

Sp3 and Sp4 Can Repress Transcription by Competing with Sp1 for the Core *cis*-Elements on the Human *ADH5/FDH* Minimal Promoter*

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The human alcohol dehydrogenase 5 gene (also known as the formaldehyde dehydrogenase gene, *ADH5/FDH*) has a GC-rich promoter with many sites at which transcription factors bind. A minimal promoter extending from –34 base pairs (bp) to +61 bp directs high levels of transcription in several different cells, consistent with the ubiquitous expression of the gene. Nearly the entire minimal promoter can be bound by Sp1. We analyzed the transcriptional regulation of *ADH5/FDH* by members of the Sp1 multigene family. Two core *cis*-elements (–22 bp to +22 bp) had the highest affinity for Sp1. Mutagenesis revealed that these *cis*-elements are critical for transcriptional activation. The zinc-finger domains of Sp3 and Sp4 also bind selectively to the core *cis*-elements. In *Drosophila* SL2 cells, which lack endogenous Sp1, the minimal promoter cannot drive transcription. Introduction of Sp1 activated transcription over 50-fold, suggesting that Sp1 is critical in the initiation of transcription. Neither Sp3 nor Sp4 was able to activate transcription in those cells, and transcriptional activation by Sp1 was repressed by Sp3 or Sp4. These data suggest that Sp3 and Sp4 can repress transcription by competing with Sp1 for binding to the core *cis*-elements. The content of Sp1, Sp3, and Sp4 in different cells may be critical factors regulating transcription of the *ADH5/FDH* gene.

The regulated transcription of a typical eukaryotic gene is governed by the combined action of multiple sequence-specific DNA-binding proteins (1, 2). The information provided by these proteins is ultimately communicated to RNA polymerase II, resulting in a precise transcription initiation frequency.

Sp1 is a well characterized sequence-specific DNA-binding protein that plays a role in the transcription of many cellular and viral genes that contain GC boxes (KRGGMGKRRY) in their promoters (3, 4). This includes numerous housekeeping genes, with high G+C content in their promoters (5). Additional human and rodent transcription factors (Sp2, Sp3, Sp4) similar in structural and transcriptional properties to Sp1 have been cloned, and form an Sp1 multigene family (6–9). Sp1, Sp3, and Sp4 have a highly conserved zinc-finger DNA binding domain close to the C terminus and contain glutamine- and serine/threonine-rich amino acid stretches in the N-terminal region (10). Sp1, Sp3, and Sp4 can bind to the same recognition

sequence (GC boxes) with identical affinity (6). Sp1 and Sp4 generally act as transcriptional activators, while Sp3 generally acts as a repressor, and rarely as an activator (10–16). Sp2 has a DNA-binding specificity different from that of Sp1, Sp3, and Sp4. The Sp1 multigene family is an important regulator of the cell cycle, differentiation, and development (9, 15–17).

We previously cloned and characterized the *ADH5/FDH*¹ gene (18, 19), which encodes the human class III χ -alcohol dehydrogenase (ADH, EC 1.1.1.1) that is also the NAD⁺/glutathione-dependent formaldehyde dehydrogenase (FDH, EC 1.2.1.1) (20). This gene is believed to be the ancestral ADH gene (21). It is important in the oxidation of various alcohols and of formaldehyde (in the presence of glutathione) (see Refs. 19 and 22–24, and references therein). *ADH5/FDH* is ubiquitously expressed, although to different levels in different cells (22, 23). The *ADH5/FDH* promoter is very different from those of other *ADH* genes, which are expressed in different tissue-specific patterns. It is very rich in GC base pairs (73% up to –200 bp; 82% in the minimal promoter) and does not contain either a TATA or CCAAT box (18). It thus has the characteristics of a housekeeping gene (25, 26). The regulation of housekeeping genes is poorly understood.

The very small DNA fragment extending to –34 bp of *ADH5/FDH* is a strong promoter in all cells tested (19). Promoter elements from –38 bp to +22 bp, flanking the transcriptional start site, are footprinted by all nuclear extracts tested, and nearly the entire minimal promoter can be bound by Sp1 (Fig. 1) (19). This is an unusual configuration of GC boxes flanking the transcription start site. Although this may lead one to expect a simple promoter, the *ADH5* promoter is surprisingly complex and the regulation of this ubiquitously expressed gene is quite complicated (19).

Here we test whether the members of the Sp1 multigene family recognize the same *cis*-acting elements on the minimal promoter, and examine the roles they play in transcriptional regulation. Analyses of DNA-protein interactions *in vitro* and *in vivo* demonstrate that the members of this multigene family compete for binding, with different effects upon transcription. These data in part explain the ubiquitous expression of the gene and how different levels of expression are achieved in different tissues. This may have significant implications in understanding how many housekeeping genes can be regulated (19, 22, 23).

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¹ The abbreviations used are: *ADH5/FDH*, alcohol dehydrogenase 5/formaldehyde dehydrogenase; Ab, antibody; bp, base pair(s); CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; fpu, footprinting unit(s); RLU, relative light unit(s); SL2, *Drosophila* Schneider line 2 cells; ZFD, zinc-finger DNA binding domain.

