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Transcriptional Inhibition of the Murine Erythropoietin Receptor Gene by an Upstream Repetitive Element

HAGOP YOUSSOUFIAN[1,2]* AND HARVEY F. LODISH[1,3]

Hematology-Oncology Division, Brigham and Women's Hospital, Boston, Massachusetts 02115[1]; Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142[2], and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[3]*

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Transcription of the murine erythropoietin receptor (EpoR) gene is inhibited by a novel repetitive element that is located upstream of the EpoR promoter. Reporter gene studies reveal that the inhibitory effect is both distance and orientation dependent. This element is a member of a family of repetitive elements specific to rodents and is present at ~10^5 copies per mouse genome. It encodes ~500- to 900-bp-long transcripts in both erythroid and nonerythroid cells. RNase protection analysis with a probe from the 5′ flanking murine EpoR gene reveals that the direction of transcription is in the sense orientation, relative to the downstream EpoR gene. We suggest that transcriptional inhibition of the EpoR promoter is mediated by read-through transcripts originating in the upstream repetitive element and that this effect may contribute to the basal level of transcription of the murine EpoR gene in erythroid cells.

Among their many functions, a number of cytokines control the development of blood cells in vertebrate species. Some regulate the proliferation or differentiation of very early, precommitted hematopoietic cells, while others act on more mature progenitors or cells committed to a specific lineage of hematopoiesis (23). Erythropoietin (Epo) is the primary cytokine that regulates the maturation and proliferation of erythrocyte progenitors (16). The biological effects of Epo are mediated through binding to the erythropoietin receptor (EpoR), which is expressed primarily on erythroid and megakaryocytic cells (6). The EpoR is a member of a growing superfamily of cytokine receptors that is intimately involved in the control of hematopoiesis (5).

Experimental models of erythropoiesis have defined two major types of Epo-responsive cells: relatively Epo-insensitive early cell types, called erythroid burst-forming units, and more mature, highly Epo-sensitive cell types, called erythroid CFU (CFUe) (16). The dramatic acquisition of sensitivity to Epo marks an important physiological stage of erythropoiesis. The mechanisms that underlie these transitions through different stages of erythropoiesis are poorly defined. Recently, the mouse EpoR gene was shown to undergo transcriptional activation upon differentiation of embryonic stem cells in vitro (34). However, the specific stage of erythropoiesis during which this activation took place was unknown. Several reports have demonstrated a change in the surface expression of EpoR with progressive differentiation of erythroid cells. There appears to be a peak in surface expression at the CFUe stage (references 10 and 33 and references therein), correlating with the heightened sensitivity of these cells to Epo. This activation may be due in part to stage-specific regulation of EpoR gene transcription.

In general, tissue-specific expression of a gene may result from its activation in appropriate tissues, its repression in other tissues, or both. In an effort to understand the genetic regulation of the EpoR gene during erythropoiesis, we and others have cloned the murine EpoR gene and have begun to delineate its regulatory elements (17, 44). Analysis of the 5′ flanking region of the murine EpoR gene showed a 0.45-kb element that directs erythroid-specific transcription of a linked reporter gene (44). This region contains a binding site for the constitutive transcription factor Sp1 (8) and one for the hematopoietic or erythroid transcription factor GATA-1 (9, 37), as well as three CACCC elements that are thought to bind constitutively expressed factors that are important for the transcription of a number of eukaryotic genes (24, 26). We have demonstrated binding activities for Sp1 and GATA-1 in this region of the promoter, as well as the *trans* activation of this otherwise erythroid-specific promoter in heterologous NIH 3T3 cells by the coexpression of GATA-1 (46). Similarly, *trans* activation of the promoter was also demonstrated in COS cells (4). More recently, we have demonstrated that the 5′ flanking region consists of a modular array of positive and negative regulatory elements. One of the negative regulatory elements profoundly inhibits the activity of the promoter. Here we describe the nature and mechanism of this inhibitory region and postulate a role for it in regulating the basal level of promoter activity.

