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Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter

[transcription factors/negative regulation/(G+C)-rich promoter]

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ABSTRACT **The murine adenosine deaminase (*ADA*) gene has a (G+C)-rich promoter that can support diverse tissue-specific gene expression. By using deletion and mutation analyses, we have identified a cis-acting "repressor" element located immediately upstream of the proximal Sp1 binding site in the *ADA* gene promoter. This repressor element was localized to a binding site for the immediate-early, serum-responsive, DNA binding factor Zif268. This Zif268 binding site partially overlaps a binding site for the general transcription activator Sp1. Disruption of the Zif268 binding site without disturbing the Sp1 binding motif abolished Zif268 binding and resulted in significantly elevated promoter function. Conversely, disruption of the proximal consensus Sp1 binding motif without disturbing the Zif268 binding site resulted in a loss of Sp1 binding at that region and greatly reduced promoter activity. Our results suggest that one function of Zif268 may be to down-regulate this type of mammalian gene promoter by competing with Sp1 for binding to the overlapping binding motif.**

A number of genes that are rapidly induced (in a matter of minutes) by the addition of serum or growth factors to quiescent cells in culture have recently been identified (1). These genes include several nuclear factor encoding oncogenes such as the *fos* (2–4) and *jun* (5–7) families of genes. Many of these "immediate-early" growth-response genes are believed to encode trans-acting factors that regulate the expression of target genes that direct a cellular response to growth induction. Recent work on the *jun* and *fos* families of genes elegantly demonstrated that the proteins encoded by these genes can bind to consensus AP1 binding motifs within various types of TATA box-containing promoters and trans-regulate promoter activity (8–11). The functions of some of these immediate-early growth-response genes, such as the *Zif268* (also known as *Egr-1*, *Krox-24*, and *NGF-1A*) (12–15) and *Nur/77* (16) genes, have not yet been unambiguously defined, although the structures of the encoded proteins suggest that they are DNA binding proteins. Speculations that such genes are also potential oncogenes have been bolstered by the recent discovery that the gene implicated in causing Wilms tumor showed significant sequence homology to the *Zif268* gene family (17).

Recently, two alternative DNA motifs [GCG(G/T)-GGGCG] that can bind to Zif268 have been identified (18). These binding motifs are present in several mammalian (G+C)-rich promoters that lack the TATA and CCAAT box motifs (18). These (G+C)-rich "housekeeping" gene promoters (19) usually initiate transcription at multiple sites and contain multiple consensus Sp1 binding sites [(G/T)-GGGCGGGGC] (20) that can interact with Sp1 to enhance promoter activity (21). The murine adenosine deaminase (*ADA*) gene promoter has a structure that is typical of classical housekeeping gene promoters (22). However, this promoter is capable of supporting highly diverse tissue-specific gene expression (19). Our analyses of this promoter revealed the presence of a "repressor" element that coincides with a Zif268 binding motif. This Zif268 binding motif partially overlaps the proximal (relative to the start codon) Sp1 binding site within the promoter. Disruption of this proximal Sp1 binding site resulted in greatly decreased promoter activity. Since Zif268 is expressed at considerable levels in the recipient cells used in the transfection assays, we hypothesize that one function of Zif268 may be to down-regulate this type of promoter by competing with Sp1 for binding at overlapping binding sites.

MATERIALS AND METHODS

Generation of the Deletion and Linker-Scanning Mutants. The 5′ and 3′ deletion mutant libraries of the *ADA* gene promoter were generated by linearizing plasmids containing the 236-base-pair (bp) promoter element with the appropriate restriction enzymes, deleting various portions of the ends using BAL-31, blunting the ends with Klenow fragment, adding a 12-bp *Xho* I linker at the end, and transfecting the recircularized plasmids into the *Escherichia coli* host (strain DH5α). Positive clones of interest were identified by hybridization with specific ^{32}P-labeled synthetic 16-mer oligonucleotides (see Fig. 1 for an example) in the presence of tetramethylammonium chloride (23). Linker-scanning mutants no. 12 and no. 13 (see Fig. 1) were generated by splicing together appropriate 5′ deletion and 3′ deletion mutants at the *Xho* I linker site. Linker-scanning mutant MluI was generated by first using the primers TATACGCGTGCCGGGGCAGC-CCGGTAAAAAAG and TATGTCGACGCGTCCGC-CCACGCTACGCCCACA in a polymerase chain reaction (PCR) (24) to synthesize the appropriate 5′ and 3′ deletion mutants, respectively. The two PCR-generated mutants were then spliced together at the *Mlu* I site to generate linker-scanning mutant MluI. All mutants generated were verified using the Sanger DNA sequencing technique (25).

