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Sp3 Represses Gene Expression via the Titration of Promoter-specific Transcription Factors*

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We have determined previously that Sp3 encodes three distinct gene products as follows: a full-length protein (Sp3) that is an activator of transcription and two isoforms (M1 and M2) derived via internal translational initiation that function as transcriptional repressors. To identify amino acids and functions required for transcriptional repression, we employed PCR-directed mutagenesis to create a panel of mutated M2 proteins. Biochemical and functional analyses of these mutated proteins indicate that functions encoded by the M2 carboxyl terminus, such as DNA binding activity and the capacity to form multimeric complexes, are not required or sufficient for transcriptional repression. Instead, a 93-amino acid portion of the *trans*-activation domain was shown to be the minimal portion of M2 required to block Sp-dependent gene expression. Transcriptional analysis of three Sp-dependent promoters showed that mutations sustained by many M2 proteins result in promoter-specific effects. Regions of M2 required for physical interactions with five TATA box-associated factors (TAF_{II}s) were mapped, and mutations that disrupt the interaction of M2 with two of these proteins, $TAF_{II}70$ and $TAF_{II}40$, were identified. We conclude that Sp3-mediated transcriptional repression is due, at least in part, to competition for promoter-specific transcription factors.

Sp1 is the founding member of a family of five transcription factors, Sp1–5, that govern the expression of a wide variety of mammalian genes (for review, see Ref. 1). Sp1 encodes a ubiquitously expressed nuclear phosphoprotein that has been divided into five sub-domains based upon their respective functions (2, 3). The Sp1 *trans*-activation domain is composed of three sub-domains termed A–C, each of which is capable of stimulating transcription if tethered to DNA via a DNA-binding domain. Sub-domains A and B are composed by serine- and threonine-rich regions as well as glutamine-rich regions. The glutamine-rich portions of A and B are believed to be required for *trans*-activation, whereas the function(s) of the serine/threonine-rich sub-regions is(are) less well understood. Domain C carries a number of charged amino acids and weakly stimulates transcription in the absence of domains A or B. Carboxyl-terminal to the domain C is a region featuring three Cys_2-His_2 zinc "fingers" required for sequence-specific DNA binding to GC-rich promoter elements. A carboxyl-terminal domain, termed D, facilitates protein multimerization and is essential for synergistic *trans*-activation of promoters with multiple Sp-binding sites. Sp1 associates with a large number of transcription-associated proteins, including components of the basal transcription complex (*e.g.* $hTAF_{II}130$/$dTAF_{II}110$ and $hTAF_{II}55$; Refs. 4–7), sequence-specific DNA-binding proteins (*e.g.* E2F, YY1, p53, and AP-2; Refs. 8–13), and transcriptional regulators (*e.g.* p107, HDAC-1, and VHL-1; Refs. 14–16). As might be expected given the variety of proteins with which it interacts, protein-binding sites have been identified throughout Sp1. For example, $hTAF_{II}130$/$dTAF_{II}110$ interact with Sp1 via its *trans*-activation domain; $hTAF_{II}55$ binds the Sp1 zinc fingers, and E2F requires the zinc finger and D sub-domains of Sp1 for protein-protein interactions.

A wide variety of extracellular stimuli have been shown to induce gene expression via discrete promoter elements bound by Sp1 and Sp3 (17–29). Moreover, subtle mutations that negate the association of Sp1/Sp3 with their cognate binding sites completely block the induction of such genes by their respective inducing agents. Although GC-rich elements within the promoters of many Sp1/Sp3-regulated genes have been identified and their necessity for induced transcription has been noted, it remains largely unclear how extracellular stimuli activate Sp1/Sp3-dependent genes. For example, in most instances treatment of cells with inducing agents does not lead to consistent alterations in (i) the abundance or subcellular localization of Sp1/Sp3, (ii) the affinity of Sp1/Sp3 for DNA, (iii) the formation of Sp1/Sp3 multimers, nor (iv) the post-translational modification Sp1/Sp3. Instead, extracellular stimuli may activate Sp-dependent genes via alterations in protein-protein interactions. For example, transforming growth factor-β induces $p15^{Ink4B}$ transcription by catalyzing the formation of protein complexes between Sp1 and members of the Smad family of transcription factors (30). Whether regulated interactions between Sp family members and other factors account for the induced transcription of additional Sp-dependent genes remains to be determined.

Several years ago we identified two novel Sp3-derived proteins, termed M1 and M2, that arise by internal translational initiation within the region of Sp3 mRNA that encodes the Sp3 B domain (31). Sp3, M1, and M2 appear to be expressed in all mammalian cells and tissues at approximately equivalent levels independent of growth status or induction by extracellular stimuli. In contrast to full-length Sp3, M1 and M2 function as potent repressors of Sp-mediated transcription, and Sp3 is at least 10-fold more sensitive to M1/M2-mediated repression than is Sp1. Given that Sp3 encodes proteins with opposing activities, we reasoned that understanding their differential

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regulation may shed light on mechanisms governing the activity of Sp-dependent promoters. To understand further the mechanism(s) by which M1/M2 repress transcription, we prepared a panel of M2 proteins carrying a limited number of random amino acid substitutions and examined their capacity to function as transcriptional regulators of three Sp-regulated promoters: *DHFR*, *p21*, and *MDR-1*. Additionally, we examined each of these mutated proteins for their capacity to bind DNA, to form multimeric complexes with Sp family members, and to bind components of the basal transcription complex. These studies have resulted in the following observations. 1) Random mutagenesis generated a panel of mutated M2 proteins that carry "loss-of-function" and "gain-of-function" mutations. 2) Many amino acid substitutions affect M2-mediated repression in a promoter-specific fashion. 3) DNA binding activity and the capacity to multimerize are not required or sufficient for M2-mediated repression. 4) The minimal region required for transcriptional repression by M2 consists of 93 amino acids of the B domain. 5) Several components of the TAF_{II} complex bind Sp1, Sp3, and M2 *in vitro*. 6) The binding of two TAF_{II}[1] proteins, $TAF_{II}70$ and $TAF_{II}40$, is compromised in several M2 mutants. We conclude from these observations that M1/M2-mediated repression occurs at least in part via the titration of one or more transcription factors that may be required in a promoter-specific fashion.

EXPERIMENTAL PROCEDURES

Cell Culture—C-33A cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and *Drosophila* Schneider line-2 (SL2) cells were a gift of Dr. Cheaptip Benyajati (University of Rochester, Rochester, NY). C-33A and SL2 cells were cultured as described previously (31–33).

Antisera—Rabbit anti-Sp3 was prepared against a GST fusion protein containing the amino-terminal 300 amino acids of Sp3, and its preparation and characterization have been described (31). Affinity-purified mouse anti-HA.11 antibody was obtained from a commercial supplier (Covance Research Products, Richmond, CA).

Expression and Reporter Plasmids—pPacSp1 was obtained from Dr. Robert Tjian (University of California, Berkeley; see Ref. 3). pPacSp3, pPacM1, pPacM2, pBSK-Sp3/flu, pCR-M1/flu, and pCR-M2/flu were prepared as described (31, 33). Constructions employed for *in vitro* translation of human and *Drosophila* TAF_{II} proteins were obtained from Dr. Robert Tjian. *DHFR*-CAT has been described (34). To generate a *DHFR*-luciferase construct, *DHFR*-CAT, 5′ (5′-GGAGATCTAGCGCGCGGCTGTACTAC-3′) and 3′ primers (5′-GGAAGCTTGCAGCCTGTACGCTGTGC-3′), and the PCR were employed to amplify *DHFR* promoter sequences. A resulting 175-bp promoter fragment was subcloned in plasmid pRL (Promega, Inc., Madison, WI). pgpLuc-B carries a 1320-bp portion of the human *MDR-1* promoter and was obtained from Dr. Kathleen Scotto (Memorial Sloan-Kettering Cancer Center, NY; see Ref. 35). p21P93-S carries a 44-bp portion of the human *p21* promoter and was obtained from Dr. Xiao-Fan Wang (Duke University Medical Center; see Ref. 19).

