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Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription

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ABSTRACT

The product of the retinoblastoma (Rb) susceptibility gene (*RB-1*) regulates expression of a variety of growth control genes via discrete promoter elements termed retinoblastoma control elements (RCEs). We have previously shown that RCEs are bound and regulated by a common set of ubiquitously expressed nuclear proteins of 115, 95 and 80 kDa, termed retinoblastoma control proteins (RCPs). We have also previously determined that Sp3 and Sp1, two members of the Sp family of transcription factors, encode the 115 and 95 kDa RCPs respectively and that Rb stimulates Sp1/Sp3-mediated transcription *in vivo*. In this report we have extended these results by determining that the 80 kDa RCP arises from Sp3 mRNA via translational initiation at two internal sites located within the Sp3 *trans*-activation domain. Internally initiated Sp3 proteins readily bind to Sp1 binding sites *in vitro* yet have little or no capacity to stimulate transcription of Sp-regulated genes *in vivo*. Instead, these Sp3-derived proteins function as potent inhibitors of Sp1/Sp3-mediated transcription. Since cell cycle- or signal-induced expression of a variety of genes, including *p21*$^{waf1/cip1}$, *p15*^{INK4B}, *CYP11A*, *mdr1* and acetyl-CoA carboxylase, have been mapped to GC-rich promoter elements that bind Sp family members, we speculate that alterations of the protein and/or DNA binding activities of internally initiated Sp3 isoforms may account in part for the regulation of such differentially expressed genes.

INTRODUCTION

Cell cycle progression is mediated in part by the carefully orchestrated transcription of growth control genes. One function of the retinoblastoma (Rb) protein, the product of a tumor suppressor gene (*RB-1*), is to couple transcription to progression of the cell cycle (1). Deletion or functional inactivation of *RB-1* is associated with outgrowth of a variety of human cancers, including retinoblastoma, osteosarcoma and breast, bladder and small cell lung carcinomas (for reviews see 2–5). Rb physically and/or functionally interacts with a number of transcription factors, including E2F, Elf-1, PU.1, ATF-2, Sp1/3 and myoD, resulting in repression or stimulation of *trans*-activation depending on the transcription factor and cell type examined (for reviews see 6,7).

Rb governs the synthesis of a subset of growth control genes, including c-*fos*, c-*myc* and *TGF*-β1, via discrete portions of their promoters, termed retinoblastoma control elements (RCEs; 8–10). Using RCEs derived from each of these Rb-regulated genes as probes, protein–DNA binding assays have identified a common set of three protein–DNA complexes (denoted 1A, 1B and 2) in mammalian cell nuclear extracts (11,12). The proteins (retinoblastoma control proteins; RCPs) bound to RCEs *in vitro* were shown to be ubiquitously expressed and to have apparent molecular weights of 115, 95 and 80 kDa. The physiological relevance of one or more of these RCE binding proteins was underscored by the observation that point mutations that abolish binding of RCPs *in vitro* also abrogate RCE-mediated transcription *in vivo*. Mutational analyses of RCEs and methylation-interference ('footprinting') assays resulted in the derivation of a 6 nt consensus sequence, 5′-GCCACC-3′, that is essential for binding of RCPs *in vitro* and RCE function *in vivo*. This consensus RCP binding site was noted to be reminiscent of GC-rich sequences (GC boxes) bound by transcription factor Sp1, and by a variety of immunochemical and biochemical criteria the 95 kDa RCP was subsequently shown to be encoded by Sp1 (12,13).

Sp1 is a ubiquitously expressed nuclear protein that was initially identified as a protein that binds and stimulates transcription of the SV40 early promoter (14,15). Since this initial observation, Sp1 has been shown to play a central role in *trans*-activation of a multitude of cellular promoters, including the aforementioned RCE-containing promoters, dihydrofolate reductase (*DHFR*), thymidine kinase (*TK*), *p21*$^{waf1/cip1}$, *p15*^{INK4B}, *GM-CSF*, *EGFR*, *IL2-Rα*, *IGF*-II, *SIS/PDGF*-B and *hIR* (13,16–25). Sp1 physically interacts with other sequence-specific DNA binding proteins; interactions with transcription factor PU.1 appear to impart cell specificity to Sp1-mediated transcription, whereas interactions with proteins such as E2F-1, BPV E2 protein or p53 result in synergistic *trans*-activation (22,26–30). In turn, physical interactions between Sp1 and components of the basal transcription machinery, such as the TATA box binding protein (TBP) and TBP-associated factors (TAFs) 110 and 55, catalyze initiation of transcription by RNA polymerase II (31–33). We and others have shown that the functional consequence of co-expression of Rb and Sp1 is stimulation ('superactivation') of Sp1-mediated transcription (12,13). Moreover, this functional interaction is likely to be physiologically significant, as Rb amino acids required for tumor

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suppression are also required for 'superactivation' of Sp1-mediated transcription (34). The precise mechanism(s) whereby Rb stimulates Sp1 *trans*-activation remains to be resolved (6).