Transient Transfection Analyses To Assay for Promoter Function. The various 5′ deletion and linker-scanning mutant promoter clones shown in Fig. 1 were inserted identically upstream of a bacterial chloramphenicol acetyltransferase (CAT) reporter gene construct and analyzed for promoter activity by means of transfection into murine Cl-1D cells using the calcium phosphate coprecipitation technique (26). CAT activity analyses were performed essentially as reported (19). Reaction times range from 40 min to 4 hr. The conversion of [^{14}C]chloramphenicol (Amersham) into acety-

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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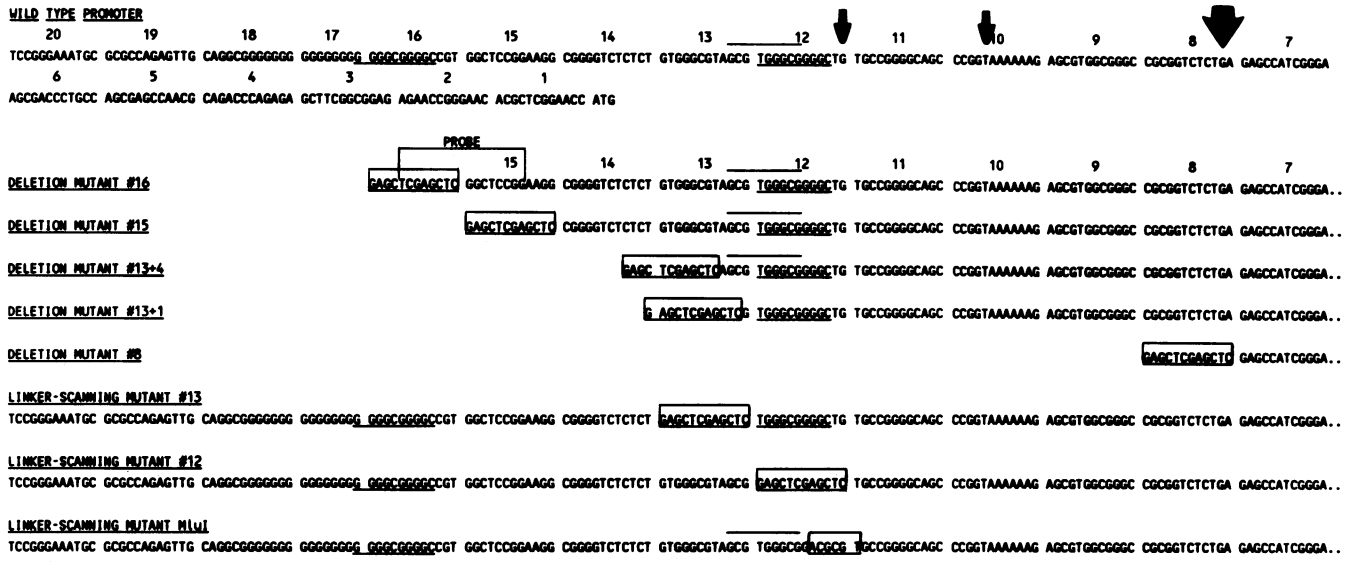

FIG. 1. Schematic illustration of the promoter sequence of the various constructs used. The wild-type promoter sequence is shown in sequence blocks of 12 bp each. The sequences of the mutant promoters are as indicated. The boxed sequences are the *Xho* I and *Mlu* I linkers. The consensus Sp1 binding sites are underlined. The Zif268 binding motif is overlined. An example of the probe sequence used to identify a 5′ deletion mutant is bracketed. The three major transcription initiation sites utilized by the wild-type promoter are indicated by arrows.

lated chloramphenicol was monitored using a Betascan betascope. All CAT activity quantitations were performed under linear reaction conditions, following dilution of the protein samples, when necessary.

RNase Protection Analyses. Transiently transfected cells were harvested 48 hr after transfection. Total RNA was isolated using the guanidinium isothiocyanate procedure as described (27) and 100 μg of the RNA was used in each RNase protection assay. The protection assays were performed as described (27) using uniformly labeled (^{32}P) probes.

