightly controlled, perhaps by more than one mechanism. In Chapter IV, I extended my analysis of Sp2 in an attempt to identify the precise mechanisms that regulate Sp2-mediated transcription. These studies revealed that one or more proteins in mammalian cell extracts could negatively regulate Sp2 DNA-binding activity *in vitro*. Subsequently, a series of *in vitro* protein/protein binding assays led to the identification of an 84 kDa protein (p84) in mammalian cell extracts that bound specifically to the B sub-domain of the Sp2 *trans*-activation domain (Fig. 8). Consistent with the notion that Sp2 forms stable protein complexes *in vivo*, size fractionation of mammalian cell extracts demonstrated that a majority of Sp2 is found within high molecular weight protein complexes (Fig. 15 B). One possibility that I have entertained is that p84, and/or other Sp2-associated proteins, actively suppress Sp2-mediated transcription via the inhibition of Sp2 DNA-binding activity/*trans*-activation. As one means to address this possibility, I attempted to clone p84 via a yeast "two-hybrid" screen. Unfortunately, this screen did not yield Sp2-binding proteins of interest. Parallel experiments that analyzed the phosphorylation state

of recombinant Sp2 protein revealed that phosphorylation is required for Sp2 DNA-binding activity. Interestingly, my examination of size-fractionated cell extracts indicates that Sp2, and a subset of Sp1/2 chimeras, are associated with an active protein kinase. Whether p84 has kinase activity and utilizes Sp2 as a substrate remains to be determined, however these are certainly attractive possibilities.

Another possible explanation for the apparent lack of Sp2 DNA-binding activity in mammalian cells is that Sp2 proteins competent to bind DNA may be sequestered within insoluble sub-nuclear domains. That is, it is conceivable that my cell extraction procedures collect only a portion of Sp2, perhaps only a soluble fraction that is not competent to bind DNA. To address this intriguing possibility, I embarked on a series of studies to examine the subcellular localization of Sp2 in comparison with Sp1-4.

5.1 INTRA-FAMILY DIFFERENCES IN SUBCELLULAR LOCALIZATION OF THE SP-FAMILY

To compare the subcellular localization of Sp-family members, COS-1 cells were transiently-transfected with expression vectors carrying human Sp1, Sp2, or Sp3 cDNAs linked to a carboxy-terminal epitope-tag derived from *Influenza* hemagglutinin. Transiently transfected cells were fixed with paraformaldehyde, challenged with a polyclonal rabbit anti-HA antibody, and analyzed by indirect immunofluorescence 48 hrs following transfection. As expected all three Sp-family members localized exclusively to non-nucleolar portions of interphase nuclei and each Sp protein was excluded from chromatin in mitotic cells (Fig. 16 and data not shown). Yet, upon close inspection Sp protein-specific differences were also noted. As shown in Fig. 16A, in addition to

generalized staining throughout transfected nuclei a significant fraction of ectopically-expressed Sp2 appeared as punctate nuclear deposits resulting in nuclei with an overall "mottled" appearance. Ectopic expression of Sp1 led to heterogeneous staining pattern that included small Sp2-like punctate deposits as well as nuclei featuring perinuclear staining as well as uniformly stained areas (Fig. 16B). In contrast to results for Sp1 and Sp2, analyses of Sp3-transfecetd cells revealed uniform staining throughout transfected nuclei (Fig. 16C).

To confirm the immunolocalization results reported thus far and to ensure that these results were not artifacts of fixation, I prepared a series of fluorescent fusion proteins whose subcellular distribution could be evaluated in live cells. Human Sp1, Sp2 or Sp3 cDNAs were sub-cloned "in-frame" downstream of the coding regions of variants of green fluorescent protein (GFP) creating pEYFP-Sp1, pEYFP-Sp2 and pEGFP-Sp3, respectively. Each of these fusion proteins were transiently transfected into COS-1 cells and their sub-nuclear distribution was examined in fixed and live cells. Consistent with results obtained by indirect immunofluorescence, each GFP-fusion protein exhibited a characteristic protein-specific sub-nuclear localization pattern in fixed cells (Fig. 17A-C). In addition to diffuse staining throughout non-nucleolar portions of the nucleus, the vast majority of cells expressing EYFP-Sp2 carried multiple punctate deposits of variable size (Fig. 17B). EYFP-Sp1 transfected cells yielded numerous, relatively small punctate deposits as well as diffusely stained nuclei (Fig. 17A), whereas nuclei of cells expressing EGFP-Sp3 were homogeneously stained (Fig. 17C). Identical patterns of nuclear staining were also noted in paraformaldehyde fixed HeLa and L929 cells as well as in live transfected COS-1 cells (data not shown and Fig. 17 D-F). This latter result indicates

that the Sp protein-specific staining patterns obtained in fixed cells are not simply a consequence of paraformaldehyde fixation.

Although our direct and indirect immunofluorescence results are entirely consistent with the notion that Sp proteins have characteristic sub-nuclear distributions I wished to determine if GFP/Sp-fusion proteins are indeed functional (*i.e.*, competent to bind DNA). To ensure that the fusion of GFP to the amino-terminus of Sp proteins does not compromise their capacity to bind DNA, COS-1 cells were transiently transfected with an EYFP-Sp2 expression vector, non-denatured nuclear extracts were prepared, and these and control extracts were subsequently incubated with a radiolabeled oligonucleotide carrying a consensus Sp2-binding site (Moorefield et al., 2004). Protein/DNA-binding ("gel-shift") reactions containing an extract prepared from EYFP-Sp2-expressing cells (*Lane*s 3 and 4, Fig. 17G) resulted in a novel protein/DNA complex not obtained in parallel reactions prepared with extracts from cells expressing EGFP alone (*Lane*s 1 and 2, Fig. 17G). To confirm that this novel protein/DNA complex results from the binding of EYFP-Sp2 to DNA, protein/DNA-binding assays were challenged with an Sp2-specific antibody. As expected, inclusion of anti-Sp2 antiserum led to a marked reduction in the abundance of this novel protein/DNA complex (*Lane* 4, Fig. 17G). As previously reported little or no soluble Sp2 DNA-binding activity can be detected in many human and mouse cell lines, and inclusion of anti-Sp2 antiserum in control reactions did not lead to the depletion of protein/DNA complexes (*Lane* 2, Fig. 17G; Moorefield et al., 2004).

Based on the direct and indirect immunofluorescence data presented thus far, I conclude that Sp1, Sp2, and Sp3 differ in their sub-nuclear distribution patterns. I

presume that the distinct localization patterns exhibited by each Sp-family member reflect their functional differences and/or the influence of regulatory pathways governing their activity.

5.2 SP2 SUB-NUCLEAR DEPOSITS ARE NOT LOCALIZED WITHIN PROMYELOCYTIC (PML) ONCOGENIC DOMAINS (PODs)

Given the unique distribution of Sp2 within a limited number of relatively large sub-nuclear deposits, I reasoned that Sp2 might localize to nuclear sub-domains termed promyelocytic (PML) oncogenic domains (PODs), nuclear domain 10 (ND10) or Kr-bodies (Seeler et al., 1999). PODs are sub-nuclear structures implicated in the regulation of cellular processes such as transcription and the response to DNA damage, and constituents such as PML and Sp100 appear as discrete foci or "dots" in direct and indirect immunofluorescence studies (Seeler et al., 1999; LaMorte, 1998; Reed et al, 2003). To determine if Sp2 is a POD constituent two experiments were performed. First, COS-1 cells were transiently transfected with an epitope-tagged Sp2 expression vector and transfected cells were stained with anti-HA and anti-PML antibodies. Second, COS-1 cells were transfected with EYFP-Sp2 and ECFP-PML or EYFP-Sp2 and ECFP-Sp100 expression vectors and the sub-nuclear distribution of ectopically expressed proteins were detected by direct immunofluorescence. As shown in Fig. 18A, although it was difficult to detect endogenous PML in COS-1 cells discrete sub-nuclear foci were noted and superimposition of anti-HA and anti-PML images indicated that Sp2 and PML do not co-localize. This conclusion was readily confirmed in transiently transfected cells receiving EYFP-Sp2 and ECFP-PML or ECFP-Sp100. As shown in Fig. 18, foci of EYFP-Sp2 and

ECFP-PML (Fig. 18B) or EYFp-Sp2 and ECFP-Sp100 (Fig. 18C) invariably localized to distinct nuclear sub-domains. I conclude from these results that nuclear foci noted Sp2-transfecetd cells do not co-localize with PML within PODs and that co-expression of Sp2 and PML or Sp100 does not recruit Sp2 to promyelocytic oncogenic domains.

5.3 SP2 NUCLEAR FOCI ARE STABLE AND IMMOBILE IN LIVING CELLS

The data reported thus far indicate that Sp2 localizes to discrete, relatively large foci within interphase nuclei and that these foci are distinct from PODs. I reasoned that the localization of Sp2 within such sub-nuclear domains might indicate that Sp2 performs its role as a regulator of gene expression within regional centers of transcriptional activity. I reasoned further that the location of such transcriptional centers might be dynamic, perhaps varying in size and/or position as a function of cell-cycle progression. To monitor the sub-nuclear distribution of Sp2 in real time, I ectopically expressed EYFP-Sp2 in COS-1 cells and used time-lapse confocal microscopy to collect fluorescent images every 10 min over an 18-hr time course. Despite obvious changes in the position of whole nuclei few if any alterations were noted in the distribution or intensity of EYFP-Sp2 foci during the 18 hrs of observation (data not shown). I conclude from these results that Sp2 nuclear foci are stable and relatively immobile in living cells.

5.4 SP2 IS ASSOCIATED WITH THE NUCLEAR MATRIX

Given that Sp2 nuclear foci appeared to be stable and immobile *in vivo*, it became of interest to determine whether their sub-nuclear distribution reflected the association of Sp2 with chromatin or with components of the nuclear matrix. The latter possibility was

particularly intriguing as numerous transcription factors, including Sp1, have been reported to associate with the nuclear matrix (Mika and Burkhard, 2005; van Wijnen et al., 1993; Zaldi, et al., 2003). As a first step I verified that I could extract chromatin from nuclei *in situ* and identify previously characterized nuclear matrix-associated proteins. COS-1 cells cultured on glass coverslips were solubilized with Triton X-100, chromatin was removed by digestion with DNaseI and extraction with 2 M ammonium sulfate, and resulting nuclear matrices were fixed with paraformaldehyde. Next, two well characterized components of the nuclear matrix, Lamin B1 and NuMa, were visualized using polyclonal antisera and indirect immunofluorescence (Reyes et al., 1997; Zaldi et al., 2001, He et al., 1990, Fey et al, 1991). As shown in Fig. 19A, anti-Lamin B1 and anti-NuMA antibodies detected their respective antigens within the nuclei of untreated COS-1 cells as well as nuclei treated with DNaseI I. In contrast to these results, a protein that does not associate with the nuclear matrix, EYFP, was noted in all cell compartments of transfected COS-1 cells but was not detected within DNaseI I-treated nuclei prepared from transfected cells.