Sp1 is a member of a burgeoning family of related transcription factors (35–37). Sp2, Sp3 and Sp4 share extensive structural and sequence homology with Sp1 and each of these Sp family members binds specifically to DNA. Sp3, like Sp1, is a ubiquitously expressed protein, whereas the distribution of Sp2 and Sp4 appears to be more limited (35,36). Given that Sp family members share similar cognate DNA binding sites, we hypothesized that Sp3, a 110 kDa protein, was likely to encode the 115 kDa RCP. This hypothesis was subsequently confirmed and we and others have shown that Sp3 stimulates transcription of RCE- and GC box-containing promoters *in vivo* (21,34). Moreover, as previously noted for Sp1, Rb co-expression leads to 'superactivation' of Sp3-mediated transcription (34). To further our understanding of Rb-mediated transcription, we have pursued identification and characterization of the remaining (80 kDa) RCE binding protein identified in nuclear extracts. Here we report that this 80 kDa RCP is encoded by Sp3 and arises via internal translational initiation within Sp3 mRNA. We also demonstrate that internally initiated Sp3-derived proteins have little or no inherent *trans*-activation activity. Instead, these novel Sp3-derived proteins function as potent inhibitors of Sp3- and Sp1-mediated *trans*-activation.

MATERIALS AND METHODS

Cell culture

Human cervical carcinoma line C-33A and *Drosophila* SL2 cells were cultured as previously described (11,12).

Plasmid constructions

pCR-M1/flu and pCR-M2/flu were prepared from plasmid pSP72-Sp3 (36; provided by Astar Winoto, University of California–Berkeley, Berkeley, CA) using PCR and one of two 5′ oligonucleotides (M1, 5′-GGGGGATCCATGACTGCAGGCATTAATG-3′; M2, 5′-GGGGGATCCATGGATAGTTCAGACAATTCA-3′) and a 3′ oligonucleotide (5′-GGGGGATCCCTAGCTAGCGTAATCTGGAACATCGTATGGGTACTCCATTGTCTCATTTCCA-3′). Resulting M1 and M2 Sp3-derived cDNAs carried N-terminal truncations of 217 and 234 amino acids respectively and an influenza hemagglutinin (HA) epitope tag at their C-termini. PCR-amplified, epitope-tagged M1 and M2 cDNAs were cloned into pCR-ScriptCamSK(+) (Stratagene Inc., La Jolla, CA) for *in vitro* transcription/translation experiments. pPacM1 and pPacM2 were constructed by subcloning M1 and M2 cDNAs from pCR-M1/flu and pCR-M2/flu into pPac, an insect cell expression vector driven by the *Drosophila actin 5C* promoter (38). pBSK-Sp3/flu was prepared by subcloning an Sp3 cDNA carrying a C-terminal HA epitope tag from CMV-Sp3/flu (34) into pBSK(+) (Stratagene Inc.). pPacSp1 and pPacSp3 expression vectors have previously been described (12,34). A GAL4–VP16 expression vector and GAL4–CAT and DHFR–CAT reporter plasmids have been previously described (16,39). A glutathione S-transferase–M1 fusion protein, GST–M1/flu, was created by subcloning the M1 cDNA from pCR-M1/flu into the *Bam*HI site of pGEX-2TK (Pharmacia Inc., San Fransisco, CA).

Antibodies

Anti-Sp3 *trans*-activation domain antiserum was prepared as described (34) using the N-terminal 300 amino acids of Sp3 as immunogen. Anti-Sp3 DNA binding domain antiserum (a kind gift of Astar Winoto) was prepared against a GST fusion protein that carries the C-terminal 415 amino acids of Sp3 (34). A polyclonal anti-Sp1 antiserum prepared against the Sp1 *trans*-activation domain has previously been described (12). Epitope-tagged proteins were detected with the anti-HA monoclonal antibody 12CA5 (12) employed as hybridoma supernatants.

Protein–DNA binding assays

Nuclear extracts from C-33A and SL2 cells were prepared as previously described (11,12). Synthetic oligonucleotides employed in protein–DNA binding assays were synthesized and purified as described (11). A c-*fos* RCE-derived oligonucleotide carrying a high affinity binding site for RCPs (5′Fos-4, 5′-CCCTTGCGCCACCCCTCT-3′) and a mutated derivative lacking this site (5′Fos-2, 5′-CCCGCGCTTCACCCCTCT-3′) have previously been described (11). Oligonucleotides were radiolabeled with [α-^{32}P]GTP and [α-^{32}P]CTP (ICN Inc., Costa Mesa, CA) and Klenow polymerase (New England Biolabs, Beverly, MA) and employed in electrophoretic mobility shift assays as described (11).

Western blotting and immunoprecipitation

Western blots using anti-Sp1 antiserum were performed as described (40). Western blots with anti-Sp3 antiserum were performed as for anti-Sp1, using a 1:2000 dilution of primary antibody and a 1:40 000 dilution of secondary antibody. Antibody–antigen complexes were detected using an enhanced chemiluminescent system (ECL; Amersham Corp., Arlington Heights, IL). Immunoprecipitations were performed as previously described (40).

