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Sp1-mediated transcriptional activation is repressed by Sp3

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Sp1, Sp3 (SPR-2) and Sp4 (SPR-1) are human sequence-specific DNA binding proteins with very similar structural features. In this report, we have analyzed Sp3 in direct comparison with Sp1. We have raised antibodies against both Sp1 and Sp3, and show that Sp3 protein, like Sp1, is expressed in various cell lines. Co-transfection experiments in different mammalian cell lines reveal that in contrast to Sp1 and Sp4, Sp3 is not able to activate several Sp1 responsive promoters. In addition, Sp3 also fails to activate reporter constructs in *Drosophila* SL2 cells lacking endogenous Sp factors. Instead, we find that Sp3 represses Sp1-mediated activation in a linear dose-dependent manner. A mutant of Sp3 lacking the DNA binding domain does not affect activation by Sp1, suggesting that the inhibition is most likely due to the competition with Sp1 for their common binding sites. To determine if any structurally similar domain of Sp3 is able to replace partially homologous domains of Sp1, we have generated chimeric proteins and tested their activation characteristics in gene transfer experiments. It appears that neither the glutamine-rich domains A and B nor the D domain of Sp1 can be replaced by the homologous regions of Sp3. Our results suggest that Sp3 is an inhibitory member of the Sp family.
Key words: repression/Sp1/Sp3/Sp4/transcription factor

Introduction

Transcriptional regulation is exerted by the combinatorial action of proteins binding to distinct promoter and enhancer elements. In the last few years, it has become evident that a limited number of *cis*-acting DNA elements are recognized not only by single factors, but by a set of different families of transcription factors, thereby acting positively or negatively on transcription (Latchman, 1991). One of the most widely distributed promoter elements are GC boxes and related motifs bound by Sp1 (Kadonaga *et al.*, 1987). In contrast to other promoter elements, it has long been thought that Sp1 is the only factor acting through these motifs.

Recently, we have cloned by recognition site screening two human factors homologous to Sp1 which we originally designated as SPR-1 and SPR-2 (Hagen *et al.*, 1992). Independently, another group has also reported cDNA clones homologous to Sp1 (Kingsley and Winoto, 1992). They have isolated two factors, Sp2 and Sp3, by low-stringency screening with the Sp1 zinc finger region. Sequence comparison revealed that their Sp3 clone is identical to SPR-2. To avoid confusion due to different nomenclatures, we renamed SPR-2 and SPR-1, which we now refer to as Sp3 and Sp4, respectively.

Northern blot analysis revealed that Sp3 mRNA is expressed ubiquitously, although the relative amount of transcripts differs moderately between various human cell lines and in mouse organs. Sp4 transcripts are also found in many cell lines, but *in vivo* the mRNA is abundant in brain and barely detectable in other organs (Hagen *et al.*, 1992).

Sp1, Sp3 and Sp4 are closely related members of a gene family encoding proteins with very similar structural features. Most significantly, the DNA binding domains of Sp1, Sp3 and Sp4 are highly conserved, and bacterially expressed subfragments recognize the GC box (GGGGCGGGC) and the GT motif (GGGTGTGGC) with identical affinities (Hagen *et al.*, 1992). Moreover, other regions of Sp3 and Sp4 also show significant homologies to Sp1 (Figure 1). All three proteins contain glutamine- and serine/threonine-rich amino acid stretches. The high degree of structural conservation between Sp1, Sp3 and Sp4 suggests that Sp3 and Sp4 are also transcriptional regulators acting through binding to the same DNA elements.

The glutamine-rich domains of Sp1 have been identified as transactivation domains (Courey and Tjian, 1988). In addition to the glutamine-rich regions A and B in the N-terminal part of the molecule, regions adjacent to the DNA binding domain (C and D) also contribute to the activation properties of Sp1 (Figure 1). Region C has only a very weak influence on activation (Courey and Tjian, 1988), whereas the most C-terminal part of Sp1 (region D) plays a key role in the ability of Sp1 to activate transcription synergistically from two adjacent binding

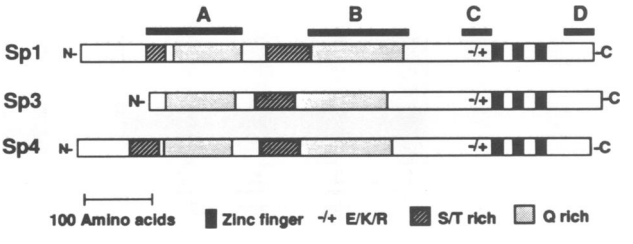

Fig. 1. Structural features of Sp3 and Sp4 in comparison with Sp1. Each differently filled box refers to the region containing a preponderance of one or several types of amino acids, which are indicated at the bottom using the single-letter code. The black lines above the drawing of Sp1 indicate the extent of the four activation domains (A–D) as defined by Courey and Tjian (1988).

sites. A Sp1 mutant lacking the D domain activates transcription from a single Sp binding site comparable to the wild-type (simple activation), but is not able to activate synergistically (strongly enhanced activation through two Sp binding sites; Pascal and Tjian, 1991). However, it should be noted that the glutamine-rich region A of Sp1 is also necessary for synergistic activation (Pascal and Tjian, 1991).

In this paper, we have embarked on a detailed analysis of the Sp3 protein in direct comparison with Sp1. We have raised specific antibodies against Sp3 and Sp1, and demonstrate that both proteins are abundant in various cell lines. In addition, we studied the basic functional characteristics of Sp3. For this, we have used co-transfection assays into mammalian cell lines. We have also used insect cells lacking endogenous Sp1-like activity to assess functional properties of Sp3 under defined conditions. Our results show that Sp3 represses Sp1-mediated activation. A mutant of Sp3 lacking its DNA binding domain does not inhibit Sp1 activity, suggesting that the inhibitory effect of Sp3 is due to its competition for Sp1 binding sites. Moreover, we have generated chimeric proteins of Sp1 and Sp3, and tested their activation characteristics in gene transfer experiments. Neither the glutamine-rich domains A and B nor the D domain of Sp1 can be replaced by the homologous regions of Sp3. Our results suggest that Sp3 is involved in regulating gene activity through repression of Sp1-mediated transcriptional activation *in vivo*.

