Sp2 DNA Binding Activity and \textit{trans}-Activation Are Negatively Regulated in Mammalian Cells\footnote{This work was supported by National Institutes of Health Grants CA53248 and GM065405 and National Science Foundation Grant IGERT 9987555. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}

K. Scott Moorefield, Sarah J. Fry, and Jonathan M. Horowitz\footnote{To whom correspondence should be addressed: Dept. of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606. Tel.: 919-515-4479; Fax: 919-515-3044; E-mail: jon_horowitz@ncsu.edu.}

\textit{From the Graduate Program in Genomic Sciences and Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606}

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Previous studies have indicated that Sp2 binds poorly to GC-rich sequences bound by Sp1 and Sp3, and further functional analyses of Sp2 have been limited. To study Sp2-mediated transcription, we employed a PCR-based protocol to determine the Sp2 consensus DNA-binding sequence (5'-GGCGGGAC-3') and performed kinetic experiments to show that Sp2 binds this consensus sequence with high affinity (225 pM) \textit{in vitro}. To determine the functional consequence of Sp2 interaction with this sequence \textit{in vivo}, we transformed well characterized Sp-binding sites within the dihydrofolate reductase (DHFR) promoter to consensus Sp2-binding sites. Incorporation of Sp2-binding sites within the DHFR promoter increased Sp2-mediated \textit{trans}-activation in transient co-transfection experiments but also revealed Sp2 to be a relatively weak \textit{trans}-activator with little or no capacity for additive or synergistic \textit{trans}-activation. Using chimeric molecules prepared with portions of Sp1 and Sp2 and the human prostate-specific antigen promoter, we show that Sp2 DNA binding activity and \textit{trans}-activation are negatively regulated in mammalian cells. Taken together, our data indicate that Sp2 is functionally distinct relative to other Sp family members and suggest that Sp2 may play a unique role in cell physiology.

The Sp subfamily of Sp/XKLF transcription factors is composed of five members, Sp1–Sp5, that govern the expression of a wide variety of mammalian genes (for reviews, see Refs. 1–3). Each member of the family carries a conserved carboxyl-terminal domain featuring three Cys2-His2 zinc fingers required for sequence-specific DNA binding to GC-rich promoter elements (GC-boxes). The amino acid sequences of regions outside of the DNA-binding domain are only modestly conserved between family members, yet each is structurally conserved, since their \textit{trans}-activation domains feature a common series of alternating serine/threonine-rich and glutamine-rich subdomains (4). The glutamine-rich subdomains of each protein are believed to be required for \textit{trans}-activation, whereas the function(s) of the serine/threonine-rich regions is less well understood.

Despite their common structure and expression in most, if not all, mammalian cells, it is abundantly clear that the functions of Sp family members are not completely overlapping. For example, mice nullizygous for Sp1, Sp3, or Sp4 exhibit profound developmental abnormalities. Sp1-deficient mice perish \textit{in utero} after embryonic day 10 and exhibit a broad range of developmental deficiencies. Consistent with the notion that loss of Sp1 function leads to a cell autonomous defect, Sp1-null embryonic stem cells contribute to the formation of early chimeric embryos, but their abundance diminishes rapidly after embryonic day 11, and such cells are absent in newborns (5). Sp3-deficient embryos are growth-retarded and survive to birth, but newborn animals invariably perish due to respiratory failure. Careful examination of Sp3 nullizygotes indicates that these animals also suffer from defects in skeletal bone ossification and tooth development (6, 7). Mice lacking Sp4 appear to develop normally \textit{in utero}, but two-thirds of newborn animals perish within 4 weeks of birth of undetermined causes (8, 9). Surviving Sp4 nullizygotes are growth-retarded, male animals fail to breed despite normal spermatogenesis, and female animals exhibit a delayed onset of puberty. Although it is likely that Sp family members share some overlapping duties, these developmental results argue rather strongly that each Sp family member is responsible for unique contributions that cannot be supplanted by other family members or other GC box-binding proteins.