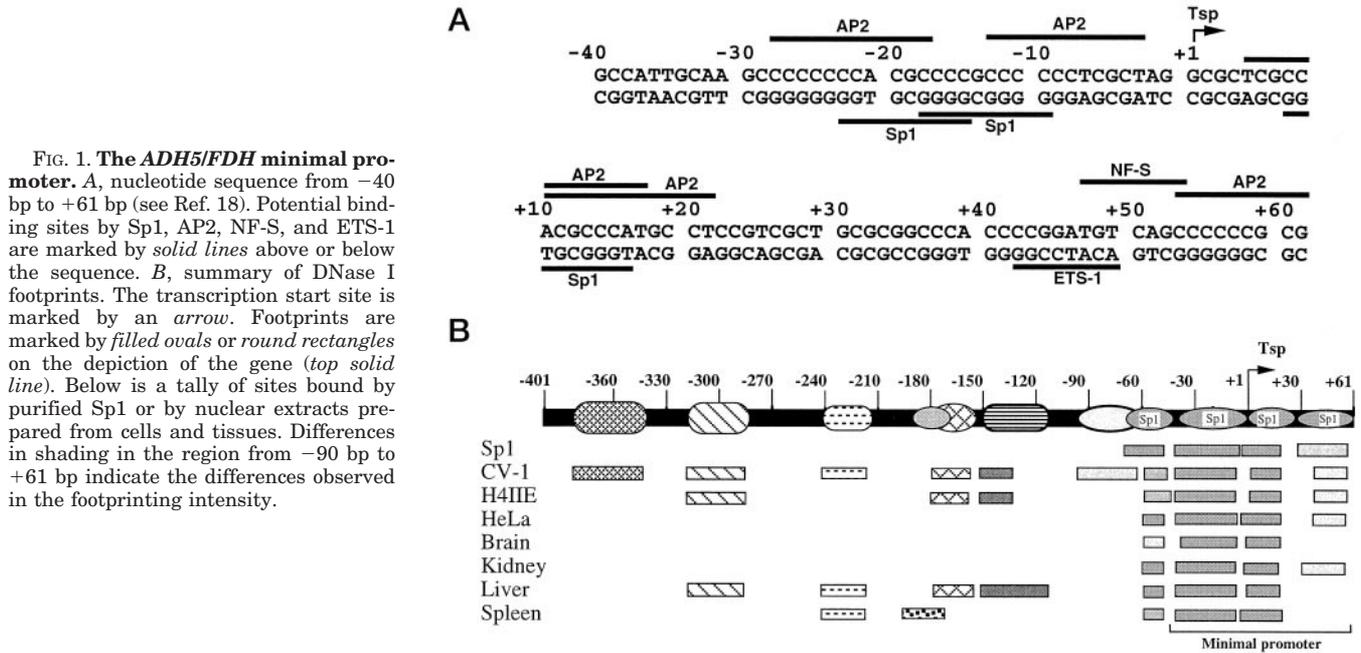


FIG. 1. The *ADH5/FDH* minimal promoter. A, nucleotide sequence from -40 bp to +61 bp (see Ref. 18). Potential binding sites by Sp1, AP2, NF-S, and ETS-1 are marked by *solid lines* above or below the sequence. B, summary of DNase I footprints. The transcription start site is marked by an *arrow*. Footprints are marked by *filled ovals* or *round rectangles* on the depiction of the gene (*top solid line*). Below is a tally of sites bound by purified Sp1 or by nuclear extracts prepared from cells and tissues. Differences in shading in the region from -90 bp to +61 bp indicate the differences observed in the footprinting intensity.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pGL luciferase, pCAT Control and pCAT Basic were purchased from Promega (Madison, WI). pCAT Control contains the SV40 promoter, enhancer, and CAT coding sequence. pCAT Basic does not have an eukaryotic promoter and enhancer. pGL luciferase contains a firefly luciferase gene driven by a cytomegalovirus (CMV) promoter in a vector called pCDNA I (Invitrogen, San Diego, CA). pCAT 5-2, which contains the *ADH5/FDH* minimal promoter (-34 bp to +61 bp) in front of the *CAT* coding region in pCAT Basic, and pCAT AX (-64 bp to +61 bp) are described elsewhere (19). pCMVSp1 and pPacSp1 were generous gifts from Dr. Robert Tjian (27). pCMVSp3, pCMVSp4, pPacSp3, and pPacSp4 were kindly provided by Dr. Guntram Suske and Dr. Luigi Lania (10, 13, 14). pRatSp1 and BTEB was kindly provided by Dr. Fujii-Kuriyama (7). pCMVSP2 was provided by Dr. Trevor Williams (28). The pPac vector used to construct pPac luciferase contains the *Drosophila* actin promoter, and was kindly provided by Dr. Carl Thummel (University of Utah).

Nuclear Extracts and Transcription Factors—Nuclear extracts from cultured HeLa cells were made according to Shapiro *et al.* (29). HeLa cell nuclear extract and the purified transcription factors Sp1 and AP2 were purchased from Promega. Antibodies to Sp1 (PEP2), Sp3, Sp4, and AP2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody to the Ku antigen was kindly provided by Dr. Westly Reeves (University of North Carolina). Flagtag antibody was purchased from Eastman Kodak Corp. Zinc-finger DNA binding domains (ZFDs) of Sp3 (from amino acid 501 to amino acid 697) and Sp4 (from amino acid 616 to amino acid 784) were prepared by subcloning polymerase chain reaction products into pCITE vector (Novagen, Madison, WI) or pT7-7 vector (obtained from Dr. Stan Tabor, Harvard Medical School). Each DNA binding domain was tagged with Flag peptide (Kodak) at the C terminus. Primers used to amplify ZFD-Sp3 are CATATGGGGGAC-CAACAACATCAAGAAGGA (5' forward) and GGGATCCTCACTTGT-CATCGTCGTCCTTGTAGTCCATTCCTCATTCCAGAAA (3' forward). Primers used to amplify ZFD-Sp4 are CATATGCCTGGCAA-GAGGCTTCAAGAGTT (5' forward) and GGGATCCTCACTTGT-CATCGTCGTCCTTGTAGTCCGAAATTCCTCATGTTGGTTGAAC (3' forward). The plasmids were transcribed and translated *in vitro* to produce functional zinc-finger DNA binding domains using the Single Tube System 2 (Novagen, Madison, WI). The resulting proteins were used for electrophoretic mobility shift assays.

DNase I Footprinting Assays—To examine DNA-protein interactions in the minimal promoter region, pCAT AX plasmid (19) was digested with *HindIII* and filled in with the Klenow fragment in the presence of [α - 32 P]dCTP. The labeled DNA was further digested with *XbaI* (a restriction site in the polylinker), and the labeled restriction fragment was purified by electrophoresis in a 4% non-denaturing polyacrylamide gel. For DNase I footprinting assays from the opposite end of the fragment, the order of digestion was reversed so that the *XbaI* site was

labeled. DNase I digestion and electrophoresis were as described previously (19). Purified Sp1 transcription factor (0.1–2 fpu), probe (40,000 cpm), and poly(dI-dC) (1 μ g) were used in each DNase I digestion reaction.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were carried out as described previously (19). Sequences of various oligonucleotides are as follows (only the top strand is shown): site A oligonucleotide (+40 bp to +60 bp): GATCCCCCGGATGTCAGCCCCCGGCC; site B oligonucleotide (+1 bp to +22 bp): GATCGCGCTCGCCACGCCAT-GCCTC; site C-1 oligonucleotide (-21 bp to 3 bp): GATCACGCCCCG-CCCCCTCGCT; site C-2 oligonucleotide (-38 bp to -22 bp): GAAT-TCATGCAAGCCCCCCC; site C-3 oligonucleotide (-29 bp to -15 bp): GAATCCCCCCCCACGCCCC. Each binding reaction was carried out in 20 μ l, and contained 10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 7% glycerol, and appropriate nuclear extract or protein (0.1–2 footprint units of Sp1, 6–36 μ g of HeLa cell nuclear extract). Where indicated, excess unlabeled competitor oligonucleotide (20–200-fold excess) and antibodies (1 μ g each) against Sp1, Sp3, Sp4, or Flag peptide was added to the binding mixture.

Transient Expression Assays in Mammalian Tissue Culture Cells—Alexander cells (human hepatoma), were grown on 10-cm dishes in minimal essential medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum. When they reached 50–60% confluence, the medium was replaced. Four hours later, CaCl_2 -DNA coprecipitates (30) were added: 6 μ g of pCAT 5-2, 3 μ g of pCMVSp1, Sp3, or Sp4, and an appropriate amount of pUC18 DNA to total 16 μ g of DNA were added to each dish. The DNA was allowed to remain on the cells for 4 h, after which the medium was removed, medium containing 20% glycerol was added for 2 min, and fresh growth medium was added. Cells were incubated for 48 h. After the plates were gently washed four times with cold phosphate-buffered saline, the cells were harvested and cell pellets were resuspended in 150 μ l of lysis buffer (100 mM KPO_4 , pH 7.8, 1 mM dithiothreitol). Cells were broken by three cycles of freezing and thawing.

We did not use reporter plasmids as internal controls because their promoters contain binding sites for Sp1 and related transcription factors. Therefore, the introduction of Sp1, Sp3, and Sp4 expression vectors into cells would also affect the internal control. Instead, we used a fixed amount of protein extract for each CAT assay. CAT assays were conducted by incubating cell extracts containing 40 μ g of protein in 200 μ l of a reaction mixture containing 0.25 M Tris-HCl (pH 7.8), 50 mM acetyl CoA, 100 nCi of [14 C]chloramphenicol, 5 mM EDTA at 37 $^\circ\text{C}$ for 4 h. The acetylation of chloramphenicol was analyzed by silica gel thin-layer chromatography and quantitated with a Fuji Phosphoimage Analyzer (Tokyo, Japan). CAT activity was expressed as relative CAT activity compared with the control (pCAT 5-2 only), and was the average of six independent experiments.

