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An Octamer Motif Contributes to the Expression of the Retinoic Acid-Regulated Zinc Finger Gene Rex-1 (*Zfp-42*) in F9 Teratocarcinoma Cells

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The message for the zinc finger gene Rex-1 (*Zfp-42*) is expressed in undifferentiated murine F9 teratocarcinoma cells and embryonic stem cells. Expression of Rex-1 is reduced at the transcriptional level when F9 cells are induced by the addition of retinoic acid (RA) to differentiate. We have isolated genomic DNA for the Rex-1 gene (*Zfp-42*), characterized the gene's structure, and mapped the gene to mouse chromosome 8. Promoter elements contributing to the regulation of the Rex-1 promoter in F9 cells have been identified. A region required for Rex-1 promoter activity in F9 stem cells contains an octamer motif (ATTTGCAT) which is a binding site for octamer transcription factor members of the POU domain family of DNA-binding proteins. Rex-1 reporter plasmids including this octamer site also exhibited reduced expression in F9 cells treated with RA. Thus, the octamer motif is a regulatory element required for the activity of the Rex-1 promoter in F9 stem cells, and this motif contributes to the negative regulation by RA of the transcription of the Rex-1 gene. As an initial confirmation of the in vivo relevance of the isolated fragment, a larger Rex-1 promoter fragment, also containing the octamer site, was able to promote expression of the bacterial *lacZ* gene in mouse embryos at the morula stage.

The development of an organism from a single cell is a complex process involving many alterations in gene expression. One substance known to influence various aspects of embryogenesis is retinoic acid (RA). RA affects the transcription of genes in part by acting through two types of nuclear receptors, retinoic acid receptors (RARs) (5, 10, 40, 53) and retinoid X receptors (44). RA-responsive elements which mediate induction and bind RARs and retinoid X receptors have been identified in genes such as Hox-1.6 (35), laminin B1 (72), the cellular retinol binding protein I (68), RARβ (11, 26), and RARγ2 (38).

The expression of other genes is reduced in the presence of RA by several different mechanisms. RA regulates some genes through binding sites for known transcription factors, such as AP-1 or octamer factors (47, 62, 64), or unknown cellular factors (80). Inhibition of the expression of these genes by RA may result from competition for DNA-binding sites, protein interactions, or reduced levels of positive regulatory proteins. Reduction of expression of the octamer factor gene oct-3/4 by RA is regulated in murine F9 cells through a two-part DNA motif bound by unknown factors in F9 cells (50). Another mechanism by which RA can inhibit expression is a reduction in mRNA stability, as is observed for the tyrosine aminotransferase message (51).

Because murine F9 teratocarcinoma stem cells respond to treatment with RA by differentiating into endoderm cells that resemble extraembryonic cells of the mouse blastocyst (69, 70), they have been utilized as a model system for studying mechanisms of RA action and early embryo development (20). We have previously identified a cDNA for Rex-1, a zinc finger gene (*Zfp-42*) whose expression is reduced at the transcriptional level as F9 cells differentiate (28). The presence of zinc finger motifs in Rex-1 suggests that Rex-1 protein binds DNA and regulates transcription. This possibility is supported by the recent identification of a protein that possesses zinc finger motifs highly similar to those in Rex-1 and that binds to transcription-regulatory elements in a broad range of cell types (17, 19, 21, 22, 37, 52, 65, 67). Rex-1 mRNA is detected in a limited range of cells and tissues: undifferentiated embryonic stem (ES) cells, mouse embryos at the blastocyst stage, trophectoderm, and meiotic germ cells of the adult mouse testis (55).

We have isolated the *Zfp-42* chromosomal gene for Rex-1 and characterized promoter elements required for expression in F9 cells. A binding site (ATTTGCAT) for octamer factors, members of the POU family of DNA-binding proteins (23, 57), is located in a region required for Rex-1 promoter activity in F9 stem cells. Rex-1 reporter plasmids containing this octamer site displayed reduced expression in F9 cells treated with RA. The octamer motif contributes to the activity of the Rex-1 promoter in F9 stem cells and to the reduction of Rex-1 expression by RA. In initial confirmation of in vivo function, a larger Rex-1 promoter fragment, also containing the octamer site, was sufficient for expression of the bacterial *lacZ* gene in mouse embryos at the morula stage.

MATERIALS AND METHODS

Cell culture and transient-transfection assays. F9 teratocarcinoma cells were cultured and transiently transfected with

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10 μg of reporter plasmid and 5 μg of a β-actin–*lacZ* plasmid per 10^6 cells by the calcium phosphate precipitation method as described previously (72, 74). For cotransfections, 1 μg of expression plasmid for RARα, -β, or -γ in the vector pSG5 was included (72). The precipitate was removed after 12 h, and medium with or without 10^{-6} M RA was added and left for 48 h. Cell extracts were prepared and assayed for chloramphenicol acetyltransferase (CAT) activity (72). To normalize for differences in transfection efficiencies, equivalent units of β-galactosidase activity were used in CAT assays. CAT activity was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). In some experiments, a Triton lysis buffer (25 mM Tris HCl [pH 7.8], 2 mM EDTA, 5 mM dithiothreitol, 15% glycerol, 1% Triton X-100) was substituted for the standard Tris-glycerol buffer and the sonication step was eliminated.

Isolation of Rex-1 genomic clones. A BALB/c mouse spleen genomic DNA library (provided by R. M. Perlmutter, California Institute of Technology, Pasadena) was plated and screened as described previously (36) with the full-length Rex-1 cDNA. By standard procedures, two distinct phage inserts were isolated, restriction mapped, and compared with F9 cell DNA Southern blot patterns (45). A BALB/c mouse liver genomic DNA library (CloneTech, Palo Alto, Calif.) was screened in the same manner with a 5′ 239-bp cDNA fragment, and an additional phage insert was chosen for further study. Sequences of regions of interest were determined as described previously (28). The presence of known transcription regulatory motifs was determined with the computer programs Dynamic (Molecular Biology Computer Research Resource of the Dana Farber Cancer Institute) and findpatterns/tfsites.dat (Genetics Computer Group, Inc., Madison, Wis.).