MATERIALS AND METHODS

Cell culture. The following murine cell lines were used. (i) Erythroid cell lines are as follows: MEL subclone 745-PC3, an Epo-independent CFUe-like cell line (21, 28); HCD57 (11), an Epo-dependent CFUe-like cell line; and CB5, an Epo-independent erythroid burst-forming unit-like cell line (36). (ii) Other hematopoietic cell lines are as follows: FDCP-1, an interleukin-3-dependent myeloid progenitor cell line (7); BaF3, an interleukin-3-dependent pre-B-cell line (27); S194, a plasmacytoma cell line (13); and WEHI-3B, a myeloid/macrophage cell line (39). (iii) Nonhematopoietic cell lines are as follows: ++/24Z, a bone marrow stromal cell line (1), and NIH 3T3, a fibroblast cell line. The erythroid and nonerythroid hematopoietic cell lines were cultured in suspension (7, 28, 36, 39). In addition, MEL and CB5 cells were also cultured as monolayers on 60-mm petri dishes precoated with fibronectin (28). The nonhematopoietic cell lines were cultured as monolayers.

* Corresponding author.

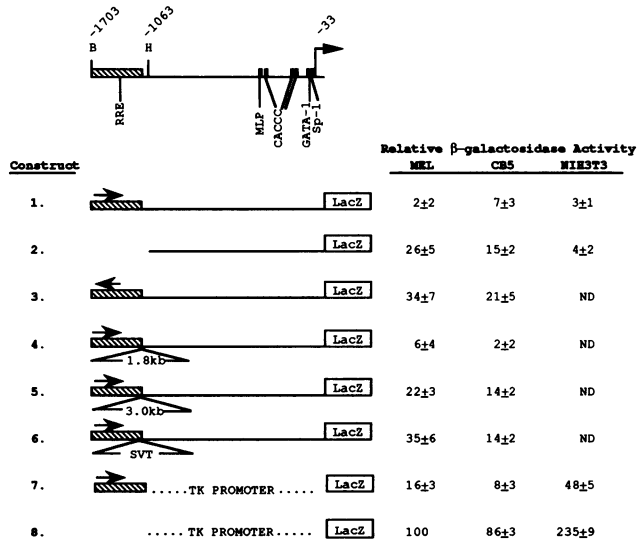

FIG. 1. Functional analysis of various constructs of the EpoR promoter. The structural organization of the EpoR 5′ flanking region, the herpesvirus TK promoter, a trimer of the simian virus 40 polyadenylation site (SVT), and heterologous spacer DNAs of mouse EpoR cDNA (1.8 kb) and human α-actinin cDNA (3.0 kb) are depicted. The numbers on the top diagram show the lengths of the fragments relative to the EpoR translation initiation site (+1, bent arrow). RRE (see text), a CACGTG binding site (MLP), a CACCC binding site, and the binding sites for GATA-1 and Sp-1 are shown. The arrow above the RRE box indicates the orientation of the box relative to the downstream fragment. The backbone of the various plasmid constructs is pLacF. Monolayer cells were transfected by a calcium phosphate precipitation technique with each expression plasmid and analyzed for β-galactosidase activity as described in Materials and Methods. The values are percentages relative to the TK promoter value in MEL cells and represent the averages of three separate transfections. B, *Bam*HI; H, *Hin*dIII; ND, not determined.

Plasmid constructions. Three types of reporter genes were used. First, for transfection of cells growing in suspension, the human growth hormone (hGH) gene was used as the reporter (35). Restriction fragments of the 5′ flanking region of the murine EpoR gene were subcloned into the polylinker region of the vector pOGH (35). Second, for MEL and CB5 cell monolayer transfections, the promoterless LacZ expression plasmid pLacF (45) was linearized at the unique Asp 718 site, and 5′ flanking EpoR fragments were subcloned into this site. Third, for COS cell transfections, a novel immunoglobulin expression vector was constructed by using Bluescript KS (full description to be reported elsewhere). By using partially overlapping mutant primers, the stop codon of open reading frame 2 (ORF-2; bases −1206 to −1204 [see Fig. 3]) was mutated into a *Bam*HI site by a technique based on the polymerase chain reaction (12). The mutated stop codon was then joined in frame to an artificial *Bam*HI site at the 5′ end of a partial cDNA for the constant regions of the murine immunoglobulin G2b (IgG2b) heavy-chain gene. The latter cDNA began with the first codon of the hinge region and spanned the two constant domains CH2 and CH3 (43a). This in-frame fusion of ORF-2 and IgG2b resulted in the introduction of an aspartic acid residue at the fusion joint. The fusion gene was then subcloned into the *Bam*HI-*Not*I sites of the Bluescript KS vector. All three parent plasmids lacked eukaryotic promoters. In other constructs, the herpesvirus thymidine kinase (TK) promoter driving either hGH (pTKGH [35]) or LacZ (Fig. 1, construct 8) was used as a positive control in the transfection experiments. Finally, different spacers were inserted at the *Hin*dIII site of construct 1 corresponding to position −1063 of the murine EpoR gene (Fig. 1). These spacers included the 1.8-kb murine EpoR cDNA (Fig. 1, construct 4) (6), a 3.0-kb human cytoskeletal α-actinin cDNA (Fig. 1, construct 5) (43), and a 0.81-kb *Hin*dIII fragment containing a tandem trimer of the simian virus 40 early polyadenylation signal (Fig. 1, SVT, construct 6) (22, 40).