Transient Expression Assays in Schneider's *Drosophila* Line 2 Cells

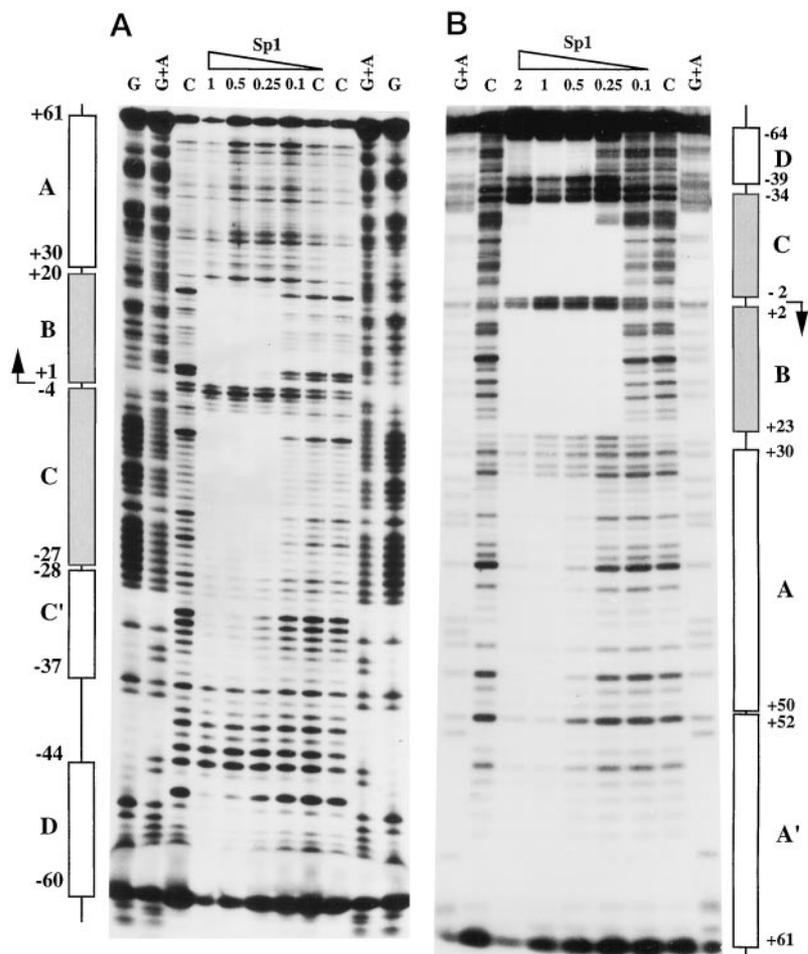


FIG. 2. DNase I footprinting with increasing amounts of Sp1. Sp1, ranging from 0.1 to 2 fpu, was added to the DNase I footprinting reactions. A, antisense strand; B, sense strand. G and G+A indicate Maxam and Gilbert sequencing reactions of the DNA fragment. C, control reaction without Sp1. The transcription start site is marked by an arrow.

(SL2)—SL2 cells were grown on 10-cm dishes in Schneider cell culture medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. When they reached 50–60% confluence, the cells were transfected. To investigate the role of Sp1, Sp3, and Sp4 on the minimal promoter, 12 μ g of pCAT 5–2, 3 μ g of pPac luciferase, varying amounts of Sp1, Sp2, and Sp3 expression vector (1–9 μ g of pPacSp1, pPacSp3, and pPacSp4) and appropriate amounts of pUC18 DNA to fill the total DNA amount to 31 μ g were transfected into the cells using the CaCl₂-DNA coprecipitation method (30). The pPac vector contains the *Drosophila* actin promoter and was not affected by the addition of Sp1 and related factors. The DNA precipitate was allowed to remain on the cells for 48 h, and then the medium was removed. After the plates were gently washed four times with cold phosphate-buffered saline, the cells were harvested and cell pellets were resuspended in 100 μ l of 1 \times reporter lysis buffer (Promega, WI). The cellular extract (10 or 20 μ l) was assayed for luciferase activity (31) to normalize plate to plate variation in transfection efficiency. Cell extracts representing 200,000 RLU of luciferase activity were then used for CAT assays. CAT assays were conducted by incubating cell extracts in 174 μ l of reaction mixture containing 0.25 M Tris-HCl (pH 7.8), 2.2 mM acetyl CoA, 100 nCi of [¹⁴C]chloramphenicol, 5 mM EDTA at 37 °C for 8 h. The acetylation of chloramphenicol was analyzed as described above. CAT activity was expressed as relative CAT activity compared with the control and was the average of three or five independent experiments. To investigate the interaction among the Sp1 multigene family, cells were transfected with 12 μ g of pCAT 5–2, 1 μ g of pPac luciferase, 1 μ g of pPacSp1, pPacSp3, or pPacSp4 (3–9 μ g) and appropriate amounts of pUC18 DNA to total 31 μ g of DNA, using the CaCl₂-DNA coprecipitation method as above. Extracts representing 67,000 RLU were used for CAT assays.

Site-directed Mutagenesis of pCAT 5–2—To investigate the role of the *cis*-elements immediately flanking the transcription start site, mutations were introduced into the Sp1 consensus sequences using a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). pCAT 5–2 M1 (CC at –12/–13 bp to AA), pCAT 5–2 M2 (CC at +13/+14 bp to AA), pCAT 5–2 M3 (CC at –12/–13 and at +13/+14 bp to AA), pCAT 5–2 M4 (CC at –12/–13 and –17/–18 bp to AA), and pCAT 5–2 M5 (CC

at –12/–13, –17/–18, and +13/+14 bp to AA) were prepared and used for transient expression assays in *Drosophila* SL2 cells. Oligonucleotides used in mutagenesis were as follows: M1: CCCCCACGC-CCCGAACCCCTCGCTAGGCG and CGCCTAGCGAGGGGTTCTGGGG-CGTGGGGGG; M2: GGCGCTCGCCACGAACATGCCTCCGTCGC and GCGACGGAGGCATGTTTCGTGGCGAGCGCC; M3: oligonucleotides used to prepare M1 and M2; M4: AAGCCCCCCCACGAACCCGAAACCCTCGCT and AGCGAGGGGTTCTGGGGGGGCTT; M5: oligonucleotides used to prepare M2 and M4.

Transient Expression Analysis of pCAT 5–2 Mutants—To investigate the importance of the core *cis*-elements in transcriptional regulation, 12 μ g each of pCAT 5–2, pCAT 5–2 M1, pCAT 5–2 M2, pCAT 5–2 M3, pCAT 5–2 M4, and pCAT 5–2 M5 were separately cotransfected into *Drosophila* SL2 cells with a mixture of the following plasmids: 1 μ g of pPac luciferase, 1 μ g of pPacSp1, and appropriate amount of pUC18 (to total 31 μ g of DNA). Transfection and analysis of the reporter gene activities were carried out as described above. CAT activity was expressed as relative CAT activity compared with the control and was the average of four independent experiments.