Genetic mapping. Two genetic crosses were typed. In the first cross, NFS/N or C58/J females were mated with *Mus musculus musculus* males, and the F_1 females were mated with *M. m. musculus* males (32). In the second cross, NFS/N females were mated with *Mus spretus* males, and the F_1 females were mated with C58/J or *M. spretus* males (1). Southern blotting was used to type DNAs extracted from the progeny of these mice for restriction enzyme polymorphisms of the Rex-1 gene, *Zfp-42*, using a 1.0-kb *Eco*RI fragment located approximately 2 kb upstream of the 5′ end of *Zfp-42*. DNAs from the same mice were also typed for other genetic markers, including *Tea*, *Plat*, and *Jund*, as described previously (8, 43, 66). *Tea* was additionally typed in the *M. spretus* cross following digestion with *Hin*dIII.

DNase I hypersensitivity assays. Preparation of Southern blots of DNA treated with DNase I for detection of hypersensitive sites has been described elsewhere (35). Blots were probed with the 1.0-kb *Eco*RI fragment used for genomic mapping (see above).

Primer extension assays. The oligomer used as a primer for primer extension analysis was complementary to Rex-1 bases 51 to 90. One nanogram of primer end labeled with ^{32}P by using polynucleotide kinase (45) was hybridized with 1.5 μg of poly(A)$^+$ RNA in 25 mM Tris HCl (pH 8.2)–250 mM KCl at 60°C for 2 h in a reaction volume of 20 μl. Five units of avian myeloblastosis virus reverse transcriptase was added in 30 μl of reverse transcriptase buffer (25 mM Tris HCl [pH 8.2], 20 mM $MgCl_2$, 7.5 mM dithiothreitol, 1 mM [each] dATP, dGTP, dCTP, and dTTP) and incubated at 50°C for 1 h. After ethanol precipitation, the pellet was resuspended in 0.1 N NaOH–2 mM EDTA and loaded onto a DNA sequencing gel. The labeled oligomer was also used as a primer for standard sequencing reaction mixtures, which were loaded in adjacent lanes.

Construction of CAT reporter plasmids. A 7.6-kb *Kpn*I-*Sal*I fragment containing Rex-1 exons 1 and 2 and approximately 4.5 kb of upstream region was subcloned into the Bluescript KS+ vector (Stratagene Cloning Systems, La Jolla, Calif.) to construct plasmid pgRex-1.14. Subclones of pgRex-1.14 deleted from the 3′ end were generated with exonuclease III and the Erase-A-Base system (Promega Biotec, Madison, Wis.) for use in sequencing and CAT reporter plasmid construction. One of the subclones, with its 3′ end in Rex-1 exon 1 at base +23, was used for construction of REXCAT plasmids in combination with the promoterless CAT vector pBC0 (7). Subclones of the −669 Rex-1 promoter construct with deletions at the 5′ end were also generated with the Erase-A-Base system, with 25 mM NaCl added to the exonuclease III digestion buffer. The CAT gene from pBC0, as a *Bam*HI-*Hin*dIII fragment, was substituted for the luciferase gene in pGL2-basic (Promega Biotec) to obtain the promoterless CAT vector pGCAT0. Selected Rex-1 promoter fragments were subcloned into this vector, which retains the simian virus 40 transcription stop signals upstream of the pGL2 polylinker sites at the 5′ end of the CAT gene.

PCR. The conditions for the reverse transcriptase polymerase chain reaction (PCR) used to assay for pseudogene expression have been described previously (55). A Rex-1 promoter fragment with point mutations in the octamer motif was generated by the recombinant PCR technique (24). Two PCRs were performed, each using one oligomer primer (prepared by National Biosciences, Plymouth, Minn.) in the Rex-1 sequence, with two mismatches which would result in the desired mutation, and one external primer matching the vector sequences outside the Rex-1 fragment. The mismatch primers were GGCATGTTCATAACTGAG and CAGTT ATGAACATGCCTC (Rex-1 bases −223 to −206 and the complement to bases −225 to −208, respectively; mismatches are underlined). The products of the two reactions were isolated from an agarose gel to remove primers and unincorporated nucleotides, mixed with each other, and used as the template for a second round of PCR amplification using only the external primers. The resulting fragment was cloned upstream of CAT and sequenced to determine that the desired point changes had been introduced.

Expression in mouse embryos of β-galactosidase driven by the Rex-1 promoter. The bacterial *lacZ* gene from pCH110 (Pharmacia, Piscataway, N.J.), including the simian virus 40 polyadenylation signals at the 3′ end, was cloned into pUC13. *lacZ* was subsequently cloned into the *Pml*I site in Rex-1 exon 2 in pgRex-1.14 (described above) as a blunted *Hin*dIII-*Nar*I fragment [retaining, at the 3′ end, the poly(A) signals and a 161-bp fragment of the pUC13 vector]. The resulting plasmid, pREX-GAL, contains, in the following order in a Bluescript (Stratagene Cloning Systems) vector backbone, approximately 4.5 kb of Rex-1 upstream region (with a unique *Nco*I site approximately 400 bp from the 5′ end), the Rex-1 transcriptional initiation sites, exon 1, intron 1, 25 bp of exon 2, the *lacZ* gene from pCH110, simian virus 40 poly(A) signals, 161 bp of pUC13 (*Eco*RI-*Nar*I), the remaining 59 bp of Rex-1 exon 2, and approximately 500 bp of intron 2 (see Fig. 4 for the Rex-1 fragment used in this construct). Plasmid DNA was digested with *Nco*I and *Sac*II (in the Bluescript linker). The isolated ~12.5-kb fragment was injected into fertilized mouse oocytes (B6CBF2/CA), and the embryos were grown in vitro in M16 medium or, alternatively, transferred into foster mothers (strain CD-1) as