PCR-mediated Mutagenesis and DNA Sequencing—Mutagenesis of M2 was performed using methods described by Zaccolo *et al.* (44). Reactions employed pGEX-M2 (see below) as template, 5′ (5′-CCTGACTTCATGTTGTATGAC-3′) and 3′ primers (5′-CAGTCACGATGAATTCTCGAGAATCCCTAGCTAGCGTAATCTG-3′), and *Taq* polymerase (Invitrogen). Three PCR cycles (92 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) were performed in a 20-μl reaction containing 4 ng of linearized template, 0.5 μM primers, 500 μM each dNTP, and 500 μM dPTP (Amersham Biosciences). An aliquot of this reaction was subsequently employed as template for an additional 22 rounds of amplification in the absence of dPTP. PCR products were digested with *Dpn*I, purified, and cloned in pCR-$Blunt_{II}$-TOPO. Expression plasmids carrying mutated M2 cDNAs were prepared by subcloning inserts from pCR-$Blunt_{II}$-TOPO into pPac (3). Sequencing of M2 mutants was performed by the North Carolina State University sequencing facility using a PerkinElmer Life Sciences AB1377 sequenator or Sequenase version 2.0 DNA polymerase following a protocol supplied by the manufacturer (Amersham Biosciences).

Bacterial and Baculovirus Expression Constructs—pGEX-Sp1, a bacterial expression construct that carries the Sp1 coding region fused in-frame with glutathione *S*-transferase (GST), has been described (36). pGEX-M2 was prepared by subcloning the M2 cDNA carried by pCR-M2/flu in pGEX-2TK (Amersham Biosciences). GST-Sp3 was prepared using pBSK-Sp3/flu, 5′ (5′-GGGGGATCCGCCACCATGAATTCCGGGCCATCGCCG-3′), and 3′ primers (5′-GGAATTCCTCCATTGTCTCATTTCCAG-3′), and the PCR. A 2142-bp amplified fragment carrying the entire Sp3 cDNA was subcloned in pGEX-2TK. pGEX-FSH15 has been described previously (36). To create GST expression plasmids carrying M2 cDNAs terminating at amino acids 103, 197, or 353, M2 cDNAs were amplified from pPacM2 using a 5′ primer (5′-GGGGGATCCATGGATAGTTCAGACAATTCA-3′), one of three 3′ primers (5′-CTACTAGACTCCTTGAAGTTG-3′, 5′-CTAACCAAGTGTGAGGGTTTC-3′, or 5′-TCAGTTAACAAACAAAAGGGCG-3′), and *Taq* polymerase. Amplified M2 cDNAs carrying premature termination codons were subcloned in pGEX-2TK. Mutated M2 GST fusion proteins were prepared by amplification from pPacM2 plasmids using 5′ (5′-GGGGGATCCATGGATAGTTCAGACAATTCA-3′) and 3′ primers (5′-GGAATTCCTCCATTGTCTCATTTCCAG-3′) and *Taq* polymerase. Amplified cDNAs were subcloned in pGEX-2TK. Baculovirus stocks encoding Sp1, Sp3, M1, and M2 were prepared using the PCR, appropriate primers, and pCMV4-Sp1/flu (37), pBSK-Sp3/flu, pCR-M1/flu, and pCR-M2/flu as substrates. Amplified cDNAs were subcloned in pFASTBacHTA and used to prepare virus stocks according to methods supplied by the manufacturer (Invitrogen).

Transient Transfections—Transient transfections for transcription assays were performed by calcium phosphate precipitation as described (31, 33). Cell extracts were prepared for analysis 48 h after transfection. The Dual-Luciferase Reporter Assay System (Promega, Inc.) was employed to quantify luciferase activity precisely as recommended by the manufacturer. Luminescence was detected in a Lumat LB 9507 luminometer (EG & G Berthold, Bad Wildbad, Germany), and results were normalized against total cell protein concentration. To prepare *Drosophila* SL2 extracts for Western blotting or protein/DNA binding assays, transient transfections were performed using SuperFect Transfection Reagent (Qiagen Inc., Hilden, Germany). Cell extracts were prepared 48 h after transfection.

Preparation of Nuclear Extracts—Nuclear extracts were prepared using methods described by Lee *et al.* (38).

Oligonucleotide Probes—Oligonucleotides were synthesized on an automated DNA synthesizer, deprotected, and partially purified through Sephadex G-25 spin columns. Radiolabeled probes for standard protein/DNA binding assays were prepared from the following oligonucleotides and their complements: GT box (39), 5′-AGCTTCCGTTGGGGTGTGGCTTCACGTCGA-3′; p21 (19), 5′-GAGCCGGGGTCCCGCCTCCTTGAGGCGGGCCC-3′; and MDR-1 (35), 5′-CAGGAACAGCGCCGGGGCGTGGGCTAGC-3′.

For quantitative protein/DNA binding assays, six double-stranded 60-mers were synthesized each carrying a single promoter-derived Sp-binding site flanked by common nucleotide sequences. The promoter-derived sequences utilized for these experiments are as follows: p21, 5′-CCCGCCTCCT-3′; MDR-1, 5′-CGCCGGGGCGTGGGC-3′; DHFR-1, 5′-AGGGCGTGGC-3′; DHFR-2, 5′-GAGGCGGGGC-3′; DHFR-3, 5′-GAGGCGGAGT-3′; and DHFR-4, 5′-TGGGCGGGGC-3′. Annealed and complementary oligonucleotides were radiolabeled and purified as described previously (31, 32).

Protein/DNA Binding Assays—Protein/DNA binding assays were performed as described previously (31, 32), and complexes were visualized by autoradiography. For quantitative protein/DNA binding assays, whole cell protein extracts prepared from baculovirus-infected Sf9 cells were incubated with a radiolabeled probe derived from the c-*fos* gene (5′-CCCTTGCGCCACCCCTCT-3′; see Ref. 32), and the resulting protein-DNA complexes were quantified *in situ* using an InstantImager (Packard Instrument Co.). Volumes of these extracts that led to half-maximal binding of this probe were then employed in assays performed in triplicate with Sp-binding sites derived from the *DHFR*, *p21,* and *MDR-1* genes and quantified *in situ*.

Western Blotting—Whole cell or nuclear extracts were resolved on denaturing polyacrylamide gels and transferred to nitrocellulose using a semi-dry transfer apparatus. Nitrocellulose filters were incubated with 5% milk in TBS-T (2.42 g/liter Tris, 8 g/liter NaCl, pH 7.6, 1 ml/liter Tween 20) from 1 h to overnight. Primary antibodies were diluted in TBS-T (anti-Sp3 at 1:2000 and anti-HA.11 at 1:1000), incubated with filters for 1 h at room temperature, and washed with TBS-T.

[1] The abbreviations used are: TAF_{II}s, TATA box-associated factors; GST, glutathione *S*-transferase; DHFR, dihydrofolate reductase; HA, hemagglutinin; dPTP, 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one triphosphate.

Filters were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T (anti-mouse, 1:10,000, Amersham Biosciences, or anti-rabbit, 1:40,000, Invitrogen) for 1 h at room temperature with gentle agitation and washed in TBS-T, and antigen-antibody complexes were detected using ECL Western blotting Detection Reagents (Amersham Biosciences).

In Vitro Transcription/Translation—In vitro transcribed/translated proteins were produced using a coupled reticulocyte lysate system (TNT; Promega, Inc.) with T3 or T7 RNA polymerase and L-[^{35}S]methionine (Tran^{35}S-label; ICN). pBSK-Sp1/flu, pBSK-Sp3/flu, pCR-M2/flu, PCR-amplified mutated M2 cDNAs, and TAF_{II} constructs were employed as templates for these reactions. Mutated M2 cDNAs in pPac were amplified and prepared for *in vitro* transcription/translation using a 5′ T7 promoter-containing primer, a 3′ primer, and the PCR.

In Vitro Protein/Protein Binding Assays—Protein binding assays were performed as described (40). GST bead-bound proteins were resolved on denaturing polyacrylamide gels and visualized by autoradiography.