Sp2 is a poorly characterized member of the Sp family. Cloned 10 years ago by virtue of its hybridization with a portion of the Sp1 DNA-binding domain, Sp2 was shown to be expressed in a number of disparate human cell lines, to have the least conserved DNA-binding domain among Sp family members (75\%), and to bind weakly to a sequence (5'-GGT-GGGTGG-3') derived from the T-cell receptor V\textit{α}11.1 promoter (10). Subsequent studies have yielded few additional clues regarding Sp2 function. Sp2 has shown little or no capacity to stimulate the transcription of promoters that are activated potently by Sp1, Sp3, and Sp4 (11–14). However, these studies did not assess whether Sp2 can bind Sp-regulated promoter elements that are \textit{trans}-activated by these additional family members. Thus, it remains unclear whether the incapacity of Sp2 to stimulate transcription springs from its inability to bind the promoters analyzed, to \textit{trans}-activate, or both. In other instances, the addition of anti-Sp2 antiserum to nuclear extracts has yielded sporadic evidence of the formation of Sp2 protein-DNA complexes on promoter elements regulated by other Sp family members (15–22). Unfortunately, these same studies did not assess the transcriptional consequence of the interaction of Sp2 with these sites \textit{in vivo}. Thus, the data in hand shed little light on the functional properties of Sp2 and its role in cell physiology.

As a first step toward understanding the functional properties of Sp2, we undertook a series of experiments to determine whether Sp2 is a transcriptional \textit{trans}-activator and to compare its capacity to stimulate transcription with Sp1 and Sp3.
Sp2-mediated Transcription

Since at the outset it was unclear what DNA sequence(s) is bound with high affinity by Sp2, we began our studies by employing a PCR-mediated technique, termed SELEX or “CASTing,” to define a consensus Sp2-binding site. This consensus site was then incorporated within well characterized, Sp-regulated elements in the DHFR promoter, and the capacities of Sp1, Sp2, and Sp3 to trans-activate this mutated promoter and a wild-type DHFR promoter were compared. Our studies identified 5′-GGGCCGGAC-3′ as the Sp2 consensus DNA-binding site, and this site is bound with high affinity (225±studies identified 5′-moter and a wild-type DHFR promoter were compared. Our activity is negligible in many human and mouse cell lines. Indeed, although Sp2 severely regulates in mammalian cells. Indeed, although Sp2 site-containing promoters were 10− or more of the four Sp-regulated elements within the DHFR promoter, and the capacity for stimulation of transcription or activation of transcription is negligible in many human and mouse cell lines. Using a protein/protein binding assay, we also show that binding of the Sp2 trans-activation domain by a novel 84-kDa cellular protein is directly correlated with the inhibition of Sp2 DNA binding activity and trans-activation.

EXPERIMENTAL PROCEDURES

Cell Culture—S9 cells were obtained from Dr. Patrick J. Casey (Duke University Medical Center, Durham, NC), and Drosophila Schneider line-2 (SL2) cells were obtained from Dr. Cheeptajit Benyajati (University of Rochester, Rochester, NY). S9 and SL2 cells were cultured as previously described (23–25). HTLA230 cells were a generous gift of Dr. Emil Bogenmann (Children's Hospital of Los Angeles, Los Angeles, CA). GoTo, SKNBE(2), T98G, K562, HCT116, HUT78, and Jurkat cells were obtained from the Duke Comprehensive Cancer Center cell culture facility (Duke University Medical Center, Durham, NC). Antisera and Western Blotting—Rabbit anti-Sp1 (K-20; sc-643) polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), mouse anti-V5 monoclonal antibody was obtained from Invitrogen, and a mouse anti-HA monoclonal antibody (anti-HA.11) was obtained from Covance Research Products (Richmond, CA).

An oligonucleotide probe was used to detect Sp family members in the crude nuclear extracts analyzed in EMSA. Purified Sp1 and Sp3 bound this consensus Sp2-binding site with significantly less affinity (700 ppt and 9.9 ns, respectively). Interestingly, incorporation of this consensus Sp2-binding site into one or more of the four Sp-regulated elements within the DHFR promoter led to only a modest increase in the capacity of Sp2 to stimulate transcription in vivo relative to the wild-type DHFR promoter. Moreover, overall levels of Sp2-mediated trans-activation of Sp2 site-containing promoters were 10−20-fold less than that of Sp1 and Sp3, respectively. Using chimeric Sp1/Sp2 proteins and the prostate-specific antigen (PSA) promoter, we show that Sp2 DNA binding activity and trans-activation are negatively regulated in mammalian cells. Indeed, although Sp2 is ubiquitously expressed, we show that Sp2 DNA binding activity is negligible in many human and mouse cell lines. Using a protein/protein binding assay, we also show that binding of the Sp2 trans-activation domain by a novel 84-kDa cellular protein is directly correlated with the inhibition of Sp2 DNA binding activity and trans-activation.

