Sp1 Trans-Activation of Cell Cycle Regulated Promoters Is Selectively Repressed by Sp3†

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ABSTRACT: The transcription factor Sp1 plays a key role in the activation of many cellular and viral gene promoters, including those that are regulated during the cell cycle. However, recent evidence indicates that Sp1 belongs to a larger family of factors which bind G/C box elements in order to either activate or repress transcription. Sp3, a member of this family, functions to repress transcriptional activation in two viral promoters, most likely by competing with Sp1 for GC box/Sp binding sites. However, the physiological role of Sp3 in the repression of endogenous cellular promoters has not been experimentally addressed. In the present study, we analyze the activity and binding of Sp3 on several eukaryotic promoters that contain G/C boxes and are known to be regulated during cellular proliferation and the cell cycle. Using antibodies specific for Sp1 and Sp3, we observe that both of these factors localize to the cell nucleus and have a similar, dispersed subnuclear distribution. Further, using gel mobility shift assays, we show that both Sp1 and Sp3 interact specifically with the histone H4 promoter. Transient cotransfections of *Drosophila* cells with Sp1 and Sp3 expression vectors and with the histone H4, thymidine kinase (TK), or dihydrofolate reductase (DHFR) promoters show that only the DHFR promoter, containing multiple functional GC boxes, displays Sp3 repression of Sp1 activation. In contrast, the single G/C boxes within the histone H4 or TK promoters, which confer transcriptional activation via Sp1 binding, are not responsive to repression by Sp3. Therefore, we demonstrate that the endogenous cellular DHFR promoter is selectively responsive to Sp3 repression. The data suggest that Sp3 may contribute to the control of proliferation- and/or cell cycle−regulated promoters depending upon the context and/or number of functional Sp1 binding sites.

Trans-activation of eukaryotic transcription requires the intricate interactions of promoter-specific gene regulatory proteins with cis-acting DNA elements and associated transcription factors, as well as with RNA polymerase. A variety of structurally related but functionally distinct protein factors belonging to a single transcription factor gene family often recognize specific DNA sequences to bring about different transcriptional responses. Surprisingly, the transcription factor Sp1, long known to activate a wide array of cellular and viral promoters [for review, see Courey and Tijan (1992)], has only recently been found to be a member of a large multigene family which bind G/C boxes in order to either activate or repress transcription (Hagen *et al.*, 1992, 1994; Kingsley & Winoto, 1992; Sogata *et al.*, 1993; Majello *et al.*, 1994). This family includes Sp3 and Sp4, both of which, like Sp1, are ubiquitous zinc finger proteins containing glutamine- and serine/threonine-rich regions and which bind DNA with similar affinities (Kingsley & Winoto, 1992; Hagen *et al.*, 1994). Other G/C box binding factors have also been described, including BTEB1 (Imataka *et al.*, 1992), BTEB2 (Sogata *et al.*, 1993), and EKLF (Miller & Bieker, 1993). The discovery of this family highlights a previously unknown level of complexity of transcriptional regulation by Sp1.

One member of the Sp family, Sp3, is believed to repress transcriptional activation in viral promoters, most likely by competing with Sp1 for GC box/Sp binding sites (Hagen *et al.*, 1994; Majello *et al.*, 1994). Repression by Sp3 *in vivo* has been studied only using either a synthetic promoter derived from the HTLVIII LTR (Hagen *et al.*, 1994) or the HIV-1 promoter itself (Majello *et al.*, 1994). The functional contribution of Sp3 repression to the regulation of endogenous cellular promoters has not been experimentally addressed.

Endogenous mammalian promoters regulated during cellular proliferation or the cell cycle serve as ideal models for the study of Sp1 activation and Sp3 repression. Many of these promoters contain either single or multiple functional G/C boxes. Also, it is well established that this class of promoter often exhibits both positive control during the cell cycle (generally between late G1 and early S phase) and downregulation in nonproliferating cells (Stein *et al.*, 1992; Azizkhan *et al.*, 1993). An excellent example is the promoter of the human H4 histone gene, which contains a single functional G/C box (Birnbaum *et al.*, 1995). This promoter undergoes a rapid and transient 3- to 5-fold activation upon entry into the S phase of the cell cycle and is repressed upon