Transfection. For suspension cells, ~50 μg of supercoiled plasmid DNA purified by CsCl centrifugation was transfected into ~1 × 10^7 to 2 × 10^7 cells by electroporation (Bio-Rad) as described previously (44). For monolayer cultures, cells were passaged 24 h prior to transfection to give 50% confluent cultures and were transfected with 20 μg of DNA over 16 h by the calcium phosphate precipitation technique (31). The medium was assayed 72 h posttransfection for hGH activity by using an immunoassay kit (Nichols Institute). For cells transfected with LacZ fusion constructs, histochemical staining for β-galactosidase activity was done as previously described (32), and LacZ-positive cells were quantitated by visual inspection under a 10× light microscope. pLacF (45) served as a negative control. Each cell type was also transfected separately with an hGH expression plasmid driven by the TK promoter to normalize for differences in transfection efficiencies (35). COS M6 cells were transfected with 2 μg of supercoiled DNA by DEAE-dextran as previously described (31). After 48 h, cells were fixed in phosphate-buffered saline (PBS)–3% paraformaldehyde, permeabilized with PBS–0.1% Triton X-100, and stained with fluorescein-conjugated goat anti-mouse IgG (Cappell).

RNA preparation and analysis. Isolation of total, cytoplasmic, or polyadenylated RNA from tissue culture cells was performed as previously described (31). Indicated amounts (see figure legends) of RNA were fractionated on 1% agarose gels and transferred to nylon membranes (Biotrans) as described by the manufacturer. Probes were labeled with [α-^{32}P]dCTP by random priming (Boehringer-Mannheim) to a specific activity of about 10^9 cpm/μg. Filters were hybridized in a solution containing 50% formamide at 42°C and washed under high-stringency conditions (31). RNase protection analysis was performed as previously described (31). Briefly, a 0.34-kb fragment encompassing the EpoR 5′ flanking region from −1527 to −1185 and containing the repetitive element was subcloned into Bluescript KS and linearized with either *Xba*I (3′ end) or Asp 718 (5′ end), two enzymes that cleave uniquely on either side of the insert within the polylinker. Synthetic RNA transcripts were prepared by using either T3 RNA polymerase (for the *Xba*I-cleaved plasmid, to generate a probe corresponding to the sense strand of the downstream EpoR gene) or T7 RNA polymerase (for the Asp 718-cleaved plasmid, to generate a probe corresponding to the antisense strand; Promega Biotech) in the presence of [α-^{32}P]dUTP. The probes were gel purified, and ~10^4 cpm was hybridized with 10 μg of total cellular RNA at 50°C for 16 h. The hybridization mixture was digested with RNase A and RNase T1, and the reaction products were resolved on 5% polyacrylamide gels.

Southern analysis. Approximately 10 μg of genomic DNA aliquots from the indicated species (see Fig. 6) was digested with *Eco*RI, fractionated on 0.75% agarose gels, transferred onto nylon membranes (Biotrans), and hybridized with DNA probes generated by random priming and labeling with [α-^{32}P]dCTP. Hybridization was performed in 50% formamide at 37°C for 16 h, and the filter was washed in 0.1× SSC

(1× SSC is 0.1 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 55°C for 1 h.