RESULTS

The DHFR, p21, and MDR-1 Promoters Possess Distinct Sensitivities to Sp-mediated Trans-activation and Repression—We have shown previously that Sp1 and Sp3 stimulate transcription of the *DHFR* promoter and that Sp1/Sp3-mediated transcription is repressed by two isoforms of Sp3, termed M1 and M2, that arise via internal translational initiation (31, 33, 37). To determine whether these results were likely to reflect a general mechanism of transcriptional regulation, we extended our analyses to the *p21* and *MDR-1* promoters, two well characterized Sp-dependent promoters of physiologic and therapeutic interest. The *p21*-luciferase construct employed in these studies includes an Sp protein-binding site that is required for transcriptional stimulation by transforming growth factor-β, calcium, or sodium butyrate, as well as a second Sp protein-binding site that is also required for induction by sodium butyrate (19, 23, 41). The *MDR-1*-luciferase construct employed includes an Sp protein-binding site that is required for induction by serum or the c-Raf kinase (42, 43).

As illustrated in Fig. 1*A*, the ectopic expression of Sp3 in *Drosophila* SL2 cells stimulated transcription of the *DHFR*, *p21,* and *MDR-1* promoters to varying degrees. *p21* transcription was stimulated 40-fold more effectively than *MDR-1* and 12-fold more than the *DHFR* promoter. Analogous results were obtained for activation of each of these promoters by Sp1 (data not shown). Consistent with results reported previously for the *DHFR* promoter, co-expression of M1 or M2 with Sp1 or Sp3 resulted in repression of *p21* and *MDR-1* transcription (Fig. 1*B* and data not shown). Interestingly, the sensitivity of each promoter to M1/M2-mediated repression was noted to be promoter-specific. The promoter most acutely sensitive to activation by Sp1/Sp3, *p21*, exhibited the least sensitivity to M1/M2-mediated repression. In turn, a promoter that is only modestly activated by Sp1/Sp3, *MDR-1*, exhibited the greatest sensitivity to M1/M2-mediated repression. Sensitivity of *DHFR* to Sp-mediated activation and repression was noted to fall between these two extremes. Given the results presented in Fig. 1, we conclude that intrinsic differences between Sp-dependent promoters influence the degrees to which they are activated by Sp1/Sp3 and repressed by M1/M2.

Trans-activation and Repression of DHFR, p21, and MDR-1 Transcription in Vivo Is Not Directly Correlated with the Capacity of Sp Proteins to Bind to DNA in Vitro—Because Sp proteins activated and repressed the *DHFR*, *p21*, and *MDR-1* to varying degrees, we wished to determine whether these effects might be accounted for by inherent differences in their capacity to bind DNA. For example, the relative sensitivity of *p21* to Sp3-mediated transcription and insensitivity to M2-mediated repression might be explained if Sp3 and M2 bind the *p21* promoter with substantially different affinities. To address

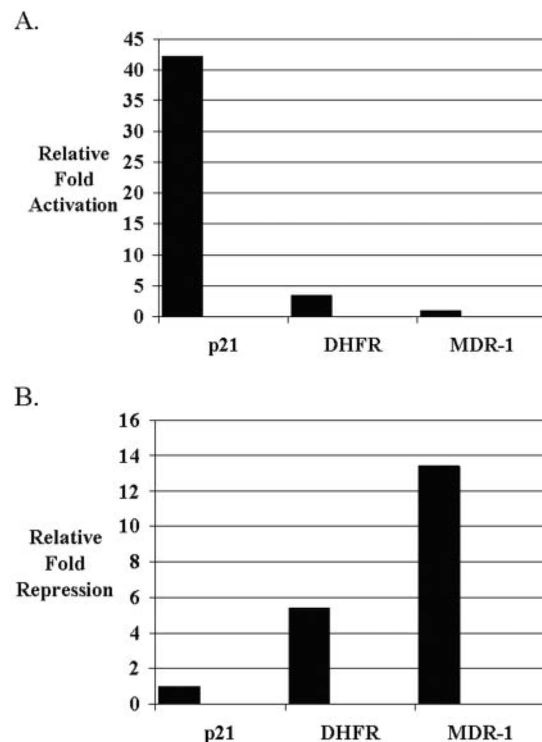

FIG. 1. **Relative fold activation and repression of the *p21*, *DHFR*, and *MDR-1* promoters by Sp3 isoforms.** *A*, relative fold activation of the *p21*, *DHFR*, and *MDR-1* promoters by Sp3. *Drosophila* SL2 cells were transiently transfected with pPac-Sp3/flu and reporter constructs, and luciferase activity was quantified 48 h later. Levels of *trans*-activation obtained from 5 to 21 independent plates of transfected cells were averaged, and fold activation of the *p21* and *DHFR* promoters was determined relative to that of *MDR-1* (set equal to 1.0). *B*, relative fold repression of the *p21*, *DHFR*, and *MDR-1* promoters by M2. *Drosophila* SL2 cells were transiently transfected with constant amounts of pPac-Sp3/flu, a 2- (*DHFR* and *MDR-1*) or 4-fold (*p21*) molar excess of pPac-M2/flu, and luciferase reporter constructs. Luciferase activity was quantified 48 h later from 5 to 21 independent plates of transfected cells, and mean levels of repression were calculated in comparison with plates transfected without M2, and fold repression of the *DHFR* and *MDR-1* promoters was determined relative to that of *p21* (set equal to 1.0).

this issue, we performed a series of quantitative protein/DNA binding assays using radiolabeled DNA probes carrying Sp-binding sites from the *DHFR*, *p21*, and *MDR-1* promoters. Recombinant baculovirus stocks encoding Sp1, Sp3, M1, or M2 proteins were prepared; Sf9 cells were infected with these viruses, and protein extracts from infected cells were normalized for DNA binding activity using a well characterized Sp protein-binding site derived from the mouse c-*fos* promoter. The volume of each protein extract that bound 50% of the c-*fos* probe was then employed in protein/DNA binding assays with six Sp protein-binding sites derived from the *DHFR*, *p21*, and *MDR-1* promoters. Each assay was performed in triplicate and quantitated *in situ*. As shown in Table I, Sp proteins bound to each probe, and the capacity of a given Sp protein to bind these DNAs varied over a 3-fold range. Interestingly, little correlation was noted between the relative capacity of Sp proteins to bind these DNAs *in vitro* and the efficiencies with which they activate or repress transcription *in vivo*. For example, Sp1 and Sp3 bound the Sp-binding site derived from the *MDR-1* promoter more efficiently than a site derived from the *p21* promoter, yet *MDR-1* is activated significantly less efficiently by both proteins *in vivo*. Similarly, Sp protein-binding sites derived from all three promoters were bound equivalently by M2, yet their sensitivity to M2-mediated repression varies over a 13-fold range *in vivo*. We conclude from these studies that the

TABLE I

Relative binding of recombinant Sp proteins to oligonucleotides carrying Sp-binding sites derived from three Sp-dependent promoters

Protein extracts were prepared from Sf9 cells infected with baculovirus stocks encoding Sp family members, and the volume of each extract required to bind 50% of a radiolabeled oligonucleotide probe derived from the c-*fos* promoter was determined. This volume of cell extract was later employed in protein/DNA binding assays with oligonucleotides derived from the *DHFR*, *p21*, and *MDR-1* promoters. Binding assays were performed in triplicate and quantified *in situ*. DNA binding activities for each protein were normalized to the amount of binding activity detected for each protein on the *p21* oligonucleotide.

Sp protein	Promoter					
	p21	*MDR-1*	*DHFR*			
			DHFR-1	DHFR-2	DHFR-3	DHFR-4
Sp1	1	2.5	2.2	1.8	1	3.0
Sp3	1	1.2	2.5	1.9	1.2	3.0
M1	1	1.5	1.7	1.2	0.6	2.1
M2	1	1	1	1	0.5	1.4

relative capacity of Sp proteins to stimulate or repress transcription is not directly correlated with the efficiency with which they interact with their cognate promoter binding sites *in vitro*.