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cellular differentiation (Stein *et al.*, 1992). Similarly, the human thymidine kinase (TK)[1] promoter is activated in proliferating cells in a cell cycle−dependent manner. This promoter contains multiple G-rich regions but apparently possesses only one functional G/C box (Flemington *et al.*, 1987; Arcot & Deininger, 1992). Finally, the promoter of the hamster dihydrofolate reductase gene, also activated during proliferation, contains multiple functional G/C boxes [Swick *et al.* (1989); Blake *et al.* (1990); for review, see Azizkhan *et al.* (1993)]. In the current study, we focus on the functional contribution of Sp1 and Sp3 to the transcriptional activation of these three promoters. The results suggest that Sp3 repression is strongly dependent on promoter context and requires multiple functional G/C boxes.

MATERIALS AND METHODS

Cell Cultures. HeLa S3 cells were grown and maintained in suspension at 3−6 × 10^5 cells per milliliter at 37 °C in Joklik-modified minimum essential medium (Gibco/BRL) supplemented with 5% fetal calf serum and 5% horse serum. ROS (rat osteosarcoma) 17/2.8 cells and IMR-90 cells were grown in F-12 medium with 5% fetal calf serum. *Drosophila melanogaster* Schneider's SL2 cells were grown in Schneider's insect medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum at room temperature. Human ME-180 cervical epithelial cells were grown as described (see legend to Figure 1).

Immunofluorescence. Human ME-180 cells were rinsed twice with room-temperature phosphate-buffered saline (PBS), and fixed at room temperature for 10 min in PBS containing 3.7% formaldehyde. After two rinses in PBS and one in PBS containing 0.5% bovine serum albumin (PBSA), cells were permeabilized in PBSA containing 0.1% Triton X-100 for 3 min at room temperature. Primary antibody (see below) diluted in PBSA was added and incubated for 1 h at 37 °C. Optimal dilutions were determined empirically. After three more rinses with PBSA, secondary antibody-fluorochrome conjugates, diluted 1:500 in PBSA, were added. Secondary antibodies were IgG-specific, cross-species absorbed, donkey anti-IgG antibodies (Jackson Immunological Laboratories, West Grove, PA). Cover slips were then incubated at room temperature for 5 min in PBSA containing 0.1% Triton and 5 μg/mL of the DNA counterstain 4′-6′-diamidino-2-phenylindole (DAPI), after which they were rinsed twice more in PBS and mounted in Pro-Long antifade reagent (Molecular Probes, Eugene, OR). Images were obtained on Kodak (Rochester, NY) Ektachrome 400 film using a Zeiss Axiofot microscope equipped for epifluorescence. The primary Sp1 antibody Sp1 (PEP 2), a rabbit polyclonal IgG raised against residues 520−538 of the human Sp1, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary Sp3 antibody was obtained by injecting rabbits with SDS−PAGE-purified, bacterially expressed Sp3 according to standard procedures (Hagen *et al.*, 1994).

Nuclear Extraction and Electrophoretic Mobility Shift Assays. Nuclear extracts from exponentially growing HeLa S3 cells or ROS cells were prepared as described previously (Dignam *et al.*, 1983). The electrophoretic mobility shift assay was performed essentially as described (Staudt *et al.*, 1986), using a TGE buffer system (50 mM Tris-Cl, 380 mM glycine, and 2 mM EDTA, pH 8.5). Binding reactions were as described previously (van Wijnen *et al.*, 1989), except that 2 μg of poly(dI-dC)·poly(dI-dC) was added, and the ^{32}P-labeled Site I probe utilized was a wild-type double-stranded oligonucleotide comprised of the proximal portion of Site I from the histone H4 FO108 promoter (Stein et al. 1992); top strand: $^{5'}$GATCTTGTCGAGAGGGCGGGGACAATTG$^{3'}$; bottom strand: $^{5'}$GATCCAATTGTCCCCGCCCTCTCGACAA$^{3'}$. The specific competitor used in this study was an Sp1 consensus oligonucleotide (top strand: $^{5'}$ATTCGATCGGGGCGGGGCGAGC$^{3'}$; bottom strand: $^{5'}$GCTCGCCCCGCCCCGATCGAAT$^{3'}$).