RESULTS

Sp1 Binding to the ADH5/FDH Minimal Promoter—We demonstrated that the fragment from –34 bp to +61 bp was able to promote transcription in all tissue culture cell lines tested (19), and defined it as an *ADH5/FDH* minimal promoter. The minimal promoter of the human *ADH5/FDH* is GC-rich (82%) and contains several consensus binding sequences for Sp1 and for AP2 protein (Fig. 1A; Refs. 18 and 19). Two Sp1 sites immediately flank the transcription start site. The region from –40 bp to +22 bp was footprinted by all nuclear extracts (19).

We characterized the minimal promoter by DNase I footprinting analysis using various amounts of Sp1 (Fig. 2). As shown in Fig. 2, Sp1 can bind to virtually all regions of the

ADH5/FDH minimal promoter, and has a very high affinity for the core *cis*-elements (boxes B and C) immediately flanking the transcription site. Sites B (+1 bp to +23 bp) and C (−4 bp to −27 bp), which immediately flank the transcription start site, had the highest binding affinity for Sp1: they began to show footprints with 0.1 fpu (Fig. 2). Site A could be bound by Sp1 at 0.5 fpu (Fig. 2B). Site D (−39 bp to −64 bp) was upstream of the minimal promoter, and was footprinted when Sp1 was above 0.25 fpu. Thus, even though Sp1 could bind to the entire minimal promoter, the affinity of the different *cis*-elements to Sp1 varied by as much as 5–10-fold. Considering their high affinity to Sp1 and locations relative to the transcription start point, the core *cis*-elements may be the most important *cis*-elements regulating transcription.

Sp1, Sp3, and Sp4 Regulate ADH5/FDH Minimal Promoter Activity in Mammalian Cells—Because the members of the Sp1 multigene family share the same binding consensus sequence (GC box), they may play important roles in the transcriptional regulation by directly interacting with the core *cis*-elements (Fig. 2, sites B and C). Thus, we tested whether the members of the Sp1 multigene family can regulate the *ADH5/FDH* gene through the minimal promoter by cotransfecting the minimal promoter-CAT fusion construct (pCAT 5–2) and the Sp1, Sp3, Sp4, and rat Sp1 expression vectors into human Alexander cells. Sp1 did not alter the promoter activity (Fig. 3), probably because there was already enough endogenous Sp1 in the cells. However, rat Sp1 significantly activated transcription. Sp3 repressed the promoter activity by 40%. Surprisingly, Sp4, a known transcription activator (16, 30), also repressed transcription by 45%. The data indicated that members of the Sp1 multigene family can regulate the transcription of the *ADH5/FDH* gene by interacting with the minimal promoter.

Proteins Interacting with the Minimal Promoter in HeLa Cell Nuclear Extract—We carried out a series of EMSAs to determine which transcription factors in a HeLa cell nuclear extract were interacting with the *ADH5/FDH* minimal promoter. We specifically tested whether Sp1, Sp3, and Sp4 can indeed interact with the *cis*-elements on the minimal promoter to give the transient expression assay data described above (Fig. 3).

Sp1 bound strongly to probe A (+40 bp to +61 bp), and the band was supershifted by the Sp1 antibody (Fig. 4A, lane 2 and 3). HeLa nuclear extract gave two main retarded bands (labeled *Sp1* and *Ku*), that could be competed by excess cold probe (lanes 9–11). One complex could be supershifted by the antibody against Sp1 (band *Sp1*, lanes 4 and 5), indicating that Sp1 in HeLa nuclear extract can interact with probe A. We suspected that the protein which produced the major fast-moving complex that was observed in all gel mobility-shift assays may be the Ku antigen, a DNA-binding subunit of the DNA-dependent protein kinase complex. Indeed, the major fast-moving complex (labeled *Ku*) was clearly supershifted by the Ku antibody (Fig. 4E) (32, 33). Ku is known to bind to the ends of DNA (34). We will not further discuss this band in the present report.

Sp1 could also bind to probe B (+1 bp to +22 bp) (Fig. 4B). A similar complex was detected in the HeLa extract, and most of this band was supershifted by the Sp1 antibody (Fig. 4B). Antibodies against Sp3 and Sp4 did not shift much of the Sp1-containing band (Fig. 4B, lanes 6–9), although some material was apparently shifted to the wells when larger amounts of extract were tested. Following a much longer exposure of the gel, very faint bands in lanes 7 and 9 appeared in a position similar to the supershifted Sp1-probe-antibody complex, which may be the Sp3- or Sp4-probe-antibody complex (data not shown). Probe B could be bound more strongly by Sp1 than was probe A, judging by the relative intensities of the Sp1 and Ku

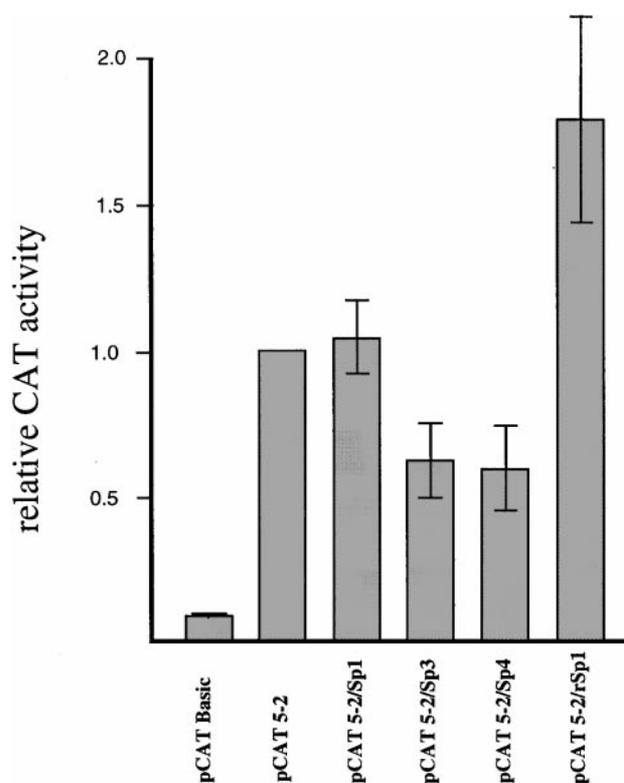


FIG. 3. Effects of Sp1, Sp3, and Sp4 on transcription in Alexander cells (of human liver origin). Six μ g of pCAT 5–2 was cotransfected with 3 μ g of various expression vectors encoding mammalian transcription factors, all driven by the CMV promoter (pCMVSp1, pCMVSp3, pCMVSp4, pCMVratSp1). pCAT Basic contained neither promoter nor enhancer, and served as a negative control. Forty μ g of total cellular protein was used for each CAT assay. The reporter activities are presented as relative CAT activity, the ratio of CAT activity of each transfection compared with that of pCAT 5–2 without cotransfected transcription factors, and are the average of six independent assays. Bars represent standard deviations.

bands (Fig. 4, B and E).

Based on DNase I footprint analysis with Sp1, we divided the upstream footprinted region (−37 bp to −2 bp: footprints C + C' in Fig. 2A, and C in Fig. 2B) into three partially overlapping probes, C-1, C-2, and C-3. Sp1 (and the Ku antigen) were the major proteins interacting with these probes (Fig. 4, C and D). Probes C-1 (−22 bp to −3 bp) and C-3 (−29 bp to −15 bp) bound strongly to Sp1, while probe C-2 (−38 bp to −21 bp) bound more weakly. We suspect that the presence of two closely positioned Sp1 binding elements enhanced the interaction between Sp1 and probe C-1.