RESULTS

Functional analysis of the EpoR gene 5′ flanking region in MEL and CB5 cells. In previous transient transfection studies we identified a 0.45-kb fragment that functions as an erythroid-specific promoter (44). More recently, we constructed a panel of deletions containing from ~3 to 0.18 kb of the EpoR 5′ flanking region and inserted them upstream of the promoterless hGH gene in an effort to delineate other potential regulatory sequences active in erythroid cells (42a). This analysis allowed us to define a region spanning ~250 bp upstream of the initiation codon ATG as a minimum erythroid-specific promoter that is responsible for basal levels of transcription in both MEL and CB5 cells. This region contains a single Sp1 consensus sequence and an inverted GATA-1 sequence, both of which are necessary for promoter activity (46). We expected that we might detect additional *cis*-acting positive regulatory elements by adding larger fragments of the 5′ flanking sequences to the proximal promoter. We were therefore surprised by our finding that sequences between the *Bam*HI site at −1703 and the *Hin*dIII site at −1063 markedly inhibited promoter activity in both MEL and CB5 cells (Fig. 1, compare constructs 1 and 2; relative values of <10% most likely reflect background activity). Placement of this 640-bp region, called rodent repetitive element (RRE), upstream of the herpesvirus TK gene promoter also completely or partially inhibited its activity in MEL, CB5, and NIH 3T3 cells (Fig. 1, constructs 7 and 8). Deletion of this sequence (Fig. 1, construct 2), inversion of its orientation relative to the EpoR gene (Fig. 1, construct 3), and an increase in its distance from the site of transcription initiation by the insertion of an ~3-kb heterologous DNA sequence (43) at the *Hin*dIII site (Fig. 1, construct 5) relieved the inhibition. By contrast, addition of a 1.8-kb spacer DNA (6) did not relieve this inhibition (Fig. 1, construct 4). However, insertion of a 0.81-kb strong transcriptional attenuator, a trimerized simian virus 40 early polyadenylation signal (22, 40), did overcome this inhibition (Fig. 1, construct 6). Taken together, these results strongly suggest that the inhibition of promoter activity is generated in *cis* from the upstream region of the mouse EpoR gene and that inhibition is both distance and orientation dependent. A plausible interpretation of these results is that a transcriptionally active element within the 640-bp region physically interferes with downstream transcriptional activation of the EpoR promoter.

Transcriptional activity of a novel repetitive element. The 640-bp *Bam*HI-*Hin*dIII fragment was used to probe Northern (RNA) blots of RNA from both erythroid and nonerythroid murine cell lines. A small, heterogenous transcript ~500 to 900 bp in size was detected in all cell lines examined (Fig. 2). In some cell lines the steady-state level of the transcript was quite high, though there was no obvious concordance between the abundance of the transcript and the expression of the EpoR (data not shown). Selection for polyadenylated RNA increased the relative abundance of the transcript, indicating that a large fraction of it is polyadenylated (Fig. 2). This notion is supported by examination of the nucleotide sequence in the 3′ region of this sequence, which shows a long region of adenines interspersed with guanines, preceded by three overlapping potential polyadenylation sequences (Fig. 3).

Inspection of the sequence from this region shows two potential ORFs (ORF-1 and ORF-2 [Fig. 3]). Comparison of these sequences with data in the GenBank showed no significant homology to mouse retroposons or to other known DNA sequences. There were also no elements suggestive of polymerase III-initiated transcription (30). However, there is a consensus TATA element beginning 37 bp

