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A Directly Repeated Sequence in the β-Globin Promoter Regulates Transcription in Murine Erythroleukemia Cells

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We have identified a previously undetected *cis*-acting element in the mouse β-major globin promoter region that is necessary for maximal transcription levels of the gene in the inducible preerythroid murine erythroleukemia (MEL) cell line. This element, termed the β-globin direct-repeat element (βDRE), consists of a directly repeated 10-base-pair sequence, 5′-AGGGCAG(G)AGC-3′, that lies just upstream from the TATA box of the promoter. The βDRE motif is highly conserved in all adult mammalian β-globin promoter sequences known. Mutation of either single repeat alone caused less than a twofold decrease in transcript levels. However, simultaneous mutation of both repeated regions resulted in a ninefold decrease in accumulated transcripts when the gene was transiently transfected into MEL cells. Attachment of the βDRE to a heterologous promoter had little effect on levels of accumulated transcripts initiated from the promoter in undifferentiated MEL cells but resulted in a threefold increase in transcript levels in induced (differentiated) MEL cells. Similarly, a comparison of the relative effects of mutations in the βDRE in uninduced and induced MEL cells indicated that the element was more active in induced cells. The increase in βDRE activity upon MEL cell differentiation and the more pronounced effects of mutations in both repeats of the βDRE have implications for the mechanism of action of the element in regulating β-globin transcription and for mutational studies of other repetitive or redundant transcription elements.

The developmental-stage and tissue-specific regulation of the mammalian β-globin locus has provided a challenging system for understanding the molecular mechanisms that mediate gene activation during terminal differentiation. An important step in understanding this regulation is identification of the *cis*-acting regulatory sequences that are required for expression of globin genes in erythroid cells. The murine erythroleukemia (MEL) cell model system for adult erythrocyte development (13) has been useful in this characterization for the adult β-globin genes. MEL cells are arrested at the proerythroblast stage of erythroid development and can be induced to terminally differentiate in vitro in a process that closely mimicks the events of normal erythropoiesis (for a review, see reference 31). MEL cell differentiation is characterized by a large increase (10- to 50-fold) in the steady-state level of β-globin mRNA. This increase is due in part to an increase in the rate of transcriptional initiation from the β-globin promoter (3, 19, 47) and in part to an increase in globin mRNA stability (44). Because transfected β-globin genes are regulated similarly to the endogenous genes when transferred into MEL cells (3, 46), it has been possible to identify *cis*-acting elements that play a role in β-globin transcriptional regulation by mutagenesis experiments (1, 4, 6, 47).

Introduction of cloned hybrid genes into transgenic mice (24, 43) and MEL cells (1, 5, 6, 47) has implicated sequences both 5′ and 3′ to the β-globin transcriptional start site in transcriptional induction of the gene during terminal differentiation and erythroid-specific expression of the gene. To evaluate the contribution of 5′ promoter sequences to this regulation in the absence of downstream elements, we previously linked the promoter to the heterologous mouse metallothionein I (MT-I) gene (6). A series of 5′ deletion mutants of the mouse β-globin promoter in this context indicated that 1,200, 300, or 106 base pairs (bp) of β-globin 5′-flanking sequences each direct transcription of equivalent amounts of correctly initiated mRNA in both transient and long-term transfection assays. In addition, all three promoters are transcriptionally induced to the same extent (10- to 20-fold) after MEL cell differentiation. On the basis of these deletion experiments, we have focused on characterizing the functional DNA sequences within the minimal −106 β-globin promoter. Three important regulatory elements within this −106 β-globin promoter were identified previously: a CACCC element (between positions −95 and −87), a CCAAT box element (positions −79 to −72), and a TATA box (positions −30 to −26). Single-base substitutions and more drastic mutations in these three elements indicate that they specify transcription levels of the β-globin gene transferred into both erythroid (6) and nonerythroid (4, 9, 15, 16, 35) cells.

In addition to these three sequence elements, a fourth region of the promoter has some interesting properties that suggested a potential role in β-globin transcriptional regulation. This region consists of an imperfect direct repeat of a 10-bp sequence, AGGGCAGGAGCCAGGGCAGAGC, located between the CCAAT and TATA elements in the mouse β-major globin promoter (between positions −53 and −32). This sequence is highly conserved among adult mammalian β-globin promoters and is also found in avian α-globin regulatory regions (Fig. 1). Four homologous or identical repeats of this sequence motif are found in the human β-globin promoter (between positions −186 and −175, −53 and −43, −41 and −32, and −22 and −13; Fig. 1). The regions of the mouse and rabbit β-globin promoters containing this repeated sequence were disrupted in previous mutagenesis studies, resulting in slight (less than twofold) decreases in transcription levels in HeLa (4, 35), 3T6 (9), and MEL (6) cells. In the experiments described here, we analyze more drastic disruptions of this sequence in an

