β-Globin dominant control region interacts differently with distal and proximal promoter elements

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We have studied the interaction between the dominant control region (DCR) and the promoter of the human β-globin gene. Expression analysis in MEL cells has revealed that the DCR contains a number of elements capable of replacing the upstream (−250 to −100) erythroid-specific region of the promoter. The DCR strongly stimulates expression from a promoter possessing only a TATA box. However, this basic level of transcription is not induced upon erythroid differentiation of the cells. Mutational analysis of the minimal (−100, noninducible) promoter shows that only the combination of the DCR and the CAC/CCAAT elements provides erythroid-specific transcription. These regions act synergistically to produce full regulated expression during erythroid differentiation.

[Key Words: β-Globin; dominant control region; transcription]

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A number of erythroid and developmental specific regulatory elements have been identified within the promoter and the two downstream enhancers of the human β-globin gene. These elements confer regulated expression on the human β-globin gene when introduced in transgenic mice or erythroid cell lines (Behringer et al. 1987; Kollias et al. 1987; Trudel et al. 1987; Antoniou et al. 1988), albeit at a level considerably below that of the endogenous globin genes. A number of ubiquitous and erythroid-specific protein-binding sites within these regulatory regions have been identified (Fig. 1; deBoer et al. 1988, Wall et al. 1988), most notably for the erythroid-specific factor NF-E1. The NF-E1 gene has recently been cloned from mouse (Tsai et al. 1989), chicken (Evans and Felsenfeld 1989), and human (Trainor et al. 1990). In addition, it has been shown that the murine NF-E1 alone is incapable of inducing regulated expression on the minimal β-globin promoter (see Fig. 1) but needs to interact with at least one ubiquitous DNA-binding protein at a neighboring site (Fig. 1, CP-1 at −160; deBoer et al. 1988).

To achieve high levels of regulated expression, an additional regulatory region located 50-60 kb upstream from the β-globin gene and, hence, distal to the entire multigene cluster, is required. When this dominant control region (DCR) is linked directly to a β-globin gene, it confers high-level, gene-copy-number-dependent expression of the gene in transgenic mice and murine erythroleukemia (MEL) cells, independent of the site of integration in the host genome (Grosveld et al. 1987; Blom van Addendelft et al. 1989). The DCR confers the same characteristics to the other genes in the cluster (α and γ-globin; Catala et al. 1989; Enver et al. 1989, Lindenbaum, unpubl.) and also the α-globin gene (Behringer et al. 1989; Hanscombe et al. 1989, Ryan et al. 1989) but does not appear to have any developmental specificity. The DCR contains four tissue-specific DNase I hypersensitive (DHS) sites over a region of 20 kb (Tuan et al. 1985; Forrester et al. 1987; Grosveld et al. 1987), which can be reduced to a small region containing only these DHS sites (Talbot et al. 1989). Further functional mapping of the DCR in MEL cells has shown that the region contains a considerable amount of redundancy (Collis et al. 1990); that is, DHS sites 2 and 3 provide ~50% of the activity but do not act synergistically, whereas sites 1 and 4 provide, at most, 10% of the activity. However, DHS sites 1 and/or 4 can act in conjunction with sites 2 and/or 3 to boost expression levels to >80% of the full DCR. Further work in transgenic mice has demonstrated that DHS site 1, 2, or 3 can promote gene-copy-number-dependent expression independently, although at levels of 30-70% of the full DCR (Fraser et al. 1990). Both DHS sites 2 and 3 have been reduced to a minimal fragment of ~300 bp, each of which contains three binding sites for NF-E1 in addition to a number of other erythroid-specific and ubiquitous proteins (Philipsen et al. 1990; Talbot et al. 1990).

