MOLECULAR AND CELLULAR BIOLOGY, Apr. 1991, p. 1935–1943
0270-7306/91/041935-09$02.00/0

Vol. 11, No. 4

Synergistic Activation of a Human Promoter In Vivo by Transcription Factor Sp1

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Received 13 August 1990/Accepted 3 January 1991

Many eucaryotic promoters contain multiple binding sites for sequence-specific DNA-binding proteins. In some cases, these proteins have been shown to interact synergistically to activate transcription. In this study, we address the possibility that the transcription factor Sp1 can synergistically activate a native human promoter in a cellular context that closely resembles that of a single-copy gene. Using DNase I footprinting with affinity-purified Sp1, we show that the human argininosuccinate synthetase (AS) promoter contains three sites that bind Sp1 with different affinities. These binding sites were mutated to abolish Sp1 binding, individually and in all possible combinations, to generate a series of AS promoter-chloramphenicol acetyltransferase (CAT) expression constructs. Mutations designed to increase Sp1 binding were also introduced at each site. The in vivo transcriptional activity of these mutant AS promoter-CAT constructs was then measured in stably transfected human RPMI 2650 cell lines. Our results show that each of the three Sp1-binding sites contributes to full activation of the human AS promoter and that the relative contribution of each site correlates well with its in vitro affinity for Sp1. More importantly, we find that the three Sp1-binding sites when present in the same promoter activate transcription to a level that is 8 times greater than would be expected given their individual activities in the absence of the other two sites. Thus, we provide direct evidence that Sp1-binding sites in their native context in a human promoter can interact synergistically in vivo to activate transcription. The ability to activate transcription synergistically may be the reason that many cellular promoters have multiple Sp1-binding sites arranged in tandem and in close proximity.

Regulation of transcription initiation by RNA polymerase II is an important means by which living cells produce specific patterns of gene expression. Although many of the molecular details remain to be elucidated, it is clear that sequence-specific DNA-binding proteins play a critical role in transcriptional control. Recently, numerous proteins that increase the rate of transcription initiation (activators) have been isolated and characterized. A common feature of eucaryotic transcriptional activators is that they often contain two domains that are essential for biological activity (29, 32). These domains have been shown to be structurally and functionally separable. One domain, the DNA-binding domain, targets the protein to a specific DNA sequence and allows for the selective activation of gene transcription. A second transcriptional activation domain can function independently of the DNA-binding domain. Several classes of activation domains have been identified, and it is believed that such domains function by making essential contacts with RNA polymerase II, with other sequence-specific DNA-binding proteins, or with proteins that are part of the general transcription machinery.

A common theme has also emerged regarding the structure of transcriptional control regions in eucaryotic genes. One feature that is characteristic of eucaryotic promoters is that they often contain multiple binding sites for the same, or for several different, sequence-specific DNA-binding proteins. These binding sites are usually located within several hundred base pairs of the transcription initiation site and, when identical, are often evenly spaced in a tandem array. Examples of this are the simian virus 40 (SV40) early promoter (12), the mouse dihydrofolate reductase promoter (10), the human Ha-c-*ras* promoter (18), and the human immunodeficiency virus promoter (17, 19). The presence of multiple factor-binding sites raises the possibility that their effects on transcription are additive or even synergistic. Indeed, synergistic activation has been observed between different *cis*-acting elements as well as between identical *cis*-acting elements within a single promoter (3, 6, 26, 34, 35). This synergism suggests that protein-protein interactions between factors, either directly or through intermediary proteins, are important for transcriptional activation (3, 26).

The studies cited above showed very clearly, with use of artificial combinations of *cis*-acting sequences, that a variety of transcription factors are capable of synergistic interactions. In this study, we investigate whether the transcription factor Sp1 does in fact synergistically activate a native human promoter in a cellular context that closely resembles that of the endogenous gene. Sp1 was one of the first mammalian transcriptional activators to be described, and it binds specifically to a G+C-rich sequence found in many viral and cellular promoters (7, 11, 20). Such promoters usually contain multiple Sp1-binding sites, suggesting that interactions between these sites may be important for transcriptional activation. In this study, we show by DNase I footprinting that the human argininosuccinate synthetase (AS) promoter contains multiple proximal Sp1-binding sites. We have performed a thorough mutational analysis of these Sp1-binding sites and have measured their effects on transcription in vivo with stably transfected genes. Our studies provide the first direct evidence that in their native context, Sp1-binding sites can interact synergistically in vivo to activate transcription.

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MATERIALS AND METHODS

Plasmids, cell culture, and DNA transfections. pmgAS-6 was derived from pmgAS-10 (1) and differs as follows: the *Hin*dIII site joining exon 4 of the human AS gene to the chloramphenicol acetyltransferase (CAT) gene and the *Bam*HI site 3′ to the CAT gene were removed. In addition, a 91-bp *Nco*I-*Stu*I fragment from pSV2cat (16), which contains the SV40 origin of replication core, was cloned into the *Nde*I site upstream of human AS promoter sequences. The SV40 origin was included to allow for replication of these plasmids in COS-7 cells and did not interfere with the analyses reported here. RPMI 2650 (ATCC CCL 30) cells were grown in Dulbecco modified Eagle medium supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 2 mM glutamine, 100 μg of streptomycin sulfate and 100 U of penicillin per ml, and 10% (vol/vol) fetal bovine serum (growth medium). Cells were grown in a humidified incubator in an atmosphere of 90% air and 10% CO_2. Plasmid DNA transfections were performed by the $CaPO_4$ precipitation method of Parker and Stark (30), with the following modifications. Approximately 10^6 cells were plated into culture dishes (90-mm diameter) 1 day prior to transfection. On the following day, the growth medium was replaced, and 3 h later the $CaPO_4$-DNA precipitate containing 5 μg of pSV2hygro and 25 μg of the test plasmid was applied to the cells. DNA precipitates were left on the cells overnight, and the next day cell monolayers were rinsed once with Dulbecco modified Eagle medium and then incubated in growth medium containing 400 μg of hygromycin B per ml (selective medium). The selective medium was changed every 3 days. After a period of 3 weeks, the number of hygromycin-resistant colonies for each transfection was recorded, and pooled cell lines were generated following trypsinization.