To determine whether Sp2 foci are associated with the nuclear matrix, COS-1 cells were transfected with an EYFP-Sp2 expression vector and nuclear matrices were prepared and analyzed by direct fluorescent microscopy. For comparison, parallel cell cultures were transfected with EYFP-Sp1 or EGFP-Sp3 expression vectors and analyzed similarly. As shown in Fig. 19B-D, each Sp-fusion protein was found in association with the nuclear matrix. However, significant differences in the numbers of resulting fluorescent nuclei were noted. To quantitate the efficiency with which each Sp-family member associates with the nuclear matrix, parallel cultures of COS-1 cells were

transfected with expression vectors encoding each Sp-fusion protein and the total numbers of fluorescent nuclei were enumerated in cultures that were or were not treated with DNaseI I. This experiment was repeated at least three times for each Sp-family member to account for plate-to-plate differences in transfection efficiency, and the ratio of fluorescent cells recovered in DNase-treated cultures relative to those recovered in untreated cultures is plotted in Fig. 19E. Consistent with the notion that Sp2 associates efficiently with components of the nuclear matrix, the vast majority (ca. 95%) of cells expressing EYFP-Sp2 gave rise to EYFP-Sp2-positive nuclear matrices. Sp1 appeared to be somewhat less efficient, as approximately 80% of transfected COS-1 cells were found to express EYFP-Sp1 in association with the nuclear matrix. In marked contrast to these results, significantly fewer (ca. 25%) cells expressing EGFP-Sp3 gave rise to EGFP-Sp3-positive nuclear matrices. It is worth noting that although EYFP-Sp1-positive nuclear matrices were recovered at relatively high frequency, these same nuclei exhibited considerably weaker staining than nuclei prepared from cells expressing EYFP-Sp2 or EGFP-Sp3. I presume that this difference in the intensity of nuclear staining reflects a relatively weaker association of Sp1 with the nuclear matrix.

To substantiate our results from *in situ* analyses, I examined further the subcellular distribution of Sp-family members using a biochemical approach. Utilizing a protocol previously described by Merriman et al. (1995) with modifications by Stein et al. (2001), I prepared whole cell and fractionated extracts from mock transfected COS-1 cells and cells transiently-transfected with expression vectors encoding Sp1-4. Cell fractions containing soluble proteins, chromatin-associated proteins, and insoluble, nuclear matrix-associated proteins were recovered, identical cell equivalents were

resolved on denaturing polyacrylamide gels, and endogenous and exogenous Sp proteins were detected by Western blotting. Consistent with results obtained in *in situ* studies, the vast majority of endogenous and exogenous Sp2 was detected in fractions carrying components of the nuclear matrix (Fig. 20). Only minor amounts of Sp2 were detected in soluble or chromatin-associated protein fractions. Sp1 exhibited a similar localization pattern. The majority of Sp1 was detected in nuclear matrix fractions, although a considerable amount of Sp1 protein was also detected within soluble protein fractions. In contrast to these results, and consistent with evidence from *in situ* analyses, Sp3 was detected uniformly in all subcellular fractions analyzed. Finally, Sp4 exhibited a localization pattern within subcellular fractions that was indistinguishable from that of Sp3. When these biochemical fractionation data are taken together with observations from indirect immunofluorescence and direct fluorescence assays, I conclude that Sp2 partitions largely to the nuclear matrix and that Sp-family members differ in their propensity to associate with the nuclear matrix.

5.5 31 AMINO ACID PORTION OF THE SP2 DNA-BINDING DOMAIN IS REQUIRED FOR ASSOCIATION WITH THE NUCLEAR MATRIX

To define amino acids required for the association of Sp2 with the nuclear matrix, a series of EYFP-Sp2 deletion mutants were generated using the PCR and each resulting fusion protein was examined *in situ* for their capacity to associate with the nuclear matrix. As shown in Fig. 21, removal of individual sub-domains or the entirety of the Sp2 *trans*-activation domain had no effect on the association of Sp2 with nuclear matrices prepared from transiently transfected COS-1 cells. These results implicated one

or more portions of the Sp2 DNA-binding domain as being necessary to target Sp2 to the nuclear matrix. To precisely delimit amino acids within the Sp2 DNA-binding domain specifying nuclear matrix attachment, I created a series of EYFP-Sp2 fusion proteins lacking individual zinc-"fingers". As shown in Fig. 21, removal of zinc-"finger" III or zinc-"fingers" II and III had no effect on the partitioning of EYFP-Sp2 to the nuclear matrix. These data indicate that at least one Sp2 nuclear matrix-targeting signal (NMTS) resides within a 31 amino acid region encompassing zinc-"finger" I as well as the first inter-"finger" linker (amino acids 518-549) of the Sp2 DNA-binding domain.

To determine if the 31 amino acid region identified by deletion analysis is necessary and sufficient to direct association with the nuclear matrix, an EYFP-fusion construct was created in which a PCR fragment encoding these amino acids was fused to the carboxy-terminus of EYFP. COS-1 cells were transiently transfected with an expression vector encoding this construction, and the distribution of EYFP in transfected cells was determined *in situ* with and without treatment with DNaseI I. Surprisingly, this 31 amino acid portion of the Sp2 DNA-binding domain was insufficient to direct association of this EYFP-fusion protein with the nuclear matrix (Fig. 21). One interpretation of these results is that amino acids outside of zinc-"finger" I and the first inter-"finger" linker are required for the association of Sp2 with the nuclear matrix. Yet, the data in hand do not rule-out the possibility that the 31 amino acids examined are poorly displayed in the EYFP construction analyzed. That is, it remains possible that the EYFP moiety blocks direct association of Sp2 amino acids with the nuclear matrix, or alters the conformation of Sp2 amino acids such that they cannot bind matrix proteins. Further constructions will need to be generated to test these alternative possibilities.

Results reported in this chapter spring from my efforts to compare the subcellular localizations of Sp-family members. These studies have documented that Sp-family members differentially associate with the nuclear matrix, and the majority of endogenous and exogenous Sp2 localizes to discrete subnuclear foci that are distinct from promyelocytic (PML) oncogenic domains (PODs). Sp2 sub-nuclear foci appear to be stable as their size, abundance, and distribution do not change appreciably during an 18 hour time course of observation. I employed a panel of EYFP-Sp2 deletion mutants to identify regions of Sp2 required for matrix association, and defined a 31 amino acid segment of the DNA-binding domain as being necessary, but perhaps not sufficient, for targeting Sp2 to the nuclear matrix. Further experiments will be required to (1) define additional portions of Sp2 that direct matrix association, (2) identify matrix components that are bound by Sp2, and (3) determine whether matrix-bound Sp2 proteins are transcriptionally active.

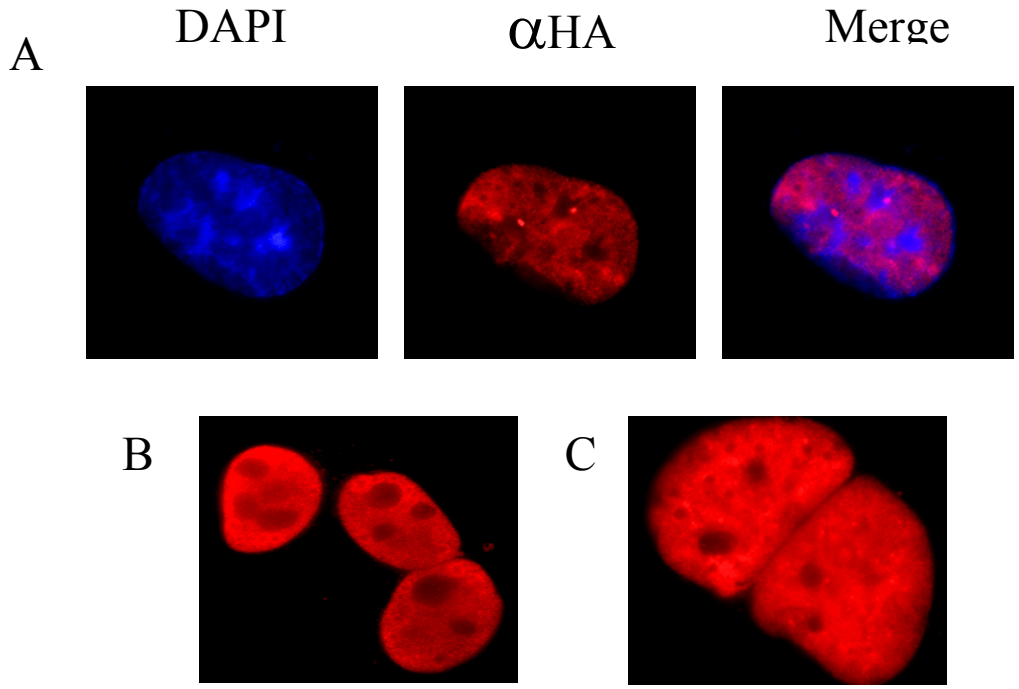
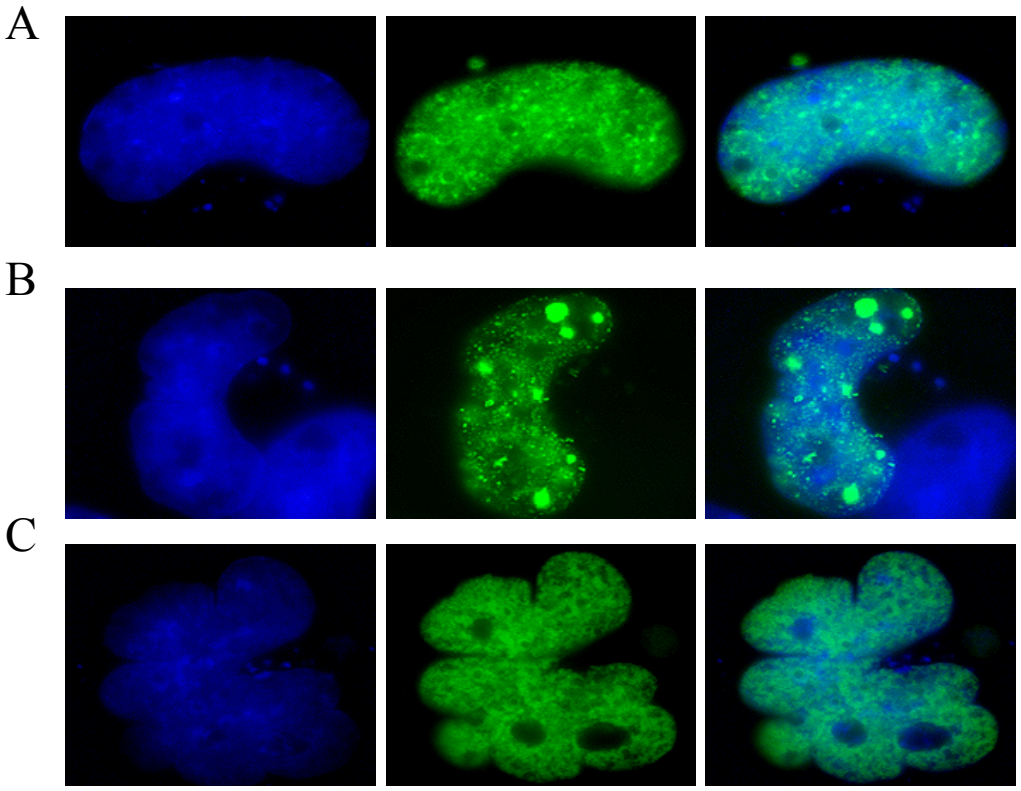
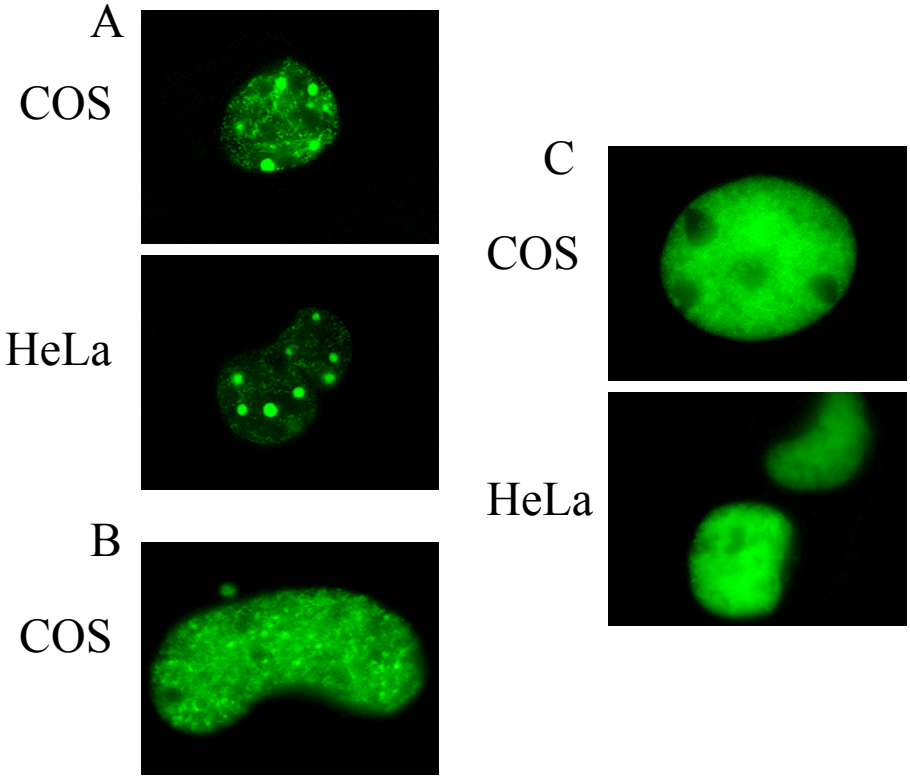