In vitro transcription/translation

In vitro transcribed/translated proteins were produced using T3 or T7 RNA polymerases (New England Biolabs), pBSK-Sp3/flu, pCR-M1/flu or pCR-M2/flu as template and a coupled reticulocyte lysate system (TNT; Promega Inc., Madison, WI). *In vitro* translated proteins were radiolabeled with a proprietary cocktail of radiolabeled amino acids (Express; New England Nuclear Inc., Boston, MA).

Transfections and CAT assays

Transient transfection of SL2 cells was performed and nuclear extracts were prepared as previously described (11,12). Chloramphenicol acetyltransferase (CAT) assays were performed using a liquid scintillation assay as previously described (41).

RESULTS

Retinoblastoma control elements (RCEs) are bound by Sp3 and Sp3-related proteins *in vitro*

We have previously shown, by photoaffinity labeling techniques, that RCEs derived from the c-*fos*, c-*myc* and *TGF*β-1 promoters are bound by three proteins *in vitro*, termed retinoblastoma control proteins (RCPs), of 115, 95 and 80 kDa (11). Using immunochemical and molecular genetic techniques we have also previously determined that two members of the Sp family of

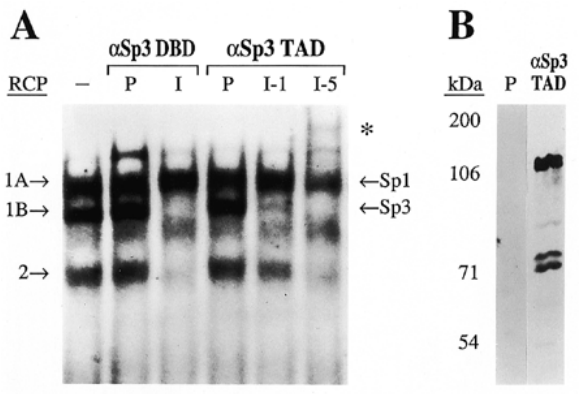

Figure 1. Sp3 and Sp3-related proteins are synthesized *in vivo* and bind to the c-*fos* RCE *in vitro*. (**A**) Protein–DNA binding assay. C-33A nuclear extracts were incubated with a wild-type c-*fos* RCE probe alone (–) or with the addition of preimmune (P) or anti-Sp3 antisera (I). Resulting RCE–RCP complexes that have previously been characterized are indicated on the left. RCE–RCP complexes 1A and 1B have previously been shown to be formed by Sp1 and Sp3 respectively and this is indicated on the right. Shown are protein–DNA binding assays performed with the addition of initial (αSP3 TAD, I-1) and subsequent (αSP3 TAD, I-5) preparations of antisera prepared against the Sp3 *trans*-activation domain as well as antiserum prepared against the Sp3 DNA binding domain (αSp3DBD, I). An asterisk indicates the position of a 'supershifted' Sp3 protein–DNA complex. (**B**) Western blots of human whole-cell extracts. Western blots of denatured C-33A extracts (100 μg) were probed with preimmune (P) or anti-Sp3 antisera (αSP3 TAD). Molecular weight markers are indicated on the left.

transcription factors, Sp3 and Sp1, encode the 115 and 95 kDa RCPs respectively (12,34). These results relied in part on an anti-Sp3 rabbit antiserum (αSp3 TAD) we had prepared against a GST fusion protein carrying the Sp3 *trans*-activation domain. Surprisingly, subsequent immunizations of this rabbit with the Sp3 *trans*-activation domain resulted in production of antiserum that also reacts with the 80 kDa protein that forms RCE–RCP complex 2 (Fig. 1A). Whereas immune serum collected following initial immunizations (Fig. 1A, lane I-1) greatly diminished the abundance of an Sp3–DNA complex (RCP 1B), aliquots of rabbit antiserum collected subsequently (Fig. 1A, lane I-5) were noted to abrogate both RCP complexes 1B and 2, leading to a novel 'supershifted' complex of decreased mobility (Fig. 1A, asterisk). These data suggested that the Sp3 *trans*-activation domain shares one or more antigenic determinants with the 80 kDa RCP. To determine whether the DNA binding domain of Sp3 is also antigenically related to that of the 80 kDa RCP, we employed a polyclonal rabbit antiserum (αSp3 DBD), prepared against the C-terminal 415 amino acids of Sp3, in protein–DNA binding assays (34). As for later aliquots of αSp3 TAD antiserum, this distinct anti-Sp3 antiserum also abrogates the detection of RCP complexes 1B and 2 (Fig. 1A). In accord with previous results, αSp3 TAD preimmune serum does not appreciably alter the abundance of RCP–RCE complexes and anti-Sp3 immune sera do not cross-react with Sp1–DNA complexes (34; Fig. 1A, RCP 1A). Taken together with our previous observation (11) that the nucleotide binding specificities of each RCP are identical, results using αSp3 TAD and αSp3 DBD antisera support the contention that the 80 kDa RCP is antigenically as well as functionally related to Sp3.