Gel Mobility-Shift Analyses. The probes used were 388-bp *Eco*RI/*Pvu* II restriction fragments that were ^{32}P labeled at the *Eco*RI site using Klenow fragment. The gel-mobility shift assays were performed as reported (18).

DNase I Footprinting Analyses. The probes used were either the 236-bp *Eco*RI/*Nco* I *ADA* gene promoter fragment or the 388-bp *Eco*RI/*Pvu* II fragment consisting of the 236-bp *ADA* promoter fused to a 152-bp CAT gene sequence. The probes were labeled at the *Eco*RI site by means of a dephosphorylation/kinase reaction (27). Recombinant Sp1 was synthesized in bacteria and partially purified as reported (28) using construct pBS-Sp1-fl obtained from R. Tjian, University of California, Berkeley. The partially purified recombinant Zif268 protein was prepared as reported (18). The DNase I footprinting reactions were carried out as described (18). Sequencing lanes of the same probes were generated using the Maxam–Gilbert procedure (27).

Immunoprecipitation Analyses. The bacterially synthesized Zif268 protein (18) was gel-purified and used to generate a rabbit anti-Zif268 antiserum. Cl-1D cells in logarithmic-phase growth were labeled for 1 hr with [^{35}S]methionine (Amersham) as described (29). Cell lysates were immunoprecipitated with preimmune or anti-Zif268 antiserum as described (30).

Gel Mobility-Shift Analysis in the Presence of Anti-Zif268 Antiserum. Logarithmic-phase Cl-1D cell extract was used in binding reactions with a kinase-labeled (27) double-stranded 20-bp oligonucleotide probe in the presence of 1 μg of sonicated salmon sperm DNA. The probe was either treated with the cell lysate alone for 10 min or treated with cell lysate for 10 min, followed by treatment with the rabbit anti-Zif268 antiserum for 20 min, prior to analysis by gel mobility-shift assay. All binding reactions were carried out at 20°C.

RESULTS

Identification of a Cis-Acting Repressor Element Located Immediately Upstream of the Proximal Consensus Sp1 Binding Site. Our previous work demonstrated that the 236-bp DNA fragment immediately upstream of the murine *ADA* gene's start codon can function as a promoter in transient gene expression assays after it is transfected into mammalian cells (22). Analysis of several 5′ deletion mutants revealed that a cis-acting repressor element was located within sequence block 13 (see Fig. 1) of the promoter. Deletion mutant clone no. 13+1 differed from deletion mutant clone no. 13+4 by having 2 additional bp deleted on the 5′ end of the promoter (see Fig. 1). These two deletion mutants displayed a 15-fold

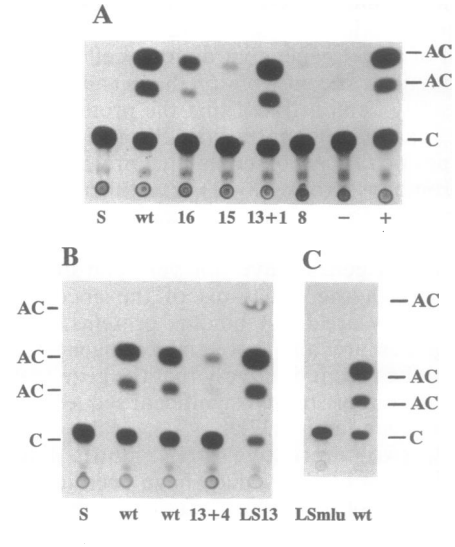

FIG. 2. CAT activity of the various promoter constructs. Samples were derived from Cl-1D cells that had been sham-transfected (S) or transfected with 20 μg each of deletion mutants nos. 16, 15, 13+4, 13+1, and 8 (indicated accordingly by number) or from linker-scanning mutant no. 13 (LS13) or MluI (LSmlu). No cell extract (−) and CAT protein (+) controls are shown. AC, acetylated chloramphenicol assay products; C, substrate chloramphenicol; wt, wild type. Five hundred micrograms of cell lysate protein was used in each reaction.

difference in promoter activity (Fig. 2 and Table 1). This indicates that the 5′ boundary of the cis-acting repressor element is located within the 2 bp lying between the 5′ ends of these two deletion mutants.