Probes B and C-1, the *cis*-elements immediately flanking the transcription start site, bound most strongly to Sp1 (Fig. 4E). Despite the consensus AP2 sites noted above, EMSA did not give any indication for AP2 binding; none of the retarded bands formed by the various probes and HeLa extract was able to be supershifted by the AP2 antibody (data not shown).

Zinc-finger DNA Binding Domains of Sp3 and Sp4 Can Bind Specifically to the Core cis-Elements—Because Sp3 and Sp4 may be present in low levels in HeLa cells (10), and the size of the retarded DNA-protein complex or DNA-protein-antibody complex overlap other bands, we were not able in the experiments shown in Fig. 4 to clearly demonstrate that Sp3 or Sp4 can bind to the minimal promoter. To investigate the possible interaction between Sp3 and Sp4 and various *cis*-elements, we prepared, by *in vitro* transcription and translation, shorter versions of the transcription factors that contain only the zinc-finger DNA binding domains tagged with Flag peptide (named ZFD-Sp3 and ZFD-Sp4). ZFD-Sp3 and ZFD-Sp4 were able to

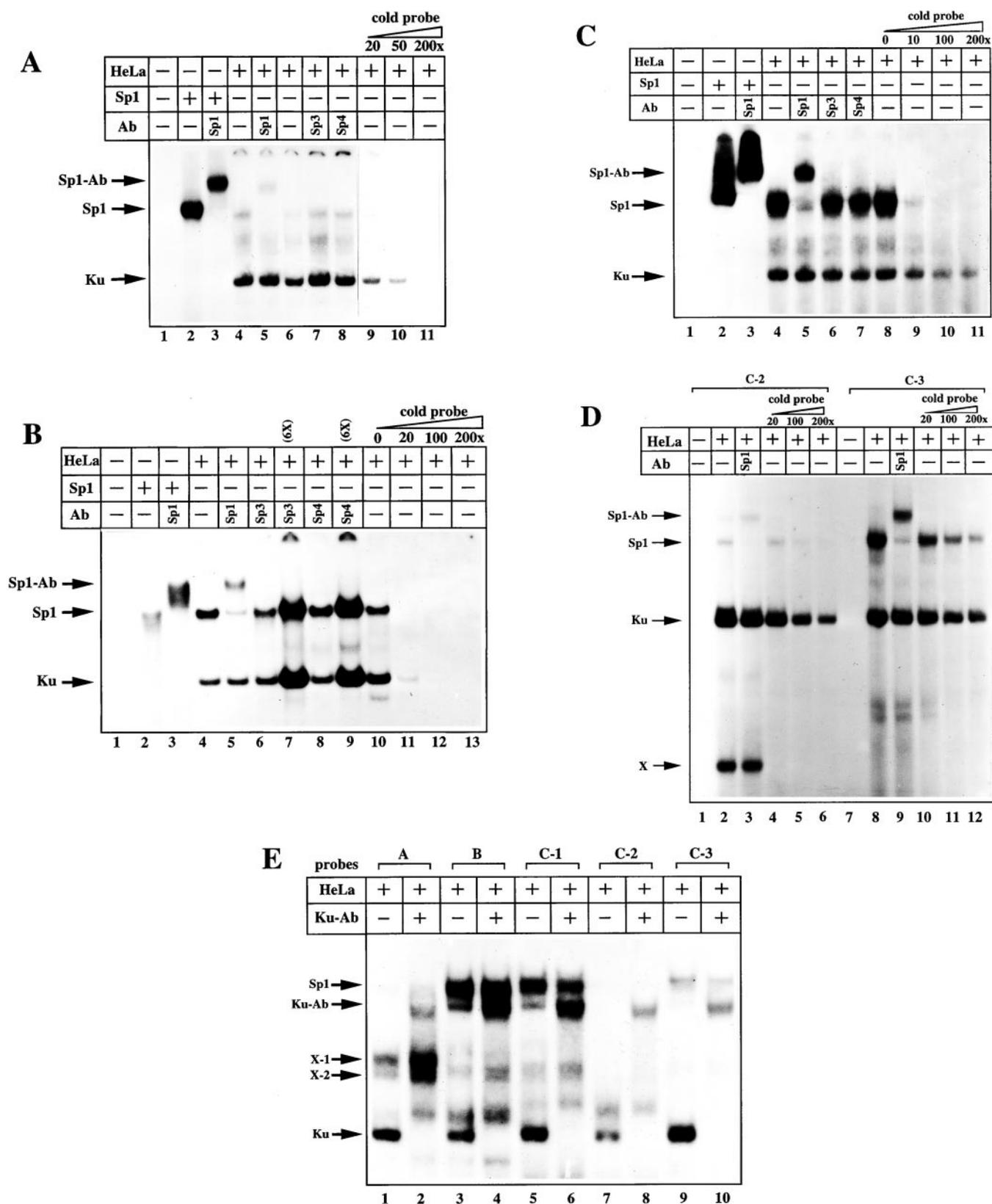


FIG. 4. EMSA with purified Sp1 and HeLa nuclear extract. One footprint unit of Sp1 or 6 to 36 μ g of HeLa nuclear extract was used. A, probe A (+40 bp to +60 bp). B, probe B (+1 bp to +22 bp). C, probe C-1 (-22 bp to -3 bp). D, probes C-2 (-38 bp to -22 bp) and C-3 (-29 bp to -15 bp). E, supershifts with antibody to Ku. Sp1-Ab band, Sp1-probe- α Sp1 Ab complex; Sp1 band, Sp1-probe complex; Ku band, Ku-probe complex; Ku-Ab band, Ku-probe- α Ku Ab complex; X band, C-1 probe-unknown protein complex; X1, X2 bands, probe-unknown protein complex enhanced by addition of Ku antibody.

bind selectively to the probe B and C-1, the two core *cis*-elements immediately flanking the transcription start site, as demonstrated in the EMSA shown in Fig. 5 (A and B, lanes 2

and 5). The retarded bands containing ZFD-Sp3 and ZFD-Sp4 were either supershifted or lost by the addition of antibodies against Sp3 or Sp4 (Fig. 5, A and B, lanes 3 and 6). Adding the

FIG. 5. Binding of Sp3 and Sp4 to the core cis-elements. A, EMSA with probe B (+1 bp to +22 bp) and ZFD-Sp3 and ZFD-Sp4. B, EMSA with probe C-1 (-22 bp to -3 bp) and ZFD-Sp3 and ZFD-Sp4. *In vitro* synthesized ZFDs of Sp3 or Sp4, tagged with Flag peptide, were used in the binding reactions. In the control lanes (lane 1 of A and B), rabbit reticulocyte extract with the *in vitro* synthesized product of the *lacZ* gene was used instead. Where noted, 1 μ g of antibodies against Sp3, Sp4, or Flag peptide was added (lanes 3, 4, 6, and 7). C, control (lane 1); ZFD-Ab, supershifted probe-ZFD-antibody complex; NS, nonspecific probe-protein complex; ZFD-Sp3, ZFD-Sp3-probe complex; ZFD-Sp4, ZFD-Sp4-probe complex.