Southern (DNA) blotting. High-molecular-weight genomic DNA was isolated from pooled cell lines by lysing cells in a solution of 10 mM Tris-HCl (pH 7.9)–1 mM EDTA–0.5% sodium dodecyl sulfate (SDS), followed by digestion with 1 mg of proteinase K per ml at 37°C overnight. Samples were extracted three times with phenol and once with chloroform and were then dialyzed overnight against a solution of 10 mM Tris-HCl (pH 7.4)–0.1 M NaCl–1 mM EDTA. Restriction digests were performed at 37°C for 16 h, using 5 μg of genomic DNA and 50 U each of *Bam*HI and *Eco*RI. Restriction digests were subjected to electrophoresis in 1% agarose, and the DNA was transferred to a filter (Gene Screen; DuPont) by standard procedures. Prehybridization was performed overnight at 65°C in 1 M NaCl–10% (wt/vol) dextran sulfate–1% SDS–100 μg of denatured salmon sperm DNA per ml. Hybridizations were performed for 24 h under identical conditions but included 5×10^5 cpm of a [^{32}P]DNA probe per ml. Following hybridization, filters were washed once in 2× SSC (1× SSC is 0.015 mM sodium citrate plus 0.15 M NaCl, pH 7.0) at room temperature, once in 1× SSC–1% SDS at 65°C, and twice in 0.5× SSC–1% SDS at 65°C. Filters were subjected to autoradiography, and the amount of radioactivity in each band was quantified with a Betagen detector.

CAT assays. CAT assays were performed as previously described (16). All assay mixtures contained equal amounts of cellular protein. The activity of all radioactive species was quantified with a Betagen detector and expressed as a percent conversion of the [^{14}C]chloramphenicol substrate. The individual contributions of each Sp1 site independent of the other two sites are represented by the CAT activities of mutants 2−3− (site 1), 1−3− (site 2), and 1−2− (site 3). The individual contributions of all Sp1 sites were summed and expressed relative to the activity of the wild-type AS promoter (100%).

DNase I footprinting analysis. DNase I footprinting reactions were performed according to a protocol kindly provided by Al Courey. The Sp1 DNA-binding reaction was performed in a total volume of 25 μl containing 25 mM HEPES (pH 7.5), 100 mM KCl, 20% (vol/vol) glycerol, 0.1% (vol/vol) Nonidet P-40, 10 μM $ZnSO_4$, 1 mM dithiothreitol, 15,000 cpm (4,000 cpm/fmol) of [^{32}P]DNA, and 10 to 20 ng of affinity-purified human Sp1 from HeLa cells (gift of A. Courey and R. Tjian). Samples were preincubated on ice for 15 min, 50 μl of 10 mM $MgCl_2$–5 mM $CaCl_2$ was added, the mixture was incubated at room temperature for 1 min, and then 1 μl of a stock DNase I (Worthington) solution was added to a give final concentration of 0.5 to 1.25 μg/ml. DNase I digestion proceeded at room temperature for 1 min, and the reaction was stopped by the addition of 90 μl of 20 mM EDTA–1% SDS–0.2 M NaCl–250 μg of yeast tRNA per ml. Samples were extracted with a mixture of phenol-chloroform (1:1), and the nucleic acid was concentrated by ethanol precipitation. Following centrifugation, the nucleic acid pellet was washed once with cold 70% ethanol, dried under vacuum, and resuspended in 5 μl of loading buffer containing 80% formamide. Samples were heated to 100°C for 3 min and subjected to electrophoresis in an 8% polyacrylamide sequencing gel.

DNA binding-gel electrophoresis assays. RPMI 2650 nuclear extracts were prepared by the method of Dignam et al. (9). Complementary 30-bp oligonucleotides representing each Sp1-binding site in the human AS promoter were annealed and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase by standard procedures (27). Binding reactions were performed by preincubating 1 to 5 μg of nuclear extract protein in 20 mM HEPES (pH 7.5)–60 mM KCl–1 mM $MgCl_2$–0.1 mM EDTA–12% glycerol–0.5 mM dithiothreitol–2 μg of poly(dI-dC) on ice for 10 min, followed by addition of the double-stranded ^{32}P-oligonucleotide and a second incubation on ice for 20 min. Samples were loaded directly onto nondenaturing 4% polyacrylamide gels (29:1 acrylamide to bisacrylamide) prepared in 45 mM Tris-borate–45 mM boric acid–0.1 mM EDTA (0.5× TBE). Electrophoresis was performed at room temperature for 3 to 4 h at a current of 15 mA. DNA binding assays using purified Sp1 were performed as described above except that reactions contained approximately 1 to 2 ng of purified protein, 10 μg of bovine serum albumin as carrier, and no poly(dI-dC). Where indicated, 0.5 μl of rabbit anti-human Sp1 antiserum (gift of S. Jackson and R. Tjian), or the corresponding preimmune serum, was added to the binding reaction prior to addition of the double-stranded ^{32}P-oligonucleotide.

Site-directed mutagenesis. Site-directed mutagenesis was performed by using a commercially available kit (United States Biochemical) that is based on the method of Kunkel (24). The single-stranded M13 mp18 DNA template used for the mutagenesis contained approximately 500 bp of human AS sequence which spans from base −150 to approximately base +350 in the human AS gene. These sequences were cloned into the M13 mp18 replicative form on a 500-bp *Hin*dIII-*Bam*HI fragment taken from pmgAS-6 (Fig. 1A). Mutants were identified by DNA sequencing according to the dideoxynucleotide chain termination method. A 500-bp *Hin*dIII-*Bam*HI restriction fragment containing the site-directed mutations was excised from the M13 mp18 replicative form, and mutant fragments were cloned between the

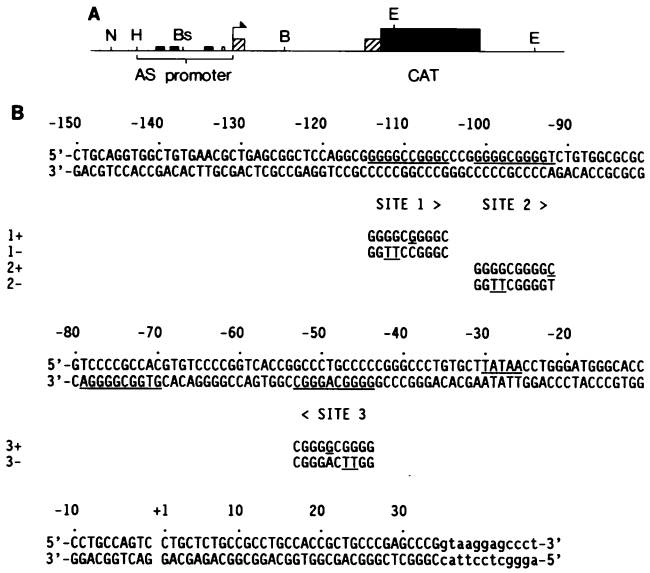

FIG. 1. (A) Structure of parental plasmid pmgAS-6. The large solid box represents the CAT gene; the hatched boxes represent sequences from exons 1 and 4 of the human AS gene; the thin line between the hatched boxes represents a hybrid intron formed between introns 1 and 3 of the human AS gene (1). The position of the human AS promoter is indicated. Sp1-binding sites are represented by the small solid boxes, and the TATA box is represented by the small open box. Restriction sites: B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I. (B) Sequence of the human AS promoter. +1 indicates the transcription initiation site. Potential Sp1-binding sites and the TATA box are underlined. The arrows indicate the orientation of each potential Sp1-binding site relative to the direction of transcription. Mutant Sp1 sites are shown below the wild-type sequence, with the base changes underlined. Promoter and exon sequences are in uppercase; intron sequences are in lowercase.