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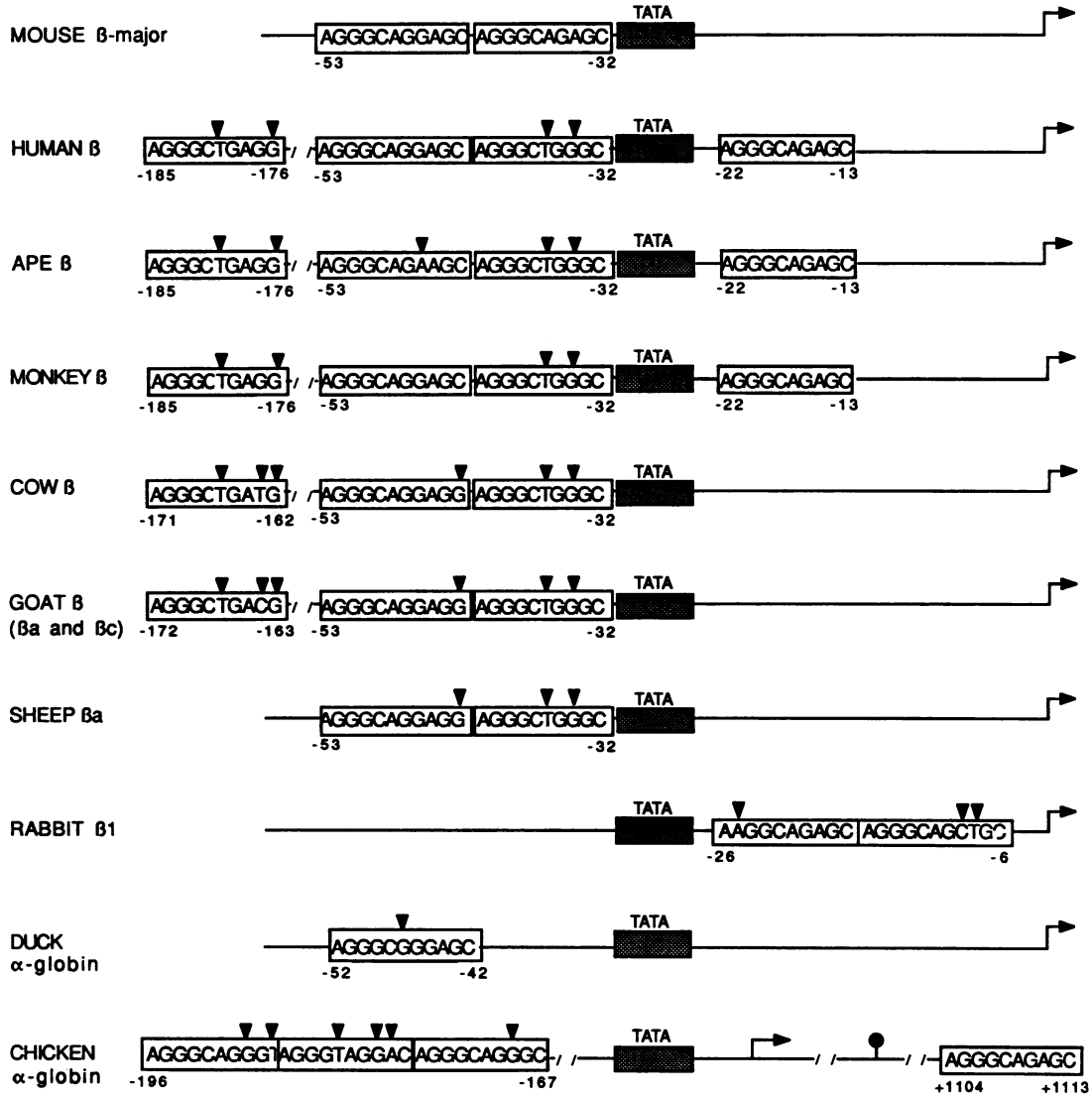


FIG. 1. Evolutionary conservation of the AGGGCAG(G)AGC motif in globin genes. The AGGGCAG(G)AGC sequences or homologous sequences found in mammalian β-globin promoters and in avian α-globin genes are shown in boxes. Numbers below the boxed regions refer to the locations of the sequence motifs relative to the transcriptional start site (designated +1). Arrowheads indicate nucleotide changes relative to the sequence found in the mouse β-major globin promoter. The TATA box designates the location of the TATA element or a putative TATA element. Symbols: //, regions of nonlinearity in the scale of the figure; ♀, end of the chicken a-globin mRNA. The sequences were compiled from a scan of the GenBank data base. References for the sequences are as follows; mouse β-major (25), human β (29, 36), ape β (37), monkey β (32), cow β (38), goat βa and βc (18), sheep β (26), rabbit β1 (17), duck α-globin (11), and chicken α-globin (10).

MEL cell transient transfection assay. Using additional 5′ deletion promoters and mutations that alter both copies of the direct-repeat motif, we demonstrate that this sequence is required for maximal transcription levels from the β-globin promoter in erythroid cells and plays a role in transcriptional activation of the β-globin gene during MEL cell differentiation.

MATERIALS AND METHODS

Construction of plasmids and mutants. Plasmids were propagated in *Escherichia coli* HB101 except where noted below, and plasmid DNA was prepared by a modification of the alkaline lysis method (42). All enzymes were purchased from New England BioLabs, Inc., and used as recommended. Most of the mutations used in this analysis were generated by the oligonucleotide-directed mutagenesis method of Kunkel et al. (28) in the *ung dut E. coli* strain CJ236. The oligonucleotides used to introduce DNA base changes by this method are summarized in Fig. 2. All in vitro-generated mutations were confirmed by dideoxy sequencing.

(i) βMT 5′ deletion series. The −106 wild-type plasmid (pβMTF [6]) contains the mouse β-globin promoter from a *Cla*I site at position −106 ($ClaI_{-106}$) to a *Bgl*II site at +26 ($BglII_{+26}$), linked to the mouse MT-I gene, $BglII_{+65}$ to $EcoRI_{+2200}$, in the pSP73 vector (27). The plasmid also contains an enhancer element contained within the 300-bp *Sac*I-to-*Pst*I fragment from the Friend spleen focus-forming virus long terminal repeat (LTR [2]); this enhancer lies at the 3′ end of the MT-I gene at the *Eco*RI site. Several mutants were analyzed in the presence and absence of this enhancer fragment. Although the mRNA signals were substantially

MUTANT	OLIGO SEQUENCE
-60	5'$_{-33}$TCTGCTCACAATCGATAGAGAGG$_{-51}$3'
rep	5'$_{-68}$TCACACAGGATAGAGCTTTACTTCTACCTTTACTCTAATATAAGGTGAGGTA$_{-17}$3'
ΔDR1	5'$_{-65}$CACAGGATAGAGCTTCTCGAGTACAGGGCAGAGCATAT$_{-28}$3'
ΔDR2	5'$_{-56}$GAGAGGGCAGGAGCCCTCGAGTCTAATATAAGGTGAGG$_{-19}$3'
MT8F	5'$_{-43}$GACTCGTCCAAGATCTATAAAGAGG$_{-19}$3'
βDREMTF	5'$_{-73}$CGCCTGCTGGGTGCAGATCTAGAGGGCAGGAGCCAGGGCAGAGCCTATAAAGAGGGCAG$_{-15}$3'

FIG. 2. Oligonucleotides used to introduce base changes. Base pair changes introduced are underlined.

higher and easier to quantitate with the enhancer present, identical relative transcription level (RTL) results were obtained under both conditions. In addition, comparison of the relative levels of inducibility of several promoters in the presence and absence of this enhancer indicated that the Friend virus LTR has only a slight effect on induction (1.2- to 2-fold at most; data not shown).

The −60 and −30 5′ deletions of the mouse β-globin promoter were constructed by introducing restriction enzyme cleavage sites (*Cla*I and *Bam*HI, respectively) at these positions in the β-globin promoter of plasmid pMHβ8 (35) by oligonucleotide-directed mutagenesis (28) and ligation of a restriction site linker, respectively. The *Cla*I$_{-60}$-to-*Bgl*II$_{+26}$ or *Bam*HI$_{-30}$-to-*Bgl*II$_{+26}$ β-globin promoters were then excised from the pMHβ8 background and cloned in place of the wild-type *Cla*I$_{-106}$-to-*Bgl*II$_{+26}$ promoter fragment in plasmid pβMTF to generate the −60 and −30 βMTF plasmids.