Data presented in Figure 1A indicate that antisera directed against distinct portions of Sp3 abrogate formation of RCE–RCP complex 2. Yet, these results do not formally prove that anti-Sp3 antisera bind the 80 kDa protein detected by photoaffinity labeling techniques within this protein–DNA complex, i.e. our results do not preclude the unlikely possibility that anti-Sp3 antisera disrupt RCE–RCP complex 2 via their interaction with an as yet undetected Sp3-related protein that is associated with the 80 kDa RCP. To determine directly whether αSp3 TAD antiserum binds proteins of ~80 kDa in addition to Sp3, denatured whole-cell extracts were analyzed in Western blots with preimmune and immune antisera. In accord with previous studies of Sp3 (36,42), a prominent protein doublet of 115 kDa is detected by anti-Sp3 serum (Fig. 1B, αSp3 TAD) but not preimmune serum (Fig. 1B, P) in Western blots of human cell extracts. In addition, and consistent with results from protein–DNA binding assays, a protein doublet of 78–80 kDa was also detected specifically by anti-Sp3 immune serum (Fig. 1B, αSp3 TAD). Identical results were obtained in immunoprecipitates of non-denatured, [^{35}S]-methionine-labeled cell extracts with αSp3 TAD antiserum or the C-terminal anti-Sp3 antiserum (αSp3 DBD) employed in protein–DNA binding assays in Figure 1A (data not shown). Moreover, similarly sized Sp3-related proteins have previously been noted by others using an independent anti-Sp3 antiserum (42). Thus, antisera prepared against distinct portions of Sp3 detect multiple antigenically related proteins in cell extracts that are similar in size to proteins that comprise RCP complexes 1B and 2.

The 78–80 kDa Sp3-related proteins are isoforms of Sp3 that arise via internal translational initiation

Since Northern analyses indicate that Sp3 encodes a single ubiquitously expressed mRNA of 4.2 kb, we reasoned that the 78–80 kDa Sp3-related proteins were unlikely to result from alternative splicing of Sp3 message or to be encoded by closely related genes (35,36). Additionally, since we have previously shown that *in vivo* expression of an epitope-tagged Sp3 cDNA leads to synthesis of a single protein of ~110 kDa (34), we concluded that the 78–80 kDa Sp3-related proteins are unlikely to arise via proteolytic degradation of Sp3. Consequently, we focused our attention on the possibility that the 78–80 kDa Sp3-related proteins might arise via internal translational initiation within Sp3 mRNA.

Scanning the Sp3 cDNA sequence, we noted two closely juxtaposed methionine residues (Fig. 2A, M1 and M2) embedded within nucleotide sequences compatible with efficient translational initiation and synthesis of proteins of ~80 kDa (43). To assess whether translation can occur from these internal methionine residues, we prepared Sp3 cRNA and translated this message in rabbit reticulocyte extracts. As shown in Figure 2B, *in vitro* translation of cRNA derived from an epitope-tagged (influenza hemagglutinin, HA) Sp3 cDNA gives rise to three prominent proteins of 115, 80 and 78 kDa. Each of these Sp3-derived proteins are precipitated by antisera prepared against the Sp3 *trans*-activation domain (αSp3 TAD) and the C-terminal epitope tag (Fig. 2B, αHA). To confirm that the 80 and 78 kDa Sp3-derived proteins initiate from the predicted internal methionine residues, we utilized Sp3 oligonucleotides and PCR to synthesize epitope-tagged, partial Sp3 cDNAs whose translation initiates at M1 and M2. As expected, translation of cRNA produced from the M1 Sp3 construct leads to synthesis of the 80 and 78 kDa Sp3-derived proteins, whereas M2-derived cRNA directs synthesis of only the 78 kDa isoform of Sp3 *in vitro* (Fig. 2B). Thus, internal