Results

Characterization of antibodies against Sp3 and Sp1

Previously, we have shown that like Sp1 mRNA, Sp3 mRNA is expressed ubiquitously and that the expression level of Sp3 varies only moderately in different cell lines (Hagen *et al.*, 1992). To test whether Sp3 and Sp1 proteins are also present in those cell lines, we have raised antibodies against bacterially expressed Sp1 and Sp3 (see Materials and methods), and have performed Western blot analysis with bacterially expressed Sp1 and Sp3. In these experiments, the antibodies against Sp3 did not cross-react with bacterially expressed Sp1 and vice versa (data not shown). In band-shift assays, bacterially expressed Sp3 generated a unique complex. This complex was supershifted by anti-Sp3 antibodies, but not by anti-Sp1 antibodies (Figure 2). Bacterially expressed full-length Sp1 produced three different complexes (Figure 2). Probably, the two faster migrating complexes are due to proteolytic degradation products of Sp1. Nevertheless, all three complexes were recognized by the anti-Sp1 antiserum. The anti-Sp3 antibodies did not influence the migration of the two faster migrating complexes, but slightly interfered with the slow-migrating complex. However, it should be noted that bacterially expressed Sp1 and Sp3 have 14 amino-terminal amino acids in common which derive from the expression vector (Studier *et al.*, 1989). Thus, it seems very likely that the reaction of the anti-Sp3 antibodies with the slow-migrating Sp1 complex is due to antibodies directed against this artificial amino-terminal sequence present in the high molecular weight complex. In addition, both antisera did not cross-react with bacterially expressed Sp4 (Figure 2). Note that in all reactions that contained antisera, including the control reaction with a pre-immune serum, an additional complex could be seen. This unspecific complex reflects the binding of a serum protein to the oligonucleotide because it was also obtained in the absence of nuclear extract (see Figure 2, lane 1).

Sp3 is abundant in mammalian cell lines and binds to GC and GT boxes

In Figure 3A, immunoblot analyses of nuclear extracts prepared from HeLa cells are shown. As expected, the

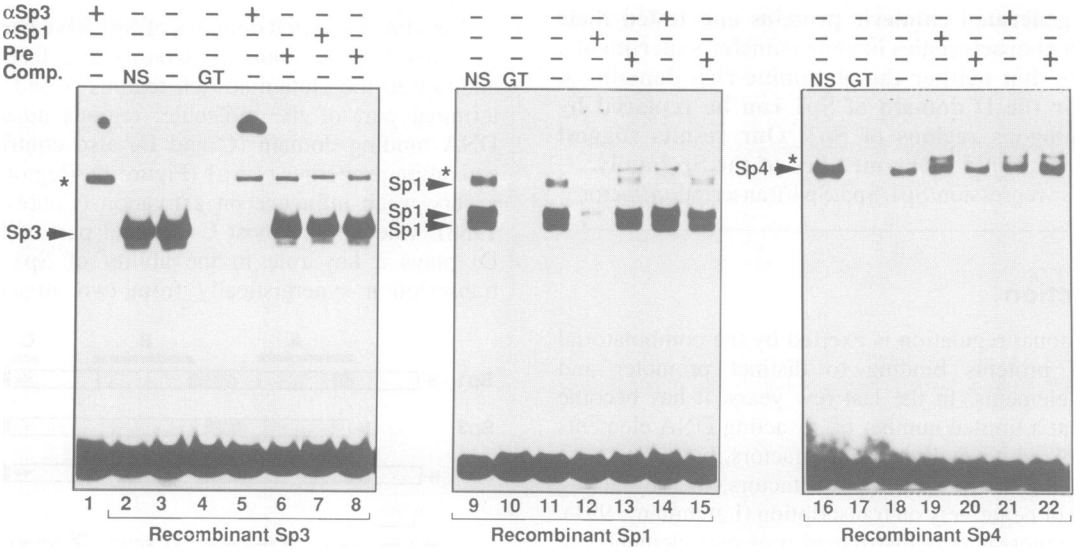

Fig. 2. Characterization of antibodies against Sp1 and Sp3. Electrophoretic mobility shift analyses (EMSAs) were performed with 0.1 ng labeled GT oligonucleotide and bacterial extracts containing Sp3 (lanes 2–8), Sp1 (lanes 9–15) and Sp4 (lanes 16–22). Antisera against Sp3 (αSp3; lanes 1, 5, 14, 21), Sp1 (αSp1; lanes 7, 12, 19) and the appropriate pre-immune sera (Pre; lanes 6, 8, 13, 15, 20, 22) were included in the binding reactions as indicated at the top. Competitions (Comp.) were performed with a 50-fold excess of specific (GT; lanes 4, 10, 17) and an unspecific (NS; lanes 2, 9, 16) oligonucleotide. Lane 1, labeled GT oligonucleotide was incubated with anti-Sp3 antibodies in the absence of bacterial extract. Sp complexes are indicated by arrows at the left of the panels. The unspecific complex which is seen already with the sera alone is indicated by an asterisk.

anti-Sp1 antiserum specifically reacted with two polypeptides of ~105 and 95 kDa in size. These two polypeptides are characteristic for Sp1 and arise from differential phosphorylation (Jackson *et al.*, 1990). The slower migrating species very likely reflects the highly phosphorylated form of Sp1 (Jackson *et al.*, 1990). The anti-Sp3 antiserum specifically recognized 97, 60 and 58 kDa polypeptides. At this stage, we do not know whether these polypeptides are due to degradation products of Sp3 or reflect different gene products (see Discussion).

To quantitate the relative amount of Sp1 and Sp3 in different cell lines, and to test if the different polypeptides reacting with the anti-Sp3 antibodies can recognize DNA, we have performed band-shift assays with crude nuclear extracts from Ishikawa, HeLa and CV-1 cells. As DNA probe, we used an oligonucleotide which contains the GT motif of the uteroglobin promoter (Suske *et al.*, 1992). This motif is bound by bacterially expressed Sp1 and Sp3 with identical affinities (Hagen *et al.*, 1992). In high-resolution gels, two strong slow-migrating and two weaker fast-migrating complexes were observed (Figure 3B) in all three cell lines. All four bands were specifically competed with oligonucleotides containing the GT or the GC motif (Figure 3B, lanes 5–11), respectively, but not with an unspecific oligonucleotide (Figure 3B, lanes 12–14). In the presence of an antiserum against Sp1, the slowest migrating complex disappeared. When the anti-Sp3 antiserum was present in the binding reaction, the other three complexes were shifted, but the slowest migration complex was unaltered. Thus, the generation of three Sp3-specific complexes coincides with the appearance of three bands in Western blots with the anti-Sp3 serum. The three complexes shifted with the anti-Sp3 antibody probably reflect DNA–protein complexes with the 97, 60 and 58 kDa polypeptides. If antisera against Sp1 and Sp3 were present in the binding reaction, all four bands disappeared (Figure 3B, lanes 4, 18, 25). There was a remaining weak band which migrated at essentially the same position as the Sp1–DNA complex. This complex may arise from endogenous Sp4.

Our band-shift data demonstrate that Sp1 and Sp3 proteins are present in Ishikawa, CV-1 and HeLa cells, and that the relative amounts of the two proteins vary moderately between these cell lines. In addition, the competition experiments show further that both proteins bind to the GT and the GC motif with very similar, if not identical, affinities, as shown previously with bacterially expressed subfragments of Sp1 and Sp3 (Hagen *et al.*, 1992).

