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Genomic Organization and Promoter Analysis of *mouse disabled 2* Gene

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The *mouse disabled 2* (mDab2) gene is a mouse homolog of the *Drosophila disabled* gene. It is markedly up regulated in retinoic acid (RA)-treated F9 cells, suggesting a role for mDab2 in the cell differentiation. To elucidate the molecular mechanisms that regulate RA-treated F9 cells specific expression of mDab2, we cloned and analyzed its genomic structure. The *mDab2* gene spans over 55 kilobases and has 13 exons. The transcription start site, mapped by primer extension and 5′RACE, was located at 53 base pairs (bp) upstream of the most 5′-end of the published cDNA. Using reporter gene transfection analysis, we found that a 1-kb mDab2 5′-flanking sequence directed a high level of promoter activity in RA-treated F9 cells but not in untreated cells. Further deletion and mutation analyses identified a direct repeat of 5′-AGGAGGCGC-3′ motif as novel positive regulatory element. Gel retardation assay showed that this element was needed to form specific DNA-protein complexes with factors present in RA-treated F9 cell extracts.
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Key Words: **retinoic acid; mDab2; F9 cells; differentiation; 5′-AGGAGGCGC-3′.**

The vitamin A derivative retinoic acid is involved in vertebrate anterior axis formation and cellular differentiation and has been shown to modulate the expression of a number of genes implicated in the control of embryonal development (1). When F9 embryonal carcinoma cells are treated with RA, it can be induced to differentiate into nonmalignant cells resembling parietal and visceral endoderm, thus facilitating the study of mouse embryogenesis (2).

Recently, we found that the expression level of *mDab 2* gene is markedly increased following RA stimulation in F9 cells (3). It was found that mDab2 is also expressed in a tissue type- and embryonic developmental stage-specific manner (3).

mDab2, a mammalian structural homolog of *Drosophila disabled,* is originally isolated from a mouse macrophage cell line, BAC 1.2F5 (4). It represents two major alternatively spliced forms, p96 and p67, which is phosphorylated on serine residues following mitogenic stimulation (4). Since its expression is lost in many tumors, it has been speculated to be a negative regulator of growth (5). The possibility of mDab2 as a tumor suppressor is supported by a recent report that it binds to Grb2, competing with Sos (6). Although the function of mDab2 has not been fully clarified, these results indicate that mDab2 may play an important role in cell differentiation and growth.

In this report we cloned and characterized the *mDab2* gene and its promoter in order to identify the genetic elements involved in the regulating pattern of *mDab2* gene expression during F9 cell differentiation.

MATERIALS AND METHODS

Cell culture. F9 cells were obtained from the American Type Culture Collection and grown and differentiated as described elsewhere (2).

Isolation and characterization of mDab2 genomic clones. A F9 mouse genomic library was constructed in λ FIX II vector (Stratagene, La Jolla, CA) as described previously (7). About 10^6 recombinant clones were screened with a DIG-labeled (Roche Diagnostics GmbH, Germany) full-length mouse mDab2 cDNA probe. Five positive clones were selected and subcloned into T7 Blue (Novagen, Madison, WI) vector for restriction mapping, sequencing and Southern blot analyses using various portions of mDab2 cDNA as probe. The genomic DNA and exon-intron junctions were determined by direct cycle sequencing (Amplitaq, Perkin-Elmer, Norwalk, CT) with primers designed from the mDab2 cDNA.

A 11-kb genomic DNA fragment containing the 5′-flanking region of mDab2 was identified by Southern blot analysis with the DIG-labeled probe, corresponding to the first 100 nucleotides of the *mDab2* gene, and a 5.2 kb *Eco*RI fragment was subcloned into pBlue-script (Stratagene). This subclone (pks-P5) was sequenced and used for the functional analysis of the mDab2 promoter.

Primer extension. The transcription start-site was mapped by primer extension using the synthetic oligonucleotides 5′-ACTCACGGACTAGCTAGTGCTTCTGTGGCG-3′, complementary to nucleotide +131 to +160 in the genomic sequence. The IRD 700 dye-labeled

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/EMBL/GenBank database under Accession No. AF220562 for the mDab2 5′-flanking region.