1 The abbreviations used are: DHFR, dihydrofolate reductase; PSA, prostate-specific antigen; SL2, Schneider line-2; GST, glutathione S-transferase; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; DAPI, 4′,6-diamidino-2-phenylindole; TSA, trichostatin A.

2 A chloramphenicol acetyltransferase reporter gene linked to the adenovirus major late promoter, Δ53MLP-CAT, was obtained from Dr. Adrian R. Black (Roswell Park Cancer Institute, Buffalo, NY) and used as a normalization control in transient transfection experiments, since its activity is not dependent on Sp family members. A CAT reporter gene carrying three multimerized copies of a consensus Sp2-binding site (pSp2-TK-CAT) was generated using complementary synthetic oligonucleotides and a reporter gene regulated by a minimal promoter derived from the Herpes simplex virus thymidine kinase gene.

Baculovirus Stocks—Baculovirus stocks encoding Sp1 and Sp3 have been previously described (24). A baculovirus stock encoding Sp2 linked at its amino terminus to a V5 (Invitrogen) epitope tag was prepared via the baculovirus expression system (New England Biolabs, Beverly, MA). A polyclonal chicken anti-Sp2 trans-activation domain antiserum was prepared against a glutathione S-transferase (GST)-Sp2 fusion protein that carries the first 496 amino acids of human Sp2. Total IgG was collected from eggs prior to and following four successive immunizations with immunogen (Avex Labs, Inc., Tigard, OR). Whole cell or nuclear extracts were resolved on denaturing polyacrylamide gels and transferred to nitrocellulose using a semidyde transfer apparatus, and antigen-antibody complexes were detected as previously described (24, 25, 27, 33, 34).

Expression and Reporter Constructs—The construction and functional characterization of pPacSp1/flu, pPacSp2/flu, and pCMV4-Sp1/flu have been previously described (23–26). An expression construct, pPacSp2/flu, carrying a 10-amino acid epitope from influenza hemagglutinin at the Sp2 carboxyl-terminus was prepared following a baculovirus expression system (New England Biolabs, Beverly, MA), a polyclonal chicken anti-Sp2 trans-activation domain antiserum was prepared against a glutathione S-transferase (GST)-Sp2 fusion protein that carries the first 496 amino acids of human Sp2. Total IgG was collected from eggs prior to and following four successive immunizations with immunogen (Avex Labs, Inc., Tigard, OR). Whole cell or nuclear extracts were resolved on denaturing polyacrylamide gels and transferred to nitrocellulose using a semidyde transfer apparatus, and antigen-antibody complexes were detected as previously described (24, 25, 27, 33, 34).

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plexes was quantified as a volume of cell extract was identified that complexed with 50% of the cells were incubated with 200 pmol of radiolabeled oligonucleotide until dilutions of cell extracts prepared from Sp2 baculovirus-infected Sf9 cells were incubated with a radiolabeled probe derived from the c-fos promoter (5′-CCCTTGCCCGC-CCCTCT-3′) (30), and resulting protein-DNA complexes were 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 assays performed in triplicate with Sp-binding sites derived from the DHFR, p21, and MDR-1 genes and quantified in situ. GST fusion proteins were induced, purified, quantified, and utilized as previously described (24, 25, 27, 33, 34).

Derivation of a Consensus Sp2-binding Site—A consensus Sp2-binding site was determined as previously described except for the incorporation of an anti-V5 monoclonal antibody (Invitrogen) to immunoprecipitate protein-DNA complexes (32).

Equilibrium Dissociation Constant (Kₐ) Determination—Calculations of Kₐ values were performed by a standard procedure. Briefly, dilutions of cell extracts prepared from Sp2 baculovirus-infected Sf9 cells were incubated with 200 pmol of radiolabeled oligonucleotide until a volume of cell extract was identified that complexed with 50% of the input probe. 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 5-fold increasing and decreasing amounts of radiolabeled probe. An excess of infected cell extract was then assayed with 200 pmol of 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 halved to determine the amount of active Sp2 in diluted protein extracts. This value was then divided by the original extract dilution factor to derive the equilibrium dissociation constant.