The Repressor Element Is Located Within a 13-bp Motif with Overlapping Consensus Sp1 and Zif268 Binding Sites. Sequence analysis of the murine *ADA* gene promoter revealed that a 9-bp sequence (GCGTGGGCG) with a 5′ boundary coincident with that of the repressor element constitutes a consensus binding motif for the early growth-responsive factor Zif268 (18). This presumptive Zif268 binding motif partially overlaps the proximal (with respect to the start codon) consensus Sp1 binding site (TGGGCGGGGC) of the promoter to form a 13-bp motif, GCGTGGGCGGGGC (see Fig. 1). A typographical error in the originally published murine *ADA* gene promoter sequence had resulted in a 2-bp transposition within the Zif268 binding motif (22). We have resequenced several independent clones of the promoter (data not shown) to confirm that the wild-type promoter sequence shown in Fig. 1 is correct.

To confirm that disruption of the presumptive Zif268 binding site located in block 13 (see Fig. 1) is sufficient to disrupt the repressor element, and to rule out the possibility that the enhanced promoter function displayed by deletion mutant no. 13+1 was merely due to the formation of a novel activating element at the junction of the linker/promoter sequences, linker-scanning mutant no. 13 was generated and analyzed. Alteration of 8 bp of the sequence in block 13 of the wild-type promoter (in linker-scanning mutant no. 13) resulted in a significant enhancement of promoter function as compared with that of the wild-type promoter (Fig. 2*B* and Table 1). These observations led us to hypothesize that Zif268 may function as a down-regulator of the murine *ADA* gene promoter by competing with the general transcription activator Sp1 (20, 21) for mutually exclusive binding to the 13-bp overlapping binding motif.

A corollary of this hypothesis is that an intact Sp1 binding site within this 13-bp overlapping motif should be important for the wild-type promoter's function. To examine this point, we synthesized the linker-scanning mutant MluI, which contains an unaltered Zif268 binding motif but a mutated overlapping Sp1 binding site. This mutant displayed significantly lower promoter activity in comparison with the wild-type promoter (Fig. 2*C* and Table 1). This showed that an intact Sp1 binding site within the 13-bp element is important.

Binding Assays To Demonstrate Sp1 and Zif268 Binding to the 13-bp Overlapping Motif. DNase I footprinting analyses

Table 1. Relative CAT enzyme activity generated by each construct in transient expression assays following transfection into C-1D cells

Construct tested	Relative CAT activity, % of wild type
Wild-type promoter	100
Deletion mutant	
No. 16	28 ± 10
No. 15	14 ± 7
No. 13+4	8 ± 3
No. 13+1	120 ± 30
No. 8	3 ± 2
Linker-scanning mutant	
No. 13	790 ± 50
No. 12	16 ± 5
MluI	0 ± 2

All values have been normalized by slot blot analysis (31) of total DNA derived from half of the transfected cells. The CAT expression level of the wild-type promoter was normalized to 100%. The values shown reflect the average of at least three separate transfection analyses performed on each construct.