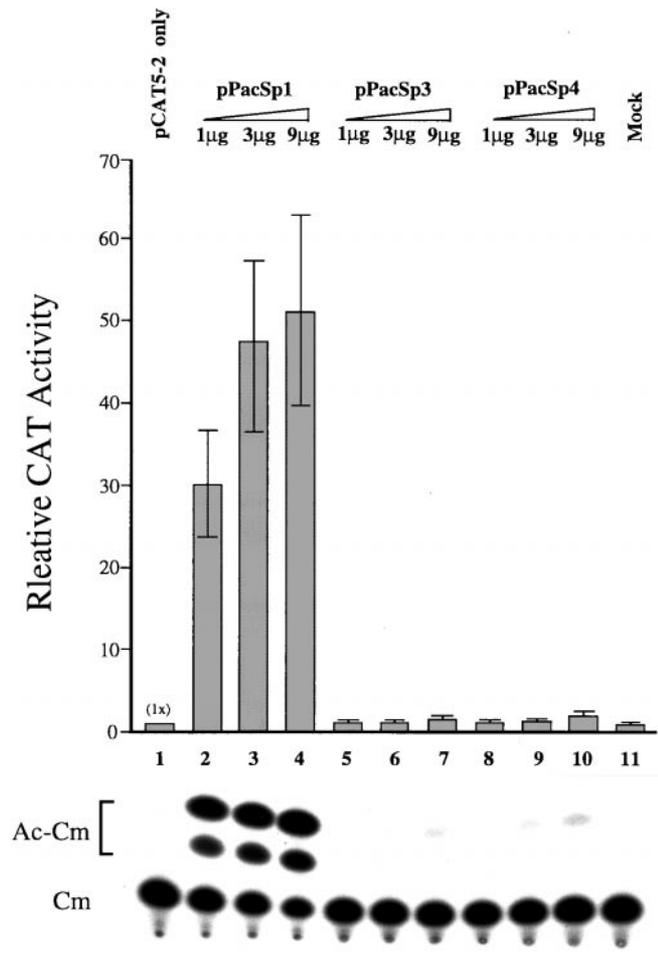
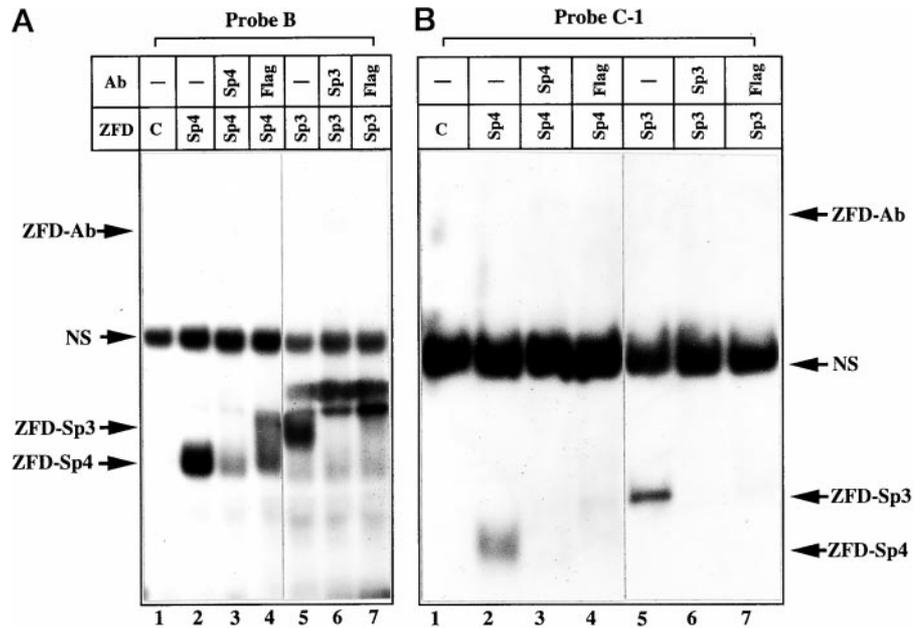


FIG. 6. Effects of Sp1, Sp3, and Sp4 on transcription in *Drosophila* SL2 cells. Twelve μ g of pCAT 5-2, 3 μ g of pPac luciferase, and varying amounts of pPacSp1, pPacSp3, and pPacSp4 (1-9 μ g) were cotransfected into *Drosophila* SL2 cells. Cell extracts representing 200,000 RLU of luciferase (from the internal control pPac luciferase) were used for CAT activity assays. Data are presented as relative CAT activity compared with the control pCAT 5-2 without cotransfected expression vector, and are the average of three independent assays. Bars represent standard deviations.

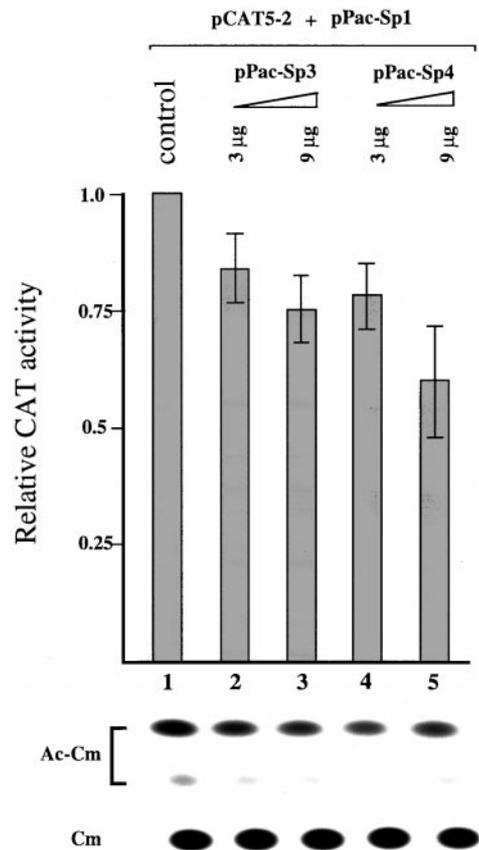


FIG. 7. Transcription activation by Sp1 can be repressed by Sp3 or Sp4 in *Drosophila* SL2 cells. Cells were transfected with 12 μ g of pCAT 5-2, 1 μ g of pPac luciferase, 3 μ g of pPacSp1, and varying amounts of pPacSp3 and pPacSp4 (3-9 μ g). Cell extracts representing 67,000 RLU of luciferase activity were used for CAT assays. Data are presented as relative CAT activity compared with the control (12 μ g of pCAT 5-2 + 1 μ g of pPacSp1), and are the average of five independent assays. Bars represent standard deviations.