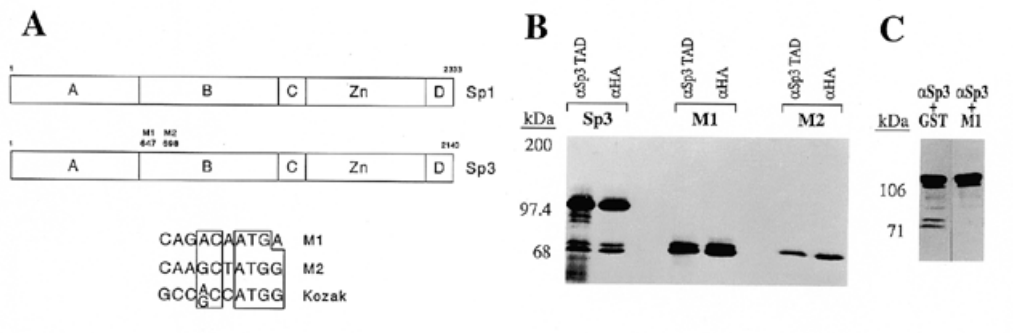

Figure 2. Internal initiation of Sp3 generates the 78–80 kDa Sp3-related proteins. (**A**) Schematic diagrams of Sp1 and Sp3. Indicated are two sites within the Sp3 *trans*-activation domain (M1, M2) that contain methionine codons embedded within consensus translation initiation sequences (Kozak). The nucleotide positions of these sites of internal translational initiation are indicated above the diagram of Sp3 relative to the first nucleotide of the Sp3 cDNA sequence reported by Kingsely and Winoto (36). Nucleotide numbers above the diagram of Sp1 refer to the first and last nucleotides of Sp1 coding sequences. (**B**) Immunoprecipitations of *in vitro* translated epitope-tagged Sp3-related proteins radiolabeled with [^{35}S]methionine. Sp3-related proteins derived from a full-length Sp3 cDNA (Sp3) or truncated Sp3 cDNAs that begin at the predicted sites of internal translational initiation (M1, M2) were incubated with anti-Sp3 (αSP3 TAD, I-5) or anti-HA (HA, 12CA5) antisera. Molecular weight markers are indicated on the left. (**C**) Western blots of denatured C-33A whole-cell extracts (120 µg) that have been probed with anti-Sp3 antiserum (αSP3 TAD, I-5) pre-incubated with an excess of GST (αSP3+GST) or GST–M1 fusion proteins (αSP3+M1). Molecular weight markers are indicated on the left.

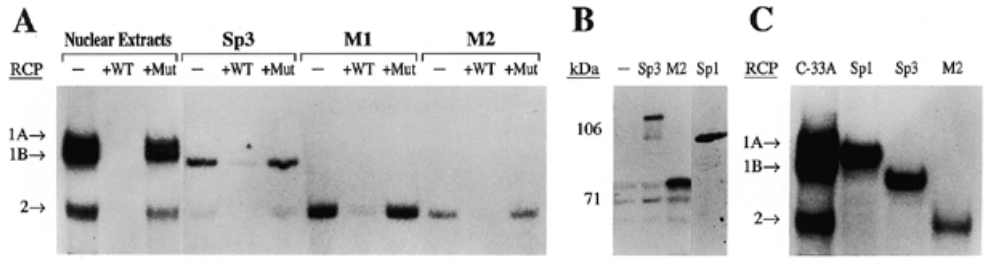

Figure 3. Internally initiated Sp3-derived proteins are karyophilic and form protein–DNA complexes that are indistinguishable from RCE–RCP complex 2. (**A**) Protein–DNA binding assays. A radiolabeled wild-type c-*fos* RCE probe was incubated with C-33A nuclear extracts or *in vitro* translated Sp3, M1 or M2 proteins alone (–) or in the presence of a 400-fold molar excess of unlabeled wild-type (+WT) or mutated (+Mut) RCE oligonucleotides. RCE–RCP complexes are indicated on the left. (**B**) Western blots of SL2 nuclear extracts prepared from untransfected cells (–) or cells transfected with equivalent amounts of pPacSp3, pPacM2 or pPacSp1 expression vectors. Blots were probed with anti-Sp3 antiserum (αSP3 TAD, I-5) or with antiserum prepared against Sp1 (Sp1). (**C**) Protein–DNA binding assay using a wild-type c-*fos* probe and C-33A nuclear extracts or nuclear extracts prepared from SL2 cells transfected as in (B). RCE–RCP complexes are indicated on the left.

translational initiation can occur at M1 and M2 *in vitro* producing proteins that are similar in size to the 78–80 kDa Sp3-related proteins synthesized *in vivo*.

To determine if the 78–80 kDa Sp3-related proteins synthesized *in vivo* are structurally identical to M1 and M2 proteins synthesized *in vitro* we wished to compare their partial proteolytic maps following cleavage with reagents such as V8 protease or cyanogen bromide. Unfortunately, such experiments could not be performed due to inefficient precipitation of radiolabeled Sp3-related proteins from cell extracts. To confirm that the 78–80 kDa Sp3-related proteins synthesized *in vivo* share antigenic determinants with M1, we prepared GST or GST–M1 fusion proteins in bacteria and incubated αSp3 TAD antiserum with an excess of these proteins *in vitro*. Following depletion for reactivity with each of these bacterial proteins, αSp3 TAD antiserum was employed to detect endogenous Sp3-related proteins in mammalian cells. As shown in Figure 2C, preincubation of αSp3 TAD antiserum with GST had little or no effect on reactivity with Sp3 or Sp3-related proteins. In contrast, preincubation of αSp3 TAD antiserum with GST–M1 results in antiserum that detects the 115 kDa Sp3 protein doublet but not the 78–80 kDa Sp3-related proteins. These data clearly indicate that αSp3 TAD antiserum binds antigenic determinants in M1 that are shared by the 78–80 kDa Sp3-related proteins synthesized *in vivo*. Moreover, these data indicate that the Sp3-related proteins do not carry antigenic determinants bound by αSp3 TAD antiserum that are encoded upstream of the M1 translational initiator.