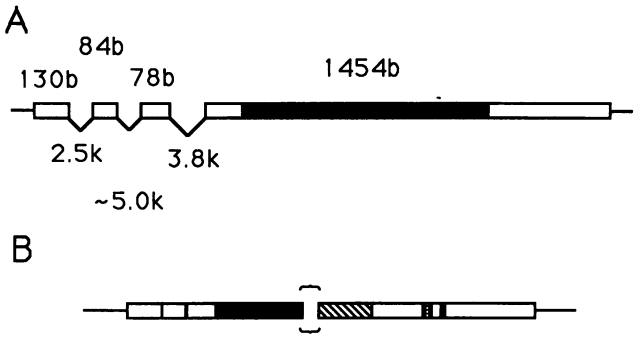

FIG. 1. Rex-1 (*Zfp-42*) genomic structure and related pseudogene (*Zfp-42ps1*). (A) The Rex-1 gene is diagrammed (not to scale), showing exon-intron boundaries. Exons 1 through 4 are shown as open rectangles. Sizes of exons (in base pairs) are indicated above the diagram. The ORF is contained within exon 4 and is shown as a black rectangle. Approximate sizes of introns (in kilobases) are given below the diagram. (B) The Rex-1-related pseudogene is diagramed (not to scale). Exons are indicated as in panel A. Brackets indicate the location of deleted region (see text). The large hatched rectangle indicates a frameshifted ORF. The crosshatched rectangles indicate insertions.

described previously (27). Recovered embryos were stained for β-galactosidase activity as described previously (31).

Nucleotide sequence accession numbers. Sequences associated with the work reported here have been submitted to GenBank and assigned the following accession numbers: M97810, Rex-1 (*Zfp-42*) promoter sequences, exon 1, intron 1, and exon 2; M97811, Rex-1 exon 3; M97812, Rex-1 exon 4; and M97813, pseudogene sequence (*Zpf-42ps1*).

RESULTS

Isolation and characterization of the Rex-1 chromosomal gene and a related pseudogene. The previously isolated Rex-1 cDNA (28) was used to screen an *Eco*RI partial-digest mouse genomic DNA library. Two nonoverlapping clones (LC-1 and LC-2) were isolated, one of which (LC-1) contained a Rex-1-related pseudogene (described below). LC-2 contained the 3′ end, but not the 5′ end, of the Rex-1 gene. A shorter cDNA probe encoding the 5′-end 239 bp was used to screen a *Sau*3A partial-digest genomic library, and a third nonoverlapping clone (LR-2), containing the 5′ end of the Rex-1 gene, was isolated. Sequences from the four exons in LC-2 and LR-2 correspond to the sequence of the Rex-1 cDNA, including a correction to the previously reported sequence (28) (three G's at positions 1463 to 1465 in the 3′ untranslated region of the cDNA should have been four G's, as confirmed by cDNA and genomic clone sequencing). The genomic structure of Rex-1 is shown in Fig. 1. Exon 4 contained the entire open reading frame (ORF) for the deduced Rex-1 protein. The minimum size of intron 2 was estimated from the results of Southern blot hybridizations (data not shown), since the entire intron was not contained in the isolated DNA.

A 5.6-kb *Eco*RI fragment from clone LC-1 hybridized to probes from both the 5′ and 3′ ends of the Rex-1 cDNA. The sequence of the hybridizing region was determined. Relative to the 1,746 bp of the Rex-1 cDNA, there were 22 point mismatches, two small insertions (29 bp following nucleotide 1357 and three T's following nucleotide 1384), and a deletion of 377 bp (reducing a 5-bp direct repeat of GTGCA to one copy and removing the intervening bases). The 5′ end corresponded to one of the major transcription initiation sites (−16; see below) for the Rex-1 gene, and a string of 9 A's occurred at the 3′ end. No intron sequences were retained. These are characteristics of a pseudogene (retrosequence) (29, 76). There were 11-bp direct repeats (ATCTAGGGTCA) at the 5′ and 3′ ends of the sequence. Southern blots of F9 cell genomic DNA were hybridized to several probes to confirm that this fragment was representative of the genome (Table 1). Probes from the 5′ and 3′ ends of the cDNA each hybridized to two bands: (i) a common one and (ii) a second one corresponding in size to Rex-1 exons 1 and 4, respectively. A probe containing cDNA bp 657 to 957, which were deleted in the potential pseudogene, hybridized to only one band, corresponding to Rex-1 exon 4. Thus, the 5.6-kb *Eco*RI fragment contained a processed pseudogene (*Zfp-42ps1*) related to Rex-1. Although the pseudogene contains sequences for three zinc finger motifs, the deduced protein encoded in a pseudogene transcript would not include zinc fingers because of a frameshift in the ORF. Pseudogene transcripts were not detected in F9 stem or RA-treated cells when a reverse transcriptase PCR assay was used (data not shown).