(ii) H4MT reference construct. The reference plasmid pH4MT was constructed in several steps as follows. The *Nar*I$_{+70}$ restriction site of the mouse histone H4 gene (gift of Rudi Grosschedl; 39) was converted to a *Bam*HI site by cleavage with *Nar*I, filling in of the site with Klenow polymerase (New England BioLabs) in the presence of deoxynucleoside triphosphates (Pharmacia, Inc.) to a blunt end, and ligation of a *Bam*HI restriction site linker (8-mer: New England BioLabs). The *Eco*RI$_{-229}$-to-*Bam*HI$_{+70}$ H4 fragment was then inserted in place of the β-globin *Cla*I-to-*Bgl*II promoter fragment in plasmid pβMTF. The resulting plasmid, pH4MTF, still contained the 300-bp enhancer fragment from the Friend virus LTR, which was removed by cleavage of pH4MTF with *Eco*RI and *Xho*I, filling in of the sites to blunt ends as described above, and recircularization of the plasmid. This yielded the reference plasmid pH4MT, which contains the mouse histone H4 promoter, *Eco*RI$_{-229}$ to *Bam*HI$_{+70}$, linked to the mouse MT-I gene, *Bgl*II$_{+65}$ to *Eco*RI$_{+2200}$, in the pSP73 vector.

(iii) β-Globin direct-repeat element (βDRE) mutations. The β-globin promoter double-point mutations were constructed by using a method (34) that involves annealing double-stranded plasmid DNAs containing the two individual single-base substitutions generated by Myers et al. (35). Heteroduplex DNAs containing the desired double-point mutations were then separated from wild-type and single-point mutant DNAs by denaturing gradient gel electrophoresis, eluted by the crush-soak (33) method, and cloned by ligation into the pMHβ8 plasmid vector and transformation into a helicase II-deficient *E. coli* strain (NK7086; gift of Nancy Kleckner; 34). The ΔDR1, ΔDR2, and REP mutations were constructed by oligonucleotide-directed mutagenesis of pMHβ8, using oligonucleotides that introduced the desired base changes (Fig. 2). ΔDR1 and ΔDR2 mutant plasmids were identified by restriction enzyme screening for the introduced *Xho*I site at position −50 (ΔDR1) or position −41 (ΔDR2). *Cla*I$_{-106}$-to-*Bgl*II$_{+26}$ β-globin promoters containing these desired mutations were then excised from the pMHβ8 background and used to replace the wild-type promoter in the pβMTF construct.

(iv) MT-I and βDRE-MT constructs. To assess whether the βDRE contributes to β-globin promoter inducibility during MEL cell differentiation, the element was cloned adjacent to a heterologous promoter. The mouse MT-I promoter was chosen for this analysis since its activity changes only slightly upon MEL cell differentiation. A small degree of inducibility (1.2-fold on average; see Table 4) was found for the pMT8F plasmid (described below) in the MEL cell transient transfection assay; this level of inducibility was not significantly affected by removal of the Friend virus LTR from the construct (data not shown).

To generate a marked MT-I construct that could be distinguished from the endogenous MT-I mRNA in a 5′ nuclease protection assay, the *Bgl*II$_{+65}$ site in the mouse MT-I gene of pmMT-1 (gift of Richard Palmiter; 14) was converted to a *Bam*HI site by cleavage with *Bgl*II and ligation of a *Bam*HI restriction site linker (8-mer; New England BioLabs) as described above for the H4 gene. This modification introduces a 12-bp insertion into the MT-I gene. The MT-I promoter was then modified by oligonucleotide-directed mutagenesis to create either a *Bgl*II restriction site at position −30 (pGCMT8) or a *Bgl*II restriction site followed by an insertion of the −53 to −32 βDRE from the mouse β-globin promoter 5′ of the MT-I TATA element at positions −51 to −30 (pGCβDREMT). The *Bgl*II$_{-30}$-to-*Xba*I$_{+215}$ MT-I fragment of pGCMT8 was then cloned in place of the *Cla*I$_{-106}$-to-*Xba*I$_{+215}$ βMT fragment of pβMTF to generate pMT8F. pβDREMTF was constructed similarly by a swap of the *Bgl*II-to-*Xba*I fragment of pGCβDREMT with the corresponding fragment of pβMTF.

Cell culture and DNA transfection. MEL *aprt*$^-$ cells (7) were grown in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal calf serum (FCS; Hyclone). Cells were transfected by a modification of the DEAE-dextran protocol of Cowie and Myers (6) as described below. MEL cells were split 18 to 24 h before transfection and replated in DME containing 10% FCS and 20 μM $CdSO_4$ (DME-FCS-Cd) onto 100-mm-diameter Primaria dishes (Becton Dickenson Labware), to which they adhere. Cadmium was added to the cell culture medium to fully induce the endogenous and transfected metallothionein genes so that all transfected plates would have comparable MT-I mRNA levels. For transfection, cells were incubated with 10

μg of supercoiled plasmid DNA (4:1 molar ratio of test to reference plasmid) in 1.1 ml of 0.5-mg/ml DEAE-dextran (Sigma Chemical Co.) in Tris-buffered saline (8) for 40 min at room temperature. After the DNA incubation, the cells were washed once with Tris-buffered saline and then treated with 0.1 mM chloroquine (Sigma) in DME-FCS-Cd for 3 to 4 h at 37°C (5 ml per plate). After the chloroquine was removed, the cells were allowed to recover for 1 h at 37°C in DME-FCS-Cd (10 ml per plate). Dimethyl sulfoxide (DMSO) treatment has a slight effect of increasing DNA uptake. Therefore, to correct for differences in transfection efficiency between DMSO-treated and untreated cells, DMSO was added to the culture medium of all cells to a final concentration of 2% for a 2- to 4-h incubation at 37°C. This brief DMSO treatment is insufficient to induce MEL differentiation. MEL cells were then removed from the Primaria dishes by gentle pipetting in culture medium. Cells were washed once by centrifugation for 5 min in an IEC clinical centrifuge, resuspension in 5 ml of culture medium, and recentrifugation as described above. The final washed MEL cell pellet (from a single 100-mm-diameter dish originally) was resuspended in DME-FCS-Cd, split into two samples, and replated onto 100-mm-diameter Primaria dishes. Then 2% DMSO was added to one of the samples to induce differentiation of the cells. Cytoplasmic RNA was prepared from the cells 44 to 48 h after the time of the first DMSO addition.