Given that M1 and M2 proteins are likely to be structurally identical to the 78–80 kDa Sp3-related proteins synthesized *in vivo*, we wished to establish whether M1 and M2 are also functionally equivalent. To determine if the DNA binding activities of M1 and M2 are similar to RCP 2, *in vitro* translated Sp3, M1 and M2 proteins were employed in protein–DNA binding assays. As shown in Figure 3A, M1- and M2-derived proteins form protein–DNA complexes that co-migrate with RCP 2 harvested from mammalian nuclear extracts. Moreover, as shown by competition experiments with wild-type (WT) and mutated (Mut) RCE oligonucleotides, the formation of M1 and M2 protein–DNA complexes is dependent on the integrity of nucleotides that are identical to those required for formation of protein–DNA complexes from nuclear extracts. To determine if Sp3 proteins that arise via internal initiation are karyophilic, stably expressed *in vivo* and capable of binding DNA as expected, we subcloned M1 and M2 cDNAs into expression vectors and transient transfections were performed in parallel with Sp3 and Sp1 using *Drosophila* SL2 cells as recipients. Nuclear extracts were prepared from transiently transfected SL2 cells and examined in: (i) Western blots for protein expression; (ii) protein–DNA binding assays (Fig. 3B and C). Each of the transfected expression constructs synthesized nuclear proteins of the predicted size that

were detected in Western blots with antisera prepared against the proteins themselves as well as their epitope tag (Fig. 3B and data not shown). Moreover, each exogenously expressed protein formed protein–DNA complexes of the expected size with radiolabeled RCE oligonucleotides (Fig. 3C and data not shown). Taken together with the immunochemical evidence cited above (Figs 1 and 2C), data from protein–DNA binding assays strongly suggest that: (i) the 78–80 kDa Sp3-related proteins detected *in vivo* are Sp3 isoforms that arise via internal translational initiation at the M1 and M2 methionine residues; (ii) internal initiation within Sp3 mRNA accounts for RCP complex 2. Since we have previously reported that RCP complex 2 appeared to be formed by a protein of 80 kDa (11), we presume that the coincident electrophoretic migration of protein–RCE complexes formed by the 78 and 80 kDa Sp3-derived proteins prevented their resolution as a protein doublet following photoaffinity labeling.

Internally initiated isoforms of Sp3 function as potent inhibitors of Sp-mediated transcription *in vivo*

Since M1 and M2 lack substantial portions of the Sp3 *trans*-activation domain, we speculated that their potency as *trans*-activators was likely to be significantly compromised. Furthermore, given that M1/M2 appear to have the same DNA binding specificity as Sp1/Sp3, we hypothesized that co-expression of M1 or M2 with Sp1 or Sp3 might interfere with Sp-mediated transcription. To examine these possibilities we utilized a well-characterized promoter–CAT construct prepared from the hamster *DHFR* promoter as a reporter gene and *Drosophila* SL2 cells as recipients to monitor Sp-mediated *trans*-activation *in vivo*. We and others have previously shown that Sp1 is a potent *trans*-activator that induces dose-dependent transcription of *DHFR* and a variety of additional mammalian promoters in SL2 cells (12,16,38,44). We have also shown that Sp3-mediated *DHFR* transcription approximates that of Sp1 (34). Consistent with the supposition that truncation of the Sp3 *trans*-activation domain might negatively effect M1/M2-mediated transcription, transient expression of M1 or M2 in SL2 cells led to little or no *DHFR*-mediated transcription above that of the reporter gene alone (Table 1 and data not shown). As might be predicted given their common nucleotide binding specificities, co-expression of M2 with Sp1 or Sp3 resulted in a profound reduction in Sp-mediated *DHFR* transcription (Table 2). Similar results were noted in parallel co-transfection experiments with M1 (data not shown). Interestingly, although Sp1 and Sp3 are expressed at equivalent levels *in vivo* (Fig. 3B), it is apparent that Sp1 and Sp3 differ in their relative sensitivity to transcriptional inhibition; Sp3-mediated transcription is at least 10-fold more sensitive to transcriptional inhibition by M1/M2 than is Sp1 (Table 2). For example, transfection of 50 or 100 ng M2 with 50 ng Sp3 was sufficient to reduce Sp3-mediated transcription by 74 and 91% respectively. In contrast, 1000 ng M2 were required to diminish Sp1-mediated transcription to equivalent levels. The negative effect of M1 and M2 co-expression on Sp-mediated transcription is not restricted to the *DHFR* promoter, as similar results were obtained using promoter–CAT constructs dependent on RCEs derived from the c-*fos*, c-*myc* and *TGF*-β1 genes (Table 2; 11,12). To confirm that transcriptional inhibition by M1/M2 is specific and not due to generalized disruption of transcription *in vivo*, co-transfection experiments with a GAL4–CAT reporter construct and a GAL4–VP16 fusion protein, a potent *trans*-activator, were performed. Consistent with the notion that Sp3-mediated transcriptional inhibition is specific, co-expression of M1 or M2 with GAL4–VP16 did not have a dose-dependent effect on VP16-mediated transcription (Table 2 and data not shown). A slight increase in VP16-mediated transcription was noted at low input amounts of M1/M2 that was not apparent with increasing concentrations of DNA. We conclude from these results that internally initiated isoforms of Sp3 do not function as stimulatory transcription factors in SL2 cells. Instead, such isoforms can function as potent inhibitors of transcription when co-expressed with Sp1 or Sp3. Furthermore, we conclude that Sp3 is significantly more sensitive to transcriptional inhibition by internally initiated Sp3 isoforms than is Sp1.