FIGURE 16: Subcellular localization of Sp1, Sp2 and Sp3 in transiently-transfected COS-1 cells detected by indirect immunofluorescence. A, COS-1 cells were transiently transfected with an expression vector encoding full length human Sp2 and expressed protein was detected by indirect immunofluorescence using an anti-HA polyclonal antibody (Y-11; center column) and Alexa Fluor 594 goat anti-rabbit secondary antibody. Nuclei were localized via DNA-staining with DAPI (left column) and superimposed images are shown in the right column. B, C, COS-1 cells expressing full length human Sp1 (B) or Sp3 (C) were prepared as in A.


A
B
C


A
COS
HeLa
B
COS
C
COS
HeLa

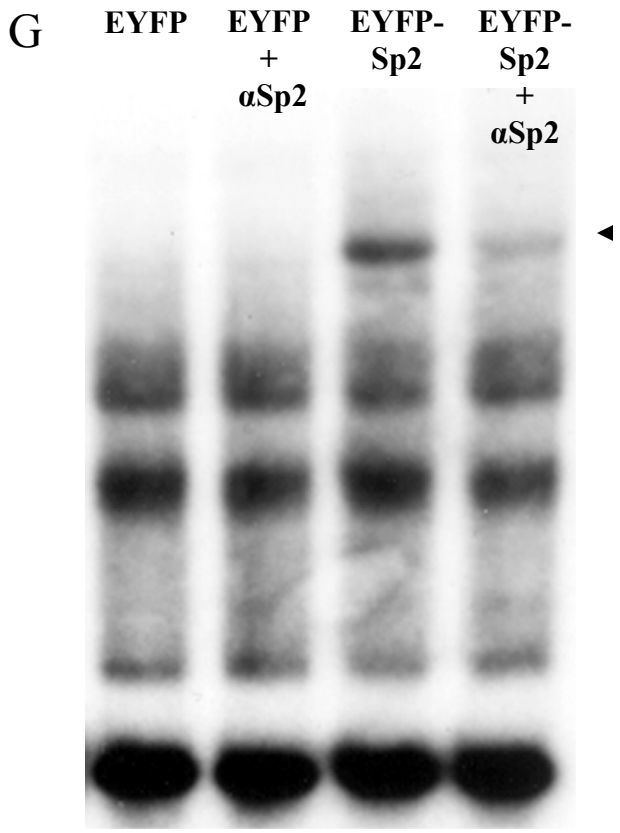


FIGURE 17: Subcellular localization of EYFP-Sp1, EYFP-Sp2, and EGFP-Sp3, and characterization of EYFP-Sp2 DNA-binding activity. A, *in situ* epifluorescence. COS-1 cells were transiently transfected with an expression vector encoding an EYFP-Sp1 fusion protein. EYFP-positive cells were identified via widefield epifluorescence (center column). Nuclei were localized via staining of DNA with DAPI (left column) and a merged image is shown in the right column. B, C, COS-1 cells expressing EYFP-Sp2 (B), or EGFP-Sp3 (C) were prepared as in A. D, E, and F, identical procedures were followed as in A (center column) to identify EYFP-Sp2 (D), EYFP-Sp1 [this is the same image as in A] (E), or EGFP-Sp3 (F) expressed ectopically in COS-1, or HeLa cells. G, protein/DNA-binding assay. Non-denatured whole cell extracts were prepared from COS-1 cells transiently transfected with EYFP-Sp2, and these and control extracts were incubated with a radiolabeled DHFR1* probe. Resulting protein/DNA complexes were resolved on a non-denaturing acrylamide gel. To confirm the identity of the novel protein/DNA complex noted in EYFP-Sp2-transfected cells, anti-Sp2 antiserum (K-20) was included in the indicated binding assays. A *closed arrowhead* indicates the position of the EYFP-Sp2/DNA complex.

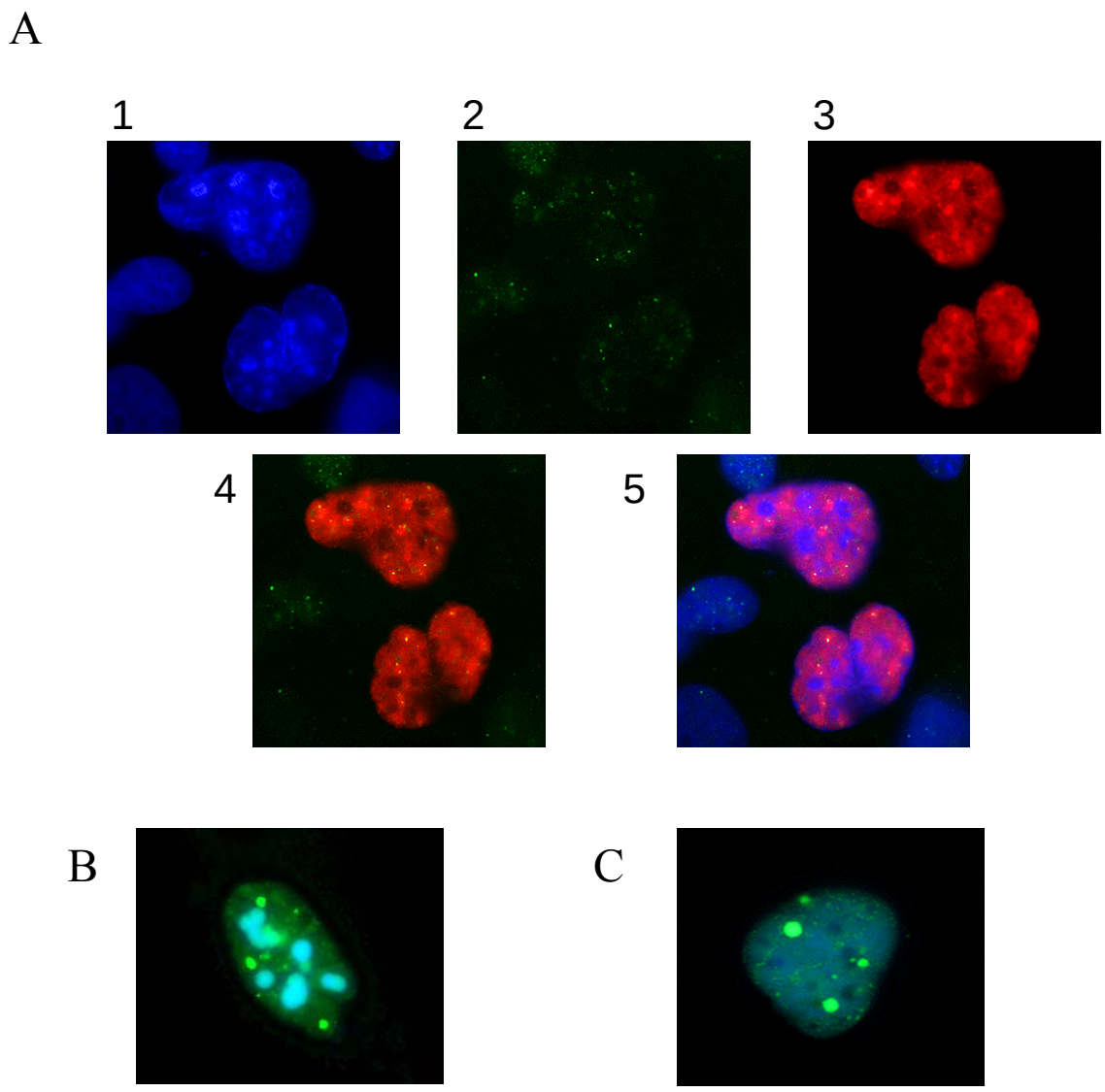
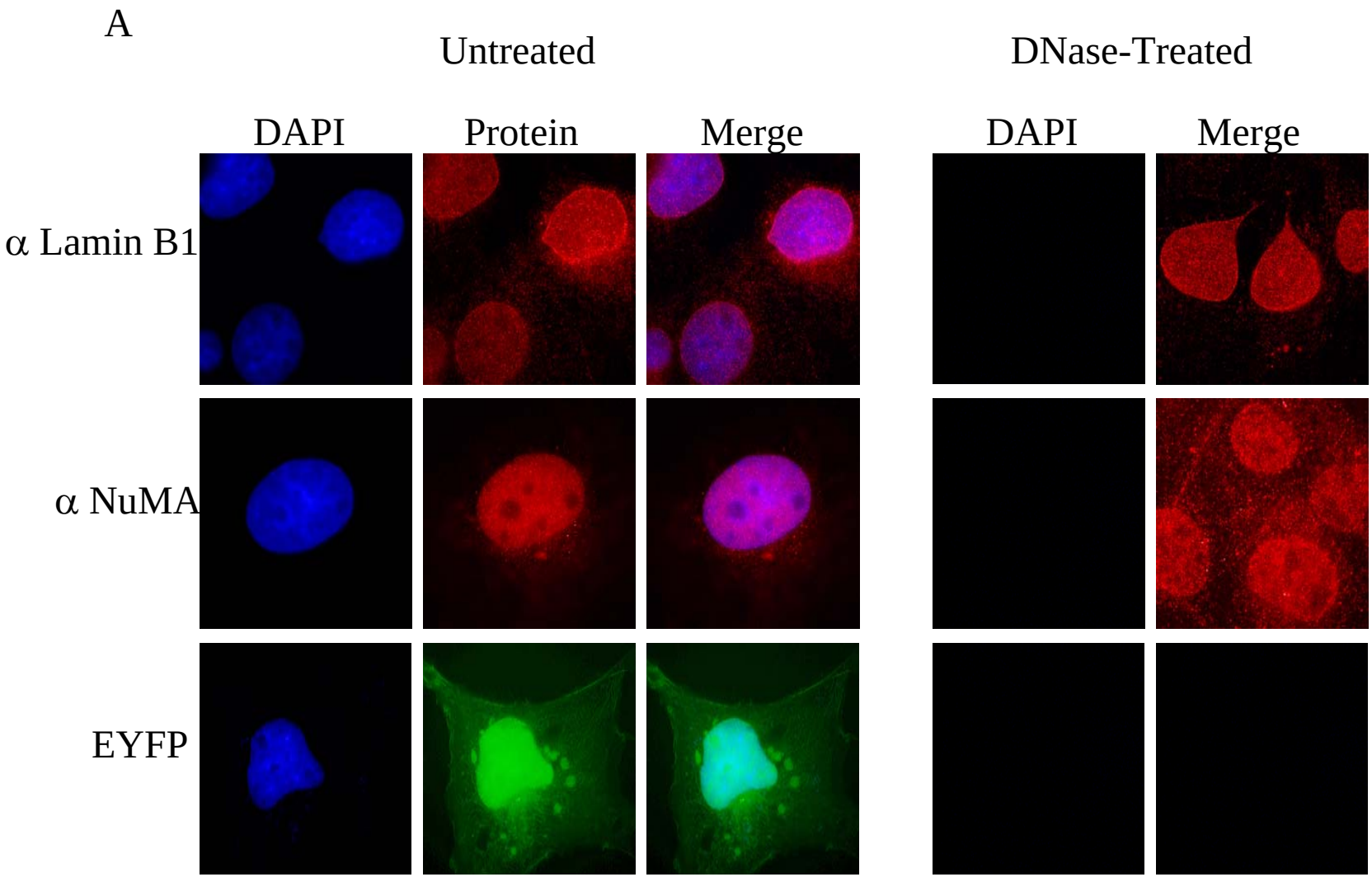