It is also important to note that the DCR possesses several properties that are not common to previously characterized cellular and viral enhancers. First, the transcriptional stimulatory function of at least three of the DHS sites (numbers 1, 2, and 4) is undetectable in
classical enhancer–trap experiments employing transient expression assays in cultured cells (Tuan et al. 1989). Thus, the DCR can only be analyzed by stable transfection into erythroid cells or in transgenic mice. Of greater significance is the ability of the DCR to confer gene-copy-number-dependent expression. To date, only the T-cell-specific “enhancer” for the CD2 gene has been shown to possess similar properties (Greaves et al. 1989). On the basis of the available information, it is not clear, however, by which mechanism the DCR is able to confer high levels of regulated expression and through which parts of the β-globin promoter it is able to exert its effect. Here, we describe expression studies in MEL cells that provide a functional analysis of the interaction between the DCR and the β-globin promoter. This constitutes the first quantitative analysis of cooperative interactions between the promoter regions and erythroid-specific regulatory elements of the human β-globin gene. Our results show that the erythroid-specific upstream part of the β-globin promoter can be replaced by a complete or partial DCR and that the DCR can interact directly with a promoter that contains only one TATA box. However, all tissue-specific stimulation of transcription is mediated through the CCAAT and the CAC box regions in the minimal, downstream promoter.

Results

Human β-globin promoter constructs

The original 40-kb human β-globin minilocus (Grosveld et al. 1987) was reduced to a 6.5-kb DCR microlocus (Talbot et al. 1989) without loss of activity. It was subsequently shown that the two downstream enhancers of the β-globin gene could also be deleted without loss of activity (Collis et al. 1990). This allowed us to study the full activity of the DCR on just the human β-globin promoter, which itself can be regulated (Antoniou et al. 1988; deBoer et al. 1988). The promoter was linked to a murine H-2Kβ reporter gene (Materials and methods; Fig. 1; Antoniou et al. 1988) and inserted into the microlocus cassette (Fig. 1; Collis et al. 1990). Deletion variants and point mutations were constructed in different combinations by use of existing (Antoniou et al. 1988; deBoer et al. 1988; Collis et al. 1990) and newly derived mutations in the promoter and DCR region of the β-globin gene.

Only the minimal promoter is essential in the presence of the DCR

The upstream part of the β-globin promoter confers inducible expression on the gene, mediated by the presence of at least one of the two NF-E1 and the [-150] CP-1-binding sites (Fig. 1; deBoer et al. 1988), although it was impossible to quantitate the contribution of each of the NF-E1 sites (deBoer et al. 1988). To overcome this limitation, the original β-globin promoter mutants that delete one or more of these upstream factor-binding sites (Fig. 2; Antoniou et al. 1988) were linked to the DCR, which confers gene-copy-number-dependent expression. Each construct was introduced into MEL cells, and three stably transformed populations were isolated for each construct. RNA was isolated before and after erythroid induction of the cell population. S1 nuclease protection analysis of the 5′ end of the hybrid β-globin H-2Kβ RNA...
Figure 2. Analysis of upstream promoter mutants of the β-globin gene within the microlocus DCR. The wild-type (β-WT) β-globin promoter (to -815) and internal deletion mutants (Δ) were linked to a murine H-2Kk reporter gene and placed within the DCR microlocus cassette (see Fig. 1). These constructs were then introduced into MEL cells, as described in Materials and methods. The S1 nuclease protection analysis of RNA (2 v-g) is shown from the resulting transfected populations of cells before (−) and after (+) 4 days of induced erythroid differentiation. The probe used to detect the 5'βH hybrid mRNA (5'βm) is a 700-bp HindIII-NcoI fragment that protects 96 nucleotides from the 5' half of the second exon of the βmaj gene (see Kollias et al. 1987). The negative control (C) is RNA from uninduced, untransfected cells. The various internal deletions tested remove one or more of the factor-binding sites within the upstream promoter (see Fig. 1); A103-120, NF-E1; A103-164, NF-E1 plus CP-1; A265-184, upstream NF-E1; A265-138, upstream NF-E1 plus CP-1.