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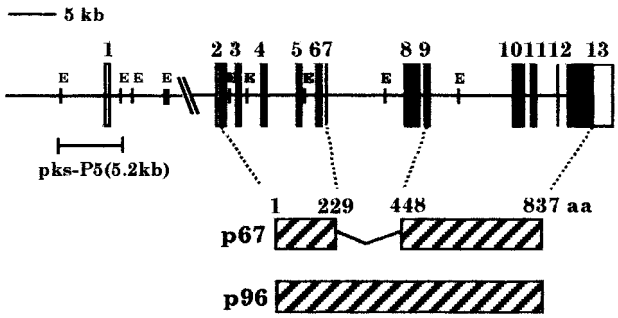

FIG. 1. Schematic diagram of the genomic structure of the mDab2 gene. Exons are indicated by black boxes numbered 1–13. The open boxes represent 5′ and 3′ untranslated regions. The open reading frames of mDab2 are indicated by hatched boxes. Exon 8, encoding the region corresponding to amino acids (aa) 230–446, is absent from the p67 form of the mDab2. Plasmid shown are pks-P5, containing 5.2 kb *Eco*RI fragment of continuous mDab2 promoter DNA. Sites for the restriction endonuclease *Eco*RI (E) are indicated.

oligonucleotides were annealed at 30°C with 2 μg of poly(A) RNA prepared from RA-treated and untreated F9 cells and extended using AMV reverse transcriptase, as described previously (7). Primer extension products, along with DNA sequencing reactions of the pks-P5 using the above oligonucleotides as primer, were electrophoresed on 6% urea-polyacrylamide gels and analyzed with Li-Cor DNA sequencer 4200 (Li-Cor, Inc., Lincoln, NE).

Construction of reporter genes for mDab2 promoter activity. Plasmid −4600/CAT6 was constructed by inserting a 4.6-kb *Hin*dIII/*Pst*I fragment of pks-P5 into *Hin*dIII/*Pst*I site of the promoterless pBLCAT6 (8). To examine the functionality of the putative promoter region within the 5′-end of the *mDab2* gene, a series of promoter/CAT reporter gene constructs were developed. Specifically, PCR amplification products were synthesized using the pks-P5 as template and primers that extended from position +57 upstream to positions −2,561, −1,257, −581, −131, and −95, respectively. The upstream primers were designed to contain a restriction enzyme site for *Sal*I and the downstream primer was designed with a restriction enzyme site for *Bam*HI, which allowed direct insertion of the the PCR products into the promoterless pBLCAT6.

Dab2P-wt/tk-CAT, Dab2P-m1/tk-CAT, Dab2P-m2/tk-CAT, Dab2P-m3/tk-CAT, Dab2P-m4/tk-CAT and Dab2-m5/tk-CAT were obtained as follows. The wild-type oligonucleotide Dab2P-wt (5′-CGAGGAGGCGCAGGAGGCGCCGGATCTCCGTGGCTTT-3′) and five mutant oligonucleotides, Dab2P-m1 (5′-CGAGGAGGgctAGGAGGCGCCGGATCTCCGTGGCTTT-3′), Dab2P-m2 (5′-CGAGGAGGCGCAGGAGGattCGGATCTCCGTGGCTTT-3′), Dab2P-m3 (5′-CGAGGtacCGCAGGAGGCGCCGATCTCCGTGGCTTT-3′), Dab2P-m4 (5′-CGAGGtacgctAGGGAGGCGCCGGATCTCCGTGGCTTT-3′) and Dab2P-m5 (5′-CGAGGtacgctAGGAGGattCGGATCTCCGTGGCTTT-3′) were synthesized as complementary pairs, made as duplexes by hybridization, and cloned in front of the TK promoter of pBLCAT5 (8). Each construct was confirmed by sequencing.