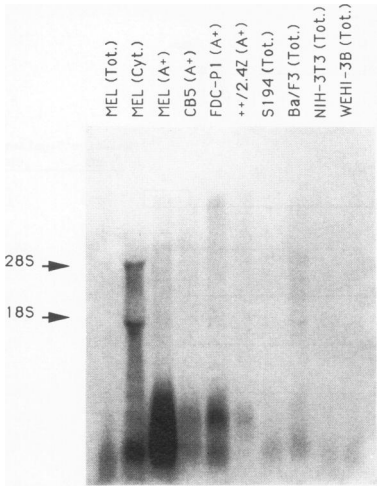

FIG. 2. Northern analysis of RNA from a variety of murine cell lines hybridized with a 640-bp *Bam*HI-*Hin*dIII probe from the 5′ flanking region (bases −1703 to −1063) of the mouse EpoR gene. The derivation of the cell lines is described in Materials and Methods. The positions of the 18S and 28S RNAs are indicated. Approximately 30 μg of total cellular (Tot.) or cytoplasmic (Cyt.) RNAs or 1 μg of poly(A)-selected (A+) RNA was loaded in each lane.

```
                -1703
                BamHI
                ggatccaaacattcaaatatatgagcctataaaagccattctcatttaaaccactgtatcataa
                                                                            *
      -1639
      atgtatgtatatatgaatgtatgtatgtattcatctatctgcctacctatctgtccatgggtgt
ORF-1 M  Y  V  Y  M  N  V  C  M  Y  S  S  I  C  L  P  I  C  P  W  V  C

      -1575
      gtcaatgcatgtatgcatatgtgaatcagtatacatacttgtatctgtgtatatgtgtatatcc
ORF-1   Q  C  M  Y  A  Y  V  N  Q  Y  T  Y  L  Y  L  C  I  C  V  Y  P
ORF-2                     *  I  S  I  H  T  C  I  C  V  Y  V  Y  I  H

      -1511
      atatatgtgtctgttcatgtatgtatgtatccatgcatctacccatgtatgtatgtatgtacgt
ORF-1  Y  M  C  L  F  M  Y  V  C  I  H  A  S  T  H  V  C  M  Y  V  R
ORF-2   I  C  V  C  S  C  M  Y  V  S  M  H  L  P  M  Y  V  C  M  Y  V

      -1447
      actatgtacttatgtatgtatatgtatgcatccacctatctctgtatttgggggtctggggat
ORF-1 T  M  Y  L  C  M  Y  M  Y  A  S  T  Y  L  C  I  W  G  V  W  G  *
ORF-2  L  C  T  Y  V  C  I  C  M  H  P  P  I  S  V  F  G  G  S  G  D

      -1383
      aaacccagggttttccacctgctaggcaagtgctgtaccactacccgccattcaactttgtttt
ORF-2 K  P  R  V  F  H  L  L  G  K  C  C  T  T  T  R  H  S  T  L  F  F

      -1319
      ttttatttttaagtatagaaagcacaggtctggggctggtgagatggctcaggggtaagagcac
ORF-2   L  F  L  S  I  E  S  T  G  L  G  L  V  R  W  L  R  G  K  S  T

      -1255
      ccgactgctcttccaaaggtccgaagttcaaatcccagcaaccacatggtagcttacaaccacctgt
ORF-2  R  L  L  F  Q  R  S  E  V  Q  I  P  A  T  T  W  *

      -1188
      aacagagatctgatgccctcttctggtgcgtctgaagacagctacagtgtacttacatatataaataaataagtctt

      -1111                                                  HindIII
      taaaaaaaaaaaagaaaagaaagaaagcacaggtctgtgatctgagcactcagaagctt
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FIG. 3. Nucleotide sequence of bases −1703 to −1063 of the 5′ flanking region of the mouse EpoR gene containing the RRE. Potential ORFs, numbers indicating the distances from the EpoR translation initiation site (+1), the first methionine residue of each ORF (in boldface), and potential polyadenylation sequences (underlined) are depicted.

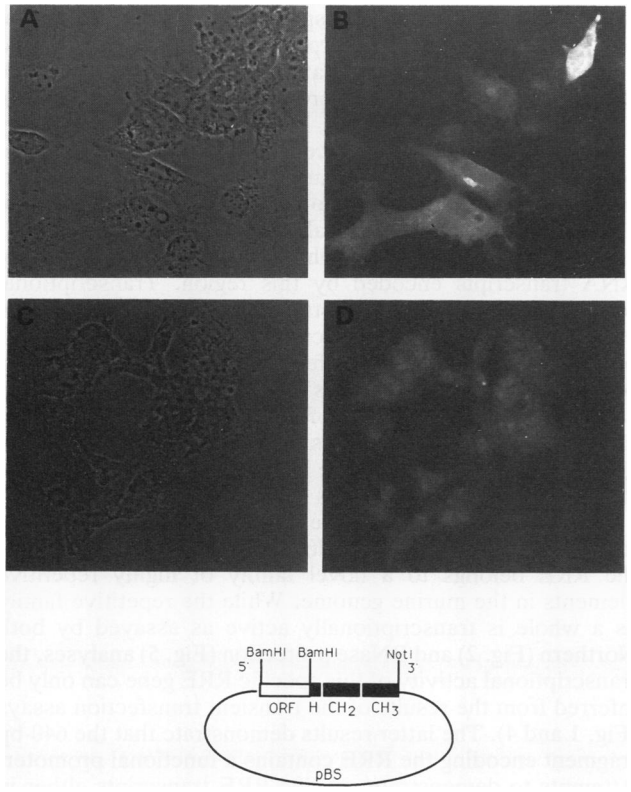

FIG. 4. Transient expression of ORF-IgG. Phase (A and C) and fluorescence (B and D) photomicrographs of COS M6 cells transfected either with a construct containing the mouse IgG2b constant domain without a eukaryotic promoter (C and D) or with the construct diagrammed at the bottom containing the latter downstream of the EpoR 5′ flanking region from bases −1703 to −1063, potentially encoding a fusion protein of ORF-2 and mouse heavy-chain IgG2b. pBS, Bluescript KS.

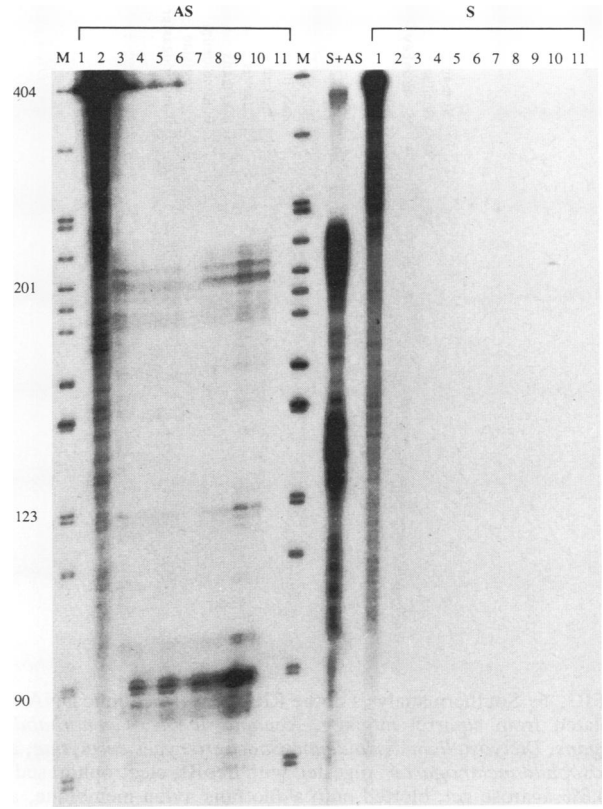

FIG. 5. RNase protection analysis of cell lines expressing RRE. The mouse EpoR 5′ flanking region from bases −1527 to −1185 containing the RRE was subcloned into the vector Bluescript KS, and RNA probes corresponding to either the sense (S) or antisense (AS) strands were prepared as described in Materials and Methods. Lanes 1 and 2, probe and tRNA in either the absence (lanes 1) or presence (lanes 2) of RNase but no other cellular RNA. The large amount of intact probe at the top of lane 1 (AS) partially overshadows lane 2, which is essentially blank. The remaining lanes contain the following total cellular RNAs in addition to carrier tRNA and RNase: CB5 (lanes 3), WEHI-3B (lanes 4), HCD-57 (lanes 5), NIH 3T3 (lanes 6), FDC-P1 (lanes 7), uninduced MEL (lanes 8), day 2-induced MEL (lanes 9), day 4-induced MEL (lanes 10), and U937 (lanes 11).