When antibodies were included in the binding reactions, 1 μL of antiserum was mixed with 9 μL of the nuclear extract or cell lysate and incubated on ice for 1 h to allow complex formation. This mixture was then added to the rest of the reaction (in the absence of DTT) and incubated for an additional 20 min at room temperature. The antibodies used were the same as those described above for immunofluoresence studies.

Transient Transfections and CAT Assays. Drosophila SL2 cells were plated in either 6-well flat bottom plates at a density of 2.5 × 10^5 cells per well in 2 mL of Schneider's insect medium, supplemented as described above (for *Drosophila* Schneider SL2 cells), or in 100-mm plates at a density of 2.5 × 10^6 cells per well in 10 mL of media the day prior to transfection. Transfection of the plasmids pSCAT (the histone H4 FO108 wild-type reporter plasmid) (Birnbaum *et al.*, 1995), pTK (Arcot & Deininger, 1992), pDHFR (Blake *et al.*, 1990), pBCAT-2 (Pascal & Tjian, 1991), pPacSp1 (Courey & Tjian, 1988), or pPacSp3, pPacDBDSp3, pCMVSp1, and pCMVSp3 (Hagen *et al.*, 1994), was accomplished with *Drosophila* cells using the calcium phosphate coprecipitation method as described (Chen & Okayama, 1988). The *Drosophila* cells were scraped off the plate 48 h after transfection and centrifuged for 3 min at 12000*g*, washed in 1 mL of cold PBS, and recentrifuged. The cell pellets were stored at −80 °C until assayed for chloramphenicol acetyltransferase activity (CAT) (Gorman *et al.*, 1982). The efficiency of transfection could not be normalized by cotransfection of a luciferase or β-galactosidase (β-gal) plasmid because the known strong promoters usually used for this purpose are activated upon Sp1 overexpression (Saffer *et al.*, 1990; Hagen *et al.*, 1994). Therefore, CAT activities are presented as percent maximal activity and are the results from two to four independent experiments using multiple DNA preparations, with each condition carried out in triplicate.

RESULTS

Sp1 and Sp3 Possess Similar Subnuclear Distributions. We initiated our analysis of Sp1 and Sp3 function by examining the *in situ* localization of these two proteins in human cervical carcinoma cells. Immunofluorescence studies using rabbit polyclonal antisera raised against Sp1 and Sp3 indicate that these factors are highly concentrated within

[1] Abbreviations: TK, thymidine kinase; BTEB 1 and 2, basic transcription element binding protein; EKLF, erythroid Krüppel-like factor; CAT, chloramphenicol acetyltransferase; DAPI, 4′-6′-diamidino-2-phenylindole; DHFR, dihydrofolate reductase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; G/C, guanosine/cytosine; β-gal, β-galactosidase; HIV-1, human immunodeficiency virus-1; HTLVIII LTR, human T lymphocyte virus; PBS, phosphate-buffered saline; TGE, Tris−glycine−EDTA buffer.