Sp1 and Sp4 but not Sp3 activate different reporter constructs

To test the activation potential of Sp3 in direct comparison with Sp1 and Sp4, we constructed expression plasmids for human Sp1, rat Sp1, Sp3 and Sp4 by fusing the appropriate cDNAs to the strong cytomegalovirus (CMV) promoter/enhancer (CMV-hSp1, CMV-rSp1, CMV-Sp3 and CMV-Sp4). As reporter constructs we have used pUGCATSV (Suske *et al.*, 1992). In this plasmid the CAT gene is driven by the uteroglobin promoter and the vector also contains the SV40 enhancer 3′ to the CAT gene (Figure 4A). The uteroglobin promoter does not contain a

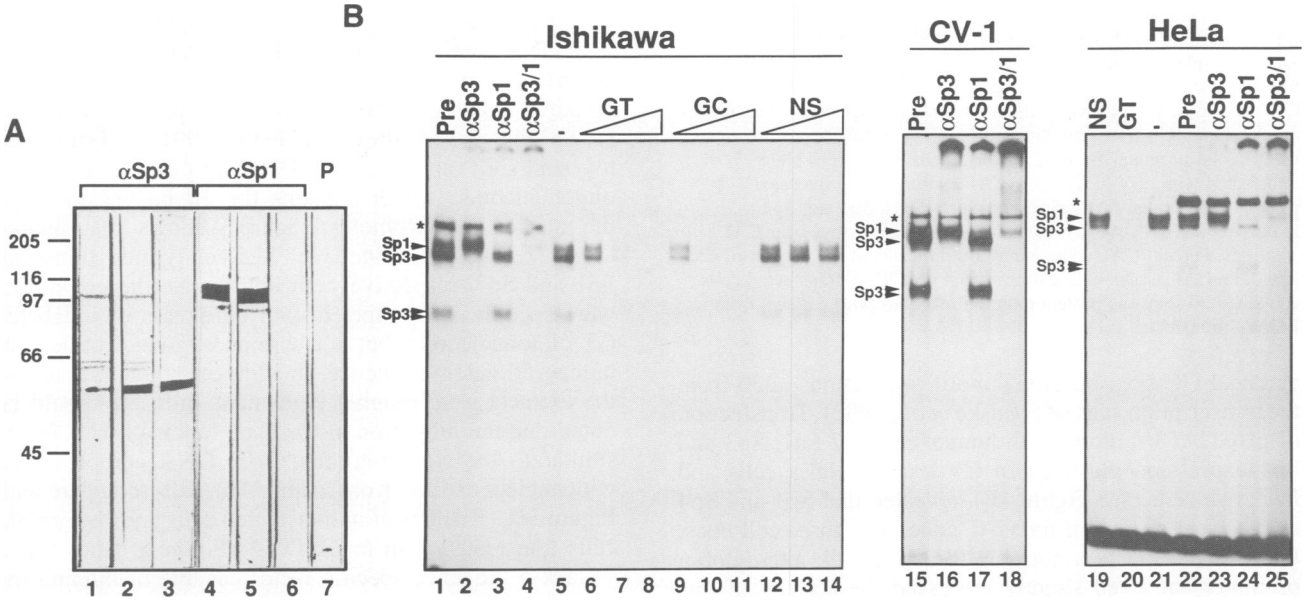

Fig. 3. Sp1 and Sp3 are abundant in various cell lines. (**A**) Immunoblot detection of Sp1 and Sp3 in HeLa cells. A nuclear extract from HeLa cells was fractionated on a 7.5% SDS–polyacrylamide gel, blotted on nitrocellulose filter and incubated with antisera against Sp3 (αSp3; lanes 1–3), Sp1 (αSp1; lanes 4–6) and the pre-immune serum of αSp1 (P; lane 7). The antisera to Sp3 and Sp1 were used without pre-treatment (lanes 1 and 4) or either pre-incubated with recombinant Sp1 (lanes 2 and 6) or Sp3 (lanes 3 and 5). (**B**) Gel-retardation analysis of nuclear extracts from Ishikawa, CV-1 and HeLa cells. Crude nuclear extracts (3 μg protein) from these cell lines were incubated with labeled GT oligonucleotide in the absence (lanes 1–5, 15–18, 21–25) and the presence of various amounts (2-, 5- and 50-fold molar excess) of unlabeled GT (lanes 6–8, 20), unlabeled GC (9–11) or non-specific (NS; lanes 12–14, 19) oligonucleotides. One microliter of pre-immune serum (Pre; lanes 1, 15, 22), serum against Sp3 (αSp3; lanes 2, 16, 23), serum against Sp1 (αSp1; lanes 3, 17, 24) or a mixture of sera against Sp1 and Sp3 (αSp3/1; lanes 4, 18, 25) was included in the binding reactions as indicated at the top. Specific complexes for Sp1 and Sp3 are indicated on the left. The non-specific complex which is also seen with the pre-immune serum is depicted by an asterisk.

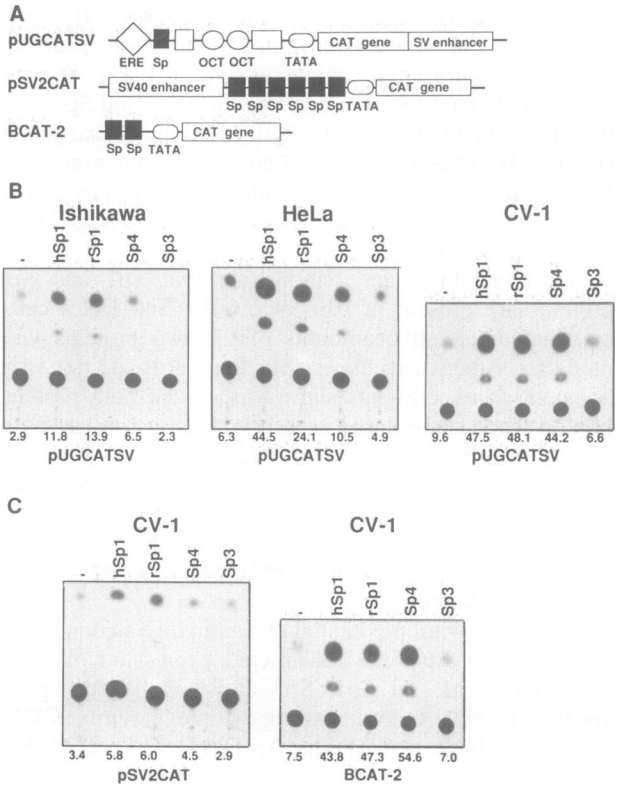

Fig. 4. Transactivation of different reporter constructs in mammalian cell lines by Sp1, Sp3 and Sp4. (**A**) Schematic illustration of the reporter plasmids pUGCATSV (uteroglobin promoter and SV40 enhancer; Suske *et al.*, 1992), pSV2CAT (SV40 promoter/enhancer; Gorman *et al.*, 1982) and BCAT-2 (two Sp1 binding sites and the E1b TATA box; Pascal and Tjian, 1991). (**B**) Transactivation of the uteroglobin promoter in different cell lines. Ishikawa, HeLa and CV-1 cells were transfected with 8 μg pUGCATSV along with 2 μg expression plasmids for human Sp1 (hSp1), rat Sp1 (rSp1), Sp4 and Sp3 as indicated. The cells were subsequently lysed and assayed for CAT activities. CAT conversions in percent are indicated at the bottom of each lane. (**C**) Transactivation of the SV40 enhancer/promoter and the artificial promoter BCAT-2 in CV-1 cells. CV-1 cells were transfected with pSV2CAT and BCAT-2 along with expression plasmids for human Sp1 (hSp1), rat Sp1 (rSp1), Sp4 and Sp3 as indicated. The cells were subsequently lysed and assayed for CAT activities. Percent CAT conversions are indicated at the bottom of each lane. The CAT assays with extracts from pSV2CAT transfected cells contained 20-fold less protein than the reactions with extracts from BCAT-2 transfected cells.

classical GC box, but a GT motif at position −230 from the transcription start site (Suske *et al.*, 1992). Transfection of pUGCATSV, along with human Sp1, rat Sp1, Sp3 and Sp4 expression plasmids in CV-1 cells, HeLa cells and Ishikawa cells (see Figure 4B), revealed that Sp1 and Sp4 enhance expression of the CAT gene in all three cell lines. In contrast, Sp3 was not able to activate the uteroglobin promoter and even slightly repressed the activity of this construct.