TABLE 1. Results of Southern blot hybridizations to F9 cell DNA with Rex-1 cDNA probes

Restriction endonuclease	Band(s) (kb) hybridizing with the following cDNA probe:			Rex-1 element detected
	2–41 bp	657–957 bp	1500–1746 bp	
*Eco*RI		8.0	8.0	Exon 4
	5.6		5.6	Pseudogene
	3.3			Exon 1
*Bam*HI	12.0		12.0	Pseudogene
	4.8			Exon 1
		3.5	3.5	Exon 4
*Hin*dIII	10.0		10.0	Pseudogene
		4.8	4.8	Exon 4
	2.1			Exon 1

The Rex-1 gene, *Zfp-42*, maps to murine Chr 8. Because of the expression pattern of Rex-1 mRNA in early murine embryogenesis and in the adult testis (55), we were interested in determining the chromosomal position of the Rex-1 gene, *Zfp-42*, relative to those of known mutations affecting embryo development or male fertility. The location of *Zfp-42* in comparison with those of other zinc finger genes could also be of interest. A 1.0-kb *Eco*RI fragment upstream of Rex-1 exon 1 was used as a probe to identify restriction enzyme polymorphisms. The gene was mapped to mouse chromosome 8 (Chr 8) by analysis of two different multilocus crosses (1, 32). The Rex-1 probe hybridized specifically to the Rex-1 gene and not to the related pseudogene. In the *M. m. musculus* cross, fragments of 7.5, 9.6, or 14.5 kb were identified in parental NFS/N, C58/J, or *M. m. musculus* mice, respectively. In the *M. spretus* cross, *Pvu*II digestion identified fragments of 7.0, 10.2, and 11.5 kb in *M. spretus*, NFS/N, and C58/J parental mouse DNAs, respectively.

Inheritance of the polymorphic fragment in both genetic crosses was consistent with expected single-gene segregation ratios, and comparison of the inheritance pattern with those of other markers demonstrated linkage to markers in the proximal region of Chr 8 (Table 2). These results are consistent with the following gene order: centromere–*Plat*–*Tea*–*Zfp-42*–*Jund*.

TABLE 2. Segregation of the restriction enzyme polymorphisms of the Rex-1 gene (*Zfp-42*), *Plat*, *Tea*, and *Jund*[a]

Progeny	Inheritance of the following allele from the F_1 parent[b]:				No. of mice	
	Plat	*Zfp-42*	*Tea*	*Jund*	Cross 1[c]	Cross 2[d]
Nonrecombinants	+	+	+	+	31	
	+	+	+	ND[e]		60
	−	−	−	−	22	
	−	−	−	ND		61
Recombinants	+	+	+	−	3	
	−	−	−	+	5	
	+	+	−	−	0	
	+	+	−	ND		2
	−	−	+	+	0	
	−	−	+	ND		5
	−	+	+	+	10	
	−	+	+	ND		9
	+	−	−	−	1	
	+	−	−	ND		10

[a] Percent recombination between restriction fragments and standard error were calculated from the number of recombinants in the total sample (18), with the following results: *Plat*, *Tea*, 31/220 = 14.1 ± 2.3; *Tea*, *Zfp-42*, 7/220 = 3.2 ± 1.2; *Zfp-42*, *Jund*, 9/73 = 12.3 ± 3.8.

[b] Scored as the presence (+) or absence (−) of the NFS/N or C58/J alleles in the *M. m. musculus* cross or of the *M. spretus* alleles in the *M. spretus* cross.

[c] (C58/J × *M. m. musculus*) × *M. m. musculus*; (NFS/N × *M. m. musculus*) × *M. m. musculus* (total of 73 mice).

[d] (NFS/N × *M. spretus*) × C58/J or *M. spretus* (total of 147 mice).

[e] ND, not determined.

The Rex-1 molecular clone thus provides a useful additional DNA marker for this region of Chr 8. Furthermore, sequence analysis of the 5′ end of the Rex-1 gene identified two upstream regions containing dinucleotide repeats. One contained the sequence $(GT)_{23}$. Another contained a complex dinucleotide repeat of $(TG)_{30}$ followed by $(CA)_{22}$. Variability in the extent of such dinucleotide repeats has been used successfully for genetic markers in both human and mouse genomic mapping (12, 75).

Rex-1 has a TATA-less promoter with multiple transcription initiation sites. The site of transcriptional initiation in the Rex-1 gene was determined by primer extension analysis. Multiple clusters of initiation sites were detected (Fig. 2). The base at the 5′ end of the originally isolated cDNA, corresponding to an initiation site of moderate strength, was arbitrarily designated nucleotide +1 for reference. Major initiation sites occurred at −27, −16, and +15 (Fig. 2). The presence of multiple initiation sites was confirmed by an RNase protection assay (data not shown). The sequence near the initiation sites (Fig. 3) does not contain a TATA box motif, although the region from −135 to −100 is GC rich and includes GC boxes, which are possible binding sites for the transcription factor Sp1 (30). The pattern of initiation sites was similar in stem and RA-treated F9 cells (Fig. 2).

DNA sites which are bound by regulatory factors are frequently hypersensitive to digestion by DNase I (14). To identify possible sites in the Rex-1 promoter, nuclei were isolated from untreated F9 cells and from cells treated with RA, with cyclic AMP and theophylline, or with RA and theophylline. Samples were incubated with increasing concentrations of DNase I. Blotted DNA from treated nuclei was probed with the Rex-1 1.0-kb *Eco*RI fragment used for chromosome mapping. Two hypersensitive sites were iden-