Mutated M2 Proteins Exhibit Promoter-specific Alterations in Their Capacities to Repress Transcription—Based on the data presented in Table I, the relative sensitivity of the *DHFR*, *p21*, and *MDR-1* promoters to M1/M2-mediated repression does not appear to be determined by the intrinsic capacity of M1/M2 to bind DNA. To confirm these data and as a first step toward defining the mechanism(s) governing M1/M2-mediated repression, we reasoned that amino acids required for transcriptional repression might be identified via a functional analysis of proteins carrying random amino acid substitutions or deletions. We predicted that the analysis of such mutants should reveal whether previously characterized functions of Sp proteins, such as DNA binding activity and/or multimerization, are required for transcriptional repression. Consequently, a limited number of amino acid substitutions were introduced into M2 using a PCR-mediated technique for random mutagenesis in which dPTP is substituted for dCTP (44). Expression plasmids carrying mutated M2 cDNAs were transiently expressed in *Drosophila* SL2 cells, and cell extracts were analyzed for the expression of M2 protein by Western blotting with anti-Sp3 antiserum. Western blots of whole cell extracts resulted in the identification of 102 independent M2 mutants that give rise to stable proteins varying in size from that of wild-type M2 (78 kDa) to ~30 kDa (Table II and data not shown).

To characterize the capacity of mutated M2 proteins to repress Sp-mediated transcription, a series of co-transfection experiments was performed with Sp3 and a *DHFR*-luciferase reporter construct. As might be expected, of the 102 mutated M2 proteins analyzed for their capacity to repress *DHFR* transcription, the vast majority (74 mutants, 73%) functioned akin to wild-type M2. Of the remaining M2 mutants, repression by nearly half (12 mutants, 43%) was found to be severely debilitated relative to wild-type M2; such mutants have less than one-tenth the activity of wild-type M2. The remaining mutants (16 mutants; 57%) exhibited varying capacities to block *DHFR* transcription, ranging from one-third to one-tenth the activity of wild-type M2. To extend this analysis, a panel of 35 mutants was assembled and analyzed for their capacity to repress *p21*- and *MDR-1* transcription. This panel included all mutants that lacked the capacity or showed diminished capacity to repress *DHFR* transcription as well as seven randomly selected mutants that repressed *DHFR* akin to wild-type M2. Interestingly, although the majority of the 35 mutants examined are partially or completely incapable of repressing *DHFR*, their activities on these additional promoters were quite variable (Table II). Three examples illustrate this point as follows. (i) Mutant 16 has a greatly diminished capacity to repress *DHFR* transcription but represses *p21* and *MDR-1* to levels akin to wild-type M2. (ii) Mutant 42 is as defective as is mutant 16 when examined as a repressor of *DHFR* transcription, yet it represses *p21* more efficiently than wild-type M2 and has a diminished capacity to repress *MDR-1*. (iii) Finally, mutant 158 exhibits wild-type activity on the *DHFR* and *p21* promoters yet is 7-fold more active than wild-type M2 on the *MDR-1* promoter. These results suggest that this panel of mutated M2 proteins has sustained loss-of-function mutations, *i.e.* alterations that diminish potency as repressors relative to wild-type M2, as well as gain-of-function mutations, *i.e.* alterations that render particular mutants more potent than wild-type M2 as transcriptional repressors. It is worth noting, however, that none of the mutated M2 proteins we examined acquired the capacity to stimulate transcription of the *DHFR*, *p21*, or *MDR-1* promoters. To extend these findings, two approaches were undertaken. First, 27 M2 mutants were sequenced in their entirety and compared with the sequence of wild-type M2. Second, a series of biochemical experiments was performed to determine whether additional functional properties of Sp family members, such as DNA and protein binding activity, were affected by the introduced mutations.

Sequence Analysis of M2 Mutants Indicates That a 93-Amino Acid Region within the M2 B Domain Is Required for Transcriptional Repression—To identify domains and amino acids required for M2-mediated transcriptional repression, 27 of the 35 mutants listed in Table II were subjected to automated DNA sequencing. As anticipated, the majority (98%) of the mutations generated by incorporation of dPTP are transitions: almost 40% are A → G mutations, G → A and T → C mutations are 23 and 21%, respectively, and 15% of the mutations are C → T. The remainder of the mutations are transversions; two G → C mutations were identified, and one mutation each of C → A, A → C, and C → G was noted. Based on previously published results we had anticipated that a limited incorporation of dPTP would introduce an average of 12 nucleotide changes per M2 cDNA, yielding on average seven amino acid changes per protein. Sequencing of resulting clones revealed that each M2 cDNA carries an average of 15 nucleotide substitutions per molecule, resulting in an average of 10 amino acid changes per M2 protein. Importantly, mutations were found to be distributed throughout the M2 cDNA (data not shown).

Because the transcriptional repression properties of many of the clones selected for sequencing are altered relative to wild-type M2, we predicted that amino acids of M2 required for repression might be mutated more frequently on average than those required for other functions. Indeed, a number of M2 amino acid positions were mutated at an above-average frequency; 23 amino acid positions were each mutated in two independent clones, and 8 amino acid positions were each mutated in three independent clones (Fig. 2). Consistent with the notion that these frequently mutated amino acids may play a role in transcriptional repression, 16 of 19 mutants that carry

TABLE II
Biochemical and functional characteristics of mutated M2 proteins

The functional properties of M2 mutants were established in transcription, protein/DNA, and protein/protein binding assays. Introduced mutations were determined by DNA sequencing.

M2 mutant	Apparent molecular mass	Relative fold repression activity[a]			DNA sequenced	DNA binding activity[b]	M2 binding activity[c]
		DHFR	p21	MDR-1			
	kDa						
2	78	$>-10_2$	$>-10_2$	ND	No	ND	−
3	78	-3_3	WT_2	ND	No	+	+
4	70	-8_4	WT_3	ND	No	ND	ND
15	78	-6_4	-3_2	ND	Yes	−	+
16	78	-8_{10}	WT_6	WT_6	Yes	−	+
20	42	$>-10_4$	-3_4	ND	Yes	−	−
33	44	$>-10_4$	-4_2	ND	Yes	−	−
37	65	$>-10_{10}$	-4_6	ND	Yes	−	−
38	78	WT_9	-5_3	-6_7	Yes	+	+
42	40	-8_8	$+3_8$	-8_7	Yes	−	−
43	33	-8_6	-3_3	-8_2	Yes	−	−
44	78	WT_4	WT_2	ND	Yes	+	+
46	78	-5_9	$+3_6$	-5_2	Yes	−	+
48	78	-4_4	-6_4	ND	Yes	−	−
50	78	$>-10_4$	-8_3	ND	No	−	ND
57	55	$>-10_{10}$	-7_7	WT_4	Yes	−	−
60	40	-9_6	WT_2	ND	Yes	−	−
63	35	$>-10_6$	-4_4	ND	Yes	−	−
68	78	-7_5	WT_7	ND	Yes	−	+
103	70	-4_5	WT_6	ND	Yes	−	−
105	78	WT_6	-3_4	ND	Yes	+	+
122	70	WT_7	$+9_2$	ND	Yes	−	+
128	78	-5_5	-7_6	$>-10_4$	Yes	−	+
138	78	-8_7	$>-10_4$	WT_{10}	Yes	−	+
158	78	WT_8	WT_7	$+7_8$	Yes	+	+
166	78	$>-10_3$	-3_2	ND	Yes	−	+
170	78	-6_2	-6_2	ND	No	−	+
172	78	-9_5	$>-10_8$	WT_2	Yes	+	+
177	33	$>-10_4$	$>-10_3$	ND	Yes	−	−
189	78	-6_7	-6_3	ND	Yes	+	+
202	78	WT_4	-4_{10}	WT_3	No	+	−
229	78	$>-10_7$	-8_5	ND	Yes	+	+
258	40	$>-10_8$	$>-10_4$	ND	Yes	−	−
259	78	$>-10_6$	-6_5	ND	No	+	ND
260	78	WT_4	WT_6	WT_3	No	+	+

[a] Negative numbers indicate fold reductions in repression activity relative to wild-type M2, and positive numbers indicate fold increases in repression activity relative to wild-type M2. Numbers of independent transient-transfection experiments performed for each M2 mutant on each promoter are indicated by subscripts. ND, not determined; WT, equivalent to wild-type M2.
[b] +, can bind DNA *in vitro*; −, cannot bind DNA *in vitro*.
[c] +, can bind GST-M2 *in vitro*; −, cannot bind GST-M2 *in vitro*; ND, not determined.