upstream of the initiation codon of ORF-1, suggesting the presence of a neighboring transcriptional start site and possibly a neighboring promoter. To further test for the transcriptional activity of this region and its potential ability to encode a polypeptide, we placed an epitope tag from the mouse IgG2b heavy-chain constant region downstream of the second ORF, after mutagenesis of its stop codon into an aspartic acid residue (see Materials and Methods). The fusion construct was inserted into a Bluescript KS vector that had no eukaryotic promoter, transfected into COS M6 cells, and examined for expression by immunofluorescence with fluorescein-conjugated goat anti-mouse IgG. Many cells transfected with the ORF-IgG construct showed high degrees of diffuse cytoplasmic staining (Fig. 4B), while those transfected with a plasmid that lacks the upstream ORF were negative (Fig. 4D). Therefore, we conclude that this 640-bp region contains a eukaryotic promoter and encodes transcripts that are capable of being translated into protein.

The transcriptional orientation of this element relative to the EpoR gene was examined by RNase protection analysis. A major protected fragment of ~90 nucleotides was observed only with an antisense probe (Fig. 5, AS) relative to the transcription of the EpoR gene (Fig. 5). It is not clear why the size of the protected fragment (~300 bp) is smaller than expected. No protected fragments are seen with the probe that would correspond to the sense strand (Fig. 5, S) of the downstream EpoR gene. Therefore, the orientation of transcripts generated from this region is in the same direction (left to right in Fig. 1 and 3) as the downstream EpoR transcript. Larger protected fragments were seen in all lanes and are thought to be artifactual. Since the RRE gene is repetitive, we cannot say whether the transcripts from this region are those from the family member upstream of the EpoR gene or from other locations in the genome.

Because of a number of differences between our published sequence of the mouse EpoR 5′ flanking region (44) and that of others (4), we have reanalyzed our data and have discovered three omissions in our published sequence in the region −448 to −465. The corrected sequence in this region is 5′(−468)GTCTTTAGGATTCTTATTTTG(−448)3′, with the corrections underlined. The remainder of the sequence is as stated previously (44).

Evolutionary conservation of the repetitive element. Southern analysis of genomic DNA from various species probed with the 640-bp *Bam*HI-*Hin*dIII fragment showed a pattern indicative of highly repetitive elements (Fig. 6). Hybridiza-

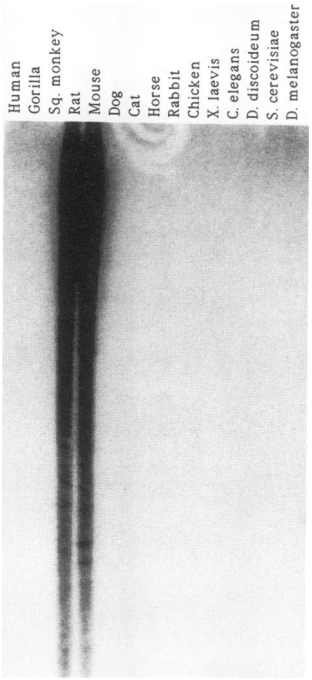

FIG. 6. Southern analysis of the RRE genes. Genomic DNA was isolated from squirrel monkey, *Xenopus laevis*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster*, digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, blotted onto a Biotrans nylon membrane, and hybridized to an α-^{32}P-labeled 640-bp *Bam*HI-*Hin*dIII probe from the 5′ flanking region of the mouse EpoR gene.

tion was observed only to rodent (including hamster) DNA (data not shown). By titration with known amounts of plasmid DNA, we estimate that the abundance of this element is $\sim 10^5$ per haploid genome (data not shown).