RNA analysis. Cytoplasmic RNA was isolated by the method of Favaloro et al. (12). Specific RNAs were detected and quantitated by S1 analysis by using 5'-end-labeled, single-stranded DNA probes (22). Probes were made by primer extension as described previously (6) and recovered by crush-soak elution (33). For S1 analysis, 20 μg of RNA was hybridized at 37°C overnight with 0.01 pmol of each probe (both the test and reference probes, except for MT-I and H4MT, which were analyzed in separate hybridization mixtures because of the closeness in size of the two protected signals). S1 digestion was carried out at 15 to 16°C for detection of signals less than 100 nucleotides in length (βMT plus H4MT signals) and at 20°C for detection of signals larger than 100 nucleotides (MT-I or H4MT alone) as previously described (12). The S1 samples were then fractionated on 10% polyacrylamide–7 M urea denaturing gels (30). Specific bands were quantitated by densitometric scanning of several autoradiographs exposed for various lengths of time on a Bio-Rad model 620 video densitometer.

RESULTS

Analysis of 5' deletion promoters. In a previous study (6), we demonstrated that mouse β-globin promoter sequences from −106 to +26 bp relative to the RNA start site were sufficient to direct expression and maximal transcriptional induction of a heterologous noninducible gene during MEL cell differentiation. Although the duplicated sequence motif, AGGGCAG(G)AGC, located between positions −53 and −32 of the mouse β-globin promoter, had been altered in this and other previous studies (4, 35), only one repeat of the sequence was disrupted in any given mutation. These mutations resulted in little or no effect on transcription levels (at most, twofold decreases). If the conserved direct-repeat sequence corresponds to a duplicated transcription element, mutation of a single copy of the element may not have been sufficient to disrupt its activity significantly; an element of this type might have gone undetected in earlier experiments. To test this hypothesis, we initially constructed two deletions at the 5' end of the −106 to +26 mouse β-globin promoter (Fig. 3A). The −106 promoter contains the three previously defined transcriptional regulatory elements, the CACCC, CCAAT, and TATA elements, as well as the direct-repeat sequence. The first deletion mutant, the −60 promoter, lacks the CACCC and CCAAT sequences and contains only the direct-repeat sequence and TATA element. The second deletion mutant lacks the duplicated sequence motif in addition to both upstream elements. This promoter extends from positions −30 to +26 and contains the TATA element.

Transcript levels from the wild-type and deletion mutant promoters linked to the mouse MT-I gene were compared after transient transfection of the plasmids into MEL cells. These constructs were introduced into MEL cells along with an internal reference plasmid, pH4MT (containing the mouse histone H4 promoter [39]), −229 to +70, linked to the same portion of the MT-I gene, +65 to +2200, used in the βMT constructs (Fig. 3A). Transfected cells were then split into two samples and replated. Terminal differentiation was induced in one of the samples by the addition of DMSO. Cytoplasmic RNA was isolated 44 to 48 h later and analyzed for correctly initiated test and reference transcripts in an S1 nuclease protection assay (Fig. 3B). The levels of βMT and H4MT mRNA were quantitated by densitometric scanning of several autoradiographic exposures and are summarized for two independent transfection experiments in Table 1.

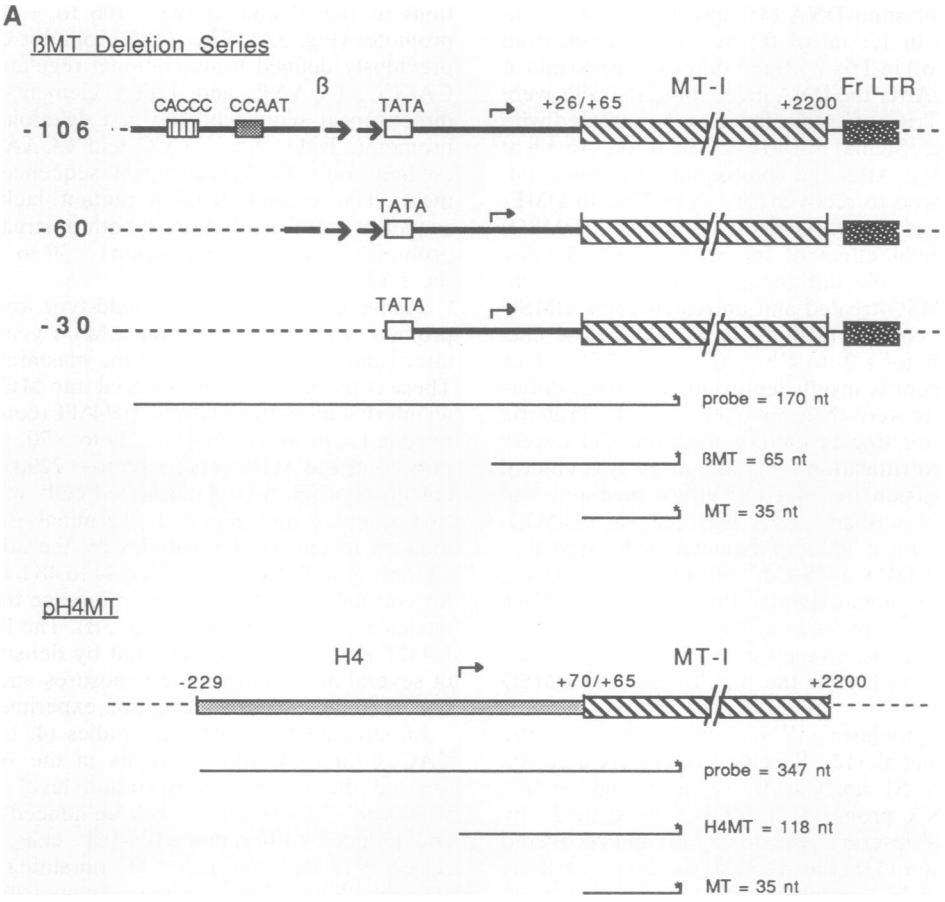
In agreement with earlier studies (4, 6), removal of the CACCC and CCAAT elements in the −60 promoter decreased the relative transcription level to about 10% of wild-type −106 levels in both uninduced (undifferentiated) and induced (differentiated) MEL cells. Removal of the region between −60 and −30 containing the direct-repeat sequence lowered transcription levels still further (Fig. 3B, lanes 3 to 6). In induced MEL cells, relative transcription levels declined 8.4-fold, on average, by deletion of the −60 to −30 sequences; in uninduced cells, a decrease of 3.4-fold was observed.