Similar results were obtained when we used other reporter plasmids (Figure 4C). pSV2CAT contains the SV40 enhancer/promoter region including the 'classical' six GC boxes. Expression of the CAT gene in pSV2CAT was stimulated weakly by co-transfected human and rat Sp1, but not by Sp3. Moreover, the reporter plasmid BCAT-2, a simple promoter construct which contains only two Sp1 binding sites from the HTLV promoter fused to the E1B TATA box (Pascal and Tjian, 1991), was also stimulated by Sp1 and Sp4, but not by Sp3.

Sp3 is expressed in transfected Drosophila Schneider cells

All mammalian cell lines we have tested contain endogenous Sp1 and Sp3. These endogenous Sp proteins, as well as other factors present in these cell lines, may obscure activities mediated by transfected Sp3. In addition, differences in the efficiency of transfection cannot be normalized by co-transfecting RSV-βgal or SV40-βgal because the activities of both promoters are influenced by co-transfected Sp1 (Saffer *et al.*, 1990). As far as we know, there exists no strong promoter whose activity is independent of Sp1 in transfection experiments. Thus, we could not study unambiguously the functional activity of transfected Sp1 and Sp3. Therefore, we decided to use *Drosophila* SL2 cells (Schneider cells) as a host for the functional analysis of Sp3. SL2 cells are particularly suited for this task because they are devoid of endogenous Sp1-like activity and have been utilized successfully in co-transfection assays to analyze Sp1 *in vivo* (Courey and Tjian, 1988; Courey *et al.*, 1989; Pascal and Tjian, 1991). An expression vector for Sp3 (pPacSp3) was constructed by fusing the appropriate cDNA to the *Drosophila melanogaster* actin 5C promoter. The expression vector for Sp1 (pPacSp1) has been described previously (Courey and Tjian, 1988).

First, we wished to examine whether Sp1 and Sp3 are expressed at comparable levels in SL2 cells after transfection. Immunoblot analysis of transfected cells is not useful for this purpose because different signal intensities obtained with different antisera would not necessarily reflect differences in the amount of protein. We therefore used DNA binding for quantitating the transient expression of Sp1 and Sp3 in SL2 cells. Crude nuclear extracts were prepared from cells transfected with expression plasmids for Sp1 and Sp3 (Andrews and Faller, 1991), and subjected to band-shift analysis. As DNA probe, we used the oligonucleotide which contains the 10 bp GT motif of the uteroglobin promoter. Figure 5 shows that unique complexes were generated with SL2 cell lysates containing Sp1 and Sp3, respectively. In both cases, the complexes were specifically competed with an excess of unlabeled GT oligonucleotide, but not with an oligonucleotide containing a random sequence. In addition, equal amounts of the extracts gave roughly equivalent shifts. It should be noted that the migration of the band obtained with Sp3 is similar to the slow-migrating Sp3−DNA complex seen with nuclear extracts from mammalian cells (compare with Figure 3B). Extracts of untransfected cells and *Drosophila* cells transfected with mock DNA (Figure 5, lanes 6 and 7) lack a sequence-specific factor capable of binding the GT motif. This experiment demonstrates that Sp1 and Sp3 are expressed with comparable efficiency in transfected SL2 cells.

Sp3 represses Sp1-mediated activation

To test the potential transcriptional activity of Sp3 in direct comparison with Sp1, we co-transfected expression constructs for Sp1 and Sp3 together with BCAT-2 as test promoter construct (see Figure 4A). This reporter construct

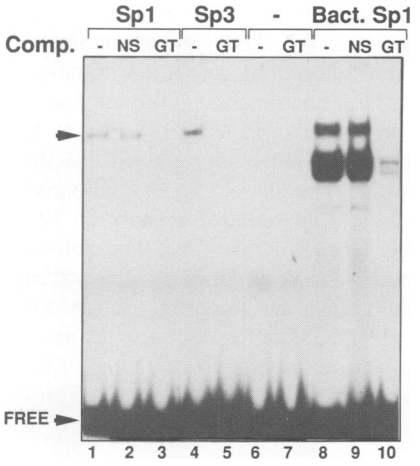

Fig. 5. Transient expression of Sp1 and Sp3 proteins in SL2 cells. Gel-retardation assays were performed with crude nuclear extracts from SL2 cells. Cells were transfected with 8 μg BCAT-2 along with 2 μg pPacSp1 (lanes 1–3), 2 μg pPacSp3 (lanes 4 and 5) and 2 μg vector (pPac) (lanes 6 and 7). In lanes 8–10, a bacterial extract containing Sp1 was used as control (Hagen *et al.*, 1992). All reactions contained 0.1 ng of labeled GT oligonucleotide. In lanes 3, 5, 7 and 10, a 50-fold molar excess of unlabeled GT oligonucleotide (GT) and in lanes 2 and 9, a 50-fold molar excess of a non-specific oligonucleotide (NS), was included in the binding reaction. Arrows indicate the free oligonucleotide and specifically retarded protein–DNA complexes, respectively.

has been successfully used to characterize activation domains of Sp1 (Pascal and Tjian, 1991) in SL2 cells. As expected, Sp1 activated the promoter in BCAT-2 several hundred-fold. In contrast, Sp3 did not activate BCAT-2. Thus, although Sp3 and Sp1 recognize the same DNA motifs *in vitro*, and both proteins are expressed equally in transfected SL2 cells, Sp3 is not able to activate BCAT-2. Given these observations, one could expect that Sp3 might prevent Sp1 activation in co-transfection experiments by competing for their common binding sites.

Before carrying out co-transfection assays with expression plasmids for Sp1 and Sp3, we titrated the Sp1 expression vector pPacSp1. A constant amount of the reporter plasmid BCAT-2 was transfected into Schneider cells along with 2, 20, 200 and 2000 ng of the Sp1 expression vector pPacSp1. The CAT activity of the resulting extracts was plotted as a function of the amount of pPacSp1 used to transfect the cells (Figure 6A). The activation of BCAT-2 by Sp1 increased rapidly between 2 and 20 ng of transfected expression plasmid pPacSp1. Higher amounts of the Sp1 expression plasmid further increased CAT activity, but with 200 ng of plasmid CAT gene expression had almost reached a plateau. Note that increasing amounts of pPacSp3 did not result in any enhanced CAT gene expression.