Indirect Immunofluorescence—One day prior to transfection, 2 × 10⁵ COS-1 cells were plated onto glass coverslips in 6-well plates and incubated at 37 °C. Cells were cultured for 48 h following transfection, and all subsequent procedures were performed at room temperature. Coverslips were transferred to 60-mm tissue culture dishes, and cells were washed for 5 min with phosphate-buffered saline (PBS) on a rotating platform. Cells were fixed in 2% paraformaldehyde plus PBS for 15 min and then washed for 5 min with PBS. Cells were subsequently permeabilized with 0.5% Triton X-100 plus PBS for 60 min and then incubated for an additional 60 min in 1% fetal bovine serum plus PBS. Cells were incubated with primary antibodies (diluted 1:500 in 1% BSA plus PBS) for 60 min and then washed three times for 5 min each in PBS on a rotating platform. Cells were incubated with Alexa Fluor 594 goat anti-mouse secondary antibody (Molecular Probes, Inc., Eugene, OR) diluted 1:500 in 1% BSA plus PBS containing DAPI (diluted 1:50,000 for 60 min and then washed three times for 5 min each in PBS on a rotating platform. Following a single 5-min wash in distilled H₂O, coverslips were mounted on glass microscope slides using Vectashield mounting medium (Vector Labs, Burlingame, CA), sealed with clear nail polish, and viewed using a Nikon TE-200 inverted epifluorescence microscope equipped with appropriate optics and filter blocks at a magnification of ×100 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.

RESULTS

Sp2 Is a Relatively Weak trans-Activator of the Hamster DHFR Promoter in Vivo—To begin to analyze Sp2 function, we 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 (23, 24, 29). 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 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). A, 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.
setting (Fig. 1B, lane 2, and data not shown). We conclude from these titration experiments that Sp2 is a relatively weak trans-activator of DHFR transcription in SL2 cells.

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, we speculated that this significant structural difference might account for Sp2’s relatively modest stimulation of DHFR transcription; i.e. we 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, we 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 we have recently reported (25). To facilitate detection of this recombinant protein, we 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 86-kDa protein that is detected with polyclonal antibodies directed against Sp2 (Fig. 2A, lane 2) and a monoclonal anti-V5 antibody (Fig. 2A, lane 4) (10). As expected, a protein of similar molecular weight was not detected with these antibodies in uninfected cell extracts (Fig. 2A, lanes 1 and 3). As is also shown in Fig. 2A, 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 nondenaturing acrylamide gels and prepared for autoradiography. As shown in Fig. 2B, 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 (25). To compare results for Sp2 with previous studies of Sp1 and Sp3, we 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 (30). 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 oligonucleotides derived from the DHFR, p21, and MDR-1 promoters. Binding assays were performed in triplicate and quantified in situ. Sp2 DNA binding activity for each probe was normalized to the amount of binding activity detected for the p21 oligonucleotide (set equal to 1).

Table 1

| Sp protein | p21 | MDR-1 | DHFR |
|------------|-----|-------|------|
|            |     |       |      |
| Sp2        | 1   | 0.01  | 0.8  |

3-fold range in vitro (25). We 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. 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, we reasoned that we 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, we 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, we predicted that Sp2 should bind with high affinity to a GC-rich nonameric sequence (35–38). 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 50-fold excess of DHFR1* was more than sufficient to eliminate a 16-nucleotide central region of random sequence, a 50-fold excess of DHFR1* was more than sufficient to eliminate the Sp2 consensus sequence show little or no capacity to stably interact with Sp2. Hence, an accurate calculation of the $K_d$ for Sp2 and 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, we 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, we attempted to determine a $K_d$ for Sp2 and DHFR1; however, due to the extremely weak association of Sp2 with this oligonucleotide, we 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, we 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) (35, 39). To quantify the extent to which the DNA-binding domains of Sp1, Sp2, and Sp3 specify differing affinities for DNA, we 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). We 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.

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, we reasoned that we had to analyze Sp2 activity using a promoter carrying high affinity Sp2-binding sites. Given that Sp2 target genes have yet to be identified, we 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, we employed oligonucleotide mutagenesis to convert one or more Sp-regulated promoter elements within the DHFR promoter to consensus Sp2-binding sites (Fig. 4A). 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. 4B). 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
A