showed that all of the upstream deletion mutants had the same inducibility and overall levels of expression per gene when compared to the complete promoter (β-WT) and the endogenous mouse βmaj-globin RNA (Fig. 2). This is in marked contrast to the results obtained with these deletion mutants in the absence of the DCR (Antoniou et al. 1988), when only the Δ103-120 and Δ184-265 constructs retained inducibility. We therefore conclude that the presence of the DCR completely abolishes the requirement for the erythroid-specific, upstream part of the promoter. This was confirmed when all of the upstream promoter was removed in a construct deleted to position −103 (Fig. 3; Table 1). To determine what parts, if any, of the minimal promoter were essential to provide full expression, we linked a further set of mutants to the DCR: the −103 mutant, which contains all the non-tissue-specific elements for expression in nonerythroid cells (Grosveld et al. 1982; Dierks et al. 1983; Myers et al. 1986); a CAC box mutant and a CCAAT box mutant, both of which were constructed by in vitro oligonucleotide-directed mutagenesis to replace 4 nucleotides essential for the binding of SP1 and TEF-2 (Xiao et al. 1987; E. Spanopoulou, unpubl.); and CP-1 (Chodosh et al. 1988), respectively (Fig. 1). In addition, we used the −77 mutant, which lacks both a functional CAC and CCAAT box. S1 nuclease protection analysis (Fig. 3; Table 1) of three independent stably transformed populations shows that the minimal promoter has the same levels as the complete (−815) promoter. Mutagenesis of the CAC or CCAAT box produces identical results; that is, the pre- and postinduction levels of transcription have decreased ~5 to 7-fold, but the inducibility has remained the same (~10-fold; Fig. 3 and inset). When both the CAC and CCAAT box are removed (−77), the preinduction level of transcription remains the same as in the single CAC or CCAAT box mutants, but inducibility is lost [Fig. 3 inset]. When the DCR is removed from this construct (−77ΔDCR, Fig. 3 inset), both pre- and postinduction levels of RNA drop to non-detectable levels. These results suggest that the DCR has a strong effect on the TATA box and initiation complex formation (>100-fold) to provide a low level of basic transcription before induction. This preinduction level is increased five- to sevenfold by the CAC and CCAAT box but only when both are present. Inducibility is mediated entirely through the CAC and CCAAT box, and the presence of only one of these is sufficient for full induction, albeit at lower total RNA levels. These data are in complete agreement with the β-globin levels observed in naturally occurring CAC box mutations (Orkin et al. 1982, 1984; Triesman et al. 1983).

The region of DHS sites 2 and 3 is essential for high levels of induction

To determine which of the DHS regions of the DCR could replace the upstream part of the β-globin promoter, we measured the activity of the DCR lacking DHS site 2 or site 3, or both sites 2 and 3 on a full (−815) promoter [Fig. 4, top] or a minimal (−103) promoter [Fig. 4, bottom]. The loss of both sites 2 and 3, but not of sites 2 or 3 alone, results in a substantial decrease in the level of transcription (down to 7%) from the full promoter. When the minimal promoter is used, the loss of sites 2 and 3, or site 3 alone, results in a decrease to <2% and 40%, respectively. The loss of site 2 alone does not result in a significant decrease [90% of full DCR]. It therefore appears that site 3 contains sequences that can substitute for the upstream promoter elements. The low levels of expression in the absence of sites 2 and 3 pre-
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Figure 3. The DCR produces full expression from the minimal β-globin promoter. The β-globin minimal promoter (-103) and variants, consisting of point mutants within the CAC and CCAAT regions, and a -77 construct, which deletes both CAC and CCAAT elements, were linked to the H-2K reporter gene in conjunction with the DCR microlocus (see Figs. 1 and 2). These constructs were then introduced into MEL cells to assess their capacity for erythroid-specific expression (see Materials and methods; Fig. 2). S1 nuclease protection analysis of RNA (2 μg) is shown from preinduced (-) and 4-day-induced (+) MEL cell populations transfected with the minimal promoter mutants and compared to the level of expression obtained with the full (-815) promoter construct (see Fig. 2). [Inset] Analysis with 40 μg of RNA from the cells carrying the CAC and -77 DCR constructs compared to a -77 βH-2K hybrid gene in the absence of the DCR (-77 ΔDCR). The probes for the βH-2K hybrid mRNA [5′βH2] and βm-globin mRNA [5′βm] were as described in Fig. 2. RNA from untransfected cells (C88) acted as a negative control. The size markers (M) are a HinfI digest of pBR322.