Next, we co-transfected Sp1 and Sp3 expression plasmids along with BCAT-2. Figure 6B shows the results of a series of co-transfections with constant amounts of pPacSp1 and increasing amounts of pPacSp3. Activation of BCAT-2 by Sp1 was repressed by co-transfected Sp3 in a linear, dose-dependent manner. This became most obvious when we used 20 ng pPacSp1 and increasing amounts of pPacSp3. Under these conditions, Sp1-mediated activation was repressed almost completely with 2000 ng of pPacSp3 (Figure 6B).

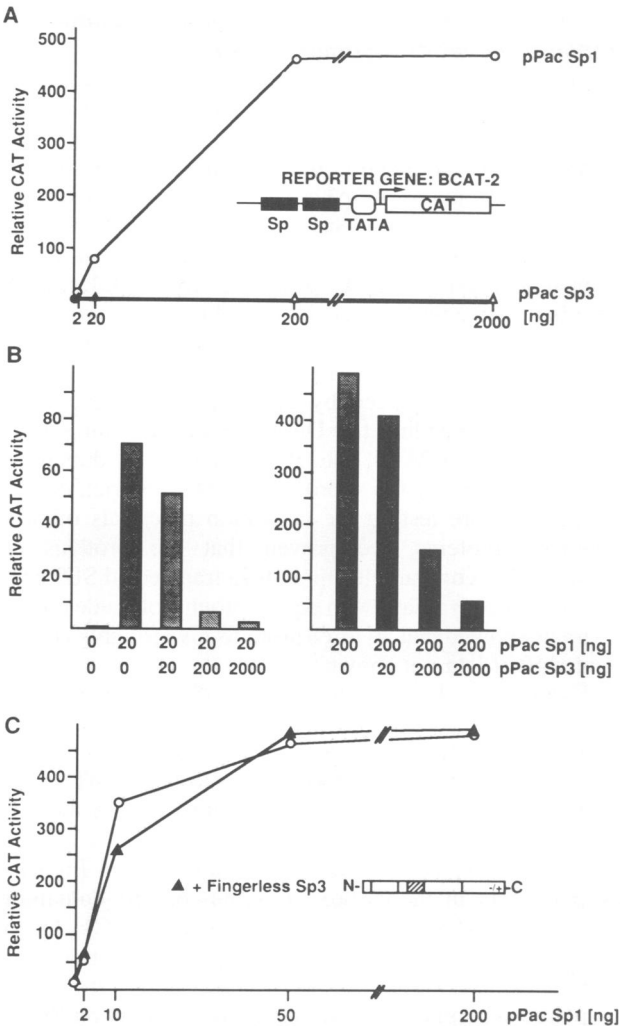

Fig. 6. Activation properties of Sp1 and Sp3 in SL2 cells. (**A**) Sp1, but not Sp3, activates transcription of BCAT-2. Eight micrograms of the reporter plasmid BCAT-2 were transfected in SL2 cells along with variable amounts of pPacSp1 and pPacSp3, as indicated. The cells were subsequently lysed and CAT activities determined as described in Materials and methods. (**B**) Sp3 represses Sp1-mediated transactivation. Eight micrograms of BCAT-2 were transfected along with constant amounts of pPacSp1 (20 or 200 ng) and increasing amounts of pPacSp3 (2, 20, 200 and 2000 ng). The cells were subsequently lysed and assayed for CAT activities. (**C**) Repression of Sp1-mediated activation by Sp3 requires the DNA binding domain. Eight micrograms of BCAT-2 were transfected with increasing amounts of pPacSp1 in the absence (open circles) and presence (solid triangles) of 1 μg of an expression plasmid for a fingerless Sp3 mutant (pPacSp3-DBD).

Next, we prompted the question whether DNA binding of Sp3 is indeed a prerequisite for inhibition of Sp1-mediated activation. We constructed an expression plasmid for a carboxy-terminal mutant of Sp3, which lacks the DNA binding domain, and co-transfected a constant amount of this fingerless Sp3 variant along with increasing amounts of Sp1. Fingerless Sp3 did not influence Sp1-mediated activation (Figure 6C). This observation strongly supports the idea that the inhibitory effect of Sp3 on transcriptional activation by Sp1 is due to the competition of both proteins for their common binding sites and does not reflect specific protein–protein interactions.

Homologous domains of Sp3 cannot functionally replace activation domains of Sp1

Although Sp1 and Sp3 appear to be structurally very similar, our transfection experiments show that they exhibit strong functional differences and that Sp3 may be considered as a repressor. To determine whether structurally similar domains of Sp3 are able to replace functionally homologous domains of Sp1, we generated three Sp1/Sp3 chimeric constructs (Figure 7) and fused them to the *D.melanogaster* actin 5C promoter. In PPAC1, the N-terminal glutamine-rich activation domains A and B of Sp1 are fused to the C-terminus of Sp3 containing the DNA binding domain of Sp3. In PPAC3, the C-terminal part of Sp3 is replaced by the homologous C-terminal part of Sp1 encoding the DNA binding domain and the D domain. In PPAC4, the glutamine-rich A domain of Sp1 is replaced by the appropriate glutamine-rich domain of Sp3. Before testing the activation potentials of these chimeric proteins, we proved that the proteins are expressed in comparable amounts in transfected SL2 cells. Gel-retardation assays with equal amounts of crude nuclear extracts showed that all three proteins give roughly equivalent shifts (data not shown).

Expression plasmids of these Sp1/Sp3 chimeras were co-transfected together with BCAT-2 into SL2 cells. To distinguish between simple and synergistic activation, we used an additional reporter construct (BCAT-1) that contains only a single Sp binding site fused to the E1B TATA box (Pascal and Tjian, 1991). The results of these co-transfection experiments are summarized in Figure 7. Replacement of the carboxy terminus of Sp1 containing the DNA binding and the D domain (in PPAC1) by the carboxy terminus of Sp3 reduced simple activation (on a reporter with a single Sp1 site) only 2-fold, but severely impaired synergistic activation (ratio of activity on BCAT-1 and BCAT-2). This result suggests that the D domain of Sp1, which is absolutely necessary for strong synergistic activation (Pascal and Tjian, 1991), cannot be replaced functionally by the equivalent domain of Sp3. Moreover, the glutamine-rich domains of Sp3 also cannot replace functionally the glutamine-rich domains of Sp1. In PPAC3, the glutamine-rich domains A and B of Sp1 are replaced by the homologous glutamine-rich domains of Sp3. This mutant protein behaved like Sp3. It activated neither BCAT-1 nor BCAT-2. Thus, the glutamine-rich domains of Sp3 do not function as activation domains under these conditions. This conclusion is further supported by the activation properties of PPAC4. In PPAC4, the glutamine-rich A domain of Sp1 is replaced by the equivalent glutamine-rich domain of Sp3. Compared with Sp1, PPAC4 had a 5-fold reduced activity on BCAT-1 and in addition the synergism was lost. The activity of PPAC4 is thus found to be very similar to the activity of an Sp1 mutant which lacks the glutamine-rich A domain (Pascal and Tjian, 1991). Taken together, the results obtained with the Sp1/Sp3 chimeras suggest that none of the domains of Sp3 can functionally replace structurally similar activation domains of Sp1.