Transient transfection assay. Activities of transfected reporter genes in RA-treated and untreated F9 cells were evaluated as follows. One day before transfection, undifferentiated F9 cells (7×10^5) were seeded on 60-mm dishes. Transfection was carried out in serum-free medium (Opti-MEM, Life Technologies, Inc.) using 10 μl of LipofectAMINE (Life Technologies, Inc.) to introduce 2.8 μg of the CAT gene expression plasmids and 0.6 μg of an internal reference plasmid (pRSVβ-gal). After transfection for 6 h, cells were trypsinized and one-third of the cells was replated on 6-well culture plates and cultured without RA. For induction of F9 cells differentiation, the remainder of the cells was divided into two 35-mm bacterial grade petri dishes and cultured in the presence of 1 μM RA. After 24 h, the media of the undifferentiated and differentiated cultures were changed to fresh medium without or with RA, respectively. The untreated cells were harvested 4 days after replating, and the RA-treated cells were harvested 2 or 4 days after replating. The CAT assays were performed according to the protocol of Gorman *et al.* (9), and CAT activities were normalized according to the results of the β-galactosidase activity in order to correct for differences in transfection efficiency. In all experiments, the values given represent the mean ± SE of at least five independent experiments.

Gel retardation assays. Preparation and incubation of nuclear extracts from RA-treated (4 days) and untreated F9 cells with ^{32}P-labeled double-stranded oligonucleotide probes, Dab2P-wt, Dab2P-m1 and Dab2P-m5, and gel retardation assay were as described previously (10).

RESULTS

Genomic organization of mDab2. To determine the genomic structure of mDab2, we screened a F9 mouse genomic library with the full-length mDab2 cDNA probe. Restriction mapping and Southern blot analyses revealed that five overlapping clones collectively encompass the entire coding sequence and further extend ~9kb upstream of the putative transcription start site. The entire *mDab2* gene is organized into thirteen exons and twelve introns spanning approximately 55 kb of genomic DNA (Fig. 1). The open reading frame for the mDab2 protein begins in the second exon and terminates at the thirteenth exon. All exons are present in p96, while exon 8 is absent in the p67 spliced form of mDab2. The size of exons, as determined by DNA sequencing and by comparison with the mDab2 cDNA, ranges from 66 to 1442 bp (Table 1). The identified exon-intron junctions (Table 1) are all in agreement with the consensus 5′-GT and 3′-AG sequences (11). The size of each intron was determined by PCR with flanking exon primers and Southern blot analyses. It was estimated to be in the range of 0.11 to ~25 kb (Table 1).

TABLE 1

Exon–Intron Organization of *mDab2* Gene

Number	Exon size	5′ splice donor	3′ splice acceptor	Intron size (kb)
1	118 bp	AGTCC **gt**gag	aat**ag** GTGTA	>25
2	189 bp	GAAAG **gt**atg	tgt**ag** GTTCT	1.3
3	141 bp	TCAAG **gt**acg	tat**ag** GGAAT	1.6
4	103 bp	GGTAA **gt**gta	gtt**ag** TTGAA	2.3
5	129 bp	AACAG **gt**aag	acc**ag** GCTGA	1.3
6	161 bp	AGCTG **gt**cgt	gca**ag** GGTGT	0.8
7	63 bp	CAACA **gt**aag	tac**ag** GAAAG	4.3
8	655 bp	CTAAG **gt**gaa	tgc**ag** TCTTC	0.62
9	153 bp	TCTAG **gt**ccg	ttc**ag** GTACG	4.5
10	636 bp	TAATG **gt**tgg	cgt**ag** AACCA	0.11
11	110 bp	CCTCT **gt**gtg	ctc**ag** GTGGT	1.9
12	66 bp	CTTCT **gt**cta	tct**ag** GAAGT	1.0
13	1442 bp			

Note. Exon sequences are shown in uppercase letters and intron sequences in lowercase letters.