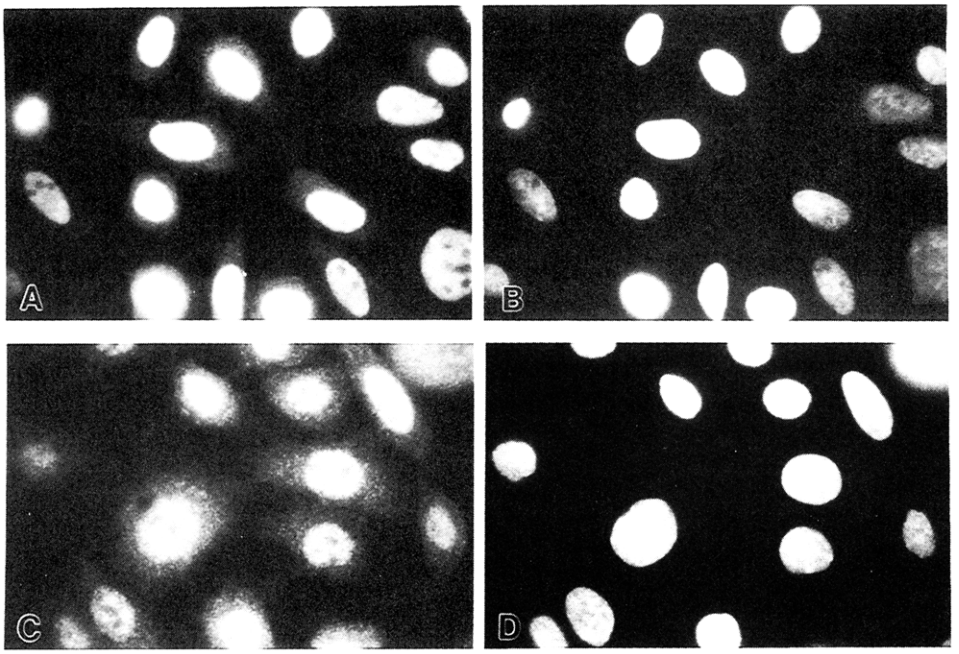

FIGURE 1: Subnuclear distributions of Sp1 and Sp3. Human ME-180 cervical epithelial cells were grown on glass coverslips in multi-well tissue culture dishes in DMEM supplemented with 10% fetal bovine serum. Immunofluorescence was carried out as described (see Materials and Methods) using DAPI staining to identify nuclei and rabbit polyclonal antisera raised against either Sp1 (A, B for the corresponding DAPI stain) or Sp3 (C, D for the corresponding DAPI stain). Controls without primary antibody displayed no staining (data not shown).

the nucleus and appear to be excluded from the nucleolus (Figure 1A–D). Both factors also display a faint and diffuse cytoplasmic staining pattern. No staining was observed in cells treated in the absence of primary antibodies (data not shown). These results are similar to those obtained using Ishikawa cells (G. Suske, unpublished). The similarities in the immunofluorescence patterns of Sp1 and Sp3 indicate that these proteins have very similar subcellular distributions. These data corroborate previous biochemical studies indicating that these two factors colocalize to the nucleus and may compete for genomic G/C box/Sp binding sites (Hagen *et al.*, 1994).

Sp3 Binds with Specificity to the Cell Cycle–Regulated Histone H4 Promoter. To establish that Sp3 binds with specificity to G/C boxes found within cell cycle–regulated promoters, we analyzed the binding of human cell nuclear proteins to an oligonucleotide probe derived from the human histone H4 promoter using the electrophoretic mobility shift assay (EMSA). Previous studies in our laboratory had demonstrated that Sp1 binds specifically to the histone H4 promoter at a single G/C box located within the proximal portion of Site I (Birnbaum *et al.*, 1995). However, not all of the HeLa cell nuclear complexes that were specifically competed with an Sp1 binding site oligonucleotide were supershifted by the Sp1 antibody (Birnbaum *et al.*, 1995). Supershift analysis of nuclear proteins derived from normal diploid fibroblasts using the histone probe (IMR-90 cells; Figure 2A) show that the Sp1 antibody caused the supershift (lane 2, complex D) of the uppermost complex (lane 2, complex A) of a doublet of bands, while two lower complexes (complexes B and C) were not affected. Hence, only complex A is mediated by Sp1. When IMR-90 nuclear proteins were incubated with an antibody generated against Sp3 (lane 3), complexes B and C were disrupted while complex A displayed a slight increase in intensity. We conclude from these results that complexes B and C are mediated by Sp3.