Mutagenesis of the direct-repeat sequence. Since the results of these deletion experiments suggested that sequences between −60 and −30 are important for maximal RNA levels from the β-globin promoter in MEL cells, we undertook a more extensive mutagenic analysis of the conserved direct-repeat sequence within this region. Double-point mutations were constructed that altered one base pair in each repeat sequence by a method that involved annealing available single-base substitutions within the direct-repeat region (35) to create heteroduplexes containing two mutations (34; see Materials and Methods). A complete replacement of the −53 to −32 sequence with a transversion mutation at each

TABLE 1. Effects of 5' deletions on transcription from the β-globin promoter in MEL cells

Construct	RTL[a]			
	Expt 1		Expt 2	
	Uninduced	Induced	Uninduced	Induced
−106	5.4	100.0	9.4	100.0
−60	0.6	8.6	1.6	12.2
−30	0.2	1.0	0.5	1.5

[a] Expressed as a percentage of the activity of the wild-type −106 promoter in induced MEL cells and corrected for transfection efficiency by normalizing to the signal of the internal reference gene, H4MT. RTL is calculated as (βMT/H4MT RNA signal)/(βMT/H4MT signal for the wild-type −106 construct in induced MEL cells) × 100.



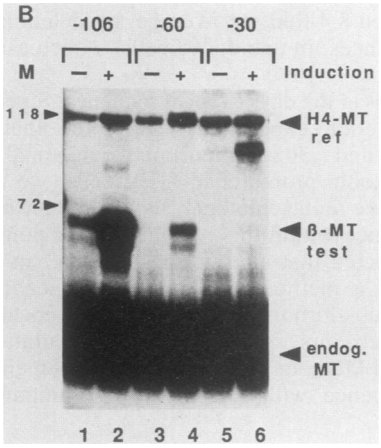


FIG. 3. Analysis of a 5′ deletion series of the mouse β-globin promoter in MEL cells. (A) βMT test and H4MT reference plasmids used in the analysis, shown along with the positions of S1 probes and sizes of protected fragments. Notations: β, fragments of the mouse β-globin promoter extending from −106 to +26, −60 to +26, or −30 to +26 as diagrammed; CACCC, CCAAT, TATA, locations of the previously identified transcriptional regulatory elements within the mouse promoter; MT-I, genomic DNA fragment containing the mouse MT-I gene; Fr LTR, 300-bp *Sac*I-to-*Pst*I fragment from the Friend virus LTR; H4, 300-bp mouse histone H4 promoter fragment; →→, location of the directly repeated sequence AGGGCAG(G)AGC within the mouse β-globin promoter; ----, pSP73 vector sequence; nt, nucleotides. (B) S1 analysis of cytoplasmic RNA from promoter deletions in uninduced (−) and induced (+) MEL cells. −106, −60, and −30 refer to βMT plasmids diagrammed in panel A; arrowheads mark positions of the designated protected fragments. M, Size markers; 118 and 72, positions of 118- and 72-nucleotide size markers. The additional S1 protected band in lane 6, migrating slightly faster than the H4MT protected band, maps to the divergence between the −30 promoter and the βMT S1 probe, which extends to position −106 and therefore corresponds to upstream start sites with the βMT plasmid.

position was also constructed. In addition, DNA sequences in the 5′ repeat and the 3′ repeat were replaced with heterologous sequences (generating the mutant promoters ΔDR1 and ΔDR2, respectively) to assess the role of the individual repeats. Mutant promoters were cloned into the βMT expression plasmid and compared with the wild-type −106 promoter in MEL cell transient transfection assays as described above. Figure 4 shows an S1 nuclease protection analysis of a panel of these mutant promoters. The nucleotide sequences of the mutant promoters and their relative transcription levels are summarized in Table 2.

When the entire −53 to −32 region was replaced with a transversion mutation at each position, transcription levels were reduced to 11% of wild-type levels in induced MEL cells (REP; Fig. 4 lanes 3 and 4). A similar decrease in transcript levels was observed with an additional mutant mouse β-globin promoter in which each nucleotide in the repeat region was changed to a sequence different from that found in the REP mutant (data not shown). Disruption of a single repeat (ΔDR1 and ΔDR2; lanes 5 to 8), on the other hand, had only a slight effect on overall transcription levels, reducing them to 57 to 60% of wild-type levels in induced cells. A greater decrease in βMT RNA levels was observed for two of the double-point mutants, −47/−35 (lanes 9 and

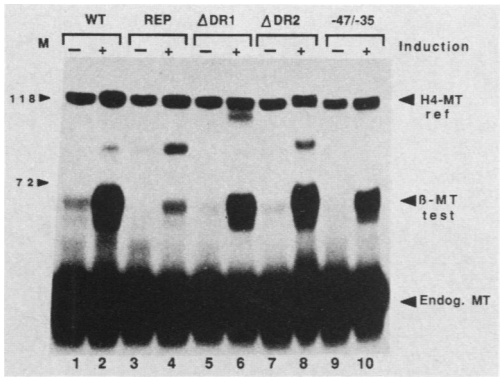


FIG. 4. Analysis of a panel of mutations within the direct-repeat sequence of the β-globin promoter in uninduced (−) and induced (+) MEL cells. Designations above the lanes refer to the promoter in plasmid pβMTF. WT, Wild-type −106 β-globin promoter; REP, transversion replacement of the −53 to −32 sequence; ΔDR1, alteration of the 5′ direct-repeat sequence; ΔDR2, alteration of the 3′ direct-repeat sequence; −47/−35, point mutations at these positions within the promoter. Arrowheads mark positions of the designated protected fragments. M, Size markers; 118 and 72, positions of 118- and 72-nucleotide size markers. Additional S1 protected bands migrating between the βMT and H4MT signals correspond to upstream starts within the βMT plasmid that map to the point of divergence between the mutant promoter template and the S1 probe.

10) and −46/−40 (data not shown) than for the single-repeat replacement mutants (ΔDR1 and ΔDR2) in which 10 to 11 consecutive bases are altered. Induced RNA levels for these two double-point mutants were reduced to 25 to 40% of wild-type levels. The three remaining double-point mutants, −51/−39, −50/−41, and −51/37, all had lesser effects (55 to 91% of wild-type levels) on βMT transcription levels.

This mutational analysis defines a new transcriptional regulatory element within the mouse β-globin promoter whose disruption by complete deletion or by transversion mutation lowers transcription levels roughly ninefold in induced MEL cells. To detect the activity of this element, it was necessary to disrupt both copies of the directly repeated 10- to 11-bp sequence. We term this element the β-globin direct-repeat element, or βDRE.