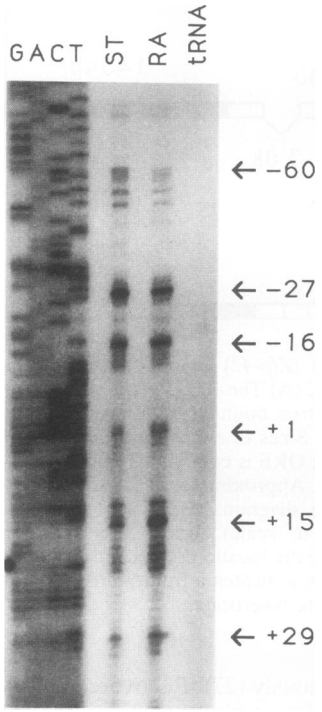

FIG. 2. Primer extension analysis. Primer extension assays were performed with RNA isolated from F9 stem (ST) and RA-treated (RA) cells to determine transcription initiation sites in the Rex-1 promoter. The oligomer used as the primer for extension was also used in sequencing reactions with the Rex-1 cDNA plasmid. Positions of major start sites are indicated (arrows) relative to the sequence ladder (GACT).

```
                                                    *
ACATGTGTGTAACAGAGGTA CTGAGATGTGACTGAGTCTC   -251

                  *
AAGGCCAGGCGATCGGGATT CAGAAGAGGCATTTGCATAA   -211

 *
CTGAGCAAGAGCCTTTGCCC CACCCTTCCACGCGGACCCA   -171

AGACAAGCGGACAGCTCTGG GTGGGTCACCTTGAAGCCAG   -131

  *
GGGCCCGCCCACATCCCCGC CCACACCCACCTTGAGCGCT   -91

TCTCATTGGGCATTCCAGCC TACCCAGCTCGGAGTTAGTT   -51

                        ↱          ↱
ACTCCGTAAGTGTGGCCGGA ACAGAGTTCGTCCATCTAAA   -11

                        ↱
AAGGGAGGGGTCCAAGGAGC TGAACTCCTAGCC          +23
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FIG. 3. Sequence of the 5′ flanking region of the Rex-1 gene. Part of the sequence of the Rex-1 promoter is shown. Major transcription sites are shown as arrows. GC boxes are underlined; the octamer motif is double underlined. Asterisks indicate the 5′ end positions (−257, −234, −204, and −125) of promoter fragments described in Fig. 4 and the text. Bases are numbered relative to the first base in the isolated Rex-1 cDNA, as discussed in the text.

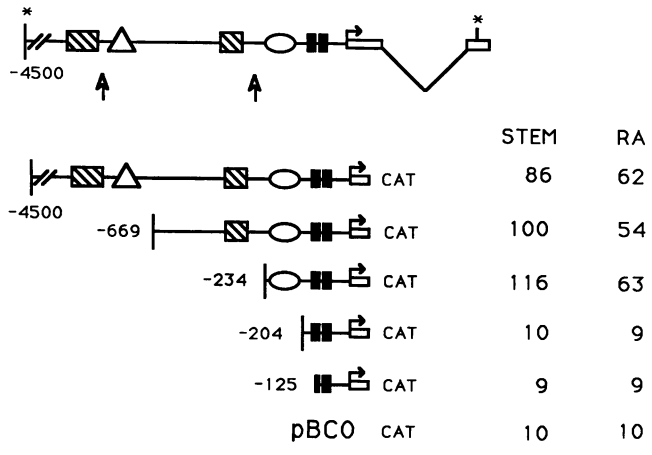

FIG. 4. Rex-1 promoter constructs and CAT assay results. Relative positions of dinucleotide repeats (hatched rectangles), the PEA-3 motif (triangles), the octamer motif (ovals), and GC boxes (solid rectangles) are indicated. Open rectangles represent Rex-1 exons 1 and 2. Asterisks mark the endpoints of the fragment used for expression of the *lacZ* gene in mouse embryos. Open-headed arrows show approximate locations of DNase I-hypersensitive sites. The 5′ endpoints of Rex-1 promoter fragments used in CAT gene reporter constructs are indicated. Results of CAT assays with F9 stem and RA-treated cells (average values from a minimum of four independent experiments) are given as percentages relative to the activity of the −669 construct in the absence of RA.

tified, one ~1.1 kb 5′ of a *Bam*HI site in intron 1, which locates the first site near exon 1, and a second approximately 0.6 kb further upstream. Both of the sites were apparent regardless of the conditions under which the cells were grown (data not shown). The locations of the sites correspond approximately to the two regions of dinucleotide repeats described above (Fig. 4). Such repeats are sometimes associated with DNase I-hypersensitive sites (41, 48). Alternatively, the DNase I-hypersensitive sites might be related to transcription factor-binding motifs present in that region, as we discuss further below (Fig. 3 and 4).

Sequences upstream of the Rex-1 gene are sufficient to promote expression of a reporter gene in F9 cells. A 7.6-kb fragment containing Rex-1 exons 1 and 2 was digested from the 3′ end with exonuclease III to obtain a fragment which encoded approximately 4.5 kb of upstream promoter region with its 3′ end at bp +23. This fragment was cloned 5′ to a promoterless CAT gene in the vector pBC0 (7) to construct the plasmid pREXCAT-4.5 (−4500 construct). In transient-transfection assays with F9 cells, pREXCAT-4.5 was active in stem cells and showed a 28% decrease in activity after treatment with RA for 48 h following transfection (Fig. 4).

Shorter 5′ end fragments were tested to determine the minimum regions required for stem cell expression and RA responsiveness (Fig. 4). A construct containing Rex-1 bases −669 to +23 was more active in F9 stem cells than the 4.5-kb construct, perhaps because more copies of the much smaller −669 construct are present in equivalent transfection preparations. Alternatively, regulatory elements located between positions −669 and −4500 may affect the F9 stem cell activity of the −4500 construct. The activity of the −669 construct decreased to 53% in the presence of RA. In nuclear runoff assays at early times, an RA-mediated decrease of the rate of transcription to 33% of stem cell level (a threefold decrease) is observed (28). Later, in fully differentiated F9 cells, the Rex-1 transcription rate decreases to less than 10% of the rate observed in stem cells, but it is not eliminated. Thus, the RA responsiveness of the −669 construct is consistent with the early decrease in transcription of the endogenous Rex-1 gene, allowing for the background level of activity seen with the promoterless CAT vector control (pBC0; Fig. 4). A fragment containing bases −234 to +23 retained stem cell and RA-responsive activity at levels similar to that of the −669 construct. However, fragments deleted to base −204 or shorter had no more activity than the promoterless CAT gene vector in either F9 stem or RA-treated cells. Thus, elements essential for promoter activity in stem cells and reduced expression in response to RA are present in the Rex-1 promoter downstream of position −234.

The Rex-1 upstream region required for stem cell activity contains an octamer factor-binding motif. Known transcriptional regulatory motifs in the DNA sequence surrounding Rex-1 exon 1 were identified by computer analysis. A potential binding site for PEA-3 was located at position −813. PEA-3 is a transcription factor present in F9 stem cells and absent in differentiated F9 cells (46, 78) and thus might contribute positively to the expression of Rex-1 in stem cells. However, deletion of this PEA-3-binding site does not affect either the stem cell or RA-responsive activity of the Rex-1 promoter fragment. GC boxes, elements associated with binding of the transcription factor Sp1 (30), were present between bases −127 and −109. The construct beginning at position −204, which retained these sites, was insufficient for Rex-1 promoter activity, suggesting that the GC boxes are not sufficient for basal activity of this promoter.