*Hin*dIII and *Bam*HI sites of pmgAS-6 following removal of the wild-type fragment.

RESULTS

Sp1 footprinting of the human AS promoter. To examine whether activation of transcription by Sp1 in human cells is synergistic, we constructed an expression plasmid driven by the human AS promoter. The AS promoter is well suited for these experiments since it contains multiple Sp1-binding sites yet is simpler than other viral or cellular promoters (25, 28). This simplicity facilitated an analysis of the role of specific *cis*-acting elements in transcription in vivo without the interference of other overlapping elements. The parental plasmid used in these experiments is shown schematically in Fig. 1A. pmgAS-6 contains 150 bp of the human AS promoter driving the expression of the bacterial CAT gene. We previously showed that only 150 bp of the human AS promoter was required to give efficient expression in human cells (1). The human AS promoter is extremely G+C rich (76%) and contains four motifs that closely resemble the 10-bp consensus binding site ([G/T][G/A]GGC[G/T]G[G/A][G/A][C/T]; 2) for Sp1 (Fig. 1B). Site 1 (−114 to −105) matches the consensus sequence except for one mismatch in the sixth position (−109), in which a C is substituted for the preferred G. Site 2 (−101 to −92) matches the consensus sequence perfectly and is identical to a previously characterized Sp1-binding site (20). Site 2 was shown to be of

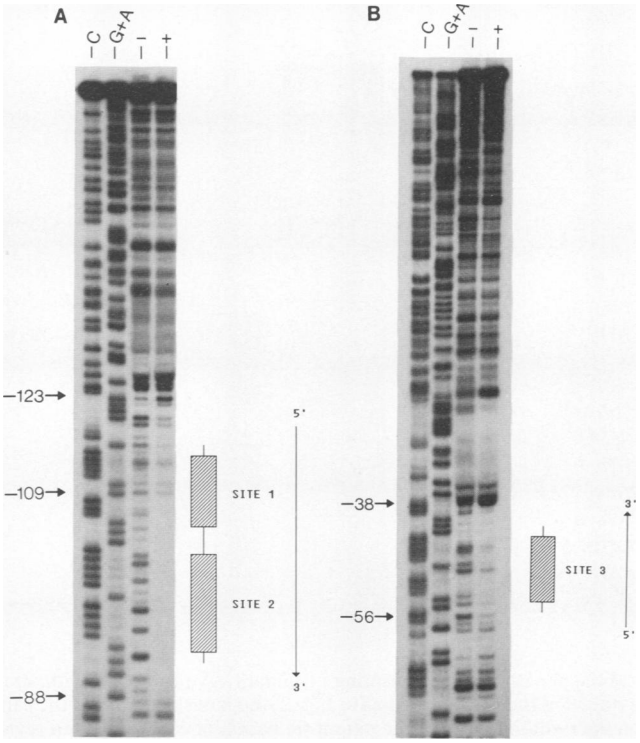

FIG. 2. DNase I footprinting of the human AS promoter with affinity-purified Sp1. (A) Footprinting of sites 1 and 2. The [^{32}P]DNA used for the footprinting analysis was a 98-bp *Hin*dIII-*Bst*EII restriction fragment of pmgAS-6 that was 5′ end labeled on the noncoding strand. −, DNase I cleavage pattern in the absence of Sp1; +, DNase I cleavage pattern in the presence of Sp1. Maxam-Gilbert sequencing ladders are shown in the left two lanes. The positions of sites 1 and 2 are indicated by the hatched boxes on the right. The extent of the protected region (in bases) is indicated by the numbers on the left. (B) Footprinting of site 3. The [^{32}P]DNA used for the footprinting analysis was a 500-bp *Hin*dIII-*Bam*HI restriction fragment of pmgAS-6 that was 5′ end labeled on the coding strand. The lanes are the same as in panel A. The position of site 3 is indicated by the hatched box on the right. The extent of the protected region (in bases) is indicated by the numbers on the left.

medium affinity compared with other known binding sites, as determined from DNase I footprinting experiments (20). Site 3 (−53 to −44), which is situated in the opposite orientation relative to sites 1 and 2, matches the consensus sequence except for one mismatch in the sixth position (−49), in which an A is substituted for the preferred G. A fourth potential Sp1-binding site (−79 to −70) is oriented similarly to site 3 and differs from the consensus sequence in two positions. Although sites 1 and 3 closely resemble the consensus sequence, they do not match any previously identified Sp1-binding sites.

To determine whether the four motifs in the AS promoter bound Sp1, we performed in vitro DNase I footprinting experiments with affinity-purified human Sp1. These experiments revealed two regions of protection corresponding to three separate binding sites for Sp1 (Fig. 2). The first region spans 36 bp between −123 to −88 and encompasses the two motifs corresponding to sites 1 and 2 (Fig. 2A). The extent of protection in this region is consistent with the presence of two binding sites, since a single Sp1-binding site will normally generate a protected region of between 15 and 20 bp