FIGURE 18: Sp2 sub-nuclear foci do not co-localize with PML or Sp100 within promyelocytic (PML) oncogenic domains (PODs). A, indirect immunofluorescence. COS-1 cells were transiently transfected with an EYFP expression vector encoding full length human Sp2. Ectopically expressed protein was detected by indirect immunofluorescence using an anti-HA polyclonal antibody (A,3), whereas endogenous PML was detected with an anti-PML polyclonal antiserum (A,2). Nuclei were detected by DNA-staining with DAPI (A,1). Superimposed images are as follows: anti-HA and anti-PML images (A,4) and anti-Sp2, anti-PML, and DAPI (A,5). B, C, *in situ* epifluorescence. COS-1 cells were transiently co-transfected with expression vectors encoding EYFP-Sp2 and ECFP-PML (B), or EYFP-Sp2 and ECFP-Sp100 (C), and ectopically expressed proteins were detected by *in situ* epifluorescence.


A
Untreated
DNase-Treated
DAPI
Protein
Merge
DAPI
Merge
α Lamin B1
α NuMA
EYFP

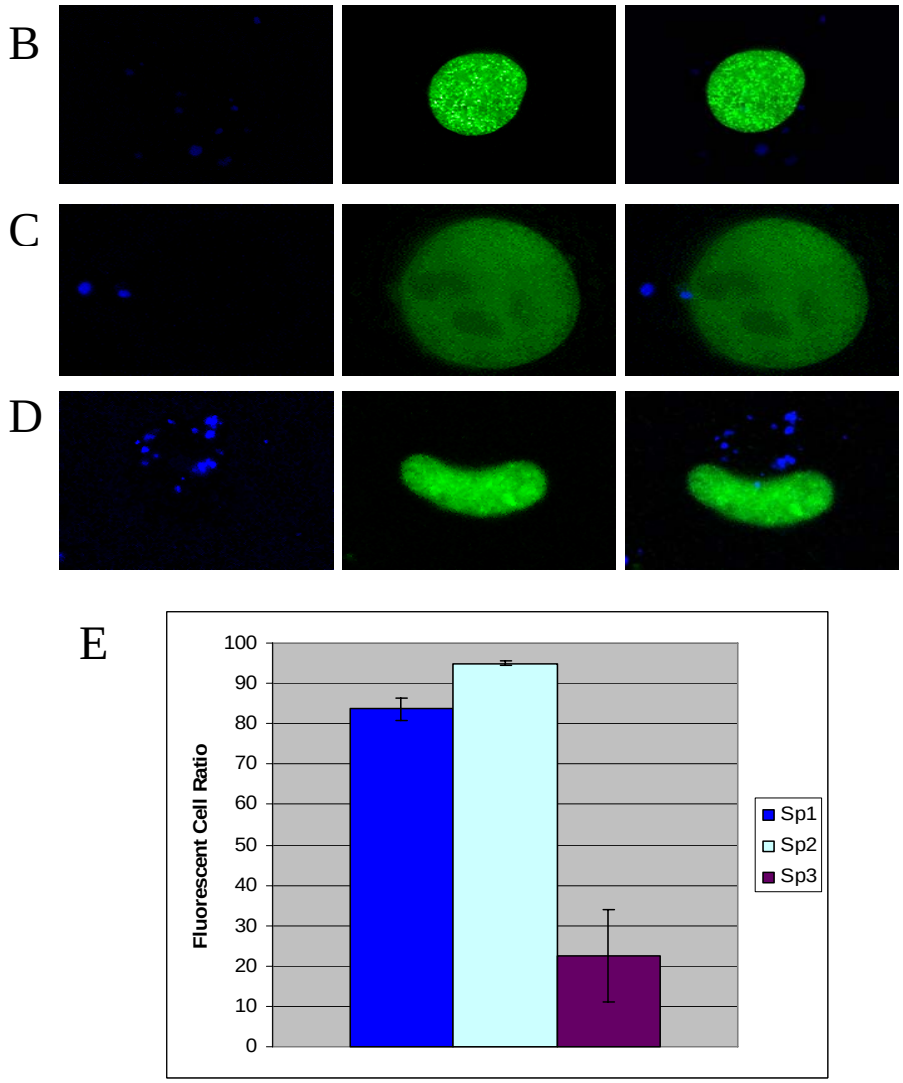


FIGURE 19: Sp-family members differentially associate with the nuclear matrix. A, indirect immunofluorescence. COS-1 cells were analyzed *in situ* by direct and indirect immunofluorescence prior to and following disruption of nuclei, chromatin digestion, and preparation of nuclear matrices. Endogenous Lamin B1 and NuMA proteins were detected with anti-Lamin B1 [what antibody?], or anti-NuMA [what antibody?]antibodies. COS-1 cells transfected with EYFP were analyzed by direct fluorescence. Nuclei were visualized by staining of DNA with DAPI. Columns of DAPI-stained nuclei (DAPI) and antibody- or EYFP-stained nuclei (Protein) are presented as well as merged images (Merge) for each protein. B, C, D, *in situ* epifluorescence. COS-1 cells were transfected with EYFP-Sp2 (B), EYFP-Sp1 (C), or EGFP-Sp3 (D), and EYFP-positive nuclei were identified via widefield epifluorescence following treatment of nuclei with DNaseI. DAPI-stained nuclei are presented (left column) as well as merged images (right column). E, COS-1 cells were transfected with expression vectors encoding Sp1, Sp2, or Sp3, and the total numbers of fluorescent nuclei detected following staining with an anti-HA antibody were quantitated *in situ* in cultures that were untreated or treated with DNaseI. The graph represents the ratio of fluorescent

cells recovered in DNaseI-treated cultures relative to untreated cultures. Each experiment was repeated in triplicate to account for plate-to-plate variations in transfection efficiency.

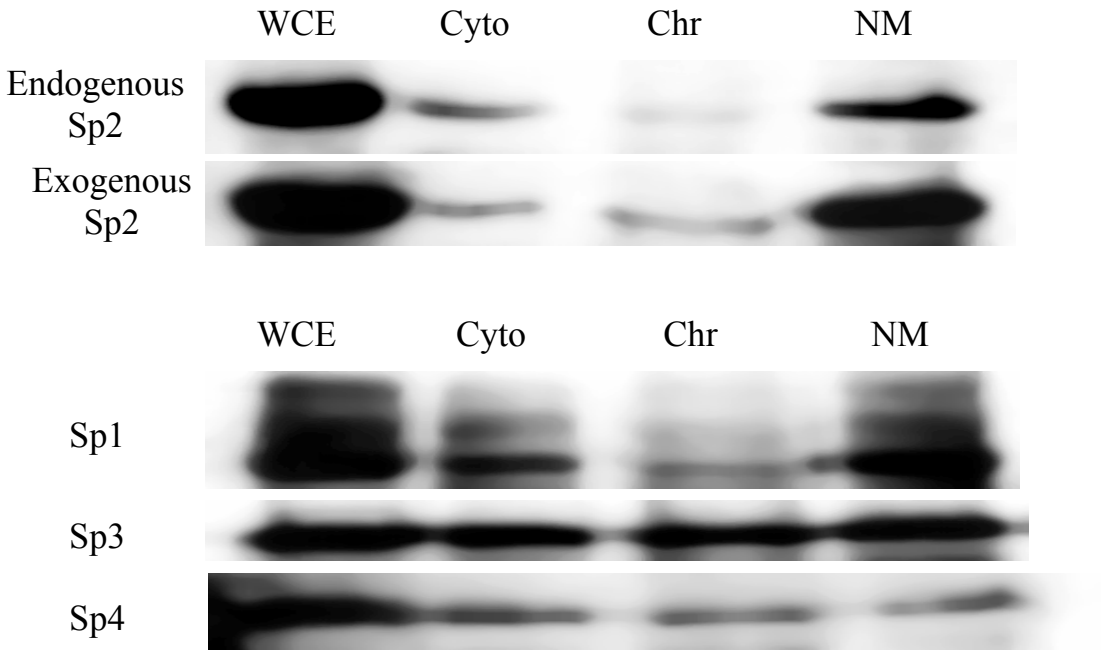


FIGURE 20: Differential association of Sp-family members with the nuclear matrix. Denatured extracts from mock transfected COS-1 cells, and COS-1 cells transiently transfected with expression vectors encoding full length human Sp1, Sp2, Sp3, or Sp4 were subjected to nuclear matrix fractionation, and cell fractions containing soluble proteins (Cyto), chromatin-associated proteins (Chr), and insoluble nuclear matrix-associated proteins (NM), were recovered. Identical cell equivalents of each fraction were resolved on denaturing polyacrylamide gels in parallel with denatured whole cell extracts prepared from transfected COS-1 cells. Resolved proteins were transferred to PVDF membranes and detected by incubation with a rabbit polyclonal anti-Sp2 antibody (K-20; top two rows), or a rabbit polyclonal anti-HA antibody (Y-11).