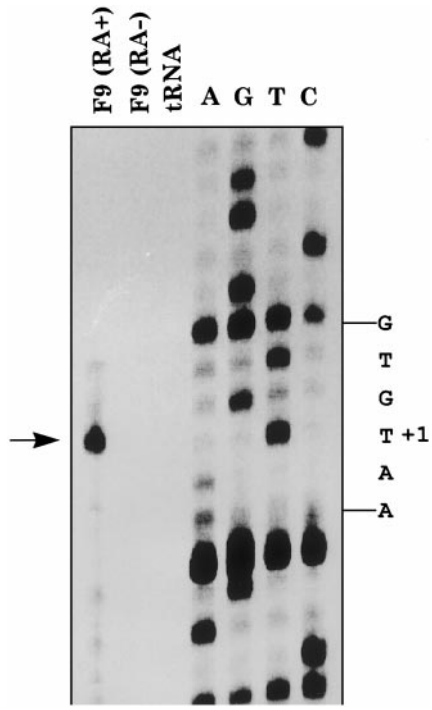

FIG. 2. Mapping of the mDab2 start-site by primer extension analysis. Products of primer extension using either 2 μg of poly(A) RNA from retinoic acid-treated (F9 +) and untreated (F9 −) F9 cells, or tRNA (tRNA), were resolved on a 6% urea–polyacrylamide gel. Lanes A, G, T, and C contained sequencing reaction products of pks-P5 with the primer used for primer extension. The primer extension product is indicated by an arrow. The sequence shown on the right corresponds to the noncoding strand.

Mapping of the start-site and putative promoter elements in the mDab2 5′-flanking region. Primer extension was performed to map the mDab2 start-site using oligonucleotide complementary to nucleotides +131 to +160 in the genomic DNA and poly(A) RNA prepared from F9 cells cultured in the absence and presence of RA (Fig. 2). The primer extension reaction yielded a single 160-bp product with RNA from RA-treated F9 cells but not from untreated cells (lanes 1 and 2). This start-site location was confirmed by 5′RACE using the RT primer 5′-CCTCGAGCATCAGGCACATC-3′ (positions +410 to +388 of the cDNA), GST1 primer 5′-AACACCATCACCTTTGAACCT-3′ (positions +360 to +340 of the cDNA) and GST2 primer 5′-ACCTGG-CCAACAAGTACTCG-3′ (positions +343 to +323) (data not shown). Taken together, these experiments revealed that a single mDab2 transcription start site is located at 53 bp upstream of the most 5′-end of the reported cDNA sequence (Fig. 2, lane 1, and Fig. 3).

To identify putative cis-acting elements that may be important in the regulation of mDab2 promoter, we cloned and sequenced the 1-kb 5′-flanking region (Fig. 3). There is no canonical TATA sequence in the mDab2 5′-flanking region; however, there is a consensus initiator motif (5′-YYYCAYYYYY-3′) at the transcription start site that may potentiate transcription from the TATA-less promoter (12). The mDab2 promoter contains several putative cis-acting elements including Sp1, NF-1, GATA-1 binding sites, and sites for basic helix-loop-helix protein, HEB. In addition, a putative glucocorticoid-responsive element (TGTCCT) and binding site of POU-homeodomain factor were found.

Promoter activity of the 5′-flanking region of the mDab2 gene. The previous study showed an increase in mDab2 mRNA levels during differentiation of F9 cells (3). We, therefore, examined whether the cloned 5′-flanking region is responsible for the transcriptional activation during differentiation of F9 cells. Undifferentiated F9 cells were transiently transfected with various mDab2 promoter/CAT constructs. The transfected cells were divided into aliquots and left undifferentiated or induced to differentiate in the presence of RA. As seen in Fig. 4, the transcriptional activity of the mDab2 promoter was markedly increased in F9 cells treated with RA for 4 days. We subsequently examined the effect of a series of 5′ deletion of the 4.6-kb fragment on the promoter activity (Fig. 4A). As shown in Fig. 4B, deletion to base −131 showed the predominant promoter activity in RA-treated F9 cell, but not in untreated cells, whereas further deletion to base −95