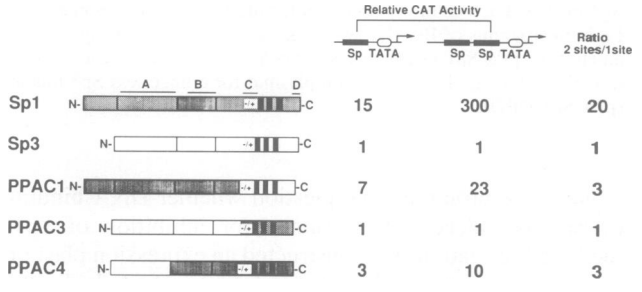

Fig. 7. Activation properties of Sp1/Sp3 chimeric proteins. Sp1, Sp3 and the chimeric proteins (PPAC1, 3, 4) are shown schematically. Black bars indicate the zinc fingers. The activation domains of Sp1 are labeled above the construct (A, B, C, D). Shaded regions of the chimeric proteins are derived from Sp1 and white regions from Sp3. Expression constructs for these proteins (200 ng) were co-transfected with either 8 μg BCAT-1 or BCAT-2 into *Drosophila* SL2 cells. The cells were subsequently lysed and assayed for CAT activities. The values represent the average of at least five independent determinations.

Discussion

Sp3 is present in nuclear extracts and binds to GT and GC boxes

Previously, we have cloned by recognition site screening with the GT1 motif two proteins (Sp3 and Sp4) which are highly homologous to the well-characterized transcription factor Sp1. In particular, the DNA binding domains of all three proteins are highly conserved and bacterially expressed subfragments of Sp1, Sp3 and Sp4 bind to GC and GT boxes with identical affinities (Hagen *et al.*, 1992). The generation of specific antibodies against Sp3 and Sp1 enabled us to explore their presence and binding characteristics in different cell lines. We show that native Sp3 and Sp1 from various cell lines bind the GC and GT box with comparable affinities. In addition, our band-shift assays demonstrate that the Sp3 protein is expressed in Ishikawa, HeLa and CV-1 cells, although at different levels. Transcripts of Sp3 have also been detected in all other cell lines tested (Hagen *et al.*, 1992; Kingsley and Winoto, 1992), as well as in all mouse organs (Hagen *et al.*, 1992). Thus, it appears that Sp3 is a ubiquitous factor.

Antibodies against Sp3 react with three distinct polypeptides

In immunoblots, three different polypeptides reacted with the anti-Sp3 serum and in band-shift assays three different complexes were observed. At this stage, we cannot exclude that the two smaller polypeptides are proteolytic degradation products of Sp3, although the relative amount of the longer and the smaller peptides did not vary between different nuclear extract preparations. Several other possibilities might also account for the presence of different polypeptides which are recognized by the antiserum against Sp3. First, they may be products of different mRNAs. Northern blot analysis does not substantiate this suggestion. Sp3 appears to be encoded by a unique transcript (Hagen *et al.*, 1992). Secondly, the anti-Sp3 serum may cross-react with yet another factor which also binds to GT and GC boxes. In addition to Sp1, Sp3 and Sp4, three other proteins, namely BTEB1, BTEB2 and EKLF, have been described which act as transcriptional activators through binding to GT/GC boxes (Imataka *et al.*, 1992; Sogawa *et al.*, 1993; Miller and Bieker, 1993). BTEB2 and EKLF mRNAs are found only in testis and in hematopoietic organs, respectively, whereas BTEB1 mRNA appears to be expressed ubiquitously. Nevertheless,

it is very unlikely that one of the smaller peptides which reacted with the anti-Sp3 antiserum is BTEB1, because the homology between Sp3 and BTEB1 is much lower compared with the homology between Sp3, Sp1 and Sp4, respectively. Thirdly, the different polypeptides reacting with the anti-Sp3 serum may reflect different translation products. This seems more likely, because *in vitro* translation of Sp3 mRNA also yielded different Sp3 products (Kingsley and Winoto, 1992). Additional experiments will be needed to clarify this issue. However, in extracts prepared from transfected Schneider cells, only the slowest migrating product is present, indicating that a unique polypeptide species is expressed in these cells.

Sp3 acts as repressor of Sp1-mediated transcription

Our functional analysis of Sp3 in direct comparison with Sp1 and Sp4 demonstrates that Sp1 and Sp4 are strong activators in mammalian cell lines, whereas Sp3 failed to activate different promoters such as the uteroglobin promoter, the SV40 promoter, as well as the artificial promoter in BCAT-2. To test if the amount of endogenous Sp1 may influence the activity of these Sp1-dependent promoter constructs, we have also performed co-transfection experiments with antisense Sp1 and Sp3 expression constructs. However, neither the antisense Sp1 construct nor the antisense Sp3 construct influenced the expression of the reporter genes (data not shown). This finding may reflect long half-life times of Sp1 and Sp3. Alternatively, the antisense Sp1 and Sp3 RNAs do not form stable hybrids with the appropriate endogenous RNAs.

Sp1 as well as Sp4 (to be published elsewhere), but not Sp3, activated different reporter constructs in the *Drosophila* Schneider cells. When Sp1 and Sp3 were co-expressed in these cells, Sp3 strongly inhibited Sp1-mediated activation. This inhibition was dependent on the presence of the DNA binding domain of Sp3. A mutant of Sp3 that lacks the C-terminal DNA binding domain did not repress Sp1-dependent transcription. Thus, it is very likely that the inhibitory effect of Sp3 is due to the competition with Sp1 for their common DNA recognition sites. Note that the fingerless mutant of Sp3 also did not enhance the synergistic activation of Sp1. In contrast, a comparable non-DNA-binding mutant of Sp1 strongly enhanced (superactivation) the synergistic transcriptional activation by the DNA-binding form of Sp1 (data not shown; Courey *et al.*, 1989; Pascal and Tjian, 1991). In addition, co-transfection of fingerless Sp1 did not render Sp3 from an inactive to an active form (data not shown).

Despite the significant homology between Sp1 and Sp3, the regions of Sp1 which are involved in activation cannot be replaced functionally by the homologous regions of Sp3. For instance, fusion of the glutamine-rich regions of Sp3 to the DNA binding domain of Sp1 did not lead to an active protein, indicating that these glutamine-rich domains do not function as activators. In contrast, fusion of the glutamine-rich segment from the *Drosophila* Antennapedia protein to the zinc fingers of Sp1 led to a strong activator protein (Courey *et al.*, 1989). In addition, when the activation domains of Sp1 are fused to the DNA binding domain of Sp3 (in PPAC1), the chimera behaved as a binding site-dependent transactivator. A finer mutational analysis will be needed to define further the amino acid residues responsible for the functional differences in the glutamine-rich domains of the two transcription factors.