To demonstrate that both Sp1 and Sp3 bind with specificity to the histone promoter using extracts obtained from other cell types as well, we analyzed HeLa cell nuclear extracts using the EMSA in which a longer gel was performed (Figure 2B). The figure shows that the three slowest migrating complexes (lane 1), similar in mobility to complexes A, B, and C observed in IMR-90 cells (Figure 2A) were specifically competed by an Sp1 consensus oligonucleotide (Figures 2B, lane 2). Several other faster migrating complexes were also competed. The uppermost complex (corresponding to A in Figure 2A) migrated to a location very similar to the major complex formed by recombinant Sp1 (lane 3). Antibody supershift analysis showed that, like with IMR-90 cells, the addition of the Sp1 antibody to HeLa cell nuclear protein caused the disruption of the uppermost complex of a doublet (lane 4, designated as Sp1 in the margin), indicating that this complex is mediated by Sp1, while the two lower complexes were not affected. The addition of the Sp3 antibody (lane 5) disrupted the two lower complexes (designated in the margin as Sp3), while the presence of this antibody and an Sp3 peptide (lane 7) resulted in the recovery of these complexes, indicating that they are mediated by Sp3. Addition of both antibodies resulted in the disruption of all three complexes (lane 6). Similar results were obtained when these HeLa cell nuclear extracts were analyzed using an Sp1 consensus oligonucleotide as the probe (Figure 2C). These results are very similar to those obtained using probes containing either a synthetic G/C motif (Hagen *et al.*, 1994), or a G/C box derived from the HIV promoter (Majello *et al.*, 1994), where a doublet of Sp1 (upper complex) and Sp3 (lower complex), as well as a single lower Sp3 complex were observed possessing very similar mobilities. In a previous study, none of the three histone H4 complexes could bind to a probe carrying a mutation that disrupts the G/C box (Birnbaum *et al.*, 1995). Therefore, taken together, these data indicate that the transcription factor Sp3, like Sp1, binds

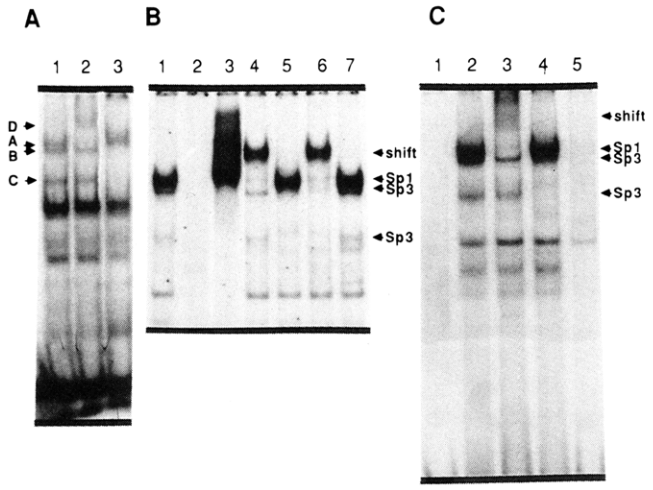

FIGURE 2: Sp3 binds specifically to the histone H4 FO108 promoter. EMSAs were carried out using a radiolabeled probe derived from either the proximal portion of the human FO108 histone H4 promoter or an Sp1 consensus sequence, both of which contain single Sp1 binding sites. (A) Antibody supershift identification of Sp1 and Sp3 in nuclear extracts derived from normal diploid fibroblasts (IMR-90 cells) using the histone H4 sequence as a probe. Lane 1: 5 μg of IMR-90 nuclear extract incubated in the absence of antibodies. Lane 2: Sp1 antibody. Lane 3: Sp3 antibody. Individual complexes (A−D, see text) were identified by their supershift behavior and are labeled on the left margin. (B) Supershift analysis of HeLa cell nuclear extracts using the histone H4 sequence as a probe. A longer gel was run in order to achieve better separation of Sp1 and Sp3. Lane 1: 5 μg of HeLa nuclear extract incubated in the absence of antibodies. Lane 2: 100-fold molar excess of an unlabeled Sp1 consensus oligonucleotide plus nuclear protein. Lane 3: 0.5 footprinting unit of recombinant Sp1 (Promega Corp.) in the absence of nuclear protein. Lane 4: Sp1 antibody plus nuclear protein. Lane 5: Sp3 antibody plus nuclear protein. Lane 6: both Sp1 and Sp3 antibodies plus nuclear protein. Lane 7: Sp3 antibody and 200 ng of anti-Sp3 control peptide (Santa Cruz Biotech.) plus nuclear protein. Individual complexes were identified by their supershift behavior and are labeled on the right margin. (C) Supershift analysis of HeLa cell nuclear extracts using the Sp1 consensus sequence as a probe. Lane 1: probe alone. Lane 2: 5 μg of HeLa nuclear extract incubated in the absence of antibodies. Lane 3: Sp1 antibody. Lane 4: Sp3 antibody. Lane 5: 100-fold molar excess of unlabeled Sp1 consensus oligonucleotide.

with specificity to the G/C box of at least one cell cycle−regulated promoter.