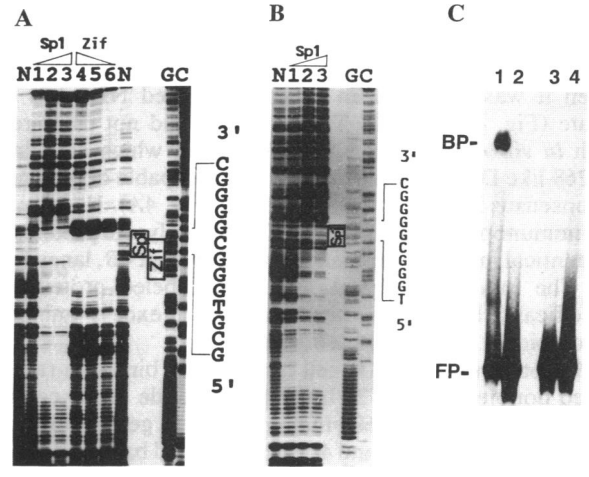

FIG. 3. (*A*) Sp1 and Zif268 bind and protect an overlapping region encompassing the 13-bp overlapping motif in DNase I footprinting analysis. *In vitro* DNase I footprinting assays were performed on the wild-type promoter using either no protein (lane N), 1 μg (lane 1), 5 μg (lane 2), or 8 μg (lane 3) of recombinant Sp1 or 3 μg (lane 4), 1 μg (lane 5), or 0.5 μg (lane 6) of recombinant Zif268 in the binding reactions prior to the DNase I treatment. The G and C sequence lanes of the same probe are as indicated. The regions of the probe protected by Zif268 and Sp1 motifs are indicated by the respective boxed areas. The location of the 13-bp overlapping motif is indicated by the bracketed sequence. (*B*) Linker-scanning mutant MluI does not bind Sp1 efficiently in the 13-bp overlapping motif in *in vitro* DNase I footprinting analysis. Zero micrograms (lane N), 1 μg (lane 1), 5 μg (lane 2), or 8 μg (lane 3) of recombinant Sp1 was used to bind to the probe prior to the DNase I treatment. The region of the probe that overlaps the Zif268 binding site and is normally protected by Sp1 in the wild-type promoter is boxed. The G and C sequence lanes of the wild-type promoter are analyzed in the same gel as shown. The relevant consensus Sp1 binding site in the wild-type promoter is indicated. (*C*) Zif268 binds specifically to the wild-type promoter but not to the mutant linker-scanning no. 13 promoter. The wild-type promoter (lanes 1 and 3) and the linker-scanning mutant no. 13 promoter (lanes 2 and 4) were used as probes in gel mobility-shift assays. The binding reactions were performed either in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of recombinant Zif268 protein. FP, free probe; BP, bound probe.

(Fig. 3*A*) confirmed that only a single binding site for Zif268 exists in the *ADA* gene promoter. This binding site overlaps the proximal Sp1 binding site within the 13-bp element (Fig. 3*A*). It appears that when Zif268 is bound to the *ADA* gene promoter, the 5′ region of the consensus Sp1 binding motif within the 13-bp overlapping element is inaccessible to other proteins, such as the DNase I used in the assay.

Mutations in the 13-bp Overlapping Motif Can Alter its Ability To Bind to Either Sp1 or Zif268. We have employed gel mobility-shift (Fig. 3*C*) and DNase I footprinting (Fig. 3*B*) techniques to demonstrate that the change in promoter activities of linker-scanning mutants no. 13 and MluI correlates with their inability to bind efficiently to Zif268 or Sp1, respectively, at the 13-bp overlapping motif. In a similar gel-mobility shift assay, we have also found that deletion mutant no. 13+1, which contains a deletion in part of the Zif268 binding motif, was similarly unable to bind to the recombinant Zif268 protein (data not shown). These results provide direct support for our hypothesis that abolishing Zif268 binding at the 13-bp overlapping motif can enhance the activity of the *ADA* gene promoter, whereas prevention of Sp1 binding to the motif will reduce the promoter's activity.

Detection of Zif268 Expression in Cl-1D Cells Growing in Logarithmic Phase. The hypothesis that the observed promoter activity differences between the wild-type promoter and linker-scanning mutant no. 13 resulted from differential Zif268 binding to the respective promoters requires the

demonstration that Zif268 is present in the Cl-1D cells used in the transfection experiments. Anti-Zif268 antiserum immunoprecipitated a single protein identical in size to Zif268 when it was treated with serum-stimulated NIH 3T3 cell lysate (Fig. 4*B*, lane 1). The antiserum did not cross-react with *in vitro* translated Krox 20 protein, which contains a Zif268-like DNA binding domain and is capable of binding to a consensus Zif268 binding motif (33) (Fig. 4*A*). However, it did immunoprecipitate a labeled Cl-1D cell lysate protein that is identical in molecular weight to Zif268 (Fig. 4*B*, lanes 1 and 2). The immunoprecipitation of this labeled protein was specifically blocked by the addition of excess unlabeled recombinant Zif268 (Fig. 4*B*, lane 3).

A protein in the Cl-1D cell lysate could bind to a radiolabeled double-stranded 20-bp oligonucleotide probe containing the consensus Zif268 binding motif in a gel mobility-shift assay [Fig. 4*C*, lanes 1 and 4–8 (the retarded band is indicated as BP)]. Competition with a 1000-fold molar excess of the unlabeled probe confirmed that formation of the bound probe (BP) band is DNA sequence specific (Fig. 4*C*, lanes 1 and 9). The presence of the BP band cannot be competitively inhibited by using a 1000-fold excess of a nonspecific double-stranded 20-bp oligomer (data not shown). This sequence-specific protein/DNA complex showed additional retardation in electrophoretic mobility in the form of super-shifted (Ss) bands when it was treated with an appropriate amount of the anti-Zif268 antiserum (Fig. 4*C*, lanes 3 and 4). These results confirmed that Zif268 is expressed in Cl-1D cells during logarithmic-phase growth.