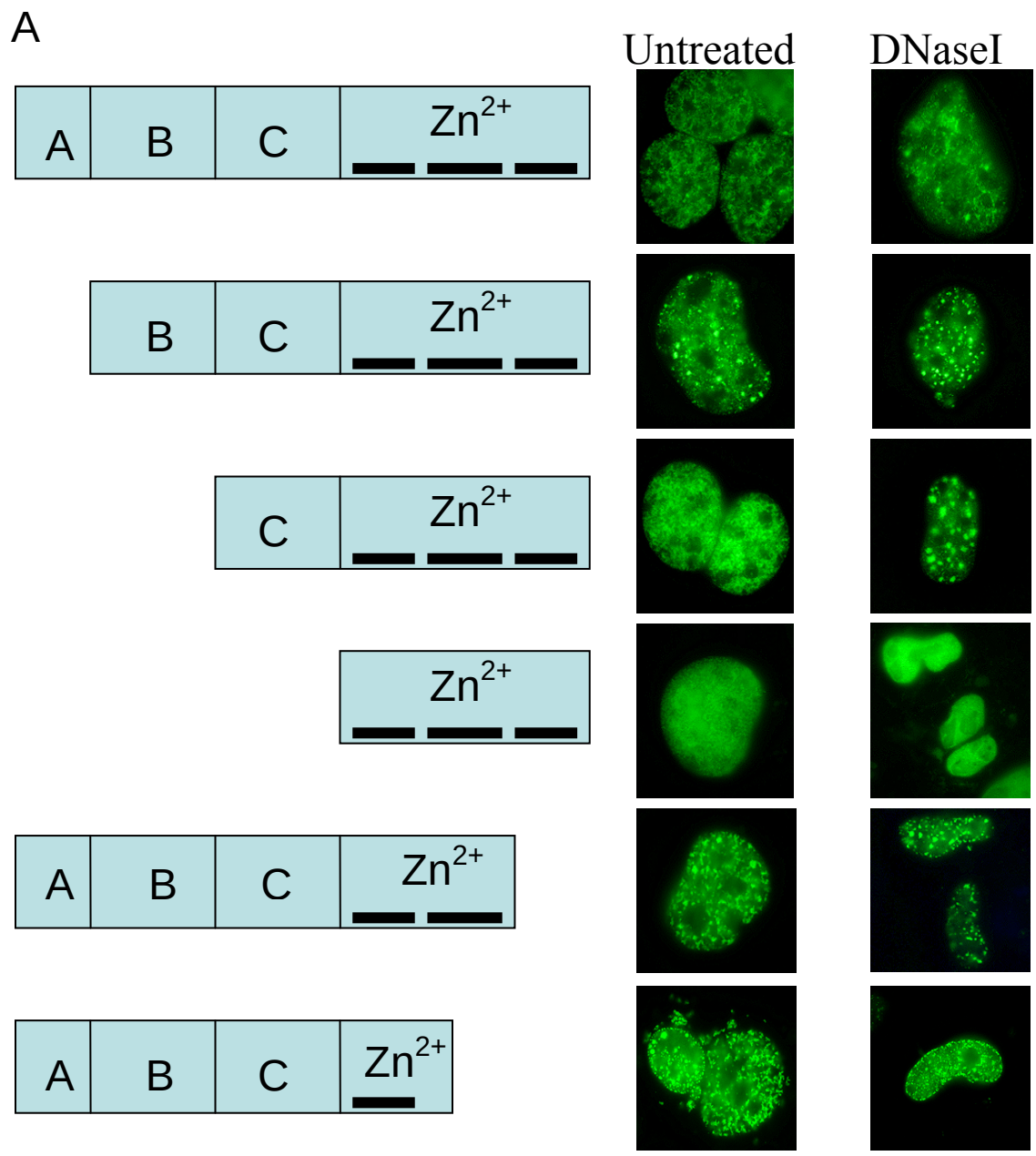

A
Untreated
DNaseI
A
B
C
Zn2+
B
C
Zn2+
C
Zn2+
Zn2+
A
B
C
Zn2+
A
B
C
Zn2+
B
Zn

FIGURE 21: Mapping of regions of Sp2 required for association with the nuclear matrix. A, *in situ* epifluorescence and schematic representing the panel of EYFP-Sp2 deletion mutants examined for association with the nuclear matrix. COS-1 cells were transiently transfected with EYFP-fusion proteins encoding full length human Sp2 or the indicated Sp2 deletion mutants. EYFP-Sp2-positive nuclei were detected in untreated nuclei as well as disrupted nuclei treated with DNaseI via widefield epifluorescence. B, indirect epifluorescence and schematic representing an EYFP-fusion protein prepared with 31 amino acids of the Sp2 DNA-binding domain. COS-1 cells were transfected with an EYFP-fusion protein encoding the 31 amino acid region encompassing zinc-"finger" I and the first inter-"finger" linker region of Sp2. Nuclei were localized via DNA-staining with DAPI (left), direct fluorescence is shown in the center column, and a superimposed fluorescent image is presented in the right column.

CHAPTER VI
Discussion

Transcriptional regulation in mammalian cells is controlled by several classes of proteins, including sequence-specific DNA-binding proteins. Members of the Sp-family of DNA-binding proteins are responsible for regulating the expression of a diverse array of mammalian genes responsible for processes such as cell cycle control, differentiation, and development such as p21, TGF-β, and DHFR. In turn, regulatory mechanisms that include post-translational modifications and interactions with other proteins dictate the activities of Sp proteins. Despite similar structures, patterns of expression, and DNA binding-site preferences, it is clear that Sp-family members share only partially overlapping functions. For example, gene ablation studies have shown that Sp1-, Sp3, and Sp4-nullizygous mice have drastically different phenotypes. Although a great deal of effort has been expended on the characterization of the biochemical and functional properties of Sp1 and Sp3, many questions remain to be resolved and we have only a superficial understanding of the properties of other Sp-family members. In an effort to fill some of these gaps, I undertook a series of studies to address the role of Sp2 in cell physiology and to compare its biochemical and functional attributes with those of Sp1, Sp3 and Sp4. Using molecular and biochemical techniques I have shown that, unlike Sp1 and Sp3, (1) Sp2 appears to function as a relatively weak activator of transcription, (2) Sp2's cognate DNA-binding sequence is distinct and more restricted from that of Sp1, (3) Sp2 DNA-binding activity and *trans*-activation are negatively regulated, potentially by two independent mechanisms, (4) Sp2 DNA-binding activity requires phosphorylation, (5) at least one Sp2 kinase can be detected within high molecular weight protein fractions, and (6) Sp2 preferentially associates with the nuclear matrix. Taken together,

my results indicate that Sp2 is a unique Sp-family member and suggest that Sp2 may play a highly specialized role in cell physiology.

Derivation and Functional Analysis of Consensus Sp2 DNA-Binding Sequence

Since Sp2 structure is similar to that of Sp1 and Sp3 and the Sp2 DNA-binding domain is closely conserved, I thought it likely that Sp2 would also function as a potent activator of transcription. Previous reports had suggested that Sp2 is a weak *trans*-activator of genes targeted by other Sp-family members, yet these same studies provided little or no evidence that Sp2 bound the promoters analyzed (Kennet et al., 2002; Moorefield et al., 2004). I began my studies by examining Sp2-mediated transcription in *Drosophila* SL2 cells, a convenient setting for such studies as these cells do not express Sp-like proteins yet have the necessary components to support Sp-directed transcription. Using a well characterized Sp-dependent gene (DHFR) as a substrate for these studies, Sp2 appeared to be a weak activator of transcription when compared to Sp1 and Sp3 (Fig. 2). Initially, I considered the possibility that structural differences between Sp2 and Sp1/Sp3 might account for their differential transcriptional activities. For example, relative to Sp1/Sp3, Sp2 encodes a truncated *trans*-activation domain lacking most of the A domain and does not encode a D domain. However, studies of Sp1 deletion mutants indicated that the B domain is sufficient to stimulate transcription and the Sp1 D domain was shown to be dispensable (Courey and Tjian, 1988; Santoro et al., 1988; Pascal and Tjian, 1991). I inferred from these results that other Sp2 properties must account for its relatively weak level of *trans*-activation. Since the Sp2 DNA-binding domain shares only 75% amino acid identity with Sp1, I hypothesized that differences in DNA-binding

site preference or affinity might account for Sp2's relatively modest capacity to stimulate DHFR transcription. This hypothesis was sustained by protein/DNA-binding studies indicating that, relative to other Sp-family members, Sp2 has a lower affinity for Sp-binding sites derived from Sp-dependent promoters (Fig 3 and Table 1). Accordingly, I decided to determine the Sp2 consensus DNA-binding sequence and compare the affinities of Sp1-3 for this sequence. Using recombinant Sp2 protein produced in baculovirus-infected Sf9 cells and a PCR-assisted protocol I (1) defined 5'-GGGCGGGAC-3' to be the consensus Sp2 binding site, (2) showed that Sp2 binds this sequence with high affinity (225 pmol), and (3) determined the nucleotide variance at each consensus nucleotide position is considerably more restricted than reported for Sp1 (Shi and Berg, 1995; Thiesen and Bach, 1991). I also showed that Sp1 and Sp3 bound the Sp2 consensus sequence with considerably less affinity (700 pmol and 8.9 nmol, respectively).

Given Sp2's unique cognate DNA-binding sequence, I reasoned that a fair analysis of Sp2-mediated transcription would require the construction of a reporter gene regulated by a promoter containing high-affinity Sp2 binding sites. Following conversion of one or more well-characterized Sp-binding sites within the DHFR promoter to consensus Sp2 DNA-binding sequences, I noted a modest increase in Sp2-mediated transcription in mammalian cells. Yet, the absolute levels of transcription remained significantly lower (10-20 fold) relative to Sp1- and Sp3-mediated *trans*-activation of the same promoter (Fig. 4). I conclude that (1) Sp2 is a relatively weak activator of transcription *in vivo*, and (2) this modest level of *trans*-activation is due to one or more mechanisms other than the absence of high-affinity DNA-binding sites.

Given that Sp2 did not stimulate transcription to levels comparable to Sp1 or Sp3, even in the context of promoters containing multiple high-affinity Sp2 binding sites, I took advantage of the modular structure of Sp-family members to define portions of Sp2 that limited its capacity to stimulate transcription. Swapping analogous portions of Sp2 with those of Sp1, I generated a series of chimeric molecules and analyzed each for DNA-binding activity and the capacity to stimulate transcription *in vivo*. These experiments proved to be extremely informative as careful analyses indicated that the Sp2 *trans*-activation and DNA-binding domains were each targets of mechanisms limiting Sp2-mediated transcription. For example, linkage of the Sp2 *trans*-activation domain to the Sp1 DNA-binding domain (*i.e.*, Sp2/1) completely eliminated *in vitro* DNA-binding activity. Similarly, linkage of the Sp2 DNA-binding domain to the Sp1 *trans*-activation domain (*i.e.*, Sp1/2, Sp1/2/1) dramatically reduced Sp-mediated transcription. Interestingly, although transcriptionally impotent the DNA-binding activities of these latter chimeras (Sp1/2, Sp1/2/1) are indistinguishable from that of Sp1.

Regulation of Sp2 DNA-Binding Activity

Since my chimera experiments indicated that the *trans*-activation and DNA-binding domains of Sp2 are each negatively regulated by one or more mechanisms, I reasoned that their activities might be governed by post-translational modifications and/or their interaction with regulatory proteins. There is considerable precedent for this supposition since the DNA-binding and *trans*-activation domains of other Sp proteins have been shown to be targets of regulatory mechanisms. As outlined in Chapter I, alterations in the DNA-binding and *trans*-activation activities of Sp1and Sp3 have been

linked to differences in post-translational modification and interactions with a diverse set of nuclear proteins (Pugh and Tjian, 1990; Sapetschnig et al., 2002; Ross et al., 2002). For example, casein kinase II-dependent (CKII) phosphorylation of the Sp1 DNA-binding domain inhibits the association of Sp1 with DNA, and phosphorylation of Sp1 by protein kinase C-γ has been shown to enhance DNA-binding activity and expression of platelet-derived growth factor β-chain (PDGF-β; Rafty and Khachigian, 2001). Glycosylation of the glutamine-rich region of the Sp1 B domain reduces its ability to stimulate transcription (Armstrong et al., 1997; Leggett et al., 1995; Roos et al., 1997; Yang et al., 2001).