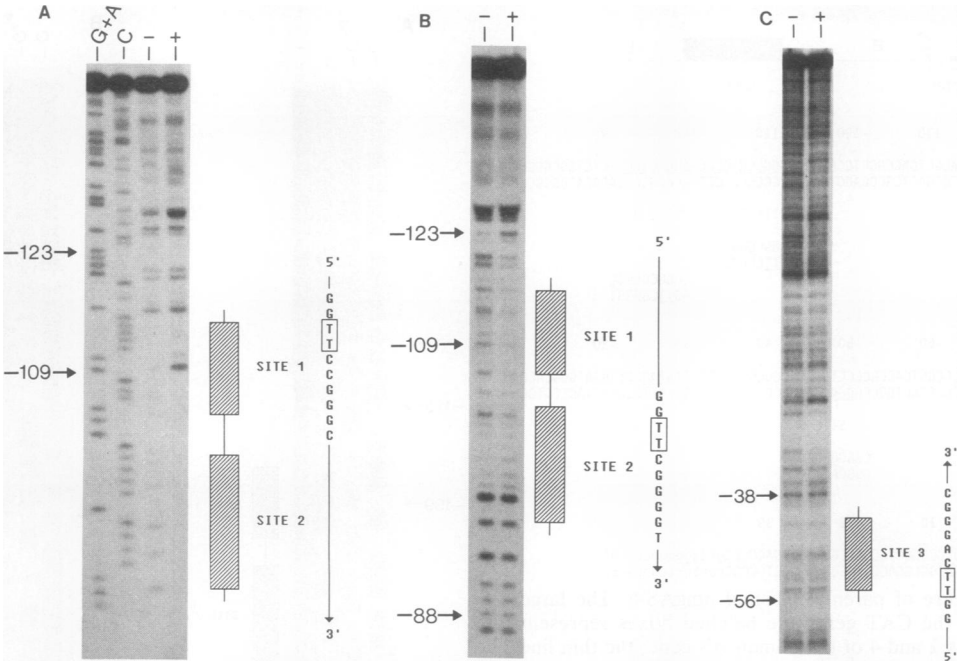

FIG. 3. DNase I footprinting of mutant AS promoters with affinity-purified Sp1. (A) Site 1; (B) site 2; (C) site 3. The DNAs are labeled with ^{32}P in the same strand as in Fig. 2; the lanes are marked the same as in Fig. 2. The double TT mutation introduced into each site is shown on the right in the box. The extent (in bases) of each protected region is indicated by the numbers on the left. The partial protection 3′ to site 3 may be due to an Sp1 half-site at −18 to −14.

(13). The 5′ end of this region showed enhanced cleavage by DNase I (bases −123 and −122). We consider this part of the Sp1-binding region since intensification of DNase I cleavages is often observed as an effect of protein binding to DNA. The degree of protection spanning from −120 to −109 (corresponding to site 1) is clearly much weaker than that spanning from −108 to −88 (corresponding to site 2). Because site 1 showed less protection than site 2, and because site 2 is a known medium-affinity binding site, we have designated site 1 in the human AS promoter as a low-affinity Sp1-binding site. The other observable DNase I footprint spans 19 bp from −56 to −38 and encompasses site 3 (Fig. 2B). Similar to site 1, site 3 showed partial protection by purified Sp1 under conditions that yield complete protection at site 2. Thus, we have also designated site 3 in the human AS promoter as a low-affinity Sp1-binding site. The fourth potential Sp1-binding site at −79 to −70 did not bind purified Sp1 (data not shown). The failure to detect binding at this site is consistent with the fact that it differs from the consensus sequence in two positions, whereas sites 1 and 3, which bind Sp1 weakly, differ from the consensus sequence in only one position. Thus, we conclude that the human AS promoter contains three Sp1-binding sites, two with low affinity and one with medium affinity for human Sp1.

Site-directed mutagenesis of the Sp1-binding sites. Although in vitro DNase I footprinting can be used to identify factor-binding sites in DNA, such experiments do not establish a direct role for these sites in the transcriptional activation of gene expression in vivo. Thus, to establish this role and to determine the relative contribution of each Sp1-binding site to activation of the human AS promoter, we generated a series of site-directed mutants designed to abolish binding of Sp1 to each of the three sites (designated 1−, 2−, and 3−) and measured their effects on transcription in vivo. As described below, these mutants were generated separately and in every possible combination. Base changes were introduced into the third and fourth positions of the consensus binding site (GG to TT; Fig. 1B), since these mutations were previously shown to abolish Sp1 binding in vitro (19). We also introduced base changes into each of the three binding sites which were designed to increase the affinity for Sp1 (designated 1+, 2+, and 3+). These mutants were constructed to determine whether mutations that increase the affinity of a binding site for Sp1 in vitro would result in a higher transcription rate in vivo. The introduced base change(s) separately converted each site to GGGGCGGG GC, a sequence that was shown previously to be a high-affinity Sp1-binding site (20).

To demonstrate that each mutation had its intended effect on Sp1 binding, we performed DNase I footprinting and DNA binding-gel electrophoresis assays, using the mutated DNA sequences as binding substrates (Fig. 3 and 4). DNase I footprinting of the mutated site 1 shows that the previously observed region of partial protection spanning from −123 to −109 corresponding to site 1 has been abolished (Fig. 3A; compare with Fig. 2A). In contrast, the region encompassing the intact site 2 (−108 to −88) still shows complete protection. Similarly, mutagenesis of site 2 abolished the complete protection normally spanning −108 to −88, which encompasses site 2, but had no effect on the partial protection spanning from −123 to −109, which corresponds to site 1 (Fig. 3B). Thus, mutagenesis of either site 1 or site 2 separately abolished binding of Sp1 to that site but did not disturb the interaction of Sp1 with the adjacent site. Taken together, these observations suggest that Sp1 does not bind cooperatively to sites 1 and 2 and are consistent with previous studies which failed to observe cooperative binding of Sp1 to adjacent sites in the SV40 promoter (13). DNase I footprinting of the mutated site 3 shows that the protected

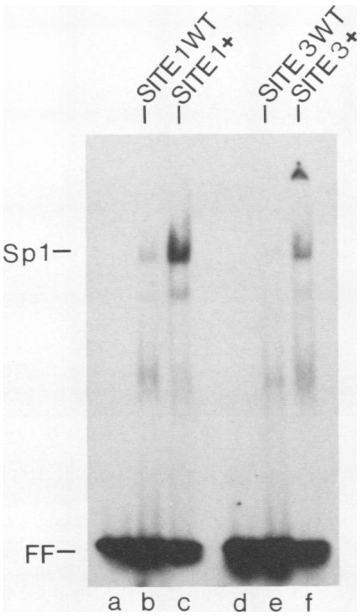

FIG. 4. DNA binding-gel electrophoresis assay of improved Sp1-binding sites. A double-stranded 30-bp ^{32}P-oligonucleotide was incubated with RPMI 2650 nuclear extract (lanes b, c, e, and f), and the complexes formed were analyzed on a nondenaturing polyacrylamide gel. Lanes: a, wild-type (WT) site 1 without nuclear extract; b, wild-type site 1; c, high-affinity site 1; d, wild-type site 3 without nuclear extract; e, wild-type site 3; f, high-affinity site 3. The specific activities of the wild-type and mutant ^{32}P-oligonucleotides were essentially the same. Positions of migration of the free fragment (FF) and the Sp1 nucleoprotein complex (Sp1) are indicated on the left.