Recently, it has been shown that the glutamine-rich activation domains of Sp1 can interact with at least two components of the TFIID complex, the TBP-associated factor dTAFII110 and TBP itself (Hoey *et al.*, 1993; Emili *et al.*, 1994). Moreover, a region of Sp1 with alternating glutamine and hydrophobic residues, which is required for the interaction with dTAFII110 and is important for mediating transcriptional activation, has been mapped. Substitution of bulky hydrophobic residues, but not of glutamine residues, within this region decreased both interaction with dTAFII110 and transcriptional activation in *Drosophila* Schneider cells (Gill *et al.*, 1994). Close inspection of the glutamine-rich region of Sp3 revealed that the glutamine-rich domains also contain bulky hydrophobic residues similar to those found in Sp1. At this stage, we do not know whether the glutamine-rich regions of Sp3 are also able to interact with dTAFII110 and TBP. *In vitro* binding studies with recombinant TBP, dTAFII110 and Sp3 will clarify this issue.

Is Sp3 an inhibitory member of the Sp family?

Although at this stage the physiological function of Sp3 remains obscure, our data suggest that its function differs significantly from that of Sp1. It is clear that Sp3 is not simply a functional equivalent of Sp1. So far, Sp3 has to be considered as an inhibitory member of the Sp family. Sp3 may play a role as a negative transcriptional regulator of basal gene expression *in vivo* by binding to appropriate DNA motifs present in many promoters. Notably, Sp3 also inhibited TAT activation of the HIV promoter (B.Majello *et al.*, submitted). The ratio of Sp1 and Sp3 molecules in a cell may contribute to the transcriptional regulation of genes with appropriate Sp binding sites by modulating their degree of activation. However, direct proof that Sp3 is indeed a physiological repressor of genes which are activated by Sp1 *in vivo* could be obtained only by creating and analyzing Sp3 knock out mice. Moreover, it remains to be established whether the ratio of Sp1 and Sp3 changes under certain conditions, e.g. during differentiation processes or during the cell cycle.

The existence of members of transcription factor families which behave as inhibitory transcription factors is a rather common phenomenon (Baeuerle and Baltimore, 1989; Baichwal and Tjian, 1990; Benezra *et al.*, 1990; Auwerx and Sassone-Corsi, 1991; Descombes and Schibler, 1991; Shi *et al.*, 1991; Garcia *et al.*, 1992; Stehle *et al.*, 1993). An example that most closely resembles Sp3-mediated inhibition of Sp1 is chicken B-myb. B-myb represses v-myb- and c-myb-mediated activation by competing for the same binding site (Foos *et al.*, 1992). Although in most cases the precise roles of these inhibitory factors are not known, their widespread occurrence suggests that regulation of gene expression frequently involves inhibitory interactions.

At this stage, however, we cannot completely exclude that Sp3 may also be an activator of certain, yet unknown genes or under certain special conditions. One might speculate that Sp3 only transactivates in combination with other transcription factors or co-activators not present in our test systems. In co-transfection experiments, BTEB1, another GC box binding factor, activates the expression

of genes with repeated GC box sequences, but represses the activity of a promoter containing a single GC box (Imataka *et al.*, 1992). The situation might be even more complex because two tissue-specific transcription factors have been described which also act through GT/GC boxes (Miller and Bieker, 1993; Sogawa *et al.*, 1993). Further investigations will be necessary to understand the functional interplay of all these transcription factors binding to the same DNA elements.

Materials and methods

Plasmid constructions

Construction of the complete Sp3 cDNA. A complete Sp3 cDNA was obtained by fusing appropriate subfragments of partial Sp3 cDNA clones (A3.1, A3.14.2, A3.15 and A3.16; see Hagen *et al.*, 1992) to each other. The resulting Sp3 cDNA clone in pBluescript II KS (pBS-Sp3) contains the coding region and 196 nucleotides of the 3′-untranslated region.

Bacterial expression plasmids. The expression plasmid for Sp1 was described previously (Hagen *et al.*, 1992). An expression plasmid for partial Sp3 encoding the 415 C-terminal amino acids was obtained by cloning a 1.4 kb *Bgl*II–*Bam*HI fragment from pBS-Sp3 into the *Bam*HI site of pET3b (Studier *et al.*, 1989).

Mammalian expression vectors. To ensure comparable expression levels of Sp1, Sp3 and Sp4, all corresponding cDNA clones were fused to the CMV promoter/enhancer. In detail, the vectors were generated as follows. CMV-hSp1 was obtained by cloning a 4.4 kb *Xba*I fragment from pBS/Sp1-fl (a gift from R.Tjian) into the *Xba*I site of pEVR2 (Matthias *et al.*, 1989). The resulting plasmid, CMV-hSp1, contains the complete coding sequence for human Sp1 and, in addition, 19 codons at the N-terminus which derive from the HSV tk gene and the polylinker. CMV-rSp1 was obtained by cloning the rat Sp1 cDNA as a *Xho*I fragment from RSVSp1r (Imataka *et al.*, 1992) into the *Hin*dIII site of the plasmid pRC/CMV (Invitrogen) via *Hin*dIII linkers. The expression vector for Sp3, CMV-Sp3, was obtained by inserting a 1.8 kb *Not*I fragment from pBS-Sp3 into the *Not*I site of pRC/CMV. To generate the expression plasmid for Sp4 (CMV-Sp4), a 3.3 kb *Hin*dIII–*Not*I fragment from pBS-Sp4 (A8.68 fused to A8O; see Hagen *et al.*, 1992) was cloned into the *Hin*dIII–*Not*I sites of pRC/CMV.

Vectors for Sp3 expression in *Drosophila* Schneider cells were generated as follows. The plasmid pPac was constructed by Mark Krasnow and generously supplied by Renato Paro (ZMBH Heidelberg). It contains 2.6 kb of the *Drosophila* actin 5C 5′-flanking region fused to the CAT gene and a poly(A) processing region. The actin 5C gene and the CAT gene are separated by a unique *Bam*HI cloning site. pPacSp3 was obtained by cloning a 2.35 kb *Not*I–*Bam*HI fragment from pBS-Sp3 into the single *Bam*HI site of pPac via decameric *Bam*HI linkers. The expression plasmid for human Sp1 (pPacSp1), a generous gift of R.Tjian, was described previously (Courey and Tjian, 1988).

The expression plasmid for the fingerless Sp3 mutant (pPacSp3-DBD) in which the 168 C-terminal codons of Sp3 are removed was obtained by cloning a 1.55 kb *Bam*HI–*Asp*718 fragment from pBS-Sp3 into the *Bam*HI site of pPac. For this, a 28-mer *Bam*HI linker (TGACTGACTGAGGATCCTCAGTCAGTCA) containing stop codons in all three reading frames was fused to the blunted *Asp*718 site.