The sequence ATTTGCAT was located within the region (−234 to −204) identified as important for expression from the Rex-1 promoter in F9 stem cells. This motif has been identified as a binding site for octamer-binding proteins, which are members of the POU family of homeobox transcription factors (23, 57). One of these octamer factors, oct-1, is constitutively present in F9 cells (61). Two other octamer factors, oct-3/4 and oct-6, have been shown to be present in F9 stem cells and absent after RA-induced F9 cell differentiation (39, 56, 61, 71). If these octamer factors regulated the expression of Rex-1, the decrease in abundance of oct-3/4 and oct-6 could account for the observed decrease in Rex-1 expression in differentiated cells, while the constitutive presence of oct-1 could explain why Rex-1 expression continues at low levels following RA treatment. In addition, octamer motifs have been implicated in the RA regulation of the fibroblast growth factor 4 (FGF4) gene (62). To investigate the possibility that the octamer site was responsible for the observed activity of the Rex-1 promoter, a fragment with point mutations introduced into the octamer site was tested in F9 cells.

PCR was used to generate a fragment containing Rex-1 bases −257 to +23, with two base changes introduced such that the octamer site was mutated to ATGTTCAT. Schöler and coworkers have shown by electrophoretic mobility shift assay that multimers of this sequence no longer bind factors in F9 cells (58). Plasmid pGoct$^-$ was constructed by cloning this PCR fragment into pGCAT0, a promoterless CAT vector. pGCAT0 includes transcription termination signals 5′ of the promoter cloning sites which help to reduce background CAT activity due to transcripts initiating at vector sequences. The Rex-1 promoter fragments with 5′ endpoints at −669 and −125 were also cloned into pGCAT0 as controls. CAT activity from pGoct$^-$ was reduced in stem cells to less than 35% of the activity of the oct$^+$ −669 construct and was not substantially reduced by RA treat-

TABLE 3. Results of CAT assays with Rex-1 promoter constructs in pGCAT0, including a construct with a mutated octamer site

Construct[a]	CAT activity[b]			
	Expt 1		Expt 2	
	Stem cells	RA-treated cells	Stem cells	RA-treated cells
−669	100	51.9	100	61.6
pGoct⁻	33.6	33.8	18.5	14.1
−125	17.5	27.8	<1	<1

[a] See text.
[b] Expressed as percentages relative to the activity of the −669 construct in the absence of RA.

ment (Table 3). The wild-type control constructs −669 and −125 in pGCAT0 showed responses comparable to those of the corresponding constructs in the pBC0 vector (Fig. 4); the −669 construct shows full promoter activity with an RA response and the −125 construct has minimal activity.

From analysis of the constructs in which the octamer motif is deleted or mutated (Table 3 and Fig. 4), we conclude that the octamer element is important for the transcription of Rex-1 in F9 cells. Although the base changes that we made in the octamer motif (pGoct⁻) do not completely eliminate CAT activity in F9 stem cells (Table 3), this mutation is the only one tested to date and may not totally prevent interactions between octamer family transcription factors and the octamer element in the Rex-1 promoter. Alternatively, other elements in the mutated fragment may contribute to basal promoter activity. In addition, the residual activity of pGoct⁻ is not further reduced in the presence of RA (e.g., it does not decrease to the level of expression of the −125 construct). Because deletion of the region that contains the octamer element (from position −234 to −204) eliminates Rex-1 promoter activity in F9 stem cells, we suggest that the octamer element is required for transcriptional activation of the Rex-1 gene in F9 stem cells and mediates the RA-associated decrease in Rex-1 transcription.

Cotransfection with RAR expression vectors does not affect the RA response of the Rex-1 promoter. Although an RA-dependent decrease in promoter activity was observed with the Rex-1 promoter constructs, the endogenous levels of RA receptor proteins might be limiting with respect to the action of RA on the constructs. If the RARs directly mediate the RA response of the Rex-1 promoter, increased levels of RARs might be required in order to observe the maximum decrease in Rex-1 promoter construct expression in the presence of RA. In some studies of genes regulated by RA, cotransfection with vectors expressing the RARs was required in order to observe an RA-mediated induction of transcription (11, 38, 72). Therefore, transient-transfection assays in the presence of cotransfected RAR expression plasmids were performed. The RA response of representative Rex-1 promoter constructs was unaffected by cotransfection with expression vectors for murine RAR-α, -β, or -γ (data not shown). Thus, either the RARs are not directly involved in the RA regulation of the Rex-1 promoter or endogenous levels of receptors in F9 cells are sufficient to mediate the observed RA response.