Flag antibody also resulted in the disappearance of the bands (Fig. 5, A and B, lanes 4 and 7). The binding of the proteins to probe B was stronger than to probe C-1, which is in line with

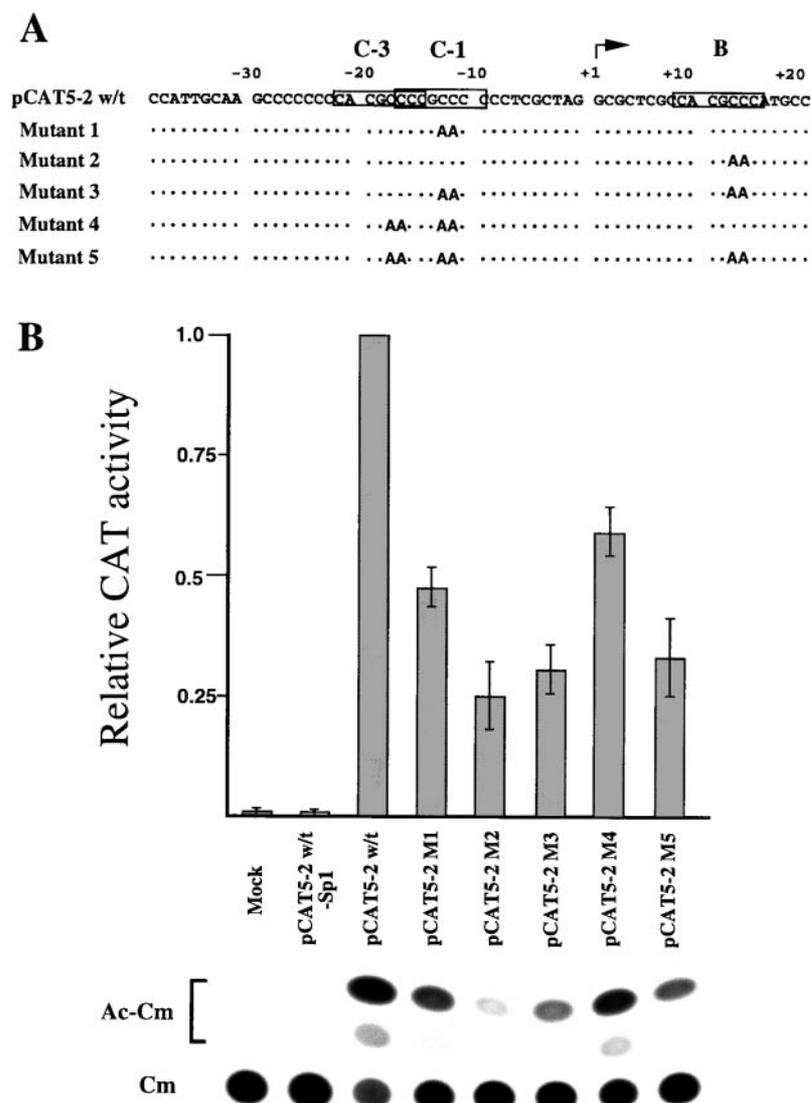


FIG. 8. The two Sp1 binding core cis-elements are the critical center in the transcriptional activation by Sp1. A, five mutant versions of pCAT 5-2 and the sites of mutations introduced by site-directed mutagenesis. B, CAT assay results of pCAT 5-2 wt and mutant versions. Twelve μg each of pCAT 5-2 w/t, pCAT 5-2 M1, pCAT 5-2 M2, pCAT 5-2 M3, pCAT 5-2 M4, and pCAT 5-2 M5 were separately cotransfected with pPac luciferase (1 μg) and pPacSp1 (1 μg). Cell extracts representing 67,000 RLU were used for the CAT assays. Data are presented as relative CAT activity (% conversion) compared with the control (12 μg of pCAT 5-2 wt + 1 μg pPacSp1) and are the average of four independent assays. Bars represent standard deviations.

data showing that the GGGCGTGG motif (probe B) has a slightly higher affinity toward Sp3 or Sp4 than to Sp1 (6). EMSA revealed that ZFD-Sp3 and ZFD-Sp4 were not able to bind to the cis-elements contained in probes A, C-2, and C-3 (data not shown).

Sp1, Sp3, and Sp4 Regulate ADH5/FDH Minimal Promoter Activity in Drosophila SL2 Cells, Which Lack Endogenous Sp1—Since Sp1 and related factors are expressed in virtually all mammalian cells, and such endogenous expression could affect the interpretation of cotransfection experiments, we decided to analyze the effects of these transcription factors in *Drosophila* SL2 cells, which are known to lack them (35, 36). We introduced the minimal promoter-CAT fusion plasmid pCAT 5-2 along with the *Drosophila* expression vectors pPacSp1, pPacSp3, and pPacSp4 into *Drosophila* SL2 cells. pCAT 5-2 alone was not able to drive transcription at all (Fig. 6, lanes 1 and 11). This demonstrated the lack of a critical transcription factor essential for the formation of the transcription initiation complex. Addition of the Sp1 expression vector, pPacSp1, drastically increased transcription in a dose-dependent manner (Fig. 6, lanes 2-4); stimulation was 51-fold at 9 μg of pPacSp1. Sp3 and Sp4 did not significantly stimulate transcription from the *ADH5/FDH* promoter. We also introduced pCAT 5-2 (12 μg) with 9 μg each of pPacSp3 and Sp4 into Schneider cells to investigate the potential interaction between Sp3 and Sp4 on the minimal promoter. Sp3 and Sp4 gave only

a very weak transcriptional activation, barely detectable over the background (data not shown). Thus, Sp3 and Sp4 function very differently than Sp1 in the transcription process.

We further investigated whether transcriptional activation by Sp1 could be repressed by either Sp3 or Sp4. pCAT 5-2 (12 μg), pPacSp1 (3 μg), and 3-9 μg of pPacSp3 or pPacSp4 were introduced into *Drosophila* SL2 cells. Transcription activation by Sp1 could be clearly repressed by both Sp3 and Sp4 (Fig. 7, lanes 2-5). Sp3 and Sp4 may abort the formation of the transcriptional initiation complex by competing with Sp1 for the same core cis-elements.

The Core cis-Elements Are Critical for Transcriptional Activation by Sp1—We tested the role of the core cis-elements in transcriptional activation by Sp1, using site-directed mutagenesis. We prepared five mutated minimal promoter constructs, with mutations introduced at one or more Sp1 binding sites (pCAT 5-2 M1 at -12/-13, GGGGCGGG \rightarrow GGTTTCGGG; pCAT 5-2 M2 at +13/+14, TGGGCGTGG \rightarrow TGTTTCGTGG; pCAT 5-2 M3 with both previous mutations, at -12/-13 and +13/+14; pCAT 5-2 M4 at -17/-18, and -12/-13; pCAT 5-2 M5 at -17/-18, -13/-12, and +13/+14) (Fig. 8A). pCAT 5-2 M1 and pCAT 5-2 M2 constructs showed reduction in transcription by 53% and 78%, respectively (Fig. 8B). Site B (+9 to +16 bp) seems to play a more important role in transcription than site C-1 (-17 to -10 bp). In the pCAT 5-2 M3 and M5 constructs, a significant reduction (greater than 70%) in tran-

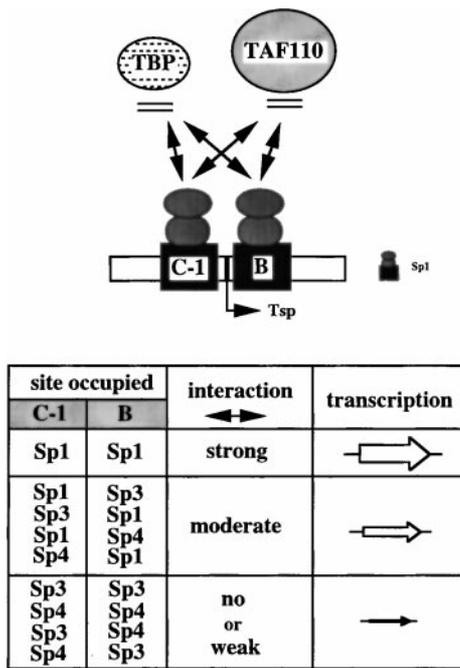


FIG. 9. A hypothetical model of the transcriptional regulation by the Sp1 multigene family at the *ADH5/FDH* minimal promoter. B and C-1 are core *cis*-elements immediately flanking transcription start point (*Tsp*, marked by an arrow). TBP, TATA-binding protein. TAF110, TBP-associated factor 110. Double arrows indicate the interaction between transcription factors (Sp1, Sp3, Sp4) and TBP or TAF110. Arrows under transcription indicate the degree of transcriptional activation.

scription resulted by having two to three mutations introduced at the core Sp1 binding sites. Comparing the activity of M4 with M1, there is little effect of the second mutation in C-3, given a mutation in C-1. This is also seen comparing M3 and M5. Thus, in the context of a promoter with a mutation in C-1, site C-3 has little or no effect.