To capitalize on the observations made with chimeric proteins, I performed a number of additional functional and biochemical experiments to define Sp2 regulatory mechanisms. An exhaustive series of protein/DNA-binding studies revealed that Sp2, DNA-binding activity was undetectable in 14 rodent and human cell lines, in primary mouse prostate tissue, and is only modestly apparent in transiently transfected cells. In marked contrast, Sp1 and/or Sp3 DNA-binding activity was readily detected in each of these settings. In a series of mixing experiments, I also showed that the DNA-binding activity of recombinant Sp2 protein is abolished by incubation with mammalian cell extracts. This loss of DNA-binding activity occurs in the absence of Sp2 protein degradation, indicating that mammalian cell extracts carry one or more active Sp2 regulators (Fig. 9). In an attempt to identify such regulators I developed an *in vitro* protein/protein-binding assay and identified a novel 84 kDa protein (p84) that bound specifically to the B domain of Sp2, but not analogous portions of Sp1 or Sp3 (Fig. 10). The gene that encodes p84 has not as yet been identified, and I believe this should be a

goal of future experimentation as its association with the Sp2 *trans*-activation domain is directly correlated with the loss of DNA-binding activity. Should p84 play an important role in the regulation of DNA-binding activity, I can envision at least two potential mechanisms by which p84 could disrupt Sp2/DNA interactions: (1) via steric hindrance or (2) post-translational modification. As the co-crystal structure of Sp2 bound to DNA has not been reported, it is plausible that its tertiary structure brings the Sp2 B domain into proximity of the Sp2 DNA binding domain. Such a configuration would be expected to bring p84 into close juxtaposition and could preclude the binding of Sp2 (or the Sp1 DNA-binding domain in a chimeric protein) to DNA. Alternatively, association of p84 with the Sp2 *trans*-activation domain distorts the tertiary conformation of Sp2 such that its DNA-binding domain is inactivated. Yet another possibility is that p84 is an Sp2-modifying enzyme. This is certainly not unprecedented as a number of kinases and phosphatases are found in association with their substrates, *e.g.,* mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase phosphatase (MKP), and the transcription factor Elk-1 have been shown to co-localize with extracellular-signal-related kinase 2 (ERK2) via MAPK-docking sites (Tanoue et al., 2002; Bardwell et al, 2003; Peters et al., 2001; Ajuh et al., 2000; Crabtree and Olson, 2002; Kawabe et al., 1997; Hong and Sarge, 1999). Consistent with this possibility, I have shown that Sp2 DNA-binding activity requires prior phosphorylation, and I have detected at least one Sp2 kinase within a high-molecular weight protein fraction carried by mammalian cell extracts. Should p84 be an Sp2-modifying enzyme, perhaps an Sp2 kinase or an Sp2 phosphatase, one might predict that it should function on targets in addition to Sp2. Indeed, this would appear to be the case as Sp1 DNA-binding activity was neutralized in

chimeric proteins. Whether other or all Sp DNA-binding domains would be similarly affected remains to be determined. It is perhaps worth mentioning here that I performed a series of *in vitro* kinase assays using immunoprecipitates prepared from cells expressing my panel of Sp1/Sp2 chimeras. These studies demonstrated that chimeras carrying the Sp2 DNA-binding domain, such as Sp2, Sp1/2, and Sp1/2/1, were phosphorylated *in vitro* more efficiently than chimeras carrying the Sp1 DNA-binding domain (data not shown). This result is particularly interesting when compared with *in vitro* protein/DNA-binding assays shown in Chapter III, which reveal that chimeric proteins containing the Sp2 DNA-binding domain are competent to form stable interactions with DNA (Fig. 5). Whether these results indicate that the Sp2 DNA-binding domain attracts more kinase activity or is a better substrate for associated kinases remains to be determined.

Implicit for my argument that phosphorylation of Sp2 is required for DNA-binding activity is evidence that Sp2 can be phosphorylated *in vivo*. Indeed, phosphoamino acid analysis of Sp2 confirmed that it is phosphorylated, with 95% of these residues being phosphoserine. I have analyzed the Sp2 DNA-binding domain for potential sites of phosphorylation using the SwissProt database (http://us.expasy.org/), and this analysis identified four serine-threonine residues within the DNA-binding domain that are predicted to be phosphorylated with greater than 95% confidence. Should phosphorylation of the Sp2 DNA-binding domain be required for DNA-binding activity, then it should be possible to use two-dimensional tryptic phosphopeptide analysis to identify critical phosphorylated amino acid. For example, comparison of two-dimensional phosphopeptide maps of Sp1 (binds DNA), Sp2 (doesn't bind DNA) and Sp1/2 (binds DNA) would facilitate the identification of the precise amino acid residues

that are differentially phosphorylated between the proteins. Identification of these critical residues might also prove helpful in the eventual identification of Sp2 kinases.

Given that phosphorylation is required for Sp2 DNA-binding activity, I attempted to identify the molecular weight of Sp2 kinase(s) by conducting *in vitro* kinase assays using mammalian cell extracts that had been fractionated via size exclusion chromatography. My analyses revealed Sp2 kinase activity within an extract fraction carrying proteins or protein complexes of 450-600 kDa. The physiological relevance of this Sp2 kinase activity remains to be determined, however I am encouraged by three additional observations. First, this Sp2 kinase activity appears to phosphorylate Sp2 specifically *in vitro*. Secondly, I showed that a considerable amount of endogenous Sp2 is detectable in the same column fractions (450-600 kDa). Although this latter observation does not prove that Sp2 associates with an active Sp2 kinase, it is a possibility that should be explored further. Finally, preliminary protein/DNA-binding assays suggest that phosphorylation of GST-Sp2 by the Sp2 kinase(s) within the high-molecular weight protein complex increases DNA-binding capacity (data not shown). How does one reconcile these kinase results with the lack of Sp2 DNA-binding activity in intact cells? One possibility worth considering is that this high-molecular weight kinase activity is quenched by p84. That is, p84 could directly interfere with this kinase activity or, if p84 is a phosphatase, compete with this kinase activity for the production of phosphorylated Sp2. Clearly, purification or the cloning of p84 and Sp2 kinases would help address these speculations.

Since (1) one or more cellular proteins appear to be capable of blocking Sp2/DNA interactions and (2) at least one protein, p84, forms a stable complex with Sp2, I

predicted that disruption of such protein/protein interactions should stimulate Sp2 DNA-binding activity. To this end I included deoxycholate in a series of extract mixing experiments with the hope that this detergent would prevent the formation of inhibitory protein/protein interactions. Disappointingly, I did not detect an appreciable increase in Sp2 DNA-binding activity in extracts treated with deoxycholate. This result may indicate that the protein/protein interactions involved are insensitive to deoxycholate treatment, or perhaps that alterations in the modification state of Sp2 do not require stable protein/protein interactions.

Since Sp2 is ubiquitously expressed but most, if not all, cell extracts are devoid of Sp2 DNA-binding activity, it would appear that Sp2 may be synthesized as an inactive precursor or be inactivated due to its association with proteins such as p84. Should this be the case, I hypothesized that stimulation of one or more common signaling pathways might stimulate Sp2 DNA-binding activity. Unfortunately, little or no change in Sp2 DNA-binding activity was noted despite treatment of cultured mammalian cells with a variety of known effectors of well-characterized signaling cascades (see Appendix II). It is certainly possible that the signaling pathways chosen have no bearing on the DNA-binding capacity of Sp2. It may also be the case that regulation of Sp2 DNA-binding is may require multiple signals and interactions with proteins that were unaffected in treatment experiments.

Regulation of Sp2-Mediated Transcription by Interactions with Co-Repressors

Studies with Sp1/Sp2 chimeras also revealed that the DNA-binding domain of Sp2 could negatively regulate *trans*-activation without inhibiting DNA-binding activity.

Given these results, I predict that one or more proteins serving as co-repressors of transcription may bind the Sp2 DNA-binding domain and in so doing interfere with the association of Sp2 with co-activators or directly block transcription via chromatin modification. Since the Sp2 DNA-binding domain is the least conserved amongst Sp proteins (75% amino acid identity), it is certainly conceivable that this sequence encodes a binding site for one or more co-repressors that do not interact with Sp1 or Sp3. Since I have shown that treatment of cells with Trichostatin A (TSA), a histone deacetylase inhibitor, did not stimulate Sp2-mediated transcription it is likely that a histone deacetylase is not involved in an Sp2-dependent chromatin modification mechanism. One intriguing possible explanation stems from a recent report suggesting Sp1-mediated transcription is negatively regulated via a mechanism that involves interactions between the Sp1 DNA-binding domain and the inhibitory domain, and co-repressors such as NcoR, BcoR, and SMRT (Lee et al., 2005). As I have not investigated functional protein/protein interactions mediated through the Sp2 DNA-binding domain, it is conceivable that the Sp2 DNA-binding domain or D domain encodes a putative inhibitory domain. Were this the case, similar to Sp1, Sp2 would be competent to bind DNA, yet the co-repressors would inhibit interaction with co-activators, such as components of the basal transcription machinery. Consistent with my observations with Sp1/Sp2 chimeras, any heterologous *trans*-activator protein containing the Sp2 DNA-binding domain would unable to stimulate transcription. Further analyses will be required to establish precisely how the Sp2 DNA-binding domain negatively regulates transcription.

Regulation of Sp2 Function via Sub-Cellular Localization

Another possible explanation for the apparent lack of Sp2 DNA-binding activity in mammalian cells is that Sp2 proteins competent to bind DNA may be localized within insoluble sub-nuclear domains. Many of the results I have discussed in this section rely on protein/DNA-binding assays performed with soluble mammalian extracts. Should the active (*i.e.*, DNA competent) fraction of Sp2 be limited to insoluble nuclear sub-domains, then my results with soluble cell extracts would be readily explained. To begin to address this possibility, I compared the subcellular localization of Sp2 with other Sp-family members. Based on indirect immunofluorescence studies and biochemical cellular fractionation, I have shown that (1) Sp-family members differentially associate with the nuclear matrix, and (2) Sp2 is almost exclusively associated with the nuclear matrix within stable, sub-nuclear foci that are distinguishable from promyelocytic (PML) oncogenic domains (PODs). Additionally, using time-lapse video microscopy I have shown that Sp2 remains stably localized within these sub-nuclear foci over an 18 hr time-course of observation. To delimit portions of Sp2 required for association with the nuclear matrix, I analyzed a series of EYFP-Sp2 deletion mutants for their subcellular localization. These studies identified a 31 amino acid region encompassing zinc-"finger" I as well as the first inter-"finger" linker of the Sp2 DNA-binding domain as being required for association with the nuclear matrix. However, it is worth pointing out that my studies have not as yet established whether these 31 amino acids are both necessary and sufficient for matrix attachment. For example, an EYFP-fusion protein carrying these amino acids alone was not competent for matrix attachment. Interestingly, amino acids that comprise the first zinc-"finger" of Sp2 are the least conserved amongst Sp-

family members (65%), perhaps accounting for its differential association with the nuclear matrix. Regardless of the amino acids required for matrix association and the matrix targets that Sp2 binds, is matrix-bound Sp2 functional**?** It is well established that matrix association is essential to the *trans*-activation function of some transcription factors, such as the Runx2/Cbfa1 family, whereas in other cases, localization to the nuclear matrix correlates with inactivation, as with NMP-2 (Zaldi et al., 2001; van Wijnen et al., 1993). Given that the vast majority of Sp2 is associated with the nuclear matrix within stable sub-nuclear foci, it is certainly possible that Sp2 is functional in this insoluble configuration. Perhaps Sp2 is associated within the nuclear matrix with one or more Sp2 kinases that stimulate its DNA-binding activity. If so, it is not unreasonable to assume that removal of Sp2 from the matrix, either by solubilization or the action of one or more signaling pathways, might result in the loss of DNA-binding activity. Perhaps this is why Sp2 DNA-binding activity is undetectable in all soluble cell extracts that I have examined. However, I favor an alternative model that incorporates each of the regulatory mechanisms (protein/protein interactions, post-translational modifications, and nuclear matrix association) that I have documented throughout my studies (Fig. 22). In this model, Sp2 is predominantly in a latent, insoluble form, tethered to the nuclear matrix. A specialized subset of intracellular signals or extracellular stimuli can liberate Sp2 and activate its DNA-binding activity via inactivation of a phosphatase (p84?), shifting the equilibrium towards Sp2 phosphorylation, disassociation form the matrix, and binding to DNA. One possibility is that this activating phosphorylation event occurs on amino acids contained within Sp2 zinc-"finger" I. Should this be the case, such a phosphorylation event might mask the nuclear matrix targeting signal (NMTS) resulting

in matrix disassociation, and facilitate Sp2 DNA-binding activity. Following the expression of Sp2-dependent genes and the dissipation of the signal that induced Sp2 DNA-binding activity, phosphatase activity is restored, leading to Sp2 dephosphorylation and inactivation of Sp2 DNA-binding activity, and ultimately to re-association with the nuclear matrix.