Sp3 Repression of Cell Cycle−Regulated Promoters. To study the effects of Sp3 on the Sp1 activation of proliferation- or cell cycle-regulated promoters, we used *Drosophila* SL2 cells. SL2 cells represent the preferred system in the study of Sp1 activation *in vivo* as they are the only higher eukaryotic cells known to provide a suitable background devoid of endogenous Sp1 (Courey & Tijan, 1988, 1992) and Sp3 (Hagen *et al.*, 1994). In these experiments, we compared the levels of transient expression from a virally derived promoter (BCAT-2) (Pascal & Tijan, 1991) known to be repressed by Sp3 (Hagen *et al.*, 1994) to those from the human histone H4 (Birnbaum *et al.*, 1995), human TK (Arcot & Deininger, 1992), or hamster DHFR (Blake *et al.*, 1990) promoters (see Figure 3). These reporter constructs were individually cotransfected into *Drosophila* cells along with plasmids expressing either Sp1 and/or Sp3 under the control of a *Drosophila* actin promoter (Hagen *et al.*, 1994).

The results of these cotransfection experiments are summarized in Figure 4. As expected from previous results, all four of these promoters were activated upon Sp1 expression

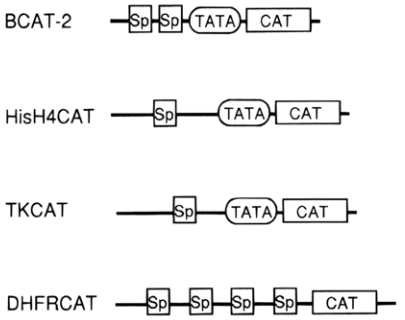

FIGURE 3: Schematic representation of the reporter plasmids used in this study. BCAT-2 contains two Sp (G/C) boxes derived from the HTLVIII LTR and the TATA box from the E1b gene (Pascal & Tjian, 1991). HisH4CAT (wtcat) encompasses 140 bp of the proximal promoter from the human histone H4 FO108 gene, including single binding sites for Sp1 and the TATA box (Birnbaum *et al.*, 1995). TKCAT encompasses −456 to +32 of the 5′ regulatory sequence of the human TK gene, which contains the TATA box, one functional Sp binding site, and several other G/C rich regions (Arcot & Deininger, 1992). DHFRCAT encompasses 220 bp of the proximal promoter from the hamster DHFR gene, including 4 functioinal Sp binding sites (Swick *et al.*, 1989; Blake *et al.*, 1990).

(Figure 4A; $p \leq 0.005$). Also as expected, the BCAT-2 promoter, containing two functional G/C boxes, demonstrated Sp3 repression of Sp1 activation (Figure 4A; $p \leq 0.0005$). However, Sp1 activtion of the histone H4 promoter, containing a single functional G/C box, displayed little, if any, repression by Sp3. Similarly, the human TK promoter, which contains multiple G-rich regions, but only a single functional G/C box, was not repressed by Sp3. On the other hand, activation of the DHFR promoter, containing multiple functional G/C boxes, was strongly repressed by Sp3 (Figure 4A; $p \leq 0.0005$). Further, Sp3 repression of Sp1 activation upon the DHFR promoter occurred in a dose-dependent manner (Figure 4B). A similar dose titration carried out using the histone promoter/reporter yielded no repression of Sp1 activation by any dose of Sp3 (data not shown). Also, coexpression of a mutated version of Sp3 in which the DNA binding domain had been deleted (Hagen *et al.*, 1994) had no effect on Sp1 activation of any of these promoters (data not shown), indicating that the DNA binding activity of Sp3 was required for its repressive function (Hagen *et al.*, 1994). Also, expression of Sp3 alone, in the absence of Sp1, had only marginal effects upon CAT activity (Table 1). Only in the case of the histone promoter did Sp3 activate expression significantly ($p \leq 0.005$). However, even in this case, activation was less than 2-fold. Therefore, taken together, these data demonstrate that, depending upon the context and/or number of functional G/C boxes present, cell cycle−regulated promoters display a selective responsiveness to the Sp1/Sp3 ratio.