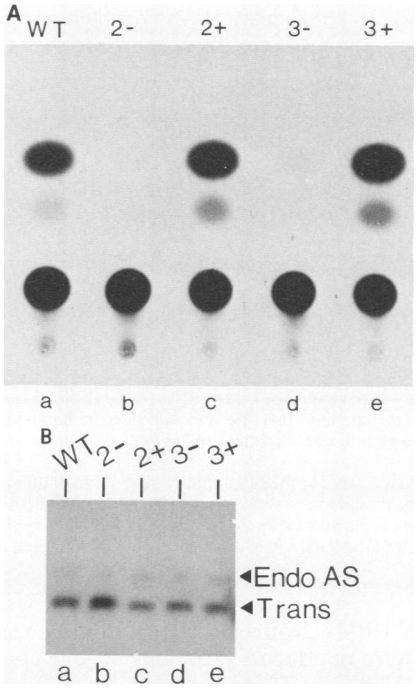

FIG. 5. Expression of mutant AS promoter-CAT constructs. (A) CAT assays. The constructs examined are indicated at the top of each lane. WT, wild type. (B) Southern blotting of genomic DNA for gene copy number. Genomic DNA from pooled cell lines was digested with *Bam*HI and *Eco*RI and subjected to Southern blot analysis. The filter was hybridized with a [^{32}P]DNA probe corresponding to the 2.3-kb *Bam*HI-*Eco*RI fragment of pmgAS-6 (see Fig. 1A). This probe contains sequences from intron 3 of the human AS gene and detects two restriction fragments; one corresponds to a 2.5-kb *Bam*HI-*Eco*RI fragment from the endogenous human AS gene (Endo AS) and the other corresponds to the 2.3-kb *Bam*HI-*Eco*RI fragment of the stably integrated transfected genes (Trans). The cell lines examined are indicated at the top of each lane. WT, wild type.

region which normally spans −56 to −38 and encompasses site 3 has been abolished (Fig. 3C).

To verify the effects of mutations designed to increase Sp1 binding, we used a DNA binding-gel electrophoresis assay (8) with double-stranded 30-bp oligonucleotides containing either the wild-type or improved Sp1-binding site sequence as the binding substrate. Nuclear extracts from human RPMI 2650 cells were used as a source of Sp1 in these experiments, since our subsequent transfection studies were performed with these cells (see below). An oligonucleotide containing a high-affinity binding sequence at site 1 (Fig. 4, lane c) gave a more intense shifted signal than did an oligonucleotide containing the wild-type sequence (lane b), indicating more avid binding of Sp1. Similar results were obtained for site 3 (compare lanes e and f). These results were substantiated by DNase I footprinting experiments with purified Sp1, which showed improved protection at sites 1 and 3 when we used DNA fragments containing the improved binding-site sequence (data not shown). Thus, we have shown by using two independent DNA binding assays that mutations that were designed to either abolish or increase Sp1 binding produced the desired and expected effects. We did not perform similar experiments for site 2 since the sequences of both the wild-type and high-affinity site 2 are identical to those of previously characterized Sp1-binding sites, and the relative affinities of these sites for Sp1 have already been established by DNase I footprinting (20).

Expression of mutant AS promoter-CAT constructs in RPMI 2650 cells. We determined the effect of each mutation on the relative transcriptional activity of the AS promoter by creating a pool of stably transfected RPMI 2650 cells expressing the wild-type plasmid (pmgAS-6) and each of the mutant plasmids. Numerous studies using transfected plasmids have shown that the level of CAT activity is directly proportional to the strength of the promoter driving the CAT gene (reference 6 and references therein). We chose to perform these studies with stable cell lines rather than with transient assays in order to monitor the effects of these mutations in a cellular context which closely resembles that of a single-copy gene. By using stable cell lines, we could avoid unnaturally high gene copy numbers and could ensure that the transfected plasmids were assembled into bona fide chromatin. Pooled cell lines were generated by cotransfecting each test plasmid with a plasmid conferring resistance to hygromycin B, followed by selection in growth media containing hygromycin. Any positional effect of the chromosomal integration site on expression of the transfected plasmids should be averaged out, since each pool of transfectants contained between 400 and 500 colonies. The level of CAT expression in each pooled cell line was quantified and then normalized to plasmid DNA copy number to correct for differences due to variations in transfection efficiency.

Compared with pmgAS-6 (Fig. 5A, lane a), mutations that abolished Sp1 binding to site 2 (lane b) and site 3 (lane d) had a dramatic effect on the transcriptional activity of the AS

TABLE 1. Summary of AS promoter mutants

Name[a]	Site[b]			Activity[c]
	1[b]	2	3	
Wild type	L	M	L	100
1−		M	L	23
2−	L		L	10
3−	L	M		21
1−2−			L	3.3
1−3−		M		7.2
2−3−	L			2.2
1−2−3−				1.5
1+	H	M	L	30
2+	L	H	L	306
3+	L	M	H	320

[a] A minus sign indicates that the site has been mutated to abolish Sp1 binding; a plus sign indicates that the site has been converted to a high-affinity site.

[b] L, Low-affinity site; M, medium-affinity site; H, high-affinity site.

[c] Based on the percent conversion of the [^{14}]chloramphenicol substrate into all acetylated products and expressed relative to that of the wild-type human AS promoter (100%). All activities are normalized to CAT gene copy number.

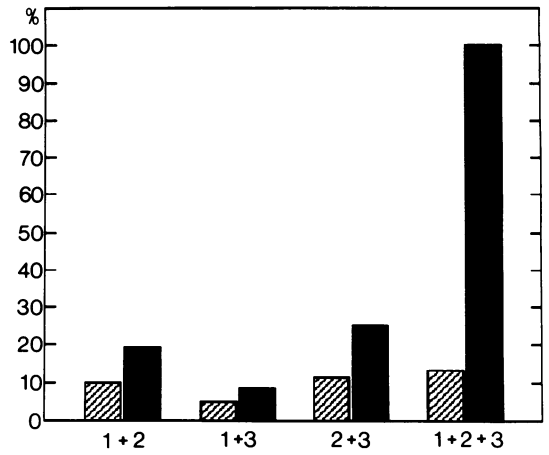

FIG. 6. Synergistic activation of the human AS promoter by Sp1. The sites included in each analysis are shown at the bottom, and the corresponding promoter activities are expressed relative to that of the wild-type AS promoter (100%). Each hatched bar represents the sum of the activities of individual Sp1-binding sites when the activities of each site is measured in the absence of the other two sites. Each solid bar represents the observed transcription rate when the sites indicated are present in the same promoter.

promoter in RPMI 2650 cells. These differences in CAT expression were not due to variations in gene copy number, since all pooled cell lines contained on average a similar number (ca. 5 copies per cell) of integrated plasmids (Fig. 5B), and all CAT values were normalized to CAT gene copy number (Table 1). Thus, the observed effect of each mutation on CAT expression is due to its effect on the activity of the human AS promoter.