| Clone | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1     | A  | T  | G  | G  | G  | C  | G  | G  | G  | A  | C  | A  | A  | A  | T  | A  | A  |
| 2     | A  | G  | T  | G  | G  | G  | C  | G  | G  | G  | A  | C  | T  | T  | A  | T  | T  | T  | T  | T  | T  | T  |
| 3     | G  | G  | G  | G  | G  | C  | G  | G  | G  | A  | C  | A  | A  | T  | T  | A  |
| 4     | G  | G  | G  | G  | G  | C  | G  | G  | G  | A  | C  | A  | A  | T  | A  |
| 5     | G  | T  | G  | T  | G  | G  | G  | C  | G  | G  | G  | A  | C  | T  | A  |
| 6     | T  | A  | G  | G  | G  | G  | G  | G  | C  | G  | G  | A  | C  | T  | A  |
| 7     | G  | G  | G  | T  | G  | C  | G  | G  | G  | A  | C  | T  | A  | A  | G  |
| 8     | A  | A  | G  | T  | G  | G  | C  | G  | G  | A  | C  | T  | T  | T  |
| 9     | A  | A  | T  | G  | G  | G  | G  | C  | G  | G  | A  | C  | T  | T  |
| 10    | T  | T  | T  | T  | T  | A  | G  | G  | G  | G  | A  | C  | T  | A  |
| 11    | A  | A  | T  | G  | G  | G  | G  | C  | G  | G  | A  | C  | T  | T  |
| 12    | G  | G  | G  | G  | C  | G  | G  | G  | G  | G  | C  | T  | A  | A  | A  | A  | A  | A  | A  | A  |
| 13    | T  | C  | A  | C  | C  | C  | G  | G  | G  | C  | G  | G  | G  | G  | C  |
| 14    | A  | T  | G  | A  | T  | A  | A  | G  | G  | A  | G  | A  | A  | C  | T  | A  |
| 15    | G  | G  | G  | C  | G  | G  | A  | C  | T  | A  | A  | A  | A  | A  |
| 16    | G  | A  | T  | T  | A  | A  | G  | G  | C  | G  | G  | A  | C  | T  | A  |
| 17    | A  | T  | T  | T  | A  | A  | G  | G  | C  | G  | G  | A  | C  | T  |
| 18    | A  | A  | T  | G  | G  | G  | G  | C  | G  | G  | A  | C  | T  | A  |
| 19    | G  | A  | T  | T  | A  | A  | G  | G  | C  | G  | G  | A  | C  | T  |
| 20    | A  | A  | A  | A  | A  | A  | A  | G  | T  | G  | G  | C  | G  | G  | A  |
| 21    | G  | A  | A  | A  | A  | A  | A  | A  | G  | T  | G  | G  | C  | G  | A  |

T  2  3  6  5  11  0  0  0  0  0  0  1  0  0  15  5  1
C  1  0  1  1  1  0  1  0  18  2  0  0  0  21  0  0  0
A  5  7  7  4  4  6  1  0  0  1  0  1  1  18  0  4  12  10
G  2  1  3  9  3  15  19  21  3  18  21  19  3  0  0  0  0  

Conesen

T  G  G  G  C  G  G  A  C  T  A  A

B

DHFR2  G  A  G  C  G  G  G  G  C
DHFR4  G  G  G  C  G  G  G  G  C
p21    A  G  G  A  G  C  G  G  G
c-fos  G  G  G  G  T  G  G  C  G  A
DHFR1  A  G  G  G  C  G  T  G  G  C
DHFR1* G  A  G  G  C  G  G  A  G  T
MDR1   C  G  C  C  G  G  G  G  G  T

C

Fig. 3. 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'-GGGCGGGAC-3'), and challenged with increasing concentrations of unlabeled DHFR1* or a related oligonucleotide, DHFR1 (5'-GGGCCTGGC-3'), derived from the DHFR promoter. An arrowhead indicates Sp2 protein-DNA complexes, and -fold excesses of unlabeled oligonucleotides are indicated at the bottom.
within DHFR3 or DHFR4 increased Sp3-directed transcription 2-fold, whereas transcription of other mutants was essentially unchanged by Sp3. To extend these results, we analyzed a series of DHFR-derivatives that carry two or more consensus Sp2-binding sites at positions of regulation by Sp family members. As shown in Fig. 4C, 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. We 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.

Sp2 Is a Relatively Weak trans-Activator of the PSA Promoter in Prostate Epithelial Cells—To extend our studies of Sp2-mediated transcription, we wished to analyze its relative capacity to stimulate transcription in mammalian cells. We 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, we 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 we have shown that PSA is stimulated by a subset of Sp family members in prostatic epithelia (28, 40–42).

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 2 S. O. Simmons and J. M. Horowitz, manuscript in preparation.
trichostatin A (TSA) 24 h following transfection. Extracts were prepared for analysis following an additional 24 h of incubation. As shown in Fig. 5A, 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. 5A). 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. We 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, we 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 vitro. In addition, the capacities of each chimera to bind DNA in vitro were also determined. As shown schematically in Fig. 5B, 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. 5B, Sp2/1D). To confirm these results, we 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 vitro at levels comparable with those of Sp1 and Sp2 (Fig. 5C). We 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. 5E). Although each parental and chimeric protein was abundantly expressed and karyophilic, we 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 were 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. 5D). In marked contrast, extracts prepared from cells transfected with Sp2, Sp2/1D, or Sp2/1/2 led to negligible amounts of protein-DNA complexes.