DISCUSSION

The expression of the EpoR on hematopoietic progenitor cells is crucial for the initiation of the erythroid differentiation cascade (38) and/or the prevention of apoptosis of committed erythroid progenitor cells by promoting their differentiation and maturation into terminally differentiated erythrocytes (15). Our results suggest that expression of the EpoR appears to be regulated by both lineage-specific and temporally specific mechanisms. We have focused on the transcriptional regulation of the EpoR and have undertaken a comprehensive analysis of the *cis*-acting regulatory elements of the murine EpoR gene. In this study, we present an analysis of a novel repetitive element upstream of the murine EpoR gene that encodes a small RNA, which apparently inhibits the transcription of the EpoR gene.

Functional analysis of the 5′ flanking region of the murine EpoR gene revealed a strong inhibitory region in a *Bam*HI-*Hin*dIII fragment spanning nucleotides −1703 to −1063. Placement of this fragment upstream of the heterologous TK promoter caused an approximately five- to sixfold reduction of promoter activity in both erythroid and nonerythroid cells. Therefore, the inhibitory activity is neither cell type specific nor specific to the EpoR promoter. Promoter activity could be restored by (i) deletion of the region from −1703 to −1063 (Fig. 1, construct 2), (ii) inversion of its orientation relative to the downstream EpoR promoter (Fig. 1, construct 3), (iii) placement of a 3-kb spacer (Fig. 1, construct 5) at position −1603, or (iv) interposition of a smaller poly(A) signal terminator (22, 40) that substantially reduces read-through from the upstream promoter (Fig. 1, construct 6). These results, taken together, identify a negative regulatory element that is both distance and orientation dependent relative to the downstream murine EpoR promoter. Furthermore, the restoration of promoter activity by the insertion of a heterologous transcriptional silencer at position −1063 strongly suggests that the inhibition may be mediated by RNA transcripts encoded by this region. Transcriptional read-through into the downstream EpoR gene could then cause interference with transcription initiation at the EpoR promoter through steric interference with either the binding or the activity of factors in this region (29). This possibility is strengthened by the results of the RNase protection assay indicating that the transcripts generated by this family of genes, including the RRE, are in the same orientation as the downstream EpoR transcripts (Fig. 5).

It is difficult to ascertain the transcriptional activity of an individual member of a repetitive gene family. In this case, the RRE belongs to a novel family of highly repetitive elements in the murine genome. While the repetitive family as a whole is transcriptionally active as assayed by both Northern (Fig. 2) and RNase protection (Fig. 5) analyses, the transcriptional activity of this specific RRE gene can only be inferred from the results of the transient transfection assays (Fig. 1 and 4). The latter results demonstrate that the 640-bp fragment encoding the RRE contains a functional promoter. Attempts to demonstrate specific RRE transcripts either in MEL cells or in COS cell transfectants by polymerase chain reaction analysis have been unsuccessful (data not shown).