Effects of βDRE mutations on β-globin promoter induction. The levels of βMT mRNA increased 10- to 20-fold after 48 h of DMSO-induced differentiation of MEL cells transfected with the wild-type −106 construct. Several lines of evidence suggest that the majority of this increase in mRNA level was due to an increase in transcriptional initiation and not to increased βMT RNA stability in induced versus uninduced MEL cells. Nuclear runoff experiments have demonstrated that the rate of transcriptional initiation at the β-globin promoter increases substantially during MEL cell differentiation (3, 19, 47). In addition, we have determined that β-globin sequences from +1 to +26, which are part of the hybrid βMT mRNA, can be removed from the βMT construct without affecting the overall level of inducibility (where inducibility is defined as the ratio of induced to uninduced mRNA; data not shown). Similarly, the MT sequences in the βMT mRNA are also present in the H4MT reference mRNA, whose activity does not change with MEL cell differentiation, suggesting that the stabilities of these mRNA sequences are similar in induced and uninduced cells. Therefore, nucleotides between −106 and +1 are likely to be the only sequences involved in the inducibility of the βMT construct. To determine whether the βDRE plays a role in inducibility, we evaluated the mutants of the βDRE described above for their relative effects on transcript levels before and after MEL cell differentiation. Even though the level of β-globin promoter inducibility was somewhat variable from transfection to transfection, a consistent effect on inducibility was observed when the βDRE sequence was disrupted. The increases in βMT RNA levels upon MEL cell differentiation for the wild-type and mutant promoters are summarized in Table 3. In addition, the mutant promoter inducibility is expressed as a percentage of the inducibility of the wild-type −106 promoter (Table 3), allowing the results from transfections with variable levels of induction to be compared.

A comparison of the relative transcription levels for the −60 versus the −30 promoter (Table 1) indicates that the effect of deleting the region containing the βDRE was more pronounced in induced cells. Therefore, when the −60 to −30 sequences were deleted, the level of promoter inducibility dropped from 73 to 31% of wild-type promoter inducibility (2.4-fold). A comparison of the effects of additional mutations within the βDRE sequence itself indicates that the two most disruptive mutants, the −47/−35 double-point mutant and the βDRE transversion replacement, REP, also had a greater effect in induced than in uninduced MEL cells. The −47/−35 mutant retained 60% of the wild-type promoter inducibility, whereas when the βDRE sequence was completely replaced, the inducibility dropped to 37% of the level of the wild-type promoter (2.5-fold), in agreement with the

TABLE 2. Effects of substitution mutations on transcription from the β-globin promoter in MEL cells

Promoter	Sequence	RTL	
		Uninduced[a]	Induced[b]
Wild type	−53 AGGGCAGGAGCCAGGGCAGAGC −32	8.7 ± 3.0	100
−47/−35	A T	3.6 ± 0.3	25 ± 4
−46/−40	A A	4.4 ± 0.5	40 ± 8
−50/−41	A C	6.5 ± 3.4	55 ± 6
−51/−37	A T	4.6 ± 0.9	65 ± 5
−51/−39	A A	6.0 ± 1.1	91 ± 5
ΔDR1	CTTCTCGAGTA	5.3 ± 0.9	60 ± 13
ΔDR2	CTCGAGTCTA	5.9 ± 0.9	57 ± 4
REP	CTTTACTTCTAACTTTACTCTA	2.6 ± 1.6	11 ± 3

[a] Calculated as (uninduced mutant βMT/H4MT signal/uninduced wild-type βMT/H4MT signal) × 8.7. Multiplication by 8.7 allows normalization of uninduced RTL values to the average wild-type uninduced RTL (8.7 = average [uninduced βMT/induced βMT signal] × 100). Normalization of uninduced values is necessary to correct for assay-to-assay variation in wild-type promoter induction. All mutant uninduced RTLs are therefore expressed as in Table 1, as a percentage of the induced wild-type βMT signal, which is arbitrarily assigned a value of 100%.

[b] Calculated as (mutant βMT/H4MT induced signal)/(wild-type βMT/H4MT induced signal) × 100.

TABLE 3. Effects of mutations on promoter inducibility

Mutant construct	Fold induction[a]	Inducibility[b]
−60	14 ± 0.4	73
−30	6 ± 0.5	31
−47/−35	12 ± 2	60
−46/−40	15 ± 5	78
−50/−41	16 ± 10	73
−51/−37	23 ± 6	123
−51/−39	23 ± 4	125
ΔDR1	18[c]	98
ΔDR2	16[c]	84
REP	9 ± 5	37

[a] Calculated as (induced βMT/H4MT signal)/(uninduced βMT/H4MT signal). The wild-type −106 value was set at 18.5-fold, the average induction value for three independent transfections in which most of the mutants were analyzed (18.5 ± 0.2). Mutant fold inductions and standard deviations are calculated from two to four independent transfections, normalizing to the wild-type fold induction value of 18.5.

[b] Expressed as a percentage of the inducibility of the wild-type −106 promoter. This value is calculated from the average RTL values in Tables 1 and 2 as follows: (average induced RTL/average uninduced RTL*) × 100, where the uninduced RTL* = (mutant uninduced βMT/H4MT signal)/(wild-type uninduced βMT/H4MT signal).

[c] Determined in only one experiment.

results of the deletion series. The other double-point mutant and single-repeat replacement promoters had more variable effects on β-globin promoter inducibility; some decreased it slightly, and others increased it somewhat. Although a consistent decline in inducibility was observed for β-globin promoters that lack the βDRE entirely (the −30 deletion mutant [Fig. 3B, lanes 5 and 6] and the βDRE replacement promoter [Fig. 4, lanes 3 and 4]), these mutant promoters were still clearly inducible. Therefore, additional elements outside the βDRE sequence motif also appear to contribute to the inducibility of the β-globin promoter after MEL cell differentiation.

Addition of the βDRE to a heterologous promoter. We have assessed potential changes in the activity of the βDRE during MEL cell differentiation by comparing the relative effects of its deletion or mutation in uninduced versus induced MEL cells. These experiments suggest that the level of inducibility of the β-globin promoter is reduced 2.5-fold when the βDRE is inactivated. To verify this result and avoid the problem of quantitation of low uninduced βMT RNA levels inherent to the analysis of these mutant promoters, we inserted the βDRE into an MT-I promoter, which is not significantly inducible in MEL cells. The βDRE was inserted 5′ to the mouse MT-I TATA element (at position −30 in a −30 to +2200 MT construct) so that the βDRE/TATA spacing found in the β-globin promoter was maintained. The levels of transcription for the pβDRE-MTF construct (diagrammed in Fig. 5A) were compared with those of the −30 MT-I promoter construct (pMT8F; −30 to +2200; Fig. 5A) in the MEL cell transient transfection assay. In four independent transfection experiments, the RTLs of the MT-I promoter in uninduced MEL cells were unaffected or only weakly affected by addition of the βDRE (Fig. 5B; lane 1 versus lane 3; RTL data summarized in Table 4). In induced MEL cells, on the other hand, the MT-I promoter containing the βDRE was expressed at 3.3-fold higher levels than the −30 promoter alone (Fig. 5B, lane 2 versus lane 4). The greater stimulation of MT-I transcription levels in induced versus uninduced MEL cells resulted in an increase in the inducibility of the βDRE-MT promoter above the level observed for the MT-I promoter alone. This experiment