Based on studies with chimeric Sp1/Sp2 proteins, we 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, we 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.

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. 5D 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. 6A, 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 bond by anti-Sp2 antisera 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 DHFR* probe. To detect Sp2-DNA complexes, we employed a commercially available anti-Sp2 peptide antibody derived from the amino terminus of Sp2 in “supershift” experiments. We also prepared an Sp2 antisera directed against the entirety of the Sp2 trans-activation domain and compared the capacity of this antisera to detect Sp2-DNA complexes with that of preimmune serum. As shown in Fig. 6B, 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 antisera 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 antisera (Fig. 6C and D, and data not shown). Thus, in striking contrast to Sp family members such as Sp1 and Sp3, we conclude from these results that Sp2 DNA binding activity is negligible in many human and mouse cell lines.

An Activity in Mammalian Cell Extracts Can Block Sp2 DNA Binding Activity in Vitro—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, we hypothesized that one or more cellular proteins may negatively regulate the capacity of Sp2 to bind DNA. To test this hypothesis, we 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. 7A). 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. 7B). As shown in Fig. 7B, recombinant Sp2 protein was not degraded following incubation with mammalian cell extracts. In sum, we 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.

An 84-kDa Mammalian Protein Specifically Binds the Sp2, but Not the Sp1 or Sp3, trans-Activation Domain via the Sp2 B Domain—Given that 1) Sp1/Sp2 chimera experiments implicated the Sp2 trans-activation domain as playing a role in the regulation of Sp2 DNA binding activity and 2) mixing experiments indicate that one or more proteins in mammalian cells regulate the association of Sp2 with DNA, we initiated a series of experiments to examine whether an endogenous mammalian protein specifically binds the Sp2 trans-activation domain.
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 into expression vectors. The synthesis of GST-Sp fusion proteins was induced in bacteria, bound to glutathione-agarose beads, and incubated with [35S]methionine-labeled extracts prepared from the indicated mammalian (HCT116, T98G, and HTLA230) or insect (SL2) cell lines, and bound proteins were resolved on acrylamide gels. GST-Sp fusion proteins, 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 [35S]methionine, loosely adherent proteins were removed by washing, and bound proteins were collected by boiling in SDS and resolved on polyacrylamide gels. As shown in Fig. 7A, each GST-Sp fusion protein bound a protein of ~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 ~100 kDa was noted sporadically in HCT116 cell extracts but not other mammalian extract analyzed (Fig. 7A, 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. 8B). We 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.

**DISCUSSION**

Sp family members play important roles in the regulation of a diverse set of biological processes, including differentiation, development, apoptosis, cell cycle progression, and oncogenesis. However, despite their importance, we currently possess only a superficial understanding of the functional attributes of most family members. Moreover, it is completely unclear which functions may be member-specific and which functions may be supplanted by those of another Sp family member. The studies...
reported herein were conducted to begin to address some of these issues by detailing the functional properties of Sp2, a poorly characterized Sp family member.

To initiate our studies, we employed recombinant human Sp2 protein and a PCR-assisted protocol to select a consensus Sp2-binding sequence from a pool of degenerate oligonucleotides. The consensus sequence we obtained, 5′-GGGGCGGGGAC-3′, differs from a sequence obtained previously for Sp1, and the breadth of sequences selected by Sp2 is considerably more restricted than that bound by Sp1. Sp2 binds its consensus sequence with high affinity (225 pm), whereas Sp1 and Sp3 bind this sequence with considerably lessened affinity, differing by as much as 40-fold. To compare the transcriptional properties of Sp2 with Sp1 and Sp3, we incorporated one or more consensus Sp2-binding sites into sites of Sp regulation within the DHFR promoter and transiently transfected these constructs with Sp expression vectors in insect cells. These studies revealed that Sp2 is a relatively weak *trans-activator in vivo* and does not synergistically *trans-activate* a promoter with multiple consensus Sp2-binding sites. Analogous results were obtained when our comparative transcriptional studies were extended to mammalian cells and a promoter derived from the human PSA gene. Moreover, using chimeric Sp1/Sp2 proteins, we showed that the Sp2 *trans-activation* and DNA-binding domains can independently down-regulate transcription when linked in *cis* with complementary portions of Sp1. Results from experiments with chimeric proteins indicate that Sp2 DNA binding activity is negatively regulated in mammalian cells, and a survey of 14 human and mouse cell lines confirmed this supposition. Finally, using an *in vitro* protein/protein binding assay we have detected a novel Sp2-specific binding protein whose physical interaction with the Sp2 B domain appears to be directly correlated with the negative regulation of Sp2 DNA binding activity and *trans-activation*. When taken together, our results indicate that Sp2 may differ in a number of important functional respects from Sp1 and Sp3 and suggest that Sp2 may play a more specialized role in cell physiology than other Sp family members.