Given the presence of open reading frames in the RRE sequence and the generation of an immunoglobulin fusion protein by transfection of COS cells, our results imply that the RRE is also translated. The cytoplasmic localization of the fusion protein (Fig. 4) does not give any further insight about the possible function of the RRE-encoded protein. It is clear, however, that the putative protein is not involved in regulating the activity of the neighboring EpoR gene in *trans*. If this were the case, the inhibitory activity would not have been orientation dependent.

At first glance, repression of a gene in a cell type in which that gene is inherently active seems paradoxical. We do not yet know about the significance of this repression in regulating EpoR gene activity during erythropoiesis. If such repression indeed plays some role in the regulation of EpoR gene activity, we envision two major scenarios by which this may be accomplished. First, the RRE locus in EpoR-expressing cells may be silent, because of either the local chromatin structure or the presence of a *trans*-acting, sequence-specific repressor that prevents the read-through of RRE transcripts. Because the RRE family is transcribed in both erythroid and nonerythroid cells, a cell type-specific repression cannot be invoked as a plausible regulatory mechanism by the RRE. Furthermore, our results are consistent only with *cis*-mediated repression, not with the generation of a *trans*-acting repressor by the RRE. Second, the RRE locus may well be active in erythroid cells, but the transcriptional repression mediated by it may not be complete, allowing a basal level of transcription from the minimum promoter. Because of the critical dependence of the minimum EpoR promoter on the presence of GATA-1 (4, 46), this mechanism could operate only in an erythroid cell background. In nonerythroid cells, the lack of EpoR promoter activity may be due to both active repression by the

RRE or other transcriptional inhibitors and the absence of GATA-1. Thus, a subtle balance may be established in erythroid cells by the competing activities of the RRE and the GATA-1. Further activation of the EpoR gene may then depend on stage-specific enhancers. In support of this model, we have recently identified such an enhancer upstream of the RRE that is able to override partially this inhibition (42a). Similar compensation of activity by enhancers over silencers has been demonstrated previously in the chick lysozyme gene (2) and possibly in the major histocompatibility complex class I gene (41).

There has been increasing awareness in recent years that, in addition to positive regulators of transcription, the control of many eukaryotic promoters involves important contributions from negative regulatory elements (19). A number of different mechanisms appear to mediate transcriptional repression and may be classified as follows: (i) transcriptional repression of specific genes by sequence-specific DNA binding proteins (reference 3 and references therein); (ii) transcriptional repression by mechanisms that produce potentially more pleiotropic effects, such as DNA methylation and higher-order chromatin organization (14); and (iii) transcriptional repression in *cis* by neighboring transcripts, generated from either the sense or the antisense strands (18, 20, 25, 29, 42). In the latter category, it is interesting to note that the transcription of another erythroid-specific gene, the human ε-globin gene, is also downregulated by the transcriptional activity of an upstream Alu element (42). Thus, transcriptional repression of tissue-specific genes by neighboring repetitive elements appears to be a more common mechanism of gene regulation than has been previously appreciated.

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