Discussion

Earlier work in this laboratory had indicated that the local enhancers of the human β-globin gene become redundant within the context of the microlocus DCR when expression is analyzed during erythroid differentiation of MEL cells (Collis et al. 1990). This suggested, but did not prove, that the DCR was functioning purely through the β-globin promoter. The results presented in this report confirm those initial observations. When the promoter of the human β-globin gene is linked to a murine major histocompatibility complex (H-2Kk) reporter gene within the microlocus cassette (see Fig. 1) and introduced into MEL cells, equal amounts of βH-2Kk and βm-globin mRNA are produced on a gene copy number basis upon induced erythroid differentiation of the transfected cells (Fig. 2). Furthermore, addition of either the gene’s internal or 3‘ enhancers (see Collis et al. 1990) upstream of the βH-2Kk hybrid gene did not produce any further stimulation of transcription (data not shown). Deletion analysis revealed that only the minimal part of the promoter is, in fact, sufficient for full regulated expression (Fig. 3, -103). Thus, the upstream region of the promoter that contains the binding sites for the erythroid-specific transcription factor NF-E1 (see Fig. 1), like the enhancers, is also made completely redundant by the DCR.

Further mutational analysis indicates that at least three types of interaction are taking place between the DCR and the minimal promoter elements (quantitated in Table 1). First, the DCR increases transcription from a purely TATA box promoter by >100-fold to produce a...
basic (but noninducible) steady-state level of mRNA (Fig. 3 inset; cf. -77 and -77ΔDCR). This indicates that the DCR stimulates the formation of the transcription initiation complex. The addition of either a functional CCAAT or CAC region to the basic TATA box promoter does not alter the preinduced level of transcription but restores full induced expression upon differentiation of the MEL cells, albeit at a reduced absolute rate [Fig. 3]. Finally, with the complete minimal promoter, both pre- and postinduced rates of transcription are increased to produce full steady-state levels of mRNA. Thus, the CAC and CCAAT regions can only cooperate with each other and the DCR in preinduced MEL cells when they are both present, whereas either one can promote full induction of transcription upon erythroid differentiation. We have yet to detect differences between proteins directly binding to the minimal promoter or the DCR in uninduced and induced MEL cells [deBoer et al. 1988; Philipsen et al. 1990; Talbot et al. 1990]. Therefore, we speculate that these differences in pre- and postinduced MEL cells reflect changes in protein factors that mediate the protein–protein interactions.

Early studies on the function of the β-globin promoter conducted in the presence of viral enhancers in either nonerythroid [Grosfeld et al. 1982; Dierks et al. 1983; Myers et al. 1986] or erythroid cells [Cowie and Myers 1988] demonstrated the importance of the CAC and CCAAT box regions as general transcription elements. The results presented here, however, are the first to demonstrate in a quantifiable manner the cooperative interactions between the minimal promoter regions and erythroid-specific regulatory elements and to distinguish the interaction between different elements in different erythroid contexts. Our results highlight the limitations of the early work described above. First, in contrast to studies employing viral enhancers in nonerythroid cells [Dierks et al. 1983], we find that the distal CAC region at −105 is not required for full expression, as the −103 deletion mutant is fully active [Fig. 3, −103]. The fact that we fail to find protein factors binding to this region argues further for its non-functional status [deBoer et al. 1988]. Of greater significance is our observation that the proximal CAC region and CCAAT box are directly involved and functionally equivalent during the DCR-mediated induced transcription on MEL cell differentiation. Thus, despite the fact that the CAC and CCAAT regions bind different protein factors, the DCR is able to interact with either of these elements in a comparable manner. Furthermore, when both are present, the CAC and CCAAT regions are able to interact synergistically, presumably not only between themselves but also with the DCR to produce full levels of expression. The CAC region has also been found to be a key component in SV40 enhancer function [Xiao et al. 1987] and in steroid hormone-regulated gene expression [Schüle et al. 1988a,b]. These observations, in conjunction with those reported here, strongly argue for a general mediatory role for this element in cell-specific gene transcription.