Table 1. *Trans*–activation of hamster DHFR promoter by Sp-family members[a]

Reporter construct	Additional DNA(s)	Relative CAT activity (±SE)[b]	*n*[c]
DHFR	–	1.0	10
	Sp1 50 ng	130 (31)	14
	Sp3 50 ng	244 (98)	18
	M1 10 ng	1.5 (0.8)	4
	M1 50 ng	1.0 (0.1)	4
	M1 100 ng	1.2 (0.2)	4
	M1 500 ng	2.0 (1.7)	4
	M2 10 ng	3.0 (0.7)	4
	M2 20 ng	3.2 (1.0)	4
	M2 50 ng	1.9 (0.5)	10
	M2 100 ng	2.9 (0.9)	4
	M2 200 ng	3.0 (0.9)	4

[a]*Drosophila* SL2 cells were transiently transfected with a DHFR–CAT reporter construct alone or in conjunction with the indicated amounts of expression constructs for Sp1 (pPacSp1), Sp3 (pPacSp3), M1 (pPacM1) or M2 (pPacM2). Resulting levels of CAT activity were quantified as previously described (12).
[b]Shown are mean values for percentage acetylation (±SE) of chloramphenicol per A_{600} of 1 μl total cell extract normalized to the amount of activity recorded for the DHFR–CAT reporter construct.
[c]*n* is the number of independent plates of transfected cells analyzed.

DISCUSSION

Rb has previously been shown to govern transcription of a subset of growth control genes, such as c-*fos*, c-*myc* and *TGF*-β1, via discrete promoter elements termed RCEs (6,7). To determine the mechanism(s) by which Rb regulates RCE function, we have sought to identify and characterize nuclear RCE binding proteins *in vitro*, determine if such proteins are required for RCE activity *in vivo* and assess the functional consequence of their interaction with Rb. We have previously determined that RCEs are bound *in vitro* by Sp1 and Sp3, two members of the Sp family of transcription factors, and that RCE-mediated transcription is stimulated by these factors *in vivo* (12,34). Moreover, co-expression of Rb and Sp1 or Sp3 leads to marked 'superactivation' of Sp1/Sp3-mediated transcription (12,34). In this report we demonstrate that: (i) Sp3 encodes at least three distinct transcription factors; (ii) each of these Sp3-related proteins binds RCEs *in vitro*; (ii) two Sp3-related proteins arise via internal translational initiation within Sp3 mRNA; (iv) internally initiated Sp3 isoforms are potent inhibitors of Sp-mediated transcription. Taken together with recent evidence that Sp1 is an alternatively spliced gene (45), our results indicate that transcriptional control of RCEs and, by extension, other promoter elements regulated by Sp family members may be significantly more complex than previously suspected.

Table 2. Inhibition of Sp-mediated, but not VP16-mediated transcription by an internally initiated isoform of Sp3[a]

Reporter construct	*Trans*-activator	Additional DNA	Relative CAT activity (±SE)[b]	*n*[c]
DHFR	Sp1 50 ng	–	1.0 (0)	14
		M2 10 ng	1.5 (0.3)	8
		M2 50 ng	1.7 (0.4)	10
		M2 100 ng	1.2 (0.2)	14
		M2 250 ng	0.6 (0.1)	2
		M2 500 ng	0.3 (0.1)	4
		M2 1000 ng	0.1 (0)	2
	Sp3 50 ng	–	1.0 (0)	18
		M2 10 ng	0.8 (0.1)	12
		M2 50 ng	0.3 (0.4)	14
		M2 100 ng	0.1 (0)	18
		M2 250 ng	0 (0)	2
		M2 500 ng	0 (0)	4
FOS	Sp3 50 ng	–	1.0 (0)	3
		M2 100 ng	0.2 (0)	3
MYC	Sp3 50 ng	–	1.0 (0)	3
		M2 100 ng	0.2 (0)	3
TGF-β1	Sp3 50 ng	–	1.0 (0)	3
		M2 100 ng	0.2 (0)	3
GAL4	VP16 10 μg	–	1 (0)	4
		M2 50 ng	1.9 (0.1)	2
		M2 100 ng	1.2 (0.1)	4
		M2 500 ng	1.3 (0)	2

[a]*Drosophila* SL2 cells were transiently transfected with DHFR–CAT, FOS–CAT, MYC–CAT, TGF-β1–CAT or GAL4–CAT reporter constructs in conjunction with the indicated amounts of expression constructs for Sp1 (pPacSp1), Sp3 (pPacSp3), M2 (pPacM2) or GAL4–VP16. Resulting levels of CAT activity were quantified as previously described (12).

[b]Shown are mean values for percentage acetylation (±SE) of chloramphenicol per A_{600} of 1 μl total cell extract normalized to the amount of activity recorded for DHFR or GAL4 reporter constructs in the absence of M2.

[c]*n* is the number of independent plates of transfected cells analyzed.