The effects of the various mutations on the activity of the human AS promoter in RPMI 2650 cells are summarized in Table 1. As mentioned above, we constructed mutants that abolished binding of Sp1 to only one site, to two sites, and to all three sites. This allowed us to determine the relative contribution of each site to the activity of the human AS promoter in the absence of the other two sites. Furthermore, this series of mutant promoters also allowed us to observe synergistic interactions between any combination of two or among all three Sp1-binding sites. The mutant designated 1−2−3− serves to provide a base line of promoter activity in the absence of Sp1 binding. Mutation of each site independently resulted in a 4- to 10-fold decrease in the activity of the AS promoter (Table 1). These data establish that each of the three Sp1-binding site is functionally operative in vivo. Ablation of site 2, which binds Sp1 with medium affinity, had a more pronounced effect on the activity of the human AS promoter than did mutagenesis of either site 1 or site 3, both of which bind Sp1 with low affinity. Thus, the effects of these mutations on transcription in vivo closely parallel their effects on Sp1 binding in vitro. Plasmids that contained mutations in two sites showed even lower levels of promoter activity, and as before, mutagenesis of site 2 had a more pronounced effect than did mutagenesis of either site 1 or site 3. For example, mutation of site 2 in the 1− plasmid to produce 1−2− resulted in a sevenfold decrease in promoter strength, whereas mutation of site 3 in the 1− plasmid to produce 1−3− resulted in a threefold decrease in promoter strength. The activity of the mutant promoter in which all three Sp1-binding sites were eliminated (1−2−3−) was only 1.5% that of the wild-type promoter, indicating that 98% of the human AS promoter activity is due to these three Sp1-binding sites.

In general, bases changes that increased Sp1 binding in vitro resulted in higher transcription rates in vivo. For example, base changes in sites 2 and 3 which increase Sp1 binding resulted in a threefold increase in promoter strength (Table 1). Increasing the affinity at site 1 gave a surprising and unexpected result. In this case, the result of increasing the affinity for Sp1 reduced the strength of the AS promoter to 30% of normal. Similar results were observed in three independent pooled cell lines. These data show that increasing the affinity of the Sp1-binding site has consequences in vivo and, with one exception, confirm our previous observations with the native sites that those exhibiting a higher affinity for Sp1 in vitro contribute more to AS promoter strength in vivo.

Sp1-binding sites interact synergistically in vivo to activate transcription. A novel aspect of the data presented in Table 1 is that they provide compelling evidence that the three Sp1-binding sites in the human AS promoter interact synergistically in vivo to activate transcription. This synergism is illustrated in Fig. 6. In every case, the observed activity when multiple sites are present in the same promoter is greater than the sum of the individual site contributions. This synergistic effect is most striking when all three sites are present in the same promoter, in which case the observed transcription rate is eight times that of the sum of the individual site contributions. These results strongly suggest that the Sp1 proteins bound at the individual sites interact in the in vivo transcription complex, either directly or through an intermediary protein, to produce a more active transcription complex.

Presence of Sp1 in RPMI 2650 nuclear extracts. Thus far, we have demonstrated that the human AS promoter contains three motifs that bind Sp1 in vitro and that all three sites are functionally operative and interact synergistically in vivo. We have also demonstrated a positive correlation between the binding affinities of Sp1 sites in vitro and their ability to activate transcription in vivo. However, it has been shown that mammalian cells contain several sequence-specific DNA-binding proteins that can recognize G+C-rich sequences similar or identical to those recognized by Sp1 (21, 23). Thus, it was important that we demonstrate that the

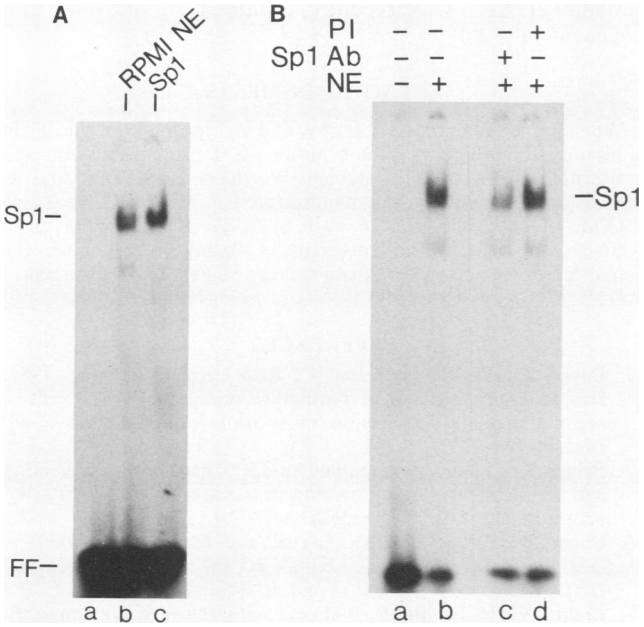

FIG. 7. DNA binding-gel electrophoresis assays. (A) Comigration of RPMI 2650 and purified Sp1 nucleoprotein complexes. A double-stranded 30-bp ^{32}P-oligonucleotide corresponding to wild-type site 2 was incubated without (lane a) or with (lane b) RPMI 2650 nuclear extract (NE) or with 1 ng of affinity-purified Sp1 (lane c), and the complexes formed were analyzed on a nondenaturing polyacrylamide gel. Positions of migration of the free fragment (FF) and the Sp1 nucleoprotein complex (Sp1) are indicated on the left. (B) Effect of anti-Sp1 antiserum on the formation of the RPMI 2650 nucleoprotein complex. The ^{32}P-oligonucleotide used in this analysis is the same as that used for panel A. A plus sign indicates that RPMI 2650 nuclear extract (NE), anti-human Sp1 antiserum (Sp1 Ab), or preimmune serum (PI) was included in the reaction mixture. The position of migration of the Sp1 nucleoprotein complex (Sp1) is indicated on the right. The Sp1 signal in lane c is 53% of that in lane d.

protein in RPMI 2650 cells which binds to the human AS promoter is in fact Sp1.