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-840 agcaaacaaattgtgagaaactataggattcccaaggtctgctctgccgg
-790 gctatccacagctgagcatcgatgaaacaccttccaatactaattcacat
-740 atctcaatatttacatgaaggaataaaaaatgctgtagaaactagcaaac
-690 aaagtcatgttatctccgtgtgggtggatatacactgaaatggtcaccaa
              GATA-1
-640 tgtagccccttatatggaggctcccctgttttgtttagcccagtttaat
-590 agcgagaatatgcgtcgagcgtgatattaaattttcaataacagggcatct
                              POU-homodomain
-540 ttttacttgggcacacatagatttccctcctgtttaagcacgttcacaat
-490 taaagaatagtgattttgtttaaaggtaacaatccagtcacatgcccagc
                                        NF-1
-440 tttagaaaggacagactggacccgggaacggaaagtcaggagtaaaggcg
-390 cgtctttgttcaaaattccccaggcggtttaggatgagttcgcagttcgc
-340 gtgccacgccactcgaagtggcaatccagggaacggatcttcaaaacgca
                              NF-1
-290 gctcagaggatcccatcctttttcctccagaatttccctttttcctgtcctt
                              NF-1                    GR
-240 acgtgcacccttttctggcccaaggcagtgtggggctcctggcccgggttg
-190 tgtgcacgctccttttttccgggtctctgcgatcgcccccagctggccggg
                                                   HEB
-140 cgcaggcgggcgaggaggaggcgccggatctccgtggctttagt
          Sp1
 -90 tcgagttaataaacagttaagtttggaagactcggcagacacgttgaggg
                                                    +1
 -40 ggagttaccaagtccaggcagcaaaaacatcccgccgcccATTTCCTGGA
                   NF-1              Sp1   Sp1
 +11 GAGTCCTCAGAGCCAGCATCTGATTAGAACCATATCTGTACCGGGAGTGG
 +61 CCGCGCGGCTATTTAATTTGCGTTCTCCGCGTCCTCGGCGCTGCAGCCTT
+101 GAGGCTTCGGGCGCGGGGAAGTCATGCTGGCGCCACAGAAGCACTAGCTA
+151 GTCCGTGAGT
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FIG. 3. mDab2 5′-flanking genomic sequences and putative *cis*-regulatory elements. The numbering of the nucleotides starts at the transcription initiation site (+ 1), which is indicated by a bent arrow. An initiator consensus element is boxed. Exon sequences are presented by uppercase letters, and intron sequences by lowercase letters. Putative *cis*-regulatory elements are underlined. A direct repeat of the 5′-AGGAGGCGC-3′ motif is indicated in boldface. The sequence of the primer used for primer extension is indicated by a double line. GR. Glucocorticoid-responsive element.