We and others have previously reported that Sp3 stimulates transcription *in vivo*, however, this observation has not proven to be universal (21,34,42,46–51). Using a cDNA isolated by Hagen *et al*. (35), a number of laboratories have reported that Sp3 has little or no capacity to function as a transcriptional *trans*-activator. Instead, based on a wide variety of transient co-transfection experiments, Sp3 has been reported to function as a repressor of Sp1-mediated transcription. Since it is now apparent that Sp3 encodes at least three distinct proteins *in vivo* and that internally initiated Sp3 isoforms function as transcriptional inhibitors, it is likely that these once disparate observations may be easily reconciled, i.e. experiments that employ Sp3 expression vectors that preferentially lead to biogenesis of full-length Sp3 protein will score Sp3 as a transcriptional *trans*-activator while expression vectors that largely yield internally initiated Sp3 isoforms will score Sp3 as a transcriptional repressor. This conclusion appears to be entirely consistent with the sizes of Sp3 proteins expressed following transient transfections with Sp3 cDNAs (34,50).

Since all Sp3 cDNAs cloned to date are partial cDNAs lacking 5′-untranslated sequences and the extreme N-terminus of Sp3 protein, we can only speculate on the mechanism governing translational initiation within Sp3 mRNA. One possibility is that the translational start site at the 5′-end of the Sp3 message is partially obscured by RNA secondary structure or embedded within a sequence that is weak relative to internal sites of translational initiation. Presumably this arrangement ensures that appropriate levels of stimulatory and inhibitory Sp3 proteins are synthesized *in vivo*. That a single mRNA can encode multiple functionally distinct transcription factors may be a more common theme for transcriptional regulation that previously realized. Like Sp3, the C/EBPα and C/EBPβ CCAAT/enhancer binding proteins have previously been noted to encode full-length proteins that function as *trans*-activators as well as internally initiated isoforms that act as inhibitors of C/EBP function (52–55). As for Sp3, internal translational initiation within C/EBPα and C/EBPβ mRNA results in elimination of a significant portion of their respective *trans*-activation domains. C/EBPβ encodes liver-enriched activating (LAP) and inhibiting (LIP) proteins and the LIP/LAP ratio, which has been shown to be developmentally and hormonally regulated, influences transcription of a variety of cellular genes. Although the precise mechanism by which LIP inhibits LAP function remains to be determined, it is likely that LIP dimerizes with LAP via their respective basic region/leucine zipper domains forming transcriptionally inactive complexes that compete with transcriptionally active LAP dimers for occupancy of DNA binding sites. It is worth emphasizing that our results provide little insight into the mechanism(s) by which internally initiated Sp3 isoforms abrogate Sp-mediated transcription. In protein–DNA binding assays we have previously shown that: (i) Sp1 and full-length and internally initiated Sp3 isoforms are equally abundant in extracts prepared from >20 mammalian cell types grown under a variety of growth conditions; (ii) Sp1 and each Sp3 isoform require identical RCE nucleotides for formation of protein–DNA complexes; (iii) on- and off-rate measurements indicate that each protein–RCE complex is equally stable *in vitro* (A.J.Udvadia and J.M.Horowitz, unpublished observations).

These results would suggest that competition for binding site occupancy between internally initiated Sp3 isoforms and Sp3 or Sp1 could account for diminished Sp-mediated transcription. Yet, if binding site competition were the sole mechanism by which Sp-mediated transcription is inhibited, equivalent levels of inhibition of Sp1 and Sp3 would be expected. Instead, Sp3-mediated transcription is reproducibly at least 10-fold more sensitive to inhibition than is transcription directed by Sp1. Given this intriguing observation, we speculate that disparate degrees of protein–protein interactions may account for the differential sensitivities of Sp1 and Sp3 to inhibition by internally initiated isoforms of Sp3. As one possibility in this regard, we note that the D domain of Sp1 has previously been shown to play an important role in its multimerization and this region is strikingly dissimilar in Sp3. Thus, amino acid differences within the D domains of Sp1 and Sp3 may facilitate higher affinity interactions between full-length Sp3 and its internally initiated isoforms. Yet another possibility springs from the recent identification of a 'repression domain' immediately upstream of the Sp3 DNA binding domain (56). It is conceivable that interactions between Sp3 molecules via this domain may be favored over those involving Sp1 and Sp3 proteins. Once complexed with Sp1 or Sp3, internally initiated Sp3 isoforms may diminish the capacity of multimeric complexes to bind DNA or prevent formation of transcriptionally active protein–DNA complexes. Consistent with the notion that multimerization of Sp proteins is required for transcriptional repression, Sp3-mediated repression has been reported to be dependent on the number of Sp3 binding sites within cellular promoters (51).

Although the mechanism by which internally initiated Sp3 isoforms inhibit Sp1/Sp3-mediated transcription remains obscure, we speculate that this mode of regulation likely plays an important role in cell cycle- and signal-induced transcription. Indeed, cell cycle- or signal-induced expression of a variety of genes, including *p21*$^{waf1/cip1}$, *p15*^{INK4B}, *CYP11A*, *mdr1* and acetyl-CoA carboxylase, has been mapped to GC-rich promoter elements that bind Sp family members *in vitro* (18,19,23,57–60). We predict that cell cycle- and signal-induced alterations of the protein and/or DNA binding activities of internally initiated Sp3 isoforms may account in part for regulation of such differentially expressed genes.

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