Identical Transcription Initiation Sites Are Utilized in Linker-Scanning Mutant No. 13 and Wild-Type Promoter. To rule out the possibility that the enhanced promoter activity observed in linker-scanning mutant no. 13 resulted from novel transcription initiation sites formed at the junctions of the linker insertion sites, we performed RNase protection assays to determine the major transcription initiation sites utilized by the mutant promoter. The results demonstrate that the identical three major transcription start sites were utilized by the wild-type promoter and the mutant promoter (Fig. 5, lanes 4 and 5) as well as the deletion mutant promoter no. 13+1 (data not shown). These results strongly imply that the enhanced promoter activities observed in these two mutant promoters resulted from an increase in transcripts initiated at the identical sites used by the wild-type *ADA* gene promoter.

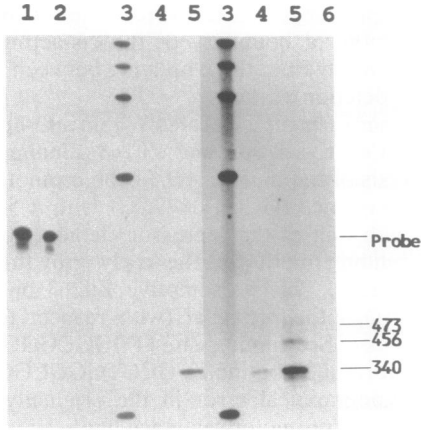

FIG. 5. Identification of transcription start sites of the wild-type and linker-scanning mutant no. 13 murine *ADA* gene promoter. RNase protection assays were performed on tRNA (lane 6) and on transcripts derived from Cl-1D cells transfected with either the wild-type promoter construct (lane 4) or the linker-scanning mutant construct no. 13 (lane 5). The three major mRNA start sites (340, 456, 473 nucleotides) are indicated. The uniformly labeled 505-nucleotide antisense transcript probes contain a 254-nucleotide CAT gene fragment fused to the 236-nucleotide wild-type (lanes 1 and 4) or linker-scanning mutant no. 13 (lanes 2 and 5) *ADA* gene promoter. The full-length probes (lanes 1 and 2) and ϕX174/*Hae* III-digested DNA markers (lane 3) are shown. The four lanes on the right were longer exposures of the same gel.

DISCUSSION

Several mammalian genes that respond rapidly to serum stimulation have been shown to encode sequence-specific DNA binding proteins (5, 18, 32). The Zif268 protein (12) is one of these early responsive gene products that are believed to be trans-acting transcription regulatory factors. Specific DNA sequences to which Zif268 binds have been recently