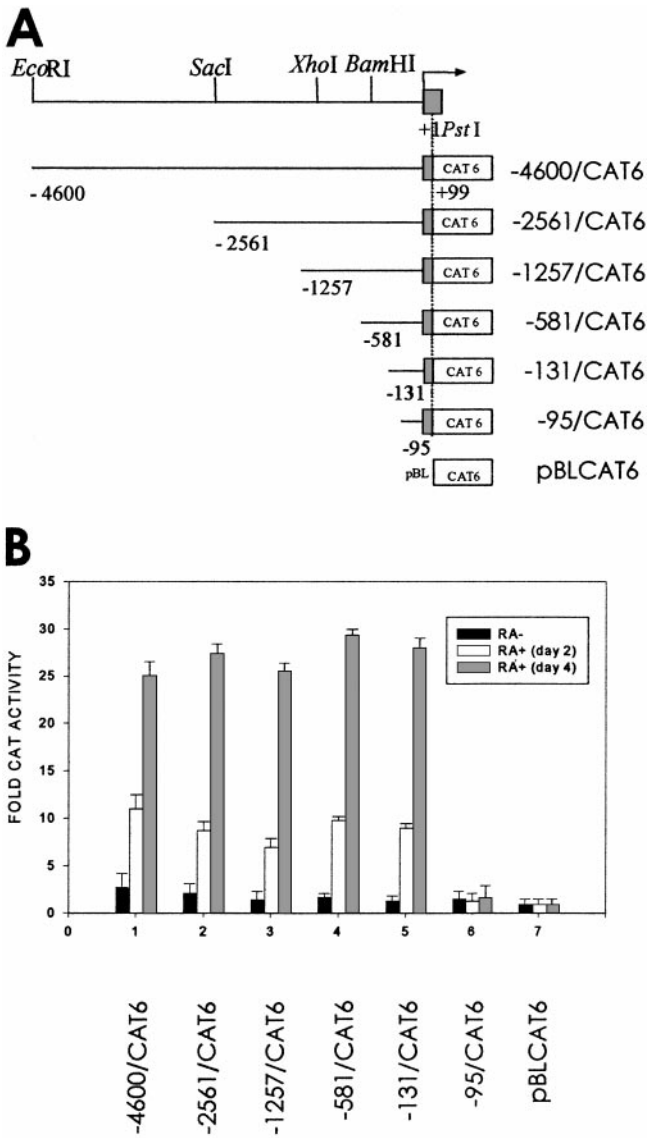

FIG. 4. Functional analysis of the mDab2 promoter. (A) mDab2 promoter constructs in pBLCAT6 used for transfections. The transcription start site is shown as +1, with arrow indicating the direction of transcription. The vertical dashed line at nucleotide position +99, relative to the transcription start site. (B) Undifferentiated F9 cells were transfected with a series of the 5′-deleted mDab2 promoter constructs. The transfected cells were divided into 3 aliquots and incubated either in the absence of RA on a tissue culture dish (untreated F9 cells, black bars) or in the presence of RA on a bacterial grade Petri dish (F9 cells treated with RA for 2 days, open bars; for 4 days, gray bars). The cells were harvested at the indicated time after replating. CAT activity was calculated as the percentage of chloramphenicol converted to the acetylated forms after these forms had been cut out of the TLC plate and the relative amounts were determined by liquid scintillation counting. Data are expressed as fold activity in relation to pBLCAT6, which was given the value of 1.0.

led to near complete ablation of promoter activity. These results indicated that the 37-bp region between bases −131 and −95 is sufficient to confer the transcriptional activation of the *mDab2* gene during the RA-induced differentiation of F9 cells.

A direct repeat of the 5′-AGGAGGCGC-3′ motif mediates RA-induced mDab2 promoter activity. A DNA sequence analysis of the 37-bp region between base −131 and −95 revealed the presence of a direct repeat of the sequences, 5′-AGGAGGCGC-3′ (Fig. 3). To determine the role of this sequence motif in mDab2 promoter activity, we designed one wild-type oligonucleotide, Dab2P-wt, and five mutant oligonucleotides, Dab2P-m1, Dab2P-m2, Dab2P-m3, Dab2P-m4 and Dab2P-5 (refer to Fig. 5A for sequences), and these oligonucleotides were inserted upstream of the TK promoter in the pBLCAT5. After 4 days of RA treatment, the CAT activity of differentiated F9 cells transfected with Dab2P-wt/tk-CAT5 was elevated 16-fold over that of the corresponding untreated cells (Fig. 5B). Mutation of either of the two AGGAGGCGC motifs (Dab2P-m1, Dab2P-m2 and Dab2P-m3) resulted in a 2-fold decrease of CAT activity, and Dab2P-m4, in which AGGCGC had been converted to TACGCT, exhibited 3-fold decrease of CAT activity. However, mutation of both of the two AGGAGGCGC motifs (Dab2P-m5) caused a dramatic reduction in CAT activity (Fig. 5B), suggesting that the direct repeat of the 5′-AGG-AGGCGC-3′ motif is important for mDab2 promoter activity in RA-treated F9 cells.

Identification of specific DNA-protein complexes from RA-treated F9 cells. Gel retardation assay was performed to test which nuclear proteins from RA-treated F9 cells could bind to the region from −131 to −95. Dab2P-wt, Dab2P-m1 and Dab2P-m5 were used as probes for the binding assays. Specific DNA-protein complexes (Fig. 6, lanes 3 and 5) were observed upon electrophoretic separation of incubation mixtures of ^{32}P-end labeled Dab2P-wt or Dab2P-m1 with extracts prepared from RA-treated F9 cells. These complexes were not observed with corresponding mutated oligonucleotide, Dab2P-m5, nor with extracts from untreated F9 cells (lanes 2 and 6). It suggested that the direct repeat of the motif 5′-AGGAGGCGC-3′, is required for the binding of trans-acting factors from RA-treated F9 cells. Further evidence for the specificity of putative trans-acting factor binding sites is shown in Fig. 6 (lane 4), where DNA–protein complexes are diminished by the presence of low level excess unlabeled probe.