Although Sp protein-DNA complexes have been analyzed exhaustively, to our knowledge, this report is the first to identify and characterize a consensus DNA-binding site using Sp proteins expressed in eukaryotic cells. Two previous binding site selection studies derived a consensus site for Sp1 using bacterially expressed partial proteins encoding the zinc finger sites of these Sp family members: a leucine at position 3 of the α-helix comprising Sp2 zinc finger I. This leucine is predicted to contact the adenine residue within the 5′-GAC-3′ triplet via hydrophobic interactions and must at least partially account for the divergence in the consensus sequences of Sp1 and Sp2 within zinc finger I. Interestingly, our results also indicate that preferred nucleotides can vary between Sp family members despite complete conservation of amino acids predicted to contact DNA. For example, the consensus nucleotides specified by zinc finger II of Sp1 and Sp2 differ, yet these proteins share identical amino acids at key positions for base contacts. We presume that such differences in nucleotide preference must arise due to interface interactions or to nearby divergent amino acids that interact with amino acids at these key positions. For example, a single amino acid difference between Sp1 (lysine) and Sp2 (alanine) exists at position 8 of the α-helix that defines zinc finger II. This same amino acid position is arginine in Sp3 and Sp4 and leucine in Sp5, perhaps suggesting that additional differences in the DNA binding specificities of Sp-family members can be expected for zinc finger II. Speculation that amino acids neighboring zinc finger contact residues can alter DNA binding specificity has been noted previously (43–46).

Yet another difference between the consensus sequences defined for Sp1 and Sp2 is the conservation of nucleotides flanking the Sp2-binding site. This conservation of sequence is particularly striking 3′ of the Sp2-binding site, where 70–90% of sequenced clones carried common nucleotides at three positions. These results suggest that high affinity Sp2-binding sites may occur within a specific context of neighboring nucleotides. In contrast to these results, two independent analyses of partial Sp1 proteins showed little or no conservation of sequence outside of the nonameric core (36, 37). Whether this diversity is specified by the Sp2 DNA-binding domain or is a consequence of our use of full-length protein for binding site selection studies remains to be determined. It is worth pointing out that although the Sp2 consensus sequence differs from those previously derived for Sp/XKLF family members, similar to the consensus sequences of zinc finger proteins, such as NGFI-A, NGFI-C, Zif268, and Egr3, the consensus Sp2-binding site is flanked by thymidine residues (35, 39, 47).

To our knowledge, this report is also the first to compare the affinities of multiple Sp family members for a consensus Sp-binding site and to relate these data to the relative transcriptional activities of these proteins *in vivo*. Kinetic measurements indicate that Sp1 and Sp3 bind the Sp2 consensus site up to 40-fold less avidly than Sp2. These data support the contention that Sp family members have significantly different DNA-binding specificities, at least *in vitro*, and argue that relatively small differences in the amino acid sequences of their DNA-binding domains determine their affinity for a given binding site. For example, despite the conservation of 89% of the amino acids comprising the Sp1 and Sp3 DNA-binding domains, our data indicate that there is a 13-fold difference in the affinity of Sp1 and Sp3 for the Sp2 consensus DNA-binding site. None of these amino acid differences involve residues that are predicted to contact DNA; however, 2 amino acid differences between Sp1 and Sp3 map to α-helices of zinc finger II (position 8) and zinc finger III (position 5), and single amino acid differences also exist within the β-sheet sheets upstream of these fingers. We presume that such amino acid differences account for the differing affinities of Sp1 and Sp3 for the Sp2 consensus site. This said, it is not at all obvious that the differences in DNA affinity that we have noted *in vitro* translate to significant differences in transcriptional activity *in vivo*. 
Pp2-mediated Transcription