We performed three different experiments to demonstrate this point. First, we compared the mobility in a nondenaturing polyacrylamide gel of the nucleoprotein complex formed with purified Sp1 with that of the complex formed with RPMI 2650 nuclear extract (Fig. 7A). This experiment shows that the major shifted species with RPMI 2650 nuclear extract (lane b) comigrates exactly with that produced with purified Sp1 (lane c), suggesting that the protein giving rise to the shift in RPMI 2650 cells is Sp1. Second, we added a small amount of rabbit anti-human Sp1 antiserum to RPMI 2650 nuclear extracts to block Sp1 binding (Fig. 7B). This antiserum was shown previously to diminish Sp1-DNA complex formation in this type of assay (36). The anti-Sp1 antiserum reduced the intensity of the shifted complex (Fig. 7B, lane c), whereas an equal amount of preimmune rabbit serum had no effect (lane d). Although we did not observe complete abolition of the RPMI 2650 nucleoprotein complex with the anti-Sp1 antiserum, we saw diminished binding in four independent experiments. Third, we fractionated the RPMI 2650 nuclear extract on a Mono S column and eluted the bound proteins with a linear 60 to 400 mM KCl gradient. The protein that produces the nucleoprotein complex shown in Fig. 7B (lane b) eluted at 255 mM KCl (data not shown), which is nearly identical to the value (0.26 M) reported for Sp1 by Briggs et al. (2). Thus, based on the fact that its binding preferences for a variety of DNA sequences match those of Sp1, on its comigration with an Sp1 nucleoprotein complex in a nondenaturing polyacrylamide gel, on its antigenicity, and on its chromatographic properties on an ion-exchange resin, we conclude that the factor present in RPMI 2650 cells which binds to the human AS promoter is authentic Sp1.

DISCUSSION

We have used DNase I footprinting with affinity-purified protein to identify three binding sites for the cellular transcription factor Sp1 in the human AS promoter. Site 2 has medium affinity for Sp1 relative to other known sites, and the sequence of this site has been found in other promoters (20). The other two sites have low affinity for Sp1 as a result of mismatches in the sixth position of the consensus sequence, which is part of the GC box core sequence (GGGCGG; 19, 20). To our knowledge these sites have not been described before, and therefore this study expands the repertoire of Sp1-binding sites. Furthermore, we have demonstrated that both of these low-affinity sites are functionally operative in vivo. This finding was based on transfection studies into human cells with mutant AS promoter-CAT genes. These experiments showed that each of the three Sp1-binding sites contributed to the activity of the human AS promoter, and together they accounted for virtually all (98%) of the promoter activity in vivo.

Generalizations drawn from studies of Sp1-responsive promoters indicate that the ability of a given Sp1 site to activate transcription correlates with its affinity for Sp1 and with its proximity to the transcription initiation site (20). Based on two lines of evidence, our data show that the affinity for Sp1 in vitro can positively correlate with transcription rates in vivo. The highest-affinity Sp1-binding site in the AS promoter is site 2, and this site contributes more to the full promoter activity than does either site 1 or site 3, both of which have low affinity for Sp1. Moreover, base changes in either site 2 or site 3 which increase Sp1 binding in vitro result in higher transcription rates in vivo. Thus, these results support the general conclusion that a higher affinity for Sp1 in vitro results in a higher transcription rate in vivo.

However, we would like to emphasize that this need not always be the case, as is clearly illustrated by our finding that a base change in site 1 of the human AS promoter which increases its affinity for Sp1 in vitro did not result in a higher transcription rate in vivo. In fact, this base change produced a threefold decrease in AS promoter activity in vivo. We speculate that this result might be due to the close proximity of sites 1 and 2 (3 bases apart) in the human AS promoter. It may be that increasing the occupancy of Sp1 at site 1 alters the nature of protein-DNA interaction at site 2 in a way that results in a lower in vivo transcription rate. There is a precedent, for example, for steric interference between proximal Sp1-binding sites in the SV40 early promoter (13). Although the basis of this unexpected result is unknown, this finding has important implications, since mutations in Sp1-binding sites have been associated with specific disease states in humans. For example, hereditary persistence of fetal hemoglobin is a disorder in humans in which one of the two fetal globin genes is expressed in adulthood, when these genes are normally inactive (4, 5, 33, 36). This phenotype has been correlated with mutations in potential Sp1-binding sites

in the promoters of the fetal gamma globin genes (4, 5, 14). Indeed, recent studies have demonstrated that naturally occurring mutations in the gamma globin promoters which are associated with hereditary persistence of fetal hemoglobin result in more avid binding of Sp1 in vitro (33, 36). Thus, in this one example, a positive correlation between increased Sp1 binding in vitro and increased gene expression in vivo has been established. However, the results presented in this study clearly demonstrate the importance of extending in vitro observations to the in vivo context.

A novel result of this study is that proximal Sp1-binding sites can interact synergistically in vivo to activate transcription in a context that closely resembles that of a single-copy cellular gene. Although synergism has been described previously for Sp1, these studies explored the ability of Sp1 to act synergistically with other transcription factors or used artificial combinations of *cis*-acting sequences (6, 34, 35). Our results confirm and extend the findings of others by showing that human Sp1 can synergistically activate transcription when its binding sites are in their native promoter context, at near-normal gene copy numbers, and when the DNA is assembled into bona fide chromatin. This was clearly illustrated by the observation that the in vivo activity of the wild-type AS promoter, which has all three Sp1 sites in the same promoter, is eight times greater than the sum of the individual site activities. This synergism was measurable but less dramatic (twofold) when only two sites appeared in the same promoter. This synergistic activation suggests that protein-protein interactions involving bound Sp1 monomers, by themselves or in combination with other proteins, are critical to establish full activation of transcription in vivo. The ability to activate transcription synergistically may be the reason that many cellular promoters have multiple Sp1-binding sites arranged in tandem and in close proximity.

The ability of Sp1 to activate transcription synergistically could be the result of cooperative binding of Sp1 monomers to DNA or due to interactions through its transcriptional activation domain. Cooperative binding to DNA has been observed with GAL4 (15), enhancer-binding proteins (31), and steroid hormone receptors (37). Courey et al. concluded that the synergistic ability of Sp1 is mediated through its activation domain, since mutants of Sp1 that are unable to bind DNA can still increase transcription when accompanied by a wild-type Sp1 protein (6). This phenomenon was referred to as superactivation. We believe that the mechanism of synergistic activation of the human AS promoter by Sp1 is likely to be similar to the mechanism of superactivation and that it is probably due to synergistic interactions at the level of activation rather than to cooperative binding of Sp1 monomers to DNA. The two sites at which one might expect to find cooperative Sp1 binding are at sites 1 and 2 in the AS promoter, since these sites are in close proximity. However, we find that the degree of Sp1 protection of the low-affinity site (site 1) in DNase I footprinting experiments is identical whether or not site 2 is present (compare Fig. 2A and 3B). The fact that Sp1 occupancy at site 2 does not improve protection at site 1 is inconsistent with cooperative binding. However, sites 1 and 2 do act synergistically in vivo to activate the human AS promoter (Fig. 6). Thus as previously proposed by Courey et al. (6), the ability of Sp1 to activate transcription may involve a protein surface that interacts with other proteins of the general transcription machinery, and this interaction may be optimal when multiple Sp1 molecules are present in the in vivo transcription complex. Evidence supporting such a proposal for the yeast activator GAL4 was recently obtained by Ptashne and colleagues (3, 26).