DISCUSSION

In this study, we determined the genomic structure of the *mDab2* gene and identified its promoter. The *mDab2* gene spans over 55-kb in mouse genome and is composed of 13 exons. All of exons are present in p96, while exon 8 is absent in p67. The previous reports showed that the alternative RNA splicing of mDab2

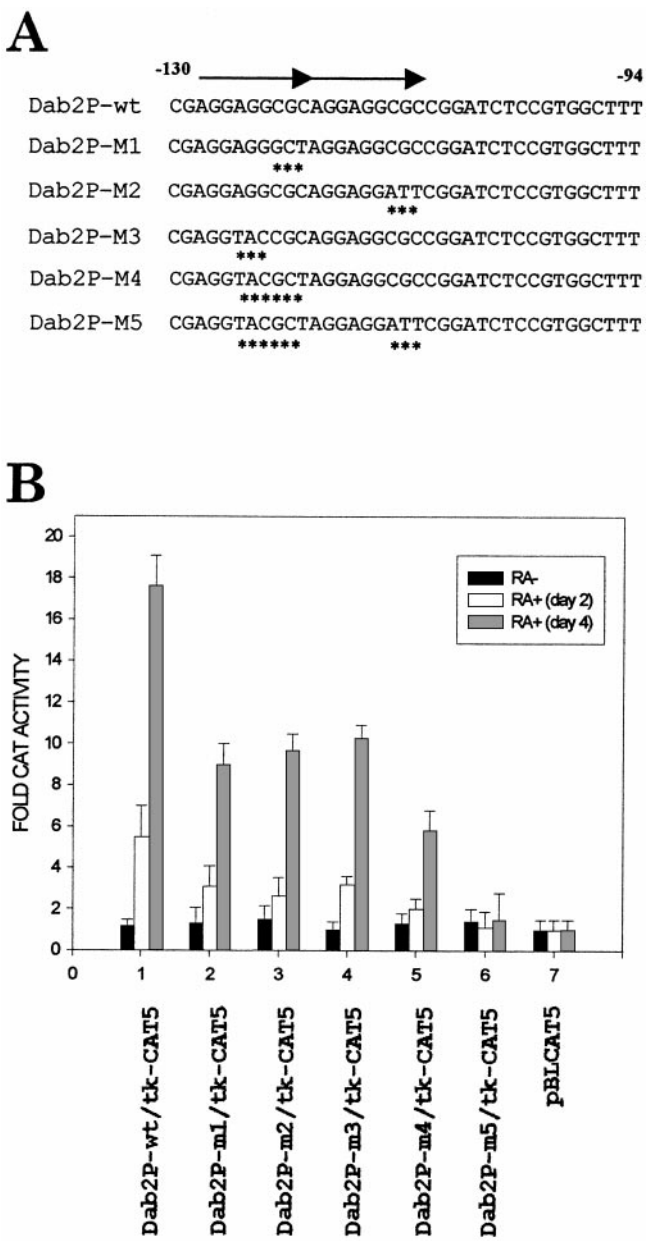

FIG. 5. Localization of a RA-treated F9 cells-specific enhancer activity to nucleotides −131 to −95 of the mDab2 promoter. (A) Oligomers, Dab2P-wt corresponds to positions −131 to −95, a direct repeat of the 5′-AGGAGGCGC-3′ motif is bold face type, and the mutated nucleotides in Dab2P-m1 to m5 are indicated by asterisk. These oligonucleotides were cloned inserted upstream of the TK promoter in the pBLCAT5. (B) Undifferentiated F9 cells were transfected with each of the constructs shown and divided into 3 aliquots and incubated either in the absence of RA (black bars) or in the presence of RA for 2 (open bars) or 4 days (gray bars). The CAT activity of each construct was expressed relative to the activity of the pBLCAT5, which was given the value of 1.0.

message should be occurring in a cell type-specific manner; p96 is highly expressed in nerve and macrophage cell (3, 4), whereas p67 is in kidney and RA-treated F9 cells (3), suggesting a functional discrepancy between two isoforms. It is possible that this discrepancy could be due to the presence or absence of exon 8 in mDab2.