Construction of Sp1/Sp3 chimeric plasmids. In plasmid PPAC1, the codons for the 597 N-terminal amino acids of Sp1 are fused to the 207 C-terminal amino acids of Sp3. First, we constructed an expression plasmid for a fingerless mutant of Sp1 (CMV-Sp1-DBD). A 1.8 kb *Bam*HI fragment from pBS-Sp1-fl (a gift from R.Tjian) was cloned into the *Kpn*I site of the mammalian expression vector pEVR3S (Matthias *et al.*, 1989) via decameric *Kpn*I linkers, resulting in CMV-Sp1-DBD. Into the unique *Sph*I site of this plasmid (cuts in the Sp1 cDNA close to the first zinc finger), a 0.9 kb *Afl*II–*Sma*I fragment from A3.1 (Hagen *et al.*, 1992) coding for the 207 carboxy-terminal amino acids of Sp3 was cloned using 12-mer *Sph*I linkers. This procedure leads to a mammalian expression vector for the chimeric protein (PP1). The appropriate expression vector for Schneider cells (PPAC1) was obtained by replacing the Sp1 cDNA in pPacSp1 (Courey and Tjian, 1988) by the 2.65 kb blunted *Sma*I–*Xba*I fragment from PP1 via 12-mer *Xho*I linkers. In plasmid PPAC3, the codons for the 142 C-terminal amino acids of Sp3 are replaced by the 223 C-terminal amino acids of Sp1. The 1.23 kb *Sac*I–*Hin*dIII Sp1 cDNA fragment encoding the 223 C-terminal amino acids of Sp1 was ligated to a 1.7 kb *Sac*I–*Hin*dIII fragment of Sp3 (from pBS-Sp3). After cutting the ligation products with *Hin*dIII, the 3.0 kb *Hin*dIII fragment was cloned into the *Hin*dIII site of the mammalian expression vector pRC/CMV, resulting in PP3. The appropriate construct for expression in Schneider cells was obtained by ligating a 2.9 kb *Xba*I fragment from PP3 into pPac via 12-mer *Xho*I linkers. For this purpose, the unique *Bam*HI cloning site of pPac was replaced by a unique *Xho*I cloning site. In plasmid PPAC4, the codons for the 507 C-terminal amino acids of Sp3 are replaced by the 485 C-terminal amino acids of Sp1. The 1.8 kb *Xba*I fragment of pRC/CMV/Sp3 (obtained from dam$^-$ DNA) was replaced by the blunted 1.5 kb *Sau*I–*Sma*I fragment from pBS-Sp1-fl using 8-mer *Xba*I linkers. The resulting plasmid (PP4) was cut with *Not*I and *Xba*I (the internal *Xba*I site of Sp3 is not cut when DNA is extracted from dam$^+$ *E.coli* strains), and cloned into pPac via *Xho*I linkers.

Anti-Sp1 and anti-Sp3 antibody preparations

Antibodies against Sp1 and Sp3 were generated by injecting rabbits with bacterially expressed Sp1 and Sp3. Appropriate Sp1 and Sp3 cDNA expression vectors (see above) were transformed into *E.coli* strain BL21(DE3)LysS (Studier *et al.*, 1989). Cells were grown in LB medium to an OD_{600} of 0.5 and expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After 2 h at 37°C, the bacteria were harvested and lysed by three rounds of freezing/thawing. Inclusion bodies were prepared by several washing steps essentially as described by Nagai *et al.* (1985). Bacterially expressed Sp1 and Sp3 present in these crude inclusion body preparations were further purified by SDS–PAGE prior to injection in rabbits. The animal treatments were performed according to standard procedures (Harlow and Lane, 1988).

Immunoblot analysis

Western blots (Towbin *et al.*, 1979) were performed according to standard procedures (Harlow and Lane, 1988). Filters were incubated with a peroxidase-conjugated pig anti-rabbit antibody and developed with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride.

Cell culture and transfections

Transfections in mammalian cell lines. Ishikawa and HeLa cells were cultured in MEM and DMEM medium, respectively, as described previously (Slater *et al.*, 1990). CV-1 cells were maintained as monolayers in MEM medium, supplemented with 25 mM HEPES, 4.5 g/ml glucose, 1 mM sodium pyruvate and 10% fetal calf serum. Ishikawa cells were transfected by the DEAE–dextran method (Cato *et al.*, 1986). HeLa and CV-1 cells were transfected by the calcium phosphate precipitation technique (DiNocera and Dawid, 1983). All cell lines were transfected with 5 μg plasmid DNA/6 cm dish. Transfected cells were harvested 48 or 72 h after transfection for CAT assays.

Transfections in SL2 cells. Schneider cells (Schneider, 1972) were maintained in Schneider medium supplemented with 10% fetal calf serum at 25°C. One day prior to transfection, cells were plated in a mixture of Schneider/DE22 medium (1:3, supplemented with 10% fetal calf serum) onto 6 cm plastic dishes at a density of 4.5×10^6 cells/plate. Cells were transfected by the calcium phosphate method described by DiNocera and Dawid (1983). Every plate received 10 μg DNA. Variable amounts of expression plasmids were compensated with the plasmid pPac. Twenty-four hours after addition of DNA the medium was replaced by Schneider medium and 24 h later the cells were washed twice with phosphate-buffered saline (PBS) and harvested.

CAT assays

For CAT assays, cells were suspended in CAT buffer A (Pothier *et al.*, 1992) and lysed by three rounds of freezing and thawing. CAT assays were carried out according to Gorman *et al.* (1982). Protein concentrations in the CAT assay and reaction times were adjusted to bring the extent of CAT conversion into a range that is linear with the CAT enzyme concentration. CAT conversion was assayed by TLC and quantitation of the acetylated and non-acetylated forms of [^{14}C]chloramphenicol performed with an automated Imaging Scanner (United Technologies Packard). The ratio of acetylated to total chloramphenicol is displayed as percentage of conversion.

Nuclear extracts and gel-retardation assays

Nuclear extracts from mammalian cell lines were prepared from one 10 cm plate according to Andrews and Faller (1991). Nuclear extracts from transfected SL2 cells were made from one half of a plate transfected for CAT assays with 2 μg expression plasmids. Gel-retardation assays were

essentially performed as described previously (Fried and Crothers, 1981; Garner and Revzin, 1986) with oligonucleotides containing the GC box and GT box binding site, respectively.

The sequences of the oligonucleotides were as follows. GC box binding site: 5′-AGCTTCCGTTGGGGCGGGGCTTCACGTCGA-3′; 3′-TCGAAGGCAACCCCGCCCCGAAGTGCAGCT-5′. GT box binding site: 5′-AGCTTCCGTTGGGGTGTGGCTTCACGTCGA-3′; 3′-TCGAAGGCAACCCCACACCGAAGTGCAGCT-5′. Unspecific oligonucleotide (NS): 5′-CAGCGACTAACATCGATCGC-3′; 3′-GTCGCTGATTGTAGCTAGCG-5′.

For supershift assays, 1 μl of the appropriate antiserum was added to the binding reaction 10 min prior to the loading of the gel.

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