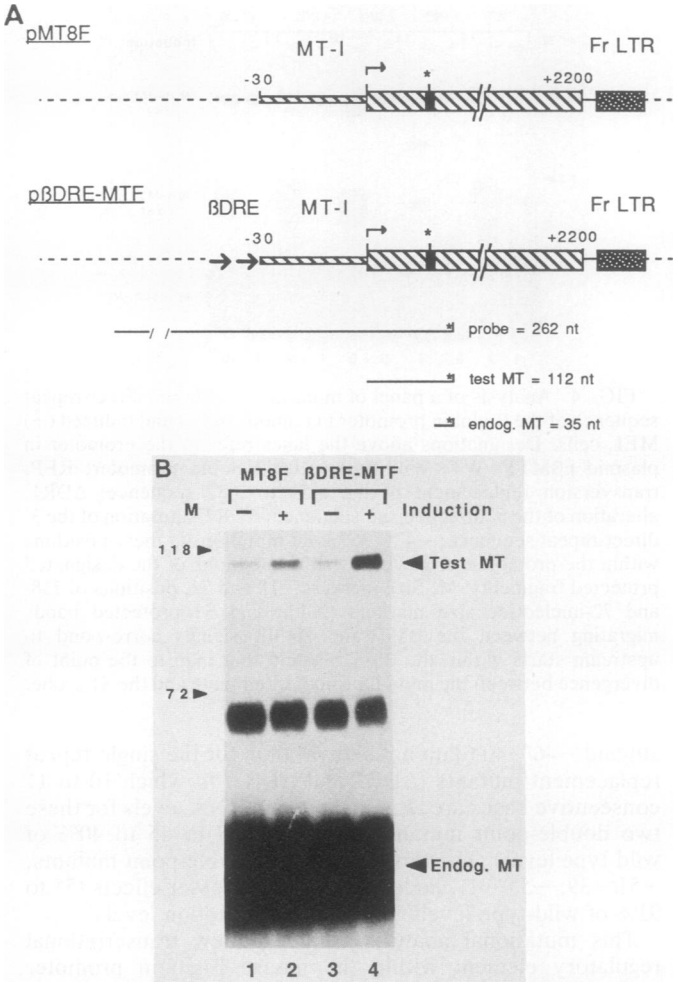


FIG. 5. Comparison of plasmids pMT8F and pβDREMTF in MEL cells. (A) Diagram of plasmids, positions of S1 probes, and sizes of protected fragments. Notations: MT-I, genomic fragment of the mouse MT-I gene containing a −30 promoter and a 12-bp insertion of a *Bam*HI restriction site linker at +65 (*); Fr LTR, 300-bp enhancer fragment from the Friend virus LTR; →→, −53 to −32 direct-repeat sequence from the mouse β-globin promoter; nt, nucleotides. S1 probe shown is homologous to the *Bam*HI-marked MT-I gene. (B) S1 analysis of pMT8F and pβDREMTF in uninduced (−) and induced (+) MEL cells. Arrowheads mark locations of protected fragments. M, Size markers; 118 and 72, positions of 118- and 72-nucleotide size markers. The additional S1 protected band migrating slightly below the 72-nucleotide size marker corresponds to a downstream start site within the MT-I gene.

suggests that the activity of the βDRE element increased roughly threefold by 48 h of MEL cell differentiation with DMSO, in agreement with the results of the deletion and mutagenesis experiments. In addition, this experiment indicates that the βDRE sequence identified in the mutagenesis experiments can function in a heterologous context, confirming that this region of the β-globin promoter is a transcriptional stimulatory element in MEL cells.

DISCUSSION

By analyzing a series of mutations that disrupt an evolutionarily conserved directly repeated sequence in the mouse β-globin promoter, we have demonstrated that this sequence

TABLE 4. Effect of the βDRE when attached to a heterologous promoter

Construct	RTL[a]		Inducibility[b]
	Uninduced	Induced	
pMT8F	82 ± 24	100	100
pβDREMTF	88 ± 22	339 ± 61	330 ± 120

[a] Calculated as (MT/H4MT signal)/(MT8F/H4MT signal in induced MEL cells) × 100.
[b] Calculated as induced (MT/H4MT signal)/uninduced (MT/H4MT signal) and presented as a percentage of the MT8F value.

is an important regulatory element required for maximum transcription levels from the β-globin promoter in erythroid cells. Interestingly, this mutational analysis indicates that the two repeats of the βDRE are largely redundant. Replacement of a single repeat with heterologous sequence lowers transcription levels less than twofold, whereas deletion or mutation of the entire −53 to −32 sequence decreases transcription levels ninefold in induced MEL cells. Although many mutations within this region of the promoter had been analyzed in earlier studies, the activity of this element was not detected because only one of the two repeats was altered at a time. These findings have implications for the design of mutational studies aimed at elucidating the regulatory DNA sequence elements in other genes, since traditional single-base substitution and linker-scanning mutagenesis approaches may be insufficient to inactivate a repeated element. Although we reexamined this region of the β-globin promoter because of its unusual features, it is likely that less obvious sequence conservation in other systems will make it difficult to identify repeated sequences by a visual examination of the DNA sequence.

Repetition of functional DNA elements has been observed in the characterization of other eucaryotic promoter and enhancer sequences. Metallothionein promoters, for example, contain several copies of a 12-bp sequence motif that mediates transcriptional induction by heavy metals. Although five such elements are present in the 5′-flanking region of the MT-I gene, two copies are sufficient to confer on the thymidine kinase promoter a level of metal regulation comparable to that found for MT-I (41). SP1-responsive promoters, such as the simian virus 40 early and herpes simplex virus IE-3 promoters, typically contain multiple SP1-binding sites, often aligned every 10 to 12 bp (each turn of the DNA helix) (21). In the case of the herpes simplex virus IE-3 promoter, the presence of multiple SP1 sites has a less than additive transcriptional stimulatory effect. In a deletion analysis of this promoter, a mutant promoter containing a single SP1 site is stimulated 20-fold by SP1. The wild-type promoter, containing five SP1 sites, is activated to only threefold-higher levels than the mutant in the presence of SP1 (20, 21).

cis-Acting DNA elements have been found to correspond to binding sites for DNA sequence-specific transcriptional regulatory factors. What, then, is the significance of the apparent redundancy of these regulatory elements? Multiple copies of a binding site for a particular transcription factor may ensure occupancy of at least a single site under various physiological conditions in vivo. Duplication would therefore be an evolutionarily simple way to increase the activity of an element consisting of a single low-affinity regulatory factor-binding site. Alternatively, it is possible that whereas two copies of the βDRE motif appear redundant in a transfection assay in a transformed cell line, more dramatic effects of mutation of a single repeat may be found in an erythrocyte or under physiological conditions that are not mimicked in the MEL cell system. Also, small (twofold or less) effects on expression levels may seem insignificant in an experimental system but have important physiological consequences for an organism. For example, a substitution mutation at the position where transcription begins from the human β-globin promoter results in a form of β-thalassemia (45); a substitution mutation at the same position in the mouse β-globin promoter causes a small (twofold) decrease in accumulated transcripts in a transient transfection assay (35). These combined results suggest that small changes in expression levels can have deleterious effects at the organismal level.