DISCUSSION

In the present study, we use coexpression experiments to demonstrate that cell cycle−regulated promoters display a selective responsiveness to the Sp1/Sp3 ratio *in vivo*. While the histone H4, TK, and DHFR promoters are all activated by Sp1, this activation can be repressed by Sp3 only in the case of the DHFR promoter. Previous studies have used synthetic promoters derived from the HTLVIII LTR (Hagen *et al.*, 1994) or the HIV-1 LTR promoter (Majello *et al.*, 1994) to characterize Sp3 repression. However, our results

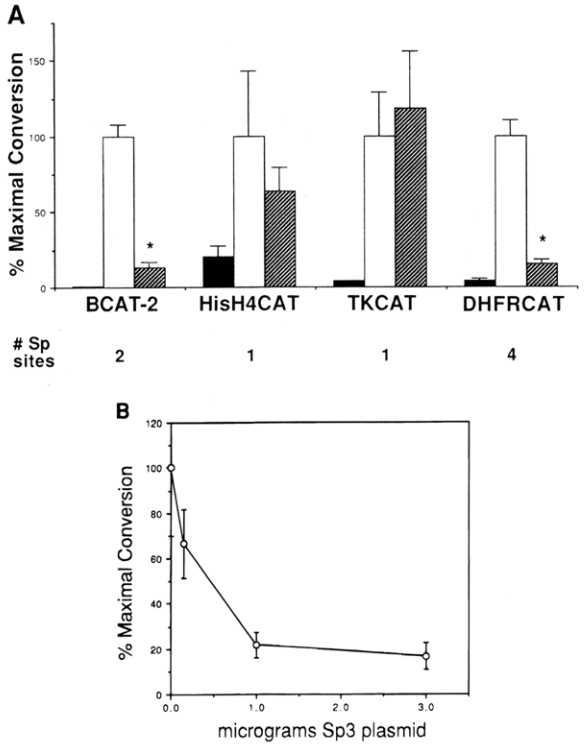

FIGURE 4: Effects of Sp1 and Sp3 expression on the activities of cell cycle−regulated reporter plasmids in *Drosophila* cells. (A) Differential control of cell cycle−regulated promoters by Sp1 and Sp3. Two micrograms of the reporter plasmids BCAT-2, HisH4CAT, TKCAT, or DHFRCAT were individually cotransfected in the absence of expression plasmid (black bars), in the presence of 15 ng of pPacSp1 (open bars), or in the presence of both 15 ng pPacSp1 and 300 ng pPacSp3 (striped bars). Each transfection was brought to a total of 5 μg of DNA with a plasmid containing the *Drosophila* actin promoter, but lacking an overexpressed open reading frame (pAct; Seto *al al.*, 1993). An asterisk over the striped bars denotes statistical significance ($p \leq 0.0005$) between pPacSp1 alone and pPacSp1 plus pPacSp3. Transfections were carried out in six-well plates. (B) Dose-responsive repression of the DHFR promoter by Sp3. Sixteen micrograms of DHFRCAT were cotransfected with 150 ng of pPacSp1 and increasing amounts of pPacSp3 as shown using the larger (100 mm) plates. DNA was brought to a total of 20 μg per transfection using the pAct plasmid. All data points represent the average of two to four separate experiments with each condition carried out in triplicate.

Table 1: Effect of Sp3 Alone on Promoter Activity in Transiently Transfected *Drosophila* Cells[a]

	mean relative CAT activity (SD)	
reporter construct	−Sp3	+Sp3
BCAT2	1.0 (.17)	1.4 (.2)
histone H4	1.0 (.25)	1.8 (.4)
TK	1.0 (.02)	1.2 (.2)
DHFR	1.0 (.1)	1.2 (.1)

[a] *Drosophila* SL-2 cells grown on six-well plates were transfected with the designated reporter plasmid in the absence or presence of 100 ng of pPacSp3. Values are normalized to the amount of activity observed for each construct in the absence of Sp3.

make the first demonstration that some endogenous cellular promoters, mediating physiological control during cell growth and differentiation, display Sp3 repression of Sp1 trans-activation. Furthermore, our studies indicate that this repression occurs in a promoter context−dependent manner.