β-Galactosidase expression is activated by the Rex-1 promoter in embryos. We have previously shown by in situ hybridization studies that Rex-1 mRNA is expressed in the early mouse embryo (55). To demonstrate that the Rex-1 promoter utilized in F9 cell experiments is active in embryos, we inserted the bacterial gene for β-galactosidase (*lacZ*) into a 7.6-kb clone containing Rex-1 exons 1 and 2 (to construct plasmid pREX-GAL). This construct produced β-galactosidase activity in F9 stem cells. A control construct that contained the same DNA fragments with the *lacZ* gene in the opposite orientation generated no activity in F9 stem cells. An isolated restriction enzyme fragment from pREX-GAL (described further in Materials and Methods) was injected into fertilized mouse oocytes. The injected oocytes were cultured in vitro or transferred to pseudopregnant females before harvest and then stained as morula stage embryos for β-galactosidase activity. In several experiments, approximately 50% of morulae (a total of 23 of 45) contained blue-stained cells (Fig. 5). Inefficient DNA fragment integration or integration into inactive regions of the genome could explain why signal was present in only half of the observed morulae, as we did not attempt to generate stable transgenic mouse lines. Thus, this large Rex-1 promoter fragment contains elements sufficient for expression in the early mouse embryo.

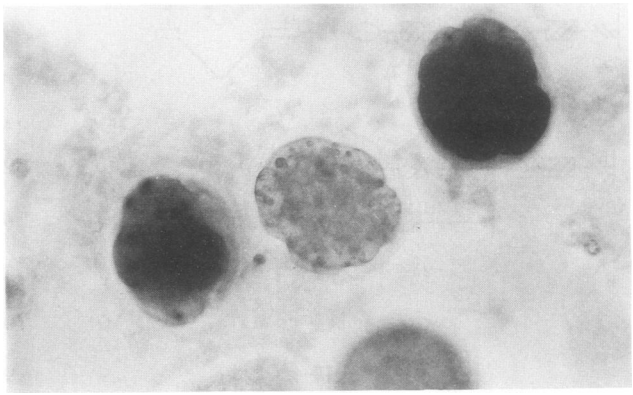

FIG. 5. Expression in mouse embryos of β-galactosidase from the Rex-1 promoter. Three morula stage embryos, representative of the range of expression observed, are shown. The blue stain indicating the presence of β-galactosidase appears dark. The zona pellucida surrounding the embryos is visible.

DISCUSSION

The Rex-1 transcript is encoded in four exons on mouse Chr 8. We have isolated genomic DNA for the Rex-1 gene (*Zfp-42*), characterized its structure, and identified its chromosomal location. As the Rex-1 gene ORF is encoded within one exon, exon 4, the four zinc fingers occur as a single unit. This appears to be the most common organization for zinc finger genes (13). Pseudogenes such as that described here (*Zfp-42ps1*) for Rex-1 have also been reported for other zinc finger genes (3).

Although zinc finger genes have been reported to occur in clusters (13), *Zfp-42* does not map near any known zinc finger genes. Two zinc finger genes, *Zfp1* and *Zfp4*, do map to the same chromosome as *Zfp-42* (mouse Chr 8), but to more-distal sites (4, 6, 25). Since the Rex-1 gene is expressed in early embryos and meiotic male germ cells, a mutation in *Zfp-42* may manifest itself with effects on embryonic survival or male fertility. However, no known mouse mutations with these phenotypes map near the Rex-1 gene. Von Melchner and colleagues reported that an inactivating mutation of the Rex-1 gene, identified in ES cells, was not

transmitted through the male germ line in chimeric mice (73). If other *Zfp-42* mutations behave similarly, identification of a naturally occurring mutant would be difficult.

Deletion of a region including an octamer motif abolishes Rex-1 promoter activity. An octamer factor-binding motif (ATTTGCAT) located within the region −234 to −204 was identified as important for expression from the Rex-1 promoter in F9 stem cells. Deletion of this region eliminates promoter activity in F9 stem cells. Mutation of the octamer motif greatly reduces CAT activity in F9 stem cells and reduces the RA-associated decrease in activity. This suggests a role for octamer factors in the regulation of the Rex-1 gene. Transfection experiments involving octamer factor expression vectors and DNA binding assays are being done to further clarify the interactions of these proteins with the Rex-1 promoter. However, other motifs in the Rex-1 promoter between positions −669 and +23 may also modulate stem cell and/or RA-responsive promoter activity.

Octamer motifs have been reported to be involved both in the glucocorticoid-associated reduction of transcription (33, 77) and in the RA-associated reduction of transcription. In addition to Rex-1, the immunoglobulin heavy chain enhancer (immunoglobulin H μ300) (39), the interleukin-2 gene (15), and the FGF4 gene (62) contain octamer motifs associated with their regulation by RA. The RA-mediated regulation of these genes and the Rex-1 promoter may be explained most simply by proposing that the expression or activity of an octamer-binding factor (or factors) is directly reduced by RA. Indeed, in F9 stem cells, two known octamer-binding factors, oct-3/4 and oct-6, are present and their binding activities decrease after treatment of the cells with RA (39, 49, 61). The promoter for the gene for one of these factors, oct-3/4, contains an enhancer element which is RA repressible, but whether the RARs play a direct role in the regulation of this promoter is as yet unclear (50). An alternative explanation for the RA-associated decrease in Rex-1 expression is that protein-protein interactions between octamer factors and RARs (or retinoid X receptors) lead to a loss of octamer factor function in a mechanism that is independent of DNA binding by the RARs. Such functional interference has been observed between oct-1 and the glucocorticoid receptor (33). The addition of RA might also affect the availability or activity of a cofactor(s) required for octamer factor function in F9 cells. Cell-specific octamer cofactors have been identified (2, 42, 54).

The four octamer-containing RA-regulated genes exhibit differences in expression that suggest that these promoters are not all regulated through the same molecular mechanism. Two of the genes, the Rex-1 and FGF4 genes, are activated by octamer motifs in the absence of RA (62; this work); the other two, the immunoglobulin H and interleukin-2 genes, IgH and IL-2 are not. In contrast to Rex-1 and FGF4, the octamer site mediates repression of the immunoglobulin H promoter in F9 stem cells (39). With respect to interleukin-2, RA interferes with induction of expression by other signalling molecules (phorbol esters and calcium ionophores) (15). This inhibition involves the RARs and may occur through a protein-protein interaction mechanism such as that described for RA inhibition of AP-1 action (34, 63, 64, 79). Therefore, the interactions between RA and proteins that bind the octamer motif for the interleukin-2 and immunoglobulin H genes may be different from those for the Rex-1 and FGF4 genes.