While I acknowledge this model is likely to be overly simplistic and many of its components are as yet unidentified, it takes into account many observations regarding the regulation of Sp2 function, while presenting a foundation on which to base further studies. Indeed, I expect further studies will show that p84 is a phosphatase, capable of maintaining Sp2 in an inactive state by removal of phosphates required for matrix dissociation and DNA-binding activity. While not all of the data presented herein is represented in my model, it stands to reason that I attempted to reconcile the majority of my findings in an effort to explain the mechanism regulating Sp2. For instance, although Sp1 is not phosphatase sensitive, yet Sp2/1 chimeras have no DNA-binding activity, it may be that in this case p84 disrupts DNA-binding via steric hindrance, or distortion of tertiary conformation, and its phosphatase activity is inconsequential. More detailed analysis of the constituent proteins of the nuclear matrix, especially those bound by Sp2, would also contribute to understanding the mechanisms regulating Sp2-mediated transcription. Particularly useful would be an Sp2 knockout mouse, which would allow us to characterize Sp2 function and possibly indicate potential target genes regulated by Sp2.

Regardless of whether my model or another is correct, what signaling pathways regulate Sp2 function and what are its transcriptional targets? One possibility is that Sp2

functions as part of a host response to viral infection. Infection by several viruses, including SV40, HIV-1, and Herpes Simplex Virus-1 (HSV-1), has been shown to induce Sp1 phosphorylation and Sp1-mediated transcription (Kim and DeLuca, 2002). It is certainly possible that Sp2 is similarly regulated, and experiments designed to detect changes in Sp2 DNA-binding activity or Sp2-mediated transcription following viral infection would address this proposition. During the course of my studies a laboratory identified Sp2 as a transcriptional repressor of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM1), a putative tumor suppressor gene, in primary prostate cells (Phan, et al., 2004). These workers reported that an increase in Sp2 expression is directly correlated with prostate epithelial cell transformation and repression of CEACAM1 expression via an HDAC-dependent mechanism. Although in my hands Trichostatin A (TSA) did little to affect Sp2-directed transcription of the prostate-specific antigen (PSA) promoter in prostate cells (Fig. 5), it is conceivable that Sp2 regulates the CEACAM1 promoter differently. Should over-expression of Sp2 be oncogenic, as the report by Phan et al (2004) implies, given the high incidence of prostate cancer in the U.S. (200/100,000 men in 2004; National Cancer Institute; http://www.nci.nih.gov/) Sp2 would be a strong candidate for cancer therapeutics.

Given its relatively restricted DNA-binding preference and the independent mechanisms that regulate Sp2 DNA-binding activity and *trans*-activation, I predict that Sp2 regulates a discrete set of target genes in response to specific cellular stimuli that are not regulated by other Sp proteins. As such, I believe that the role of Sp2 in cell physiology may be significantly more specialized than other Sp proteins. Unfortunately,

the identification of Sp2 target genes may prove to be quite challenging due to the array of mechanisms that regulate Sp2 transcription in mammalian cells.

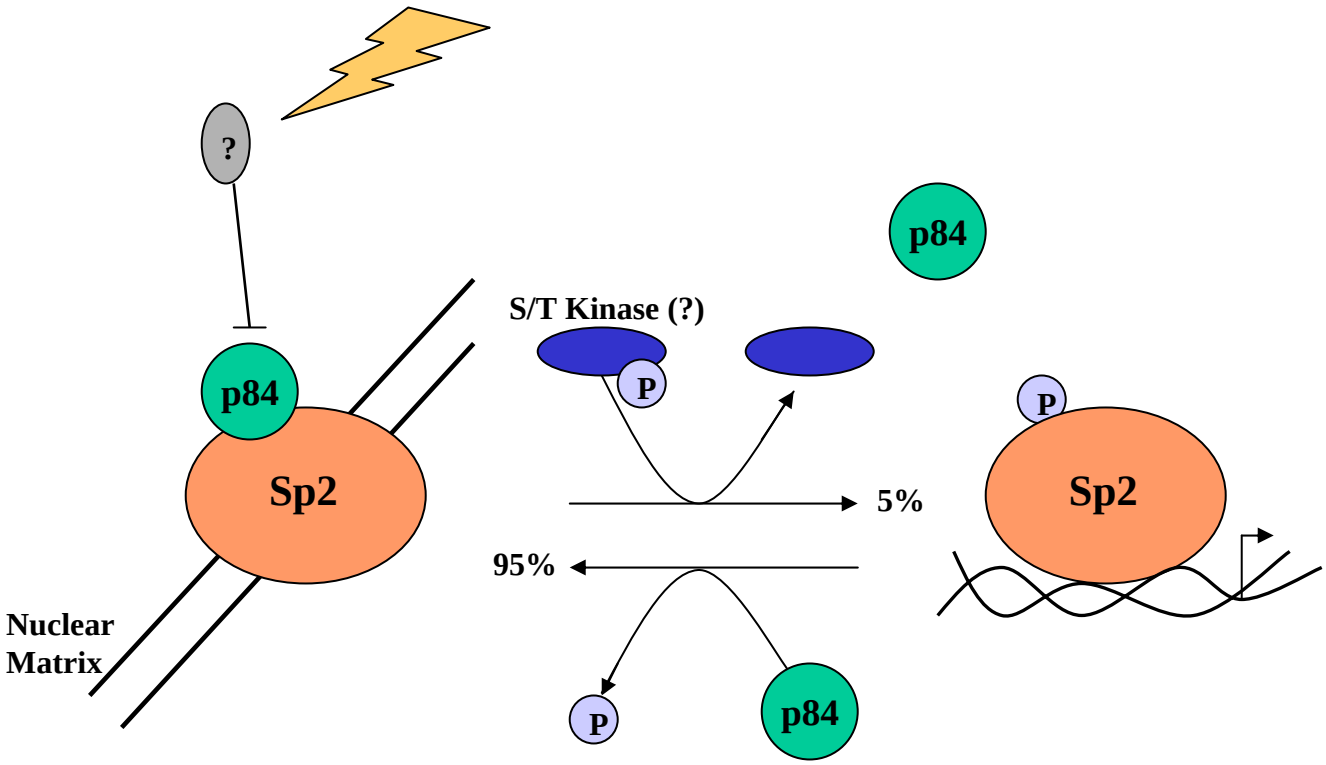


FIGURE 22: Model for regulation of Sp2-mediated gene expression in mammalian cells.

APPENDIX I:

Phosphorylation of Sp-Family Members within Inter-"Finger" Linkers is Insufficient for Exclusion from Mitotic Chromatin

While comparing the subcellular localization of Sp proteins by indirect immunofluorescence, it became apparent that each was excluded from mitotic chromatin. That is, Sp proteins were not associated with chromatin in prophase (prior to the break-down of the nuclear envelope) and did not re-associate with chromatin until telophase (following the reconstitution of daughter nuclei). Previous workers had reported that many, but not all, sequence-specific DNA-binding proteins are displaced from mitotic chromatin and this observation is consistent with the general lack of transcriptional activity during mitosis. The mechanisms that regulate mitotic exclusion have yet to be defined, nor is it understood why some factors are excluded and others remain chromatin-associated. Some have suggested that phosphorylation of conserved threonine residues within the inter-"finger" linker regions may be required for mitotic exclusion of zinc-"finger" proteins (Martinez-Balbas et al., 1995; Dovat et al., 2002; Jantz and Berg, 2004). *In situ* indirect immunofluorescence assays performed in COS-1 cells, HeLa, cells, and L929 cells demonstrated that Sp1-3, and all Sp1/Sp2 chimeric proteins, are excluded from mitotic chromatin (data not shown). To determine if threonine phosphorylation within the inter-"finger" linker regions of Sp proteins is required for mitotic exclusion, I used oligonucleotide-mediated mutagenesis to substitute isoleucines for each of the threonines of interest (Fig. 23). I chose to assess the affect of these amino acid substitutions on the mitotic localization of an Sp1/Sp2 chimera (Sp1/2) that carries the *trans*-activation domain of Sp1 and the DNA-binding domain of Sp2. As reported in

Chapter III, I had previously shown that this chimera is competent to bind DNA *in vitro* and thus I could expect that it is competent to bind chromatin *in vivo*. Given that the inter-"finger" linker has been shown to play an important role in the stabilization of zinc-"finger" structure, I anticipated that the substitution of isoleucine for threonine might reduce Sp1/2 DNA-binding activity. As shown in Fig. 24, replacing the conserved threonine within each inter-"finger" linker with isoleucine had a combinatorial affect on Sp1/2 DNA-binding activity. Substitution of isoleucine for each threonine led to a decrease in Sp1/2 DNA-binding activity, yet even the double-mutant was still somewhat able to bind DNA (Fig. 24, compare *Lane*s 3, 5, 7, and 9). Fig. 25 demonstrates that, akin to wild type Sp2, substitution of both threonines with isoleucines did not prevent mitotic exclusion. Thus, it appears that threonine phosphorylation is not required for the exclusion of Sp proteins, and by extension most zinc-"finger" proteins, from mitotic chromatin.