Incorporation of Sp2-binding sites within the DHFR promoter affected Sp1- and Sp3-directed transcription similarly despite the 13-fold difference in their affinity for such sites. At least two possible explanations may account for these results. First, the conditions under which affinity measurements were derived in vitro may not closely mimic those encountered in the intracellular milieu. Perhaps the topological constraints of chromatin or the “anchoring” of Sp proteins to DNA by nearby trans-acting factors reduce differences in DNA affinity that are accentuated when oligonucleotides and recombinant proteins are assayed in vitro. Second, differences in DNA affinity may only have a modest impact on overall levels of Sp-mediated transcription. Instead, Sp-dependent gene expression may rely more heavily on differences specified by the trans-activation domains of Sp proteins or combinatorial interactions with promoter-specific transcription factors. Indeed, results obtained in insect and mammalian cells are entirely consistent with the proposition that the trans-activation domains of Sp proteins are functionally distinct. For example, incorporation of four consensus Sp2-binding sites within the DHFR promoter resulted in only a modest (2–3-fold) increase in Sp2-mediated transcription in insect cells, and this level of trans-activation remains 10–20-fold below that directed by Sp1 and Sp3. These results are consistent with conclusions from previous studies of Sp2-mediated transcription of several TATA-less and TATA-containing promoters (11–14). However, results that we have obtained with chimeric Sp1/Sp2 proteins are perhaps even more compelling. These studies indicate that the Sp2 trans-activation domain carried by chimeras such as Sp2/1 and Sp2/1D negatively regulates transcription when linked to the Sp1 DNA-binding domain. Interestingly, the mechanism by which the Sp2 trans-activation domain negatively regulates transcription appears to involve the inhibition of DNA binding activity. This is a novel finding and one that is entirely consistent with our further observation that many human and mouse cell lines exhibit negligible amounts of Sp2 DNA binding activity. How might the Sp2 trans-activation domain limit the formation of Sp2-DNA complexes? Although we can only speculate on this issue, results from protein/protein binding assays indicate that the Sp2 trans-activation domain is bound by a novel 84-kDa protein (p84) that does not interact with analogous portions of Sp1 and Sp3. One mechanistic possibility is that the binding of Sp2 by p84 may sterically hinder the association of Sp2 and Sp1/Sp2 chimeras, such as Sp2/1 and Sp2/1D, with DNA or otherwise distort the DNA-binding domain such that it cannot recognize DNA. Alternatively, p84 might modify Sp2, Sp2/1, and Sp2/1D such that their DNA binding activity is minimized. It is worth noting that Sp2 DNA binding activity appears to require phosphorylation, and thus one might speculate further that p84 may have phosphatase activity. Should this be the case, p84 might be akin to phosphatases such as calcineurin, PRL-1, and protein phosphatase 1 that associate with specific transcription factors and regulate their activity (48–52). If so, we would predict that Sp2 exists in most if not all mammalian cells in an inactive, p84-associated form. Presumably, stimulation of one or more signal transduction pathways would liberate Sp2 from p84, leading to activation of downstream target genes.

Studies with Sp1/Sp2 chimeras have also revealed that the Sp2 DNA-binding domain can negatively regulate transcription when linked to portions of Sp1. Chimeras such as Sp1/2 and Sp1/2/1 are clearly stable, karyophilic proteins capable of binding DNA, yet like Sp2, each only marginally stimulates transcription in vivo. How might the Sp2 DNA-binding domain limit trans-activation activity? Again, although we can only speculate on this issue, one possibility is that the Sp2 DNA-binding domain attracts one or more co-repressors to protein-DNA complexes. The DNA-binding domain of Sp2 is the most divergent among Sp family members (75% homology), and thus it is conceivable that it encodes binding sites for one or more co-repressors that are not carried by proteins such as Sp1 and Sp3. Should this be the case, such putative co-repressors are unlikely to be histone deacetylases, since treatment with TSA does not significantly stimulate Sp2-mediated transcription. Why are the trans-activation and DNA-binding domains of Sp2 negatively regulated by two presumably independent mechanisms? One strong possibility is that the role of Sp2 in cell physiology may be significantly different than other Sp-family members. A more specialized subset of signal transduction pathways may converge on Sp2 than proteins such as Sp1 or Sp3, and until triggered Sp2 is maintained in a latent, tightly regulated form. Given its novel consensus binding sequence and relatively restricted set of DNA-binding specificities, it is also conceivable that Sp2 may regulate a discrete set of target genes that are not serviced by other Sp family members. The identification of such target genes may prove to be quite challenging given the widespread mechanisms that negatively regulate Sp2 DNA binding activity and trans-activation in mammalian cells.

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