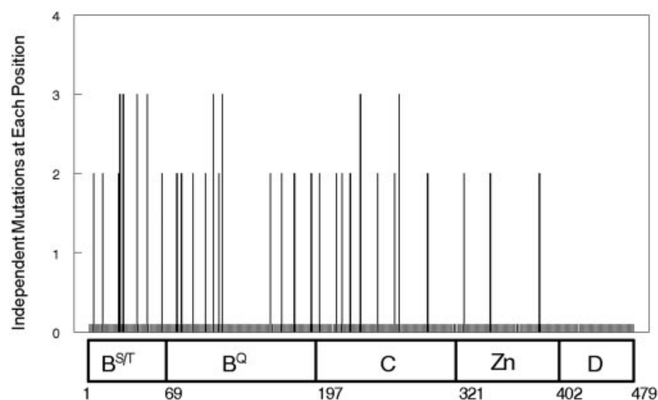

FIG. 2. **Distribution of mutational hot spots in 27 M2 mutants.** Amino acids mutated in two or three independent M2 mutants are illustrated above a schematic representation of the domain structure of M2. Amino acid positions demarcating each domain are indicated relative to the initiation of M2 translation. $B^{S/T}$, serine- and threonine-rich sub-domain; B^Q, glutamine-rich sub-domain; *C*, C domain; *Zn*, zinc finger domain; *D*, D domain.

mutations at one or more of these mutational "hotspots" are essentially inactive as repressors on one or more of the promoters examined. As indicated in Fig. 2, the vast majority of these mutational "hotspots" map outside of domains that harbor carboxyl-terminal functions such as DNA binding and protein multimerization. Instead, two-thirds of the amino acids mutated in three independent clones and more than half of the amino acids mutated in two independent clones are located within the $B^{S/T}$ and B^Q sub-domains, regions that account for only 19% of the amino acids that compose M2. It is worth mentioning that the frequency of mutants carrying mutations within amino-terminal hotspots is identical to full-length and nonsense codon-containing M2 mutants. Because the majority of the mutational hotspots identified in M2 are located within the B domain, these data suggest that one or more functions encoded by this region are required for transcriptional repression. This supposition would appear to be supported by biochemical analyses of M2 mutants that carry "nonsense" mutations (see below).

Twelve of 27 sequenced mutants were found to carry nonsense mutations within the M2 B or C domains or within the amino terminus of the zinc "finger" region. Yet approximately half of the truncated proteins elaborated by these mutants were quite active as transcriptional repressors when analyzed on the *p21* or *MDR-1* promoters (Table II). For example, translation of mutants 42 and 57 terminates within the amino-terminal portion of the M2 B^Q domain (amino acids 82 and 94, respectively), yet each are capable of repressing Sp-mediated transcription of the *p21* or *MDR-1* promoters. Based on these

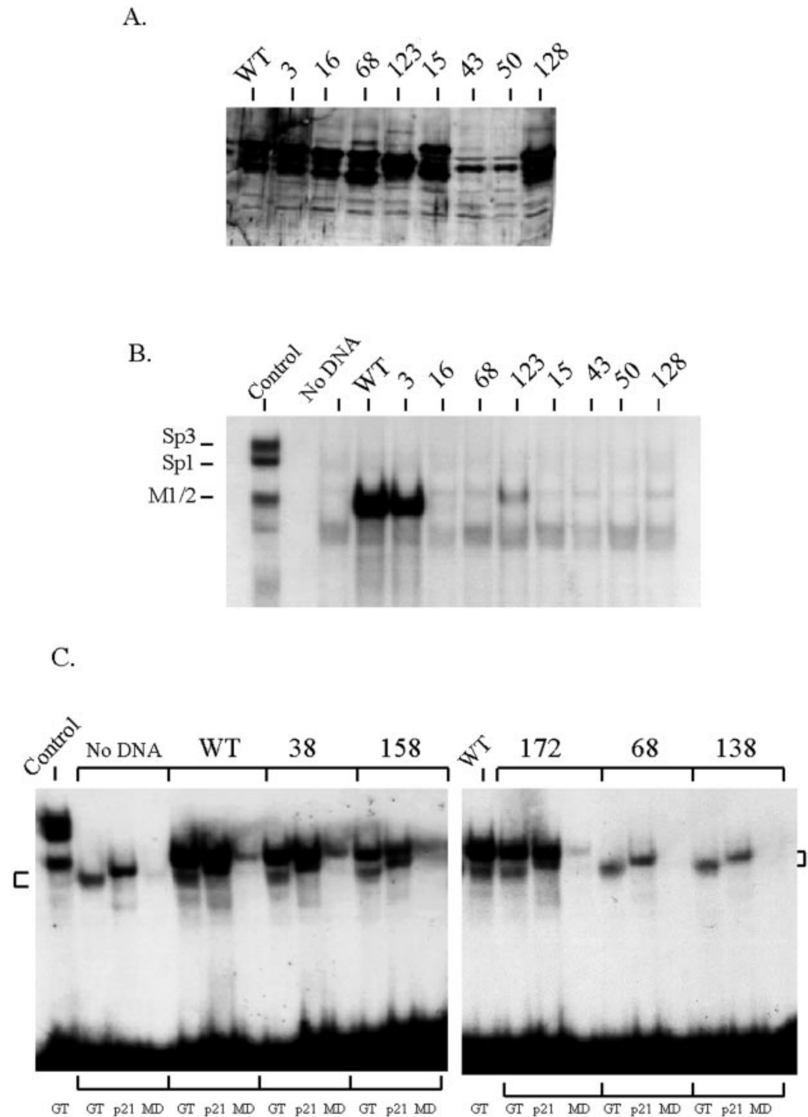

FIG. 3. **Wild-type and mutated M2 protein expression and DNA binding activity in nuclear extracts prepared from transfected *Drosophila* SL2 cells.** *A*, Western blot of nuclear extracts probed with anti-HA antibody 12CA5. Nuclear extracts were prepared from SL2 cells transfected with wild-type (*WT*) or mutated M2 constructs. Mutant numbers are indicated at *top*. *B*, DNA binding activity of wild-type and mutated M2 proteins. A radiolabeled GT box probe was incubated with the above nuclear extracts, a nuclear extract prepared from untransfected SL2 cells (*No DNA*), and a nuclear extract prepared from human C-33A cervical carcinoma cells (*Control*). Protein-DNA complexes generated by Sp3, Sp1, and M1/M2 are indicated at the *left*. Mutant numbers are indicated at *top*. *C*, DNA binding activity of wild-type and mutated M2 proteins. Radiolabeled probes derived from the *p21* (p21) and *MDR-1* (MD) promoters as well as a GT box (*GT*) probe were incubated with above nuclear extracts, a nuclear extract prepared from untransfected SL2 cells (*No DNA*), and a nuclear extract prepared from human C-33A cervical carcinoma cells (*Control*). Mutant numbers are indicated at *top*. *Brackets* to the *left* and *right* indicate protein-DNA complexes generated by endogenous *Drosophila* proteins.

results, we conclude that the minimal region of M2 required for transcriptional repression of the promoters examined includes amino acids 1–93 (corresponding to amino acids 235–328 of Sp3). This minimal region for transcriptional repression spans the serine- and threonine-rich sub-domain and 25 amino acids of the glutamine-rich sub-domain of the M2 B region.