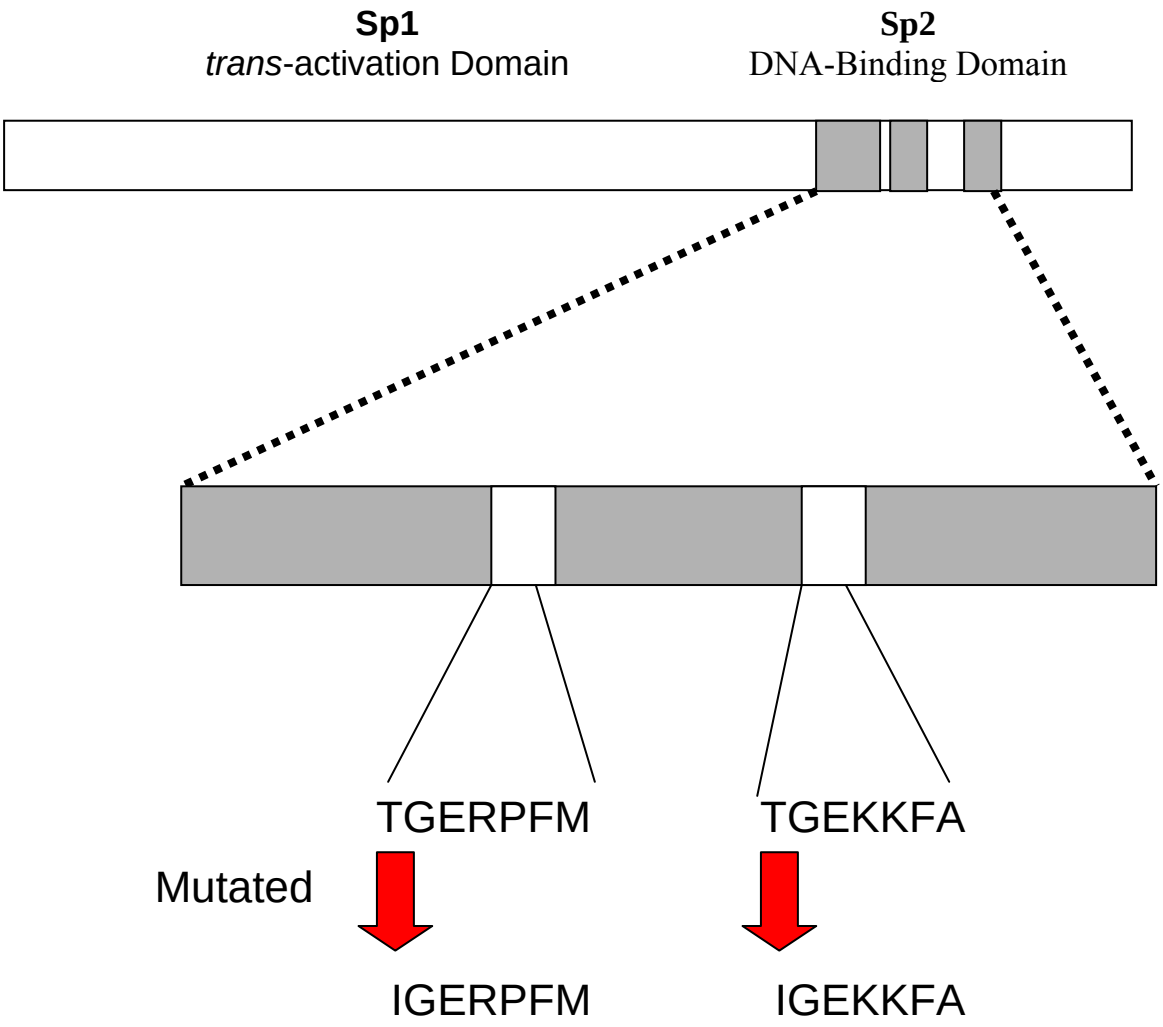


FIGURE 23- Schematic diagram indicating Sp1/2 linker region amino acid substitutions. The *trans*-activation domain of Sp1 consists of domains A, B and C. The Cys_2-His_2 zinc-"finger" DNA-binding domain is derived from Sp2. Zinc-"fingers" are shown in gray. Enlarged images indicate wild-type inter-"finger" linker regions and threonine to isoleucine amino acid substitutions.

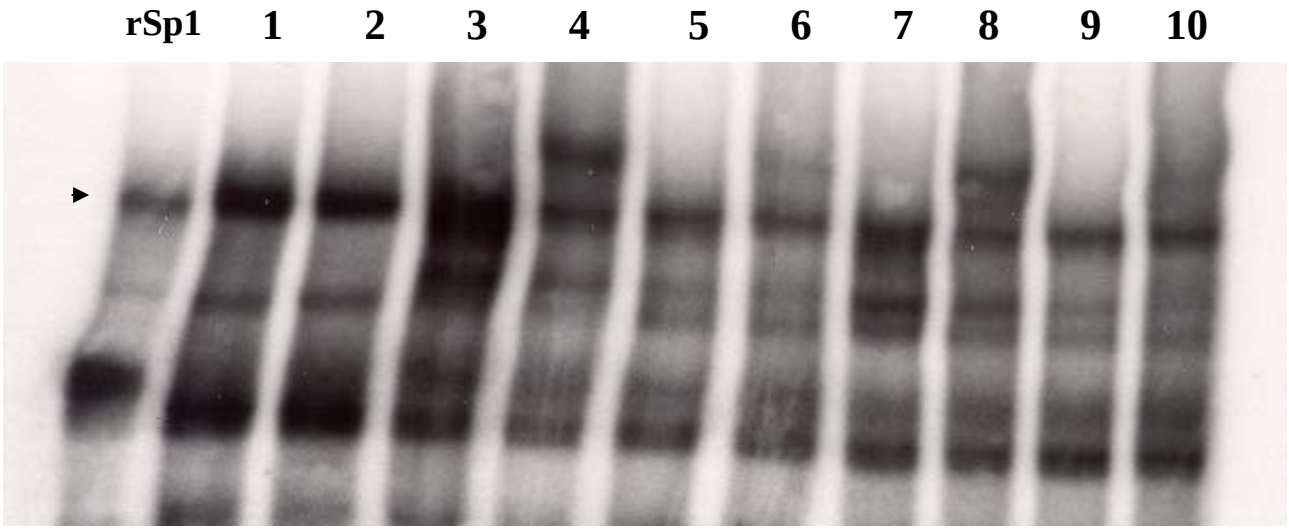


FIGURE 24- Characterization of protein/DNA complexes formed by wild-type and mutated chimeric Sp1/2 constructs in COS-1 extracts. Non-denatured extracts were prepared from mock-transfected COS-1 cells and cells transfected with Sp1, Sp1/2, or Sp1/2 proteins harboring single or double linker mutations. COS-1 extracts and baculovirus-expressed recombinant Sp1 protein (rSp1) were incubated with a radiolabeled DHFR1* probe, and protein-DNA complexes were resolved on a non-denaturing acrylamide gel. The d*ark arrowhead* indicates the position of Sp1/DNA complexes. Protein/DNA complexes were identified via the addition of antisera (Y-11) against the HA epitope attached to the carboxy-termini of each protein. *Lane 1*, mock-transfected COS-1; *Lane 2*, mock-transfected COS-1 plus anti-HA; *Lane 3*, Sp1/2; *Lane* 4, Sp1/2 plus anti-HA; *Lane 5*, Sp1/2 with linker 1 mutation; *Lane 6*, Sp1/2 with linker 1 mutation plus anti-HA; *Lane 7*, Sp1/2 with linker 2 mutation; *Lane* 8, Sp1/2 with linker 2 mutation plus anti-HA; *Lane 9*, Sp1/2 with linker 1 and 2 mutations; *Lane 10*, Sp1/2 with linker 1 and 2 mutations plus anti-HA.

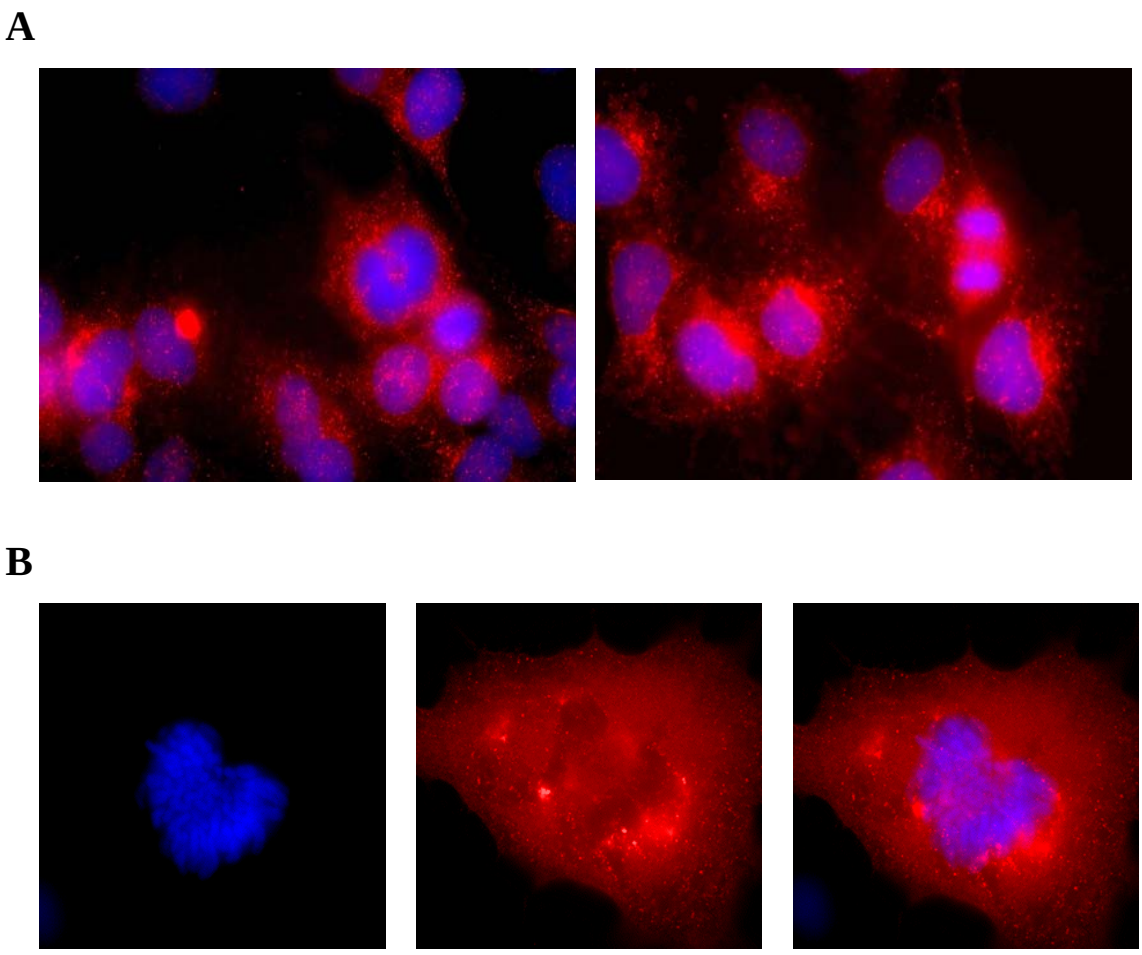


FIGURE 25: Subcellular localization of wild type Sp2 and mutated Sp1/2 in transiently-transfected COS-1 cells. A, COS-1 cells were transiently transfected with an expression vector encoding full length human Sp2 and detected by indirect immunofluorescence using an anti-HA polyclonal antibody (Y-11) and Alexa Fluor 594 goat anti-rabbit secondary antibody. DNA was stained with DAPI, and merged images are shown. B, COS-1 cells expressing Sp1/2 harboring mutations in both linker regions were analyzed with an anti-HA antibody (center column). DNA was stained with DAPI (left column) and superimposed images are shown in the right column.

APPENDIX II:

TABLE 2- Treatments Attempted to Activate Sp2 DNA-Binding Activity (see Fig. 11)

Treatment	Conditions	Affect on Sp2 DNA-Binding
Oxidative Stress	0.1 M H_2O_2, 37°C, 20'	No Change
Ethanol Stress	7% EtOH, 37°C, 20'	No Change
Osmotic Stress	0.7M NaCl, 37°C, 15'	No Change
Heat Shock Stress	43°C, 10'	No Change
phorbol 12 –myristate 13-acetate (TPA)	10 nM, 3 hrs., 37°C	No Change*
Dibutyryl cAMP (db cAMP)	1 mM, 3 hrs., 37°C	No Change*
Calcium ionophore A23187	3 μM, 0,1,4,8,or 20 hours, 37°C	No Change
Neuronal Growth Factor (NGF)	100 ng/ml NGF in DMEM containing 1% horse serum, 37°C, 3 days	No Change

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