Of the reporter constructs used in this study, the virally derived BCAT2, and the cellular histone H4, TK, and DHFR promoters, only those containing multiple functional G/C boxes (DHFR and BCAT2) were repressed by Sp3. Sp3 repression of the DHFR promoter is not correlated with the absence of a TATA box because the virally derived BCAT2 promoter, which does contain a functional TATA box, is also repressed by Sp3. Further, it is unlikely that the selective repression observed in the current study results from differences in the relative affinities of Sp1 and Sp3 for these different G/C boxes, as it is known that Sp1 and Sp3, which contain very highly conserved, functionally interchangeable zinc finger DNA binding domains, recognize several different G/C boxes with very similar affinities (Hagen *et al.*, 1992; 1994; Majello *et al.*, 1994; Dennig et al., 1995). Rather, repression by Sp3 appears to be dependent upon the promoter context and/or number of functional G/C boxes present. Quite interestingly, recent experiments indicate that a synthetic viral promoter containing two G/C boxes from HTLVIII (BCAT2) is more sensitive to Sp3 repression of Sp1 activation than a construct containing only one of these G/C boxes (BCAT1; G. Suske, unpublished). Taken together, therefore, these findings are consistent with the concept that Sp3 repression of Sp1 activation occurs in promoters containing multiple G/C boxes and in which multiple bound Sp1 molecules mediate synergistic activation.

While our study demonstrates that different cellular promoters are differentially responsive to Sp3 repression, the mechanism of this repression remains unknown. While Sp3 repression apparently requires Sp3 binding to G/C boxes (Hagen *et al.*, 1994; Majello *et al.*, 1994), we speculate that repression may be related to the synergistic activation by Sp1. Sp1 activation is a complex mechanism involving not only protein−DNA interactions, but also the interactions of multiple modular domains of Sp1 with coactivator proteins (Courey & Tijan, 1992; Gill *et al.*, 1994). Synergistic activation may require the higher-order interaction of Sp1 tetramers to facilitate stabilization of DNA loops (Pascal & Tjian, 1991; Courey & Tjian, 1992); however, this process apparently does not involve the cooperative binding of Sp1 molecules (Pascal & Tjian, 1991). Synergistic activation requires glutamine-rich activation domains A and B, and the carboxy terminal domain D (Pascal & Tjian, 1991; Courey & Tijan, 1992). These domains cannot be replaced functionally by homologous regions from Sp3 (Hagen *et al.*, 1994); however, the DNA binding domains of Sp1 and Sp3 appear to be functionally interchangeable (Hagen *et al.*, 1994). Therefore, we offer the speculation that Sp3 repression of Sp1 synergistic activation may result from a stochastic process involving competition for any of the Sp1 binding sites required for synergism.

The Sp family of G/C box binding proteins is now known to include both activators and repressors of transcription. Activators include Sp1 itself, along with Sp4 (Hagen *et al.*, 1994), BTEB2 (Sogata *et al.*, 1993), and EKLF (Miller & Bieker, 1993). On the other hand, BTEB1 is believed to activate expression from promoters containing multiple G/C boxes, but repress promoters with only a single G/C box (Imataka *et al.*, 1992). Sp3 is now known to possess a repressor function upon multiple G/C boxes. A recent report (Udvadia *et al.*, 1995) suggests that Sp3, like Sp1, may also activate some but not all promoter fragments containing an RCE (retinoblastoma control element), and that Rb may enhance this activation. Although our results (Table 1) and those presented elsewhere (Hagen *et al.*, 1994; Majello *et al.*, 1994) show little, or at best modest (less than 2-fold)

activation by Sp3 on a variety of viral and cellular promoters, a functional interaction between Sp1 or Sp3 and Rb may have important implications to the function of the Sp family during the cell cycle. The coordinated function of the Sp family remains to be investigated.

An important question that arises from our study is why some genes have evolved multiple G/C boxes, regulated by both Sp1 synergism and Sp3 repression, whereas other genes have not. Sp1 and its family of transcription factors are very important in the regulation of the DHFR promoter, where the multiple G/C boxes are involved in serum-responsive transcriptional activation (Azizkhan *et al.*, 1993). Therefore, genes that have obtained the ability to become highly transactivated by Sp1 may have also evolved the capacity to be repressed by Sp3 in order to control the levels of gene expression more stringently.

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