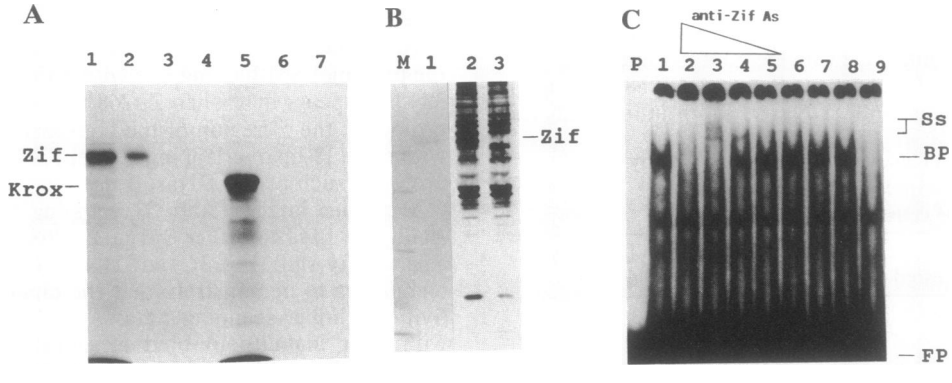

FIG. 4. Electrophoretic analyses of proteins immunoprecipitated by the rabbit anti-Zif268 antiserum. (*A*) Anti-Zif268 antiserum does not cross-react with the murine Krox 20 protein. Five microliters of *in vitro* translated ^{35}S-labeled Zif268 was loaded either without antiserum (lane 1), after immunoprecipitation with the anti-Zif268 antiserum in the absence (lane 2) and the presence (lane 3) of 2 μg of unlabeled gel-purified recombinant Zif268 protein, or after immunoprecipitation with preimmune serum (lane 4). Five microliters of *in vitro* translated ^{35}S-labeled Krox 20 was loaded either without antiserum (lane 5), after immunoprecipitation with the anti-Zif268 antiserum (lane 6), or after immunoprecipitation with preimmune serum (lane 7). The locations of the Zif268 (Zif) and Krox 20 (Krox) proteins are indicated. (*B*) Zif268 can be detected in Cl-1D cells growing in logarithmic phase. Protein markers (lane M), ^{35}S-labeled Zif268 protein (Zif) purified from NIH 3T3 cells by immunoprecipitation with the anti-Zif268 antiserum (lane 1), and ^{35}S-labeled Cl-1D cell lysate (5 μl) immunoprecipitated with the same antiserum in the absence (lane 2) or presence (lane 3) of 2 μg of unlabeled recombinant Zif268 protein are shown. (*C*) Gel mobility-shift and antibody-directed "super-shift" analyses of Cl-1D cell lysate. The ^{32}P-labeled 20-mer probe containing a consensus Zif268 binding motif was used to bind to 6 μl of Cl-1D cell lysate (lanes 1–9) in the presence of 1 μl (lane 2), 0.1 μl (lane 3), 0.01 μl (lane 4), or 0.001 μl (lane 5) of anti-Zif268 antiserum (anti-Zif As), 1 μl of preimmune serum (lane 6), 1 μl (lane 7) or 0.01 μl (lane 8) of anti-134 antiserum [an antiserum to the protein 3CH134 (32), which has no homology with Zif268], or a 1000-fold molar excess of unlabeled probe (lane 9). FP, unbound "free probe;" BP, sequence-specific "bound probe;" Ss, "super-shifted" probe.

identified and these motifs have been found to be located in several mammalian (G+C)-rich gene promoters (18). However, the role of the interaction between Zif268 and its binding site in regulating promoter function has not yet been conclusively established.

The murine *ADA* gene promoter is one of several mammalian (G+C)-rich promoters that can support highly diverse tissue-specific and developmental stage-specific gene expression (19, 33). Our deletion analyses of this promoter revealed the presence of a cis-acting repressor element that was mapped to a region containing a 13-bp (GCGTGGGCGGGGC) sequence that encompasses overlapping binding motifs for Zif268 (GCGTGGGCG) and Sp1 (TGGGCGGGGC). By competing with the general transcription factor Sp1 for binding to this type of overlapping motif, Zif268 may act as a negative regulator of this type of (G+C)-rich promoter by blocking the binding of Sp1 to its own recognition sequence. If this hypothesis is correct, then the relative concentrations of Sp1 and Zif268 within specific cell types, or factors that affect the binding of Zif268 or Sp1 to DNA, could conceivably modulate transcriptional activity of mammalian promoters that contain such overlapping binding motifs. Interestingly, a similar 13-bp overlapping motif (GCGGGGGCGGGGC) has also been found within the (G+C)-rich murine *Hox 1.4* gene promoter (33). It is probably not coincidental that the *Hox 1.4* gene promoter can also support diverse tissue-specific and developmental stage-specific gene expression.

Interestingly, when the Sp1 and Zif268 binding sites within the 13-bp element were disrupted (as in linker-scanning mutant no. 12), significant residual promoter activity above that exhibited by MluI was observed (Table 1). This suggests that the suppressive effect of Zif268 can be exerted by the binding of Zif268 alone, regardless of whether the Zif268 binding motif overlaps a functional Sp1 site.

Because *Zif268* is a member of a gene family that shows highly conserved DNA binding domains, it is certainly possible that other members of this gene family, such as *Krox-20* (or *Egr-2*), may encode proteins that can also bind to the Zif268 binding motif found in the murine *ADA* gene promoter (33). Although our results cannot preclude the possibility that the repressor effect that we observed resulted from the binding of proteins encoded by other members of the *Zif268* gene family, the super-shift experiment does rule out the possibility that the Krox 20 protein is the sole responsible factor. Because the rapid down-regulation of many immediate-early, serum-responsive genes requires newly synthesized proteins (1, 12), it has always been expected that one of the immediate-early serum-responsive factors must be a negative regulator of these genes. The observations that Zif268 expression is rapidly induced by serum stimulation and that Zif268 binding sites are found in the promoters of many early serum-responsive genes certainly make Zif268 a likely candidate as a negative promoter regulator.

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