The Rex-1 and FGF4 genes are both actively transcribed in undifferentiated embryonal carcinoma cells, and their expression is reduced when RA is added to induce differentiation of the cells (9, 28). In addition, fragments which mediate reduced expression of reporter genes following the addition of RA have been identified for each gene, and octamer motif elements are present in these fragments (62; this work). In the Rex-1 gene, the octamer motif is located near the multiple transcription initiation sites (around position −220), while the motif is found in the 3′ untranslated region of the FGF4 gene. However, additional regulatory elements or factors must be involved in the regulation of these genes, as their patterns of expression differ in other cell lines. For example, FGF4 mRNA is expressed and RA regulated in the P19 embryonal carcinoma cell line, in which the Rex-1 gene is not expressed (9, 28, 55, 62). This may be related to variations in the differentiation potential of the two cell lines, as we have discussed elsewhere (55). The octamer factors (oct-1, oct-3/4, oct-5, and oct-6) found in F9 cells are also present in P19 cells (49, 61, 71). Thus, the differential expression of Rex-1 and FGF4 may reflect the activities of other regulatory factors or octamer cofactors (2, 42, 54) present in the cells. In addition, both genes are expressed in ES cells, and their expression decreases with RA addition (55, 62). FGF4 expression is unaffected when the ES cells are allowed to differentiate by removal of leukemia inhibitory factor/differentiation-inhibiting activity (62). In contrast, Rex-1 expression decreases rapidly when leukemia inhibitory factor/differentiation-inhibiting activity is removed or when the cells are induced to differentiate by culturing them as aggregates (55). Thus, in ES cells, reduced expression of FGF4 specifically accompanies the addition of RA, while reduced expression of Rex-1 correlates with differentiation. Further comparison of the regulatory elements of these two genes may help to identify additional elements or factors which are involved in RA-specific regulation and the establishment of expression patterns as totipotent cells begin to restrict their developmental potential.

Rex-1 and octamer factor expression patterns partially overlap in the embryonic mouse and the adult mouse. The expression patterns of oct-3/4, oct-6, and Rex-1 mRNAs in the early embryo and in adult mouse tissues have been described previously (55, 60, 61, 71). In the adult, both Rex-1 and oct-6 are expressed in the testes; however, oct-6 is also expressed in the brain, where Rex-1 is not detected (55, 71). Adult oct-4 expression is restricted to oocytes, where Rex-1 mRNA also is not detected (55, 60, 61). The oct-4 and Rex-1 expression patterns are similar in the preimplantation mouse embryo (55, 60). Following implantation, Rex-1 mRNA is restricted to extraembryonic and trophoblast-derived tissues, while oct-4 is absent in those tissues but is expressed in the embryonic ectoderm (55, 60). oct-6 is expressed at later times in the developing neural ectoderm (71). Just as the octamer motif alone cannot completely account for the regulation of the Rex-1 promoter observed in F9 cells, the expression of known octamer factors cannot account for the observed pattern of Rex-1 mRNA in vivo. However, the effects of oct-3/4 on octamer motif-containing promoters in vitro are reported to depend on the concentration of the factor in cells. Intermediate levels of oct-3/4 activate transcription, while high levels of the factor repress expression from the same reporter construct (59). Thus, octamer factors may activate the Rex-1 promoter in blastocyst and earlier-stage embryos and repress its expression in specific regions at later times if the concentration of octamer factors varies embryonically and affects regulation. Alternatively, cofactors, as observed in B cells (2, 42, 54), may be involved in regulating the specificity of expression due to octamer motifs. The levels of such factors may prove to be limiting.

oct-1, which is expressed in all cells and tissues examined (57), may be involved in basal transcription of the Rex-1 promoter, although it is unlikely to contribute to specific patterns of regulation. Additional independent factors or regulatory motifs are likely to be required in order to establish the full expression pattern of Rex-1.

Transcription from the embryonic genome begins at the two-cell stage in the mouse embryo (16). Because of the pattern of Rex-1 mRNA expression, the Rex-1 promoter is a candidate for early embryonic activation. We have shown that a large promoter fragment is sufficient for expression of β-galactosidase by the morula stage. This fragment is likely to contain regulatory elements not included in the fragments tested with the CAT reporter gene in F9 cells, as the *lacZ* construct contains approximately 3.0 kb of additional Rex-1 genomic DNA, including all of intron 1. Such additional sites may be involved in determining the pattern of Rex-1 expression in the early embryo or in meiotic male germ cells.

Further studies with early embryos and transgenic mice of reporter gene constructs controlled by Rex-1 genomic fragments will determine whether the Rex-1 promoter is activated at earlier stages of embryonic development and may aid in the identification of elements essential for establishment of cell fate commitment and patterns of embryonic expression.

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