Unexpectedly, mutating both sites B and C (in pCAT 5–2 M3 and M5) did not knock out the transcription completely (Fig. 8B). The residual transcription (22–30%) observed in pCAT 5–2 M3 and M5 might be caused by some residual binding of Sp1 to the mutated elements, or to *cis*-elements outside of this core. We investigated the possibility that the newly mutated sequence may be recognized by endogenous *Drosophila* transcription factors. We were not able to observe any transcriptional activation with these mutated constructs in the absence of Sp1, suggesting that *Drosophila* transcription factors were not capable of stimulating these mutated promoters (data not shown).

DISCUSSION

We investigated the regulation of transcription of the *ADH5/FDH* minimal promoter by members of the Sp1 multigene family member. DNase I footprinting analysis and EMSA showed that the *cis*-elements on the minimal promoter differ in their binding affinity toward Sp1 and related transcription factors; the affinities vary by as much as 5–10-fold. Sp1 can preferentially bind to the core *cis*-elements immediately flanking the transcription start site, sites B and C-1, extending from –22 bp to +20 bp. Sp1 is a strong transcriptional activator of this promoter, acting through these sites. Sp3 and Sp4 do not significantly activate this promoter, and compete with Sp1 for the key sites, leading to a reduction in transcription. These data provide the first demonstration that Sp4 can act as a repressor.

Analyses of the DNA-protein interactions on the minimal

promoter, using both purified proteins and HeLa cell nuclear extract, demonstrate that Sp1 is the major transcription factor binding to the *cis*-acting elements. Although virtually the entire promoter region can be footprinted by 0.5 fpu of Sp1, the core *cis*-elements flanking the transcription start site have the highest binding affinity toward Sp1 (5–10-fold higher than the other sites; Figs. 2 and 4). Binding of Sp1 or related factors to this unique arrangement of *cis*-acting elements may allow the nucleosome surrounding the minimal promoter to enter into an “open” state by actively displacing the histone (37–39). Since Sp3 and Sp4 were reported to bind to the same *cis*-elements that are bound by Sp1 (*i.e.* GC boxes), we examined the binding of Sp3 and Sp4 to this promoter. The zinc-finger DNA binding domains of both Sp3 or Sp4 can bind selectively to the core *cis*-elements, but not elsewhere in the minimal promoter (Figs. 4 and 5). Therefore it is very likely that competition among Sp1 multigene family members and the interaction among them are mainly occurring on the core *cis*-elements *in vivo*.

To investigate the roles of Sp1 family members in transcriptional regulation, we carried out transient expression assays using the minimal *ADH5/FDH* promoter-CAT fusion constructs. In human Alexander cells, which contain endogenous Sp1, rat Sp1 but not human Sp1 stimulated transcription (Fig. 3), probably because there is already enough endogenous Sp1. Sp3 inhibited transcription, as might be expected given prior reports of its function as a repressor (10, 14). Surprisingly, given its usual function as a transcriptional activator (14), Sp4 also inhibited transcription (Fig. 3).

To better analyze the transcriptional roles of the Sp1 family members, we carried out further assays in *Drosophila* SL2 cells, which do not express endogenous Sp1 (35, 36). pCAT 5–2, a minimal promoter construct, was not able to drive transcription at all in these SL2 cells (Fig. 6). Addition of Sp1 (by cotransfection) potently activated transcription, more than 50-fold. This demonstrates that Sp1 is a critical factor in transcriptional initiation at this promoter. Mutations in the core *cis*-elements resulted in substantial reductions of the transcription (Fig. 8), indicating that these elements are critical for transcriptional initiation. Considering the particular location of Sp1 binding sites relative to the transcription start site and other reports on the interaction between Sp1 and TBP or TAF110 (11, 26, 40–43), Sp1 may play a critical role in the formation or recruitment of the transcription initiation complex onto the core promoter (Fig. 9). Synergistic activation by Sp1 is often made possible by having two or more Sp1 sites located next to each other in a promoter (10, 44). However, the two core *cis*-elements in the *ADH5/FDH* promoter did not show synergism (Fig. 8).

We investigated whether Sp3 or Sp4 could activate transcription in the absence of Sp1, using SL2 cells. There was little or no transcription, even in the presence of the highest amount of cotransfected expression vectors (Fig. 6). This suggests that Sp3 and Sp4 lack the ability to interact with TBP or TAF110 in this promoter context. We also tested whether transcriptional activation by Sp1 can be affected by Sp3 or Sp4. Transcriptional activation by Sp1 was repressed by Sp3 or Sp4 (Fig. 7). The transcriptional repression by Sp3 or Sp4 is surprisingly similar to the results obtained with the mutant constructs where one of the two core *cis*-elements is destroyed by site-directed mutagenesis. Because Sp3 or Sp4 cannot themselves activate transcription on this particular promoter, the occupation of one of the two sites by Sp3 or Sp4 and the other by Sp1 is like the situation where only one site is occupied by Sp1 due to the mutation introduced. By having one site occupied by Sp3 (or Sp4), or by having one Sp1 binding site destroyed, a significant drop in transcription can result. The drop might be more

dramatic if higher levels of Sp3 or Sp4 were induced by the expression vectors; others have reported that the levels achieved are not high (10). In contrast, even at 20 ng of added DNA, pPac-Sp1 expresses Sp1 sufficiently to activate transcription strongly (10).

These data suggest that Sp3 and Sp4 can act as repressors by competing with Sp1 for binding to the core *cis*-elements and preventing the formation of the transcription initiation complex. Sp4 had not previously been shown to repress transcription. Based on these findings, we propose a hypothetical model on the transcriptional regulation at the *ADH5/FDH* minimal promoter (Fig. 9). The two core *cis*-elements are the critical center for transcriptional initiation and activation by Sp1. These elements can be preferentially bound by Sp1, Sp3, and Sp4. If only Sp1 occupies these elements, it can interact with TBP or TAF110 and can promote strong transcription. By having two core binding sites for Sp1, transcription activation by Sp1 is ensured and strong transcription is made possible. If the core sites are occupied by Sp3 or Sp4 alone, the interaction between the transcription factor with one or more components of general transcription machinery is either absent or weak, which can result in little or no transcriptional activation. If one site is occupied by Sp1 and the other by Sp3 or Sp4, the transcription activation will be relatively low compared with the situation where two sites are occupied by Sp1 only.

Therefore, the cellular content of Sp1, Sp3, and Sp4 and their interactions on the minimal promoter may be critical factors influencing transcription of the *ADH5/FDH* gene in various human tissues (22, 23). This model may also be applicable to many housekeeping genes with GC boxes located either in the proximal promoter or around the transcription start site, as is the case in the *ADH5/FDH* gene.

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**Sp3 and Sp4 Can Repress Transcription by Competing with Sp1 for the Core *cis*
-Elements on the Human *ADH5/FDH* Minimal Promoter**

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