The presence of multiple adjacent elements of a single sequence suggests that more than one molecule of the same factor, or multimers of a factor, bind to the element. The concurrent binding of multiple factors may cause a greater stimulation of transcription initiation than could be achieved by binding of a single factor by allowing two or more factors to make simultaneous contacts with RNA polymerase or other regulatory factors. If a dimer of the putative βDRE factor normally interacts with the directly repeated sequence in the β-globin promoter, the dimer might still be capable of binding to a single repeat when one of the two repeats is mutated. In this case, inactivation of the element would require disruption of both half-sites, and the direct-repeat element would appear redundant in a transfection assay. By characterizing the binding of the βDRE factor to a promoter containing one versus two copies of the βDRE motif in a footprinting assay, it should be possible to address this model.

The specific nucleotides within the AGGGCAG(G)AGC motif that are critical for βDRE activity have not yet been fully determined. In this study, alteration of the G at positions −47 and −35 to an A and T, respectively, causes the greatest decrease in βMT transcription levels. In addition, the tested base changes at positions −51, −39, and −37 have only slight effects on transcription levels, although other base changes at these positions might have different effects. To map the endpoints and the critical nucleotides for function of this redundant element, it will be necessary to first replace one repeat with heterologous sequence and then sequentially alter base pairs in the second motif.

As the activity of this element was not detected in several mutagenesis studies of the β-globin promoter, it is possible that because of its repeated nature, it may also be overlooked in a mutational analysis of another gene. A scan of the GenBank data base reveals perfect AGGGCAG(G)AGC sequence motifs in the regulatory regions of a number of vertebrate genes expressed in differentiated erythroid, lymphoid, or muscle cells. Transfection studies in HeLa and K562 (a leukemic cell line that expresses embryonic and fetal but not adult globin genes) cell lines indicate that function of the βDRE is not restricted to cells of the adult erythroid lineage; βDRE mutant RTLs in these two cell types are similar to those found in MEL cells (data not shown). Therefore, it seems likely that this regulatory sequence in the mouse adult β-globin gene will be functional in other contexts and cell types.

In contrast to response elements that mediate transcriptional induction of a promoter after a specific stimulus (e.g., heat shock, steroid hormone stimulation, or heavy-metal exposure), transcriptional activation of the β-globin promoter during erythroid differentiation does not appear to be mediated by a single discrete response element. In addition

to elements further upstream and downstream (1), our data suggests that within the −106 promoter alone, several regulatory elements act in concert to bring about the large increase in β-globin promoter activity that occurs during MEL cell differentiation. The analysis of 5′ deletions of the β-globin promoter described here suggests that three separate regions of the −106 to +26 promoter contribute to the overall 10- to 20-fold inducibility. When both the CACCC and CCAAT elements are deleted in the −60 promoter, a small (1.4-fold) drop in inducibility of the β-globin promoter is observed. An additional 2.5-fold drop in inducibility occurs when the βDRE is removed, indicating that the element contributes significantly to induction. This conclusion is also supported by experiments showing that addition of the βDRE to a heterologous noninducible promoter causes that promoter to become inducible. Finally, the minimal −30 to +26 region also contributes significantly to induction of the promoter (4.6-fold on average), which is likely due to the TATA element.

Several models can be invoked to account for the differential activity of a *cis*-acting element in differentiated versus undifferentiated MEL cells. Two distinct factors capable of recognizing the regulatory sequence motif may be present in MEL cells before and after differentiation. The differentiation-specific factor, therefore, would be predicted to have a greater stimulatory effect on the rate of transcriptional initiation or to bind to its recognition site with higher affinity than the factor found in uninduced cells. Alternatively, an increase in the abundance or activity of a single factor could account for an increase in regulatory element activity during MEL cell differentiation. Heat shock induction of the human hsp70 promoter, for example, is mediated by the binding of heat shock transcription factor to an element within the promoter. The level of this binding activity increases five- to sevenfold after heat shock in a rapid manner that is independent of protein synthesis, suggesting that a preexisting factor is modified in some way to allow DNA binding and transcriptional activation (23).

A comparison of the effects of addition of the βDRE to either a minimal MT-I or β-globin promoter (−30, each containing its TATA element) indicates that the βDRE increases the relative inducibility of each promoter to about the same extent. However, the βDRE stimulates transcription to a greater overall extent on the homologous β-globin TATA element. In induced MEL cells, it increases the rate of transcriptional initiation ninefold from the β-globin TATA, as compared with only a threefold stimulation of initiation from the MT-I TATA element. Curiously, the βDRE affects transcription levels from the β-globin TATA in both uninduced and induced cells, whereas in the context of the MT-I promoter, the βDRE has little effect on transcription levels in MEL cells before differentiation. It is possible that different TATA factors recognize the β-globin and MT-I TATA motifs, as has been postulated in other systems (40, 41a), and that the βDRE factor stimulates transcriptional initiation more efficiently in association with the β-globin TATA factor. The relative locations of the βDRE (−53 to −32) and the TATA element (−30 to −26) indicate that the factors recognizing these DNA sequences are likely in close physical proximity to one another. Although a systematic study has not yet been done, our initial analysis suggests that βDRE function may be sensitive to spacing alterations between the TATA element, the transcriptional start site, or both. The βDRE was evaluated at other positions further upstream in the MT-I promoter and in upstream positions in the histone H4 promoter, and no effects of the element were detected (data not shown). Together with the finding that two copies of the βDRE motif have a less than additive transcriptional stimulatory effect, the activity of this element seems in contrast to that of an enhancer element. Enhancer elements function in a distance- and orientation-independent manner and, when multimerized, often gave greater than additive activity. The βDRE, therefore, would be predicted to affect the rate of transcriptional initiation in a manner mechanistically distinct from that of an enhancer element.

A mechanistic understanding of the regulation of β-globin transcriptional initiation during erythroid development will require purification and characterization of the sequence-specific DNA-binding factors that mediate transcriptional regulation of the gene. Toward this end, we have identified a specific βDRE-binding activity that increases in abundance three- to fivefold with MEL cell differentiation (data not shown). Our current efforts are focused on purification and further characterization of this binding activity and its role in regulation of β-globin gene expression in the terminal stages of erythroid differentiation.

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