Although the data described thus far indicate that residues within the B portion of the M2 *trans*-activation domain appear to be required for M2-mediated transcriptional repression, amino acids within other domains are likely to play significant roles in determining the degree to which a given promoter is repressed. For example, mutant 122 represses *DHFR* transcription akin to wild-type M2 but is 9-fold more effective as a repressor of *p21* transcription. Similarly, mutant 158 represses *DHFR* and *p21* transcription akin to wild-type M2 but is 7-fold more active as a repressor of *MDR-1*. As might be predicted given their potent activities as transcriptional repressors, each of these mutants carries a wild-type M2 B domain. Yet each of these mutants carries mutations that dramatically alter their activity as transcriptional repressors. Translation of mutant 122 terminates within its zinc finger region, and mutant 122 also carries single amino acid substitutions within the C and zinc finger regions. Mutant 158 is wild-type in length and carries single amino acid substitutions within its C and zinc finger regions distinct from those carried by mutant 122. Because mutations outside of the M2 B domain can alter the capacity of mutated M2 proteins to repress particular Sp-dependent promoters, we conclude that such mutations affect regions of M2 that may determine promoter-specific interactions. This conclusion encouraged us to determine whether such mutations alter the capacity of mutated M2 proteins to bind DNA or form multimeric protein complexes.

DNA Binding Activity Is Not Required for M2-mediated Repression—To determine whether carboxyl-terminal M2 mutations affect the capacity to bind DNA, mutated M2 proteins were characterized for their DNA binding activity via an *in vitro* protein/DNA binding (gel shift) assay. Wild-type or mutated M2 proteins were expressed ectopically in *Drosophila* SL2 cells, and nuclear extracts prepared from transfected cells were incubated with a radiolabeled high affinity Sp protein-binding site (GT box) that has been characterized in detail (39). Mutated M2 proteins examined include (i) mutants competent to repress transcription of the *DHFR*, *p21,* and/or *MDR-1* promoters, *e.g.* mutants 3, 16, 68, and (ii) mutants with little or no capacity for repression of Sp-mediated transcription, *e.g.* mutants 15 and 128. To ensure that equivalent levels of wild-type and mutated M2 proteins were assayed, protein expression was monitored via Western blotting of nuclear extracts (Fig. 3*A* and data not shown). As illustrated in Fig. 3*B*, although some mutated M2 proteins retain the capacity to bind a high affinity

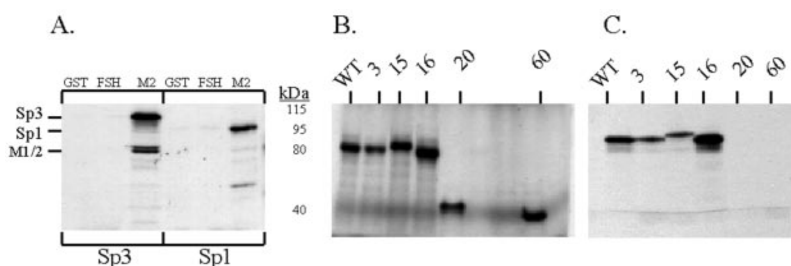

FIG. 4. **Formation of multimeric protein complexes by wild-type and mutated M2 proteins.** *A*, interaction of Sp1 and Sp3 with GST fusion proteins. Radiolabeled and *in vitro* translated Sp1 (*right panel*) and Sp3 (*left panel*) proteins were incubated with glutathione-agarose beads bound to GST-M2 (*M2*), GST-FSH15 (*FSH*), or GST (*GST*) alone, and radiolabeled bead-bound proteins were resolved by electrophoresis. *In vitro* translated proteins are identified at *left*. *B*, *in vitro* translation of wild-type and mutated M2 proteins. Wild-type (*WT*) and mutated M2 proteins were radiolabeled via *in vitro* translation and resolved by electrophoresis. Mutant numbers are indicated at *top*. *C*, interaction of wild-type and mutated M2 proteins with GST-M2. Radiolabeled *in vitro* translates shown in *B* were incubated with GST-M2, and bead-bound proteins were resolved by electrophoresis. Mutant numbers are indicated at *top*.

Sp-binding site, many mutated M2 proteins exhibited undetectable or greatly reduced DNA binding activity relative to wild-type M2. As indicated in Table II, many mutated M2 proteins that exhibit little or no *in vitro* DNA binding activity are quite competent to repress Sp-mediated transcription *in vivo*. For example, mutant 16 exhibits little or no DNA binding activity *in vitro*, yet this mutant represses the *p21* and *MDR-1* promoters as efficiently as wild-type M2. Similarly, mutant 68 gives rise to little or no protein-DNA complexes *in vitro* yet is competent to repress *p21* akin to wild-type M2. Finally, three mutants, 42, 46, and 122, that repress *p21* transcription more potently than wild-type M2 also exhibit little or no DNA binding activity *in vitro*. It is also quite apparent from the data in Table II that the capacity to bind DNA is not sufficient to repress transcription. For example, mutants 38, 172, 189, 229, and 259 each bind DNA efficiently, yet their capacity to repression transcription is severely impaired.

To ensure that these *in vitro* protein/DNA binding results are not specific to the Sp-binding site examined, similar assays were performed using radiolabeled probes carrying well characterized Sp protein-binding sites derived from the *p21* and *MDR-1* promoters. Consistent with results for the GT box probe, many mutated M2 proteins that have lost the capacity to bind Sp-binding sites from the *p21* or *MDR-1* promoters *in vitro* retain activity as transcriptional repressors of these same promoters *in vivo* (Fig. 3*C* and Table II). Additionally, for those mutated proteins that retain the capacity to bind DNA little correlation was noted between their relative activity as DNA-binding proteins and their capacity to repress transcription (Table II). We conclude from these results that DNA binding activity, at least as revealed by *in vitro* protein/DNA binding assays, is not required for M2-mediated transcriptional repression.

Protein Multimerization Is Not Required for M2-mediated Repression—The Sp1 D domain is located immediately downstream of the DNA-binding domain, and this 80-amino acid region has been shown to be essential for protein multimerization and synergistic *trans*-activation of promoters carrying multiple Sp-binding sites (2). Although Sp3 has yet to be shown to form multimers *in vitro* or *in vivo*, all Sp3 isoforms carry a region carboxyl-terminal to their DNA-binding domains that is similar in size and amino acid composition with the Sp1 D domain. Should Sp3 isoforms be competent to form multimers *in vivo*, we reasoned that the formation of multimeric complexes containing M1 and/or M2 proteins might "poison" such complexes and thus account for Sp3-mediated transcriptional repression. In keeping with this reasoning, we hypothesized that alterations in transcriptional repression by mutated M2 proteins might reflect their increased or decreased capacity to join such multimeric protein complexes. Accordingly, an *in vitro* protein/protein binding assay was developed, and wild-type and mutated M2 proteins were examined for their capacity to multimerize with wild-type M2 protein.

To begin these studies, a bacterial fusion protein was prepared that links GST with wild-type M2 protein. GST-M2 protein harvested from bacteria was bound to glutathione-agarose beads and incubated with radiolabeled *in vitro* translated Sp1 or Sp3. As negative controls for these binding studies, radiolabeled Sp1 and Sp3 proteins were also incubated with GST alone as well as GST-FSH15, an irrelevant GST fusion protein. Consistent with the notion that M2 can associate with Sp proteins, Sp1 and all isoforms of Sp3 bound to GST-M2 but not to GST or GST-FSH15 (Fig. 4*A*). To extend these studies, wild-type or mutated M2 proteins were radiolabeled via *in vitro* translation and were assayed similarly. As shown in Fig. 4, *B* and *C*, wild-type M2 and mutants such as 3, 15, and 16 bound GST-M2 *in vitro*, whereas mutants such as 20 and 60 have lost this function. Consistent with the hypothesis that the Sp3 D domain may facilitate multimerization, it is worth noting that mutants 20 and 60 carry premature "stop" codons that eliminate 322 and 283 carboxyl-terminal amino acids, respectively, including their D domains. Similar to results for DNA binding activity, the capacity of M2 proteins to multimerize, at least *in vitro*, is not required for repression of Sp-mediated *trans*-activation (Table II). For example, mutant 42 does not bind GST-M2, and although its capacity for repressing *DHFR* or *MDR-1* is compromised, it is 3-fold more active than wild-type M2 as a repressor of *p21*. Mutant 57 does not bind GST-M2 but represses *MDR-1* as efficiently as wild-type M2, although it has a diminished capacity for repression of *p21* and does not repress *DHFR*. Finally, mutant 103 does not bind GST-M2 but retains at least some capacity to repress Sp-mediated transcription of *DHFR* and *p21*. In summary, *in vitro* multimerization and DNA binding activity do not appear to be required or sufficient for M2-mediated transcriptional repression. Although we believe it is highly likely that these functional defects are also manifest *in vivo*, it is worth noting that we cannot discount the possibility that the intracellular milieu may completely or partially ameliorate these functional deficiencies.