ACKNOWLEDGMENTS

We are indebted to A. Courey and S. Jackson in the Tjian laboratory for providing affinity-purified Sp1 protein and anti-Sp1 antiserum and for technical advice. We thank B.-S. Yang and R. Pogulis for comments on the manuscript.

G.M.A. was supported in part by a genetics training grant fellowship awarded to the University of Michigan. This work was supported in part by an American Cancer Society grant awarded to S.O.F.

REFERENCES

1. **Boyce, F., G. M. Anderson, C. Rusk, and S. Freytag.** 1986. Human argininosuccinate synthetase minigenes are subject to arginine-mediated repression but to *trans* induction. Mol. Cell. Biol. **6**:1244–1252.
2. **Briggs, M., J. Kadonaga, S. Bell, and R. Tjian.** 1986. Purification and biochemical characterization of the promoter-specific transcription factor Sp1. Science **234**:47–52.
3. **Carey, M., Y.-S. Lin, M. Green, and M. Ptashne.** 1990. A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. Nature (London) **345**:361–364.
4. **Collins, F., C. Boehm, P. Waber, C. Stoeckert, S. Weissman, B. Forget, and H. Kazazian.** 1984. Concordance of a point mutation 5′ to the ^{G}gamma globin gene with ^{G}gamma beta^{+} hereditary persistence of fetal hemoglobin in the black population. Blood **64**:1292–1296.
5. **Collins, F., C. Stoeckert, G. Sergeant, B. Forget, and S. Weissman.** 1984. ^{G}Gamma beta^{+} hereditary persistence of fetal hemoglobin: cosmid cloning and identification of a specific mutation 5′ to the ^{G}gamma gene. Proc. Natl. Acad. Sci. **81**:4894–4898.
6. **Courey, A., D. Holtzman, S. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59**:827–836.
7. **Courey, A., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55**:887–898.
8. **Crothers, D.** 1987. Gel electrophoresis of protein-DNA complexes. Nature (London) **325**:464–465.
9. **Dignam, J., R. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **5**:1475–1489.
10. **Dynan, W., S. Sazer, R. Tjian, and R. Schimke.** 1986. Transcription factor Sp1 recognizes a DNA sequence in the mouse dihydrofolate reductase promoter. Nature (London) **337**:573–576.
11. **Dynan, W., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35**:79–87.
12. **Gidoni, D., W. Dynan, and R. Tjian.** 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature (London) **312**:409–413.
13. **Gidoni, D., J. Kadonaga, H. Barrera-Saldana, K. Takahashi, P. Chambon, and R. Tjian.** 1985. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. Science **230**:511–517.
14. **Gilman, J. N. Mishima, X. Wen, F. Kutlar, and T. Huisman.** 1988. Upstream mutations associated with a modest elevation of fetal hemoglobin expression in human adults. Blood **72**:78–81.
15. **Giniger, E., and M. Ptashne.** 1988. Cooperative DNA binding of the yeast transcriptional activator GAL4. Proc. Natl. Acad. Sci. USA **85**:382–386.
16. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
17. **Harrich, D., J. Garcia, F. Wu, R. Mitsuyasu, J. Gonzalez, and R. Gaynor.** 1989. Role of Sp1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **63**:2585–2591.

18. **Ishii, S., R. Kadonaga, J. Brady, G. Merlino, and I. Pastan.** 1986. Binding of the Sp1 transcription factor by the human Harvey ras 1 proto-oncogene promoter. Science **232**:1410–1413.
19. **Jones, K., J. Kadonaga, P. Luciw, and R. Tjian.** Activation of the AIDS retrovirus promoter by the cellular transcription factor Sp1. Science **232**:755–759.
20. **Kadonaga, J., K. Jones, and R. Tjian.** 1986. Promoter-specific activation of RNA polymerase II transcription by Sp1. Trends Biochem. Sci. **11**:20–23.
21. **Kagemaya, K., and I. Pastan.** 1989. Molecular cloning and characterization of a human DNA binding factor that represses transcription. Cell **59**:815–825.
22. **Karin, M., A. Haslinger, H. Holtgreve, R. Richards, P. Krauter, H. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature (London) **308**:513–519.
23. **Kim, C., C. Heath, A. Bertuch, and U. Hansen.** 1987. Specific stimulation of a simian virus 40 late transcription in vitro by a cellular factor binding the simian virus 40 21-base-pair repeat promoter element. Proc. Natl. Acad. Sci. USA **84**:6025–6029.
24. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
25. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) **325**:369–372.
26. **Lin, Young-Sun, M. Carey, M. Ptashne, and M. Green.** 1990. How different eucaryotic transcriptional activators can cooperate promiscuously. Nature (London) **345**:359–360.
27. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46**:795–805.
29. **Mitchell, P., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
30. **Parker, B., and G. Stark.** 1979. Regulation of SV40 transcription: sensitive analysis of the RNA species present early in infection by virus or virus DNA. J. Virol. **31**:360–369.
31. **Poellinger, L., B. Yoza, and R. Roeder.** 1989. Functional cooperativity between protein molecules bound at two distinct sequence elements of the immunoglobulin heavy-chain promoter. Nature (London) **337**:573–576.
32. **Ptashne, M.** 1988. How eucaryotic transcriptional activators work. Nature (London) **335**:683–689.
33. **Ronchi, A., S. Nicolis, C. Santoro, and S. Ottolenghi.** 1989. Increased Sp1 binding mediates erythroid-specific overexpression of a mutated (HPFH)-gamma globin promoter. Nucleic Acids Res. **17**:10231–10241.
34. **Schule, R., M. Muller, C. Kaltschmidt, and R. Renkawitz.** 1988. Many transcription factors interact synergistically with glucocorticoid receptors. Science **242**:1418–1420.
35. **Strahle, U., W. Schmid, and B. Schutz.** 1988. Synergistic interaction of the glucocorticoid receptor with transcription factors. EMBO J. **7**:3389–3395.
36. **Sykes, K., and R. Kaufman.** 1990. A naturally occurring gamma globin gene mutation enhances Sp1 binding activity. Mol. Cell. Biol. **10**:95–102.
37. **Tsai, S., M. Tsai, and B. O'Malley.** 1989. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. Cell **57**:443–448.