Primer extension and 5′RACE analysis identified a single transcription start site located at 53 bp upstream of 5′ end of the published cDNA (4). The mDab2 promoter lacks a consensus TATA or CCAAT box. Instead, it possesses a consensus initiator motif and Sp1 binding sites that are thought to be critical for initiation of transcription in TATA-less promoter (13).

The marked increase in the mDab2 promoter activity during the RA-induced differentiation of F9 cells agreed well with the results obtained by the Northern hybridization experiment (3). The promoter region located to −131 to −95 was sufficient to confer the responsiveness, and the promoter activity had been elevated significantly by day 4. Since the differentiation of F9 cells was shown to be induced irreversibly by 4-day treatment with RA (2), it is highly likely that the increased expression of mDab2 is critical for the process.

Unlike many RA-inducible genes, this 37-bp promoter region lacks consensus RA responsive elements, suggesting that the activity of the *mDab2* gene expression in response to RA may be indirect. Rather, the 37-bp promoter region contains a direct repeat of the

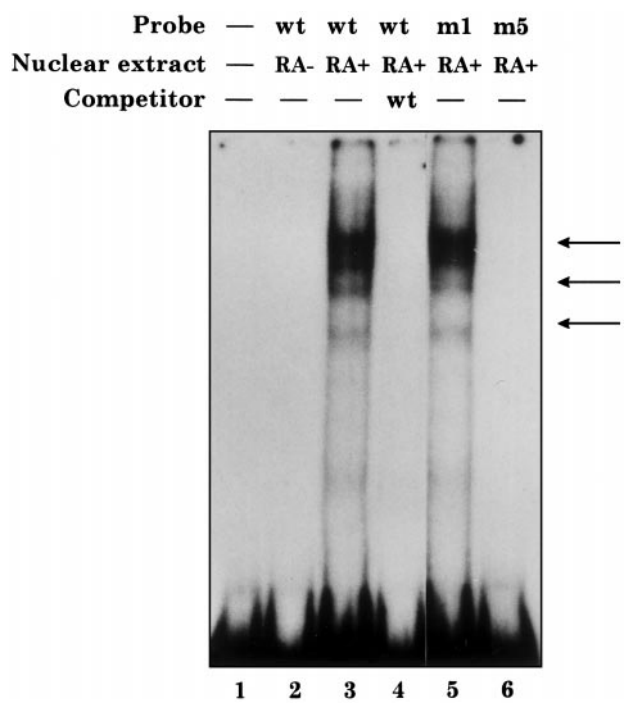

FIG. 6. Gel mobility shift assay. ^{32}P-end-labeled oligonucleotides Dab2P-wt (Wt), Dab2P-m1 (m1) and Dab2P-m5 (m5) were incubated with nuclear extracts from RA-treated (RA +) and untreated (RA −) F9 cells. Arrowheads mark DNA-protein complexes unique to RA-treated F9 cells. The DNA–protein complexes formation is inhibited in the presence of 20-fold molar excess unlabeled Dab2P-wt competitor (lane 4).

5′-AGGAGGCGC-3′. The mutation analyses and gel retardation assays clearly demonstrate that the direct repeat is an essential cis-element for the RA-treated F9 cells-specific promoter activity of mDab2. This sequence, 5′-AGGAGGCGC-3′, is not identical to any consensus sequence reported thus far. Maybe there is a novel trans-acting factor that binds to the sequence. Further experiments are needed to precisely define the cis-acting elements and transcription factors involved in regulating of the *mDab2* gene expression.

Our isolation and characterization of a functional mDab2 promoter will facilitate new investigations into the mechanisms by which cell-signaling factor influences a transcriptional event of this RA-induced F9 cells differentiation-specific gene.

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