M2 Binds TATA Box-binding Protein-associated Factors, and Interactions with $TAF_{II}70$ and $TAF_{II}40$ Are Disrupted or Diminished in Some M2 Mutants—Because M2-mediated repression does not require DNA binding activity or multimerization, we reasoned that interactions between M2 and one or more cellular proteins might account for repression of Sp-mediated transcription. Given that the C, zinc finger, and D domains are not absolutely required for transcriptional repression, Sp-binding proteins that interact with carboxyl-terminal portions of Sp proteins, such as E2F and AP-2, were considered to be unlikely candidates (8–10). Instead, components of the basal transcrip-

tion complex, such as TATA box-binding protein-associated factors (TAF_{II}s), presented themselves as attractive candidates because (i) TAF_{II}s have been shown to participate in Sp-mediated *trans*-activation, and (ii) one member of the TAF_{II} complex (TAF_{II}110) interacts with Sp1 via glutamine-rich domains, including a hydrophobic region that is closely conserved with an analogous portion of the B domain of Sp3, M1, and M2 (7).

To examine interactions between Sp family members and TAF_{II} proteins, a series of *in vitro* protein/protein binding assays were performed that combined GST fusions prepared with Sp family members and radiolabeled *in vitro* translated TAF_{II} proteins ($TAF_{II}30\alpha$, -30β, -32, -40, -70, -110, -150, and -250). To ensure that these assays would reflect differences in the affinity of particular TAF_{II}s for GST-bound proteins, equivalent amounts of bacterially expressed GST fusion proteins were bound to beads. As illustrated in Fig. 5*A*, some TAF_{II} proteins (30α, 30β, and 32) exhibited little or no capacity to bind Sp family members, whereas other TAF_{II} proteins (40, 70, 110, 150, and 250) bound readily and specifically. Some differences in the binding of TAF_{II} proteins were noted between Sp family members. For example, TAF_{II}250 and TAF_{II}70 appear to bind Sp3 and M2 more efficiently than Sp1 (Fig. 5*A*).

Because previous reports that have mapped interactions of TAF_{II}s with Sp family members have limited their analyses to Sp1, we performed a series of protein/protein binding assays that mapped regions of M2 required for interactions with TAF_{II}s 40, 70, 110, 150, and 250. As shown in Fig. 5*B*, three truncated GST-M2 fusion proteins were employed for these studies as follows: (i) a wild-type M2 protein whose translation terminates within the amino terminus of B^Q (amino acid 103; Δ103), (ii) a wild-type M2 protein whose translation terminates at the carboxyl terminus of B^Q (amino acid 197; Δ197), and (iii) a wild-type M2 protein whose translation terminates within the second zinc finger (amino acid 353; Δ353). Equivalent amounts of GST alone, GST-M2, and each truncated GST-M2 fusion protein were employed in *in vitro* protein/protein-binding reactions with radiolabeled *in vitro* translated TAF_{II} proteins. As shown in Fig. 5*C*, TAF_{II}250 bound full-length M2 and Δ353 but did not interact with Δ197 or Δ103. Binding by TAF_{II}250 to Δ353 was greatly diminished relative to M2, and thus amino acids at the extreme carboxyl terminus of M2 appear to be required for efficient interactions with TAF_{II}250. Similar results were noted for TAF_{II}150, as binding was restricted to full-length GST-M2 indicating that the carboxyl-terminal portion of the M2 zinc finger and D domains (amino acids 353–479) is required for interactions. TAF_{II}110 bound full-length M2, Δ353, and Δ197 but not Δ103. Thus, at least one binding site for TAF_{II}110 resides within the M2 B^Q domain between amino acids 103 and 197. This result is entirely consistent with previous reports that identified a binding site for TAF_{II}110 within a hydrophobic region of Sp1 that is conserved in M2 (amino acids 159–174; see Ref. 7). Finally, TAF_{II}70 and -40 bound Δ353, Δ197, and Δ103 as efficiently as M2, and thus at least one binding site for these proteins maps to amino acids 1–103, corresponding to the $B^{S/T}$ and amino-terminal B^Q subdomains. In summary, these binding assays revealed that several TAF_{II} proteins are capable of interacting specifically with Sp family members *in vitro* and that binding sites for TAFs are distributed throughout M2. It is worth mentioning that our mapping results identify the minimal regions of M2 required for association with TAF_{II} proteins and that additional binding sites may also exist.

Should physical interactions between M2 and one or more TAF_{II}s play a role in M2-mediated transcriptional repression, one would predict that mutants exhibiting altered patterns of repression should also exhibit altered TAF_{II} binding activity.

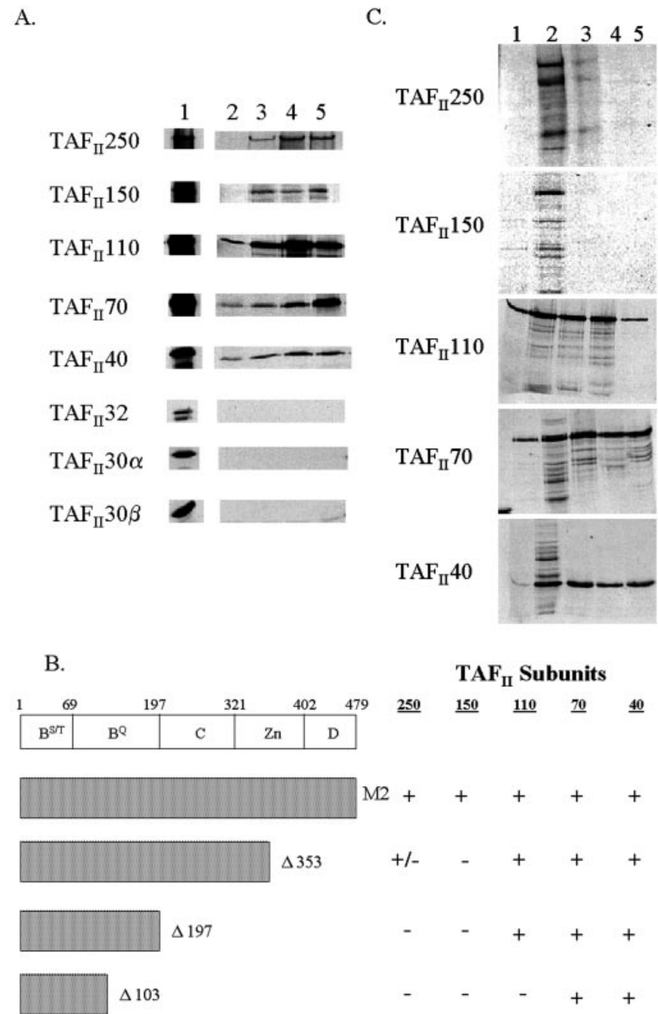

FIG. 5. **Characterization of protein complexes between Sp family members and TAF_{II} subunits.** *A*, protein complexes between Sp family members and TAF_{II} subunits. Radiolabeled and *in vitro* translated TAF_{II} subunits were incubated with glutathione-agarose beads bound to equivalent amounts of GST (*lane 2*), GST-Sp1 (*lane 3*), GST-Sp3 (*lane 4*), or GST-M2 (*lane 5*). For comparison, *lane 1* contains 10% of the radiolabeled protein applied to beads in each of *lanes 2–5.* Bead-bound proteins were resolved by electrophoresis through acrylamide gels. *B*, summary of M2 amino acids required for interaction with TAF_{II} subunits. A schematic diagram of the sub-domain structure of M2 and amino acids that demarcate these sub-domains are illustrated at the *upper left. Below* this diagram is illustrated wild-type M2 and the structure of three truncated M2 derivatives (Δ353, Δ197, and Δ103) employed for mapping experiments. + indicates binding akin to wild-type M2; − indicates no binding activity; +/− indicates partial binding activity relative to wild-type M2. *C*, protein complexes between TAF_{II} subunits and wild-type or truncated M2 derivatives. Radiolabeled and *in vitro* translated TAF_{II} subunits were incubated with glutathione-agarose beads bound to equivalent amounts of GST (*lane 1*), GST-M2 (*lane 2*), GST-M2Δ353 (*lane 3*), GST-M2Δ197 (*lane 4*), or GST-M2Δ103 (*lane 5*). Bead-bound proteins were resolved by electrophoresis through acrylamide gels.

As a first step toward determining whether M2 mutations affect TAF_{II} interactions, a set of mutated M2 proteins were fused to GST and examined in an *in vitro* protein/protein binding assay with *in vitro* translated TAF_{II} proteins. As illustrated in Fig. 6, TAF_{II}110 bound each mutated M2 protein examined as well as wild-type M2, whereas the binding of TAF_{II}70 and TAF_{II}40 was diminished for a subset of these same mutants. Mutants 68, 138, 158, and 172 showed a significant decrease in their capacity to bind TAF_{II}70 *in vitro*, and the capacity of mutant 138 to bind TAF_{II}40 was also diminished. Consistent with mapping results presented in Fig. 5, some mutants with

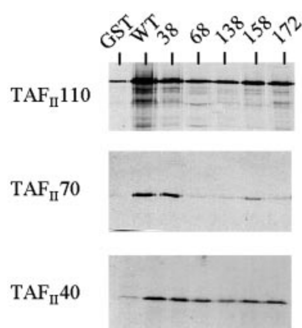

FIG. 6. **Characterization of protein complexes between TAF_{II} subunits and wild-type or mutated M2 proteins.** Radiolabeled and *in vitro* translated TAF_{II} subunits were incubated with glutathione-agarose beads bound to equivalent amounts of GST (*GST*), wild-type GST-M2 (*WT*), or M2 mutants fused to GST. Mutant numbers are indicated at *top*. Bead-bound proteins were resolved by electrophoresis through acrylamide gels.

diminished capacity to bind $TAF_{II}70$ or $TAF_{II}40$ carry mutations within regions of M2 required for interactions with these proteins. For example, mutants 68 and 138 exhibit diminished capacities to bind $TAF_{II}70$ and $TAF_{II}40$, and these mutants carry amino acid substitutions within three mutational hotspots located within the minimal region (amino acids 1–103) of the M2 B domain required for binding by $TAF_{II}70$ and $TAF_{II}40$. Interestingly, mutants 158 and 172 do not carry mutations within the minimal region of M2 required for binding $TAF_{II}70$ and $TAF_{II}40$. Instead, these mutants carry mutations within the C and/or zinc finger sub-domains perhaps indicating the positions of additional binding sites for these TAF_{II} proteins. We conclude from results presented in Fig. 6 that mutations carried by some M2 mutants disrupt interactions with specific TAF_{II} proteins. Whether the disruption of these protein interactions contributes to the novel patterns of transcriptional repression exhibited by these mutants remains to be determined.

DISCUSSION

Transcription of a wide variety of mammalian genes has been shown to be stimulated by Sp1 and Sp3 in transient expression assays. Additionally, the integrity of many promoter elements bound by Sp1 and Sp3 has been shown to be necessary for cell cycle-regulated and induced transcription. Given their functional similarities, common target genes, and ubiquitous expression, Sp1 and Sp3 might be viewed as entirely redundant transcriptional regulators. Yet this supposition is challenged by the finding that Sp3 encodes functionally distinct proteins and by studies of nullizygous animals lacking Sp1 or Sp3 function. It is abundantly clear that in addition to a transcriptional activator, Sp3 encodes two transcriptional repressors that we have termed M1 and M2. Experiments reported here were undertaken to identify Sp3 amino acids and functions required for transcriptional repression and to begin to determine the mechanism(s) by which Sp-dependent genes are repressed. As a consequence of these studies, we have established that DNA binding activity and the capacity to form multimeric protein complexes are not required for transcriptional repression. Instead repression requires a discrete portion of the Sp3 B domain (amino acids 235–328) that includes serine/threonine-rich and glutamine-rich amino acids. Our studies also indicate that mutations carried by M2 that disrupt transcriptional repression of one Sp-dependent promoter do not necessarily block repression of others, suggesting that transcriptional repression may require promoter-specific interactions. Finally, five TAF_{II} proteins were found to bind M2 *in vitro,* and we have identified several M2 mutants whose interaction with two TAF_{II} proteins, $TAF_{II}70$ and $TAF_{II}40$, is compromised. Taken together, our results strongly suggest that Sp3-mediated transcriptional repression proceeds, at least in part, via the titration of one or more promoter-specific *trans*-acting factors.

Although Sp1 is an alternatively spliced gene, Sp3 is unique among Sp family members in its utilization of internal translational initiation to synthesize multiple proteins (31, 45). Unlike other transcription factors, such as C/EBPβ, that utilize internal translational initiation to produce functionally distinct proteins, the relative abundance of Sp3 isoforms does not vary as a function of cell proliferative index or signal transduction. Indeed, the abundance of Sp3, M1, and M2 appears to remain constant in all cells, and thus regulation of Sp3-dependent genes is likely to be dictated by one or more post-translational mechanisms. Two potential mechanisms that appear to be ruled out by our studies are competition between Sp3 isoforms for promoter occupancy and the "poisoning" of transcriptionally active Sp multimers by M1 and/or M2. Our survey of mutated M2 proteins identified many that lack the capacity to bind DNA or to multimerize, yet these same mutants were competent to block transcription of at least some of the promoters examined. Indeed, some of these mutants were more potent repressors of transcription than wild-type M2. Instead, the identification of the amino-terminal 93 amino acids of M2 as the minimal region required for repression strongly suggests that Sp3-mediated repression proceeds via competition for one or more key *trans*-acting proteins that are required for promoter activity. This 93 amino acid region harbors the entirety of the serine/threonine-rich region carried by M2 as well as the first 25 amino acids of the glutamine-rich domain. Other than serving as sites of post-translational modification, little is known about the precise contributions of serine/threonine-rich regions to the functions of Sp family members. The primary amino acid sequences of these sub-domains are very poorly conserved between Sp family members. Yet the amino acid sequence of any given family member is highly conserved across animal species, suggesting that these regions are functionally important. When analyzed for their capacity to function as *trans*-activation domains, serine/threonine-rich regions exhibit little or no activity on their own although they can regulate transcription when linked to the glutamine-rich domains of Sp family members (36, 45). Given these results and our findings that (i) many hotspot mutations map to the M2 $B^{S/T}$ region and (ii) at least two TAF_{II} proteins, $TAF_{II}70$ and $TAF_{II}40$, are likely to bind M2 via this region, we speculate that serine/threonine-rich domains may regulate Sp-mediated transcription via physical interactions with components of the basal transcription complex. Consistent with the notion that $TAF_{II}70$ and $TAF_{II}40$ may be physiologically relevant targets of M2 function, several M2 mutants carry mutations that perturb these physical interactions. Moreover, N-CoR, yet another transcriptional repressor, has been shown to require contacts with $TAF_{II}70$ and -40 to inhibit transcription (46). Based on our results, we propose that Sp1/Sp3 and M1/M2 may compete for interactions with proteins such as $TAF_{II}70$ and 40, and it is this competition that helps determine the expression level of a given Sp-dependent gene. We predict that extracellular signals that induce Sp-mediated transcription may do so in part by relieving this competition, perhaps by favoring interactions between Sp1/Sp3 and proteins such as $TAF_{II}70$ and -40. In apparent accord with this proposal, we have shown that several TAF_{II} proteins bind Sp1 and Sp3 in addition to M2 and some TAF_{II} proteins, such as $TAF_{II}250$ and $TAF_{II}70$, bind differentially to Sp family members. Whether $TAF_{II}70$ and -40 are

relevant physiological targets for Sp family members remains to be determined.

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