The observation that DCR can work purely through the minimal promoter elements that bind ubiquitous transcription factors also explains why the DCR can reprogram nonglobin heterologous promoters to express in an erythroid-specific manner. For example, the herpes simplex virus (HSV) thymidine kinase [Talbot et al. 1988] and murine Thy-1 [Blom van Assendelft et al. 1989] promoters become inducible in MEL cells in the presence of the DCR, as does that for murine MHC H-2Kk [O. Hanscombe et al., unpubl.]. All of these promoters possess a functional CCAAT or CAC region. Interestingly, the only promoter we have tested that does not respond to the DCR is the murine histone H4 promoter. This promoter does not contain either a CAC or CCAAT region, although it appears to contain an Sp1-binding site [Seiler-Tuyns and Birmstiel 1981]. This indicates that the crucial factor that binds to the β-globin

**Figure 4.** Effect of deleting DHS sites 2 and 3 from the DCR. The full (−815, top) and minimal (−103, bottom) promoter βH-2K hybrid genes were linked to either the complete micro locus DCR, as described previously (see Figs. 1, 2, and 3), or to DCR deletion mutants lacking site 2 (Δ2), site 3 (Δ3), or both sites 2 and 3 (Δ2/3). Analysis of RNA from MEL cell populations transfected with these constructs was exactly as described in Fig. 2. RNA from untransfected cells (C88) acted as a negative control.

**Figure 5.** DCR sites 1 and 4 confer and enhance erythroid-specific expression. Expression from the full (−815) and minimal (−103) promoter βH-2K genes linked to DCR sites 1 and 4 (HSS-1/4, see Fig. 4) was compared to that from constructs lacking any DCR elements (ΔDCR). RNA (20 μg) from MEL cell populations transfected with these constructs was analyzed by S1 nuclease protection (Fig. 2) for βH-2K mRNA (A, 5′BH). (B) Analysis with 2 μg RNA for βGlobin mRNA sequences to assess the level of induced erythroid differentiation of the cells. Each pair of lanes is RNA from preinduced and 4-day-induced cells, respectively. RNA from untransfected cells (C88) acted as a negative control. Size markers (M) were a HinfI digest of pBR322.
CAC region and can mediate erythroid-specific expression may not be Sp1 but TEF-2 [Xiao et al. 1987].

Our data show that the deletion of either DHS site 2 or 3 in the presence of the full promoter [Fig. 4, top] did not cause a significant drop in βH-2K mRNA levels. The same deletions with the minimal promoter [Fig. 4, bottom], however, showed a substantial decrease to 40% of full levels when site 3 was deleted. The implications of these observations are at least twofold. First, although the upstream promoter is no longer required to confer erythroid-specific transcription per se, it can still interact synergistically with a DCR lacking DHS site 3 to increase transcription (see also Fig. 5 and below). In addition, these results show that site 3 is more capable than site 2 of producing high-level transcription in the absence of the upstream promoter. This difference in the erythroid capabilities between DHS sites 2 and 3 can best be explained at present by the complement of protein factors that bind to these two regions. A “core” site 2 region of ~250 bp, which retains full functional activity in MEL cells, has been characterized [Philipsen et al. 1990]. This element is comprised of three tandemly repeated sequences containing one NF-E1-binding site juxtaposed with a G-rich region 30 bp downstream. The G-rich sequence resembles the CAC region of the promoter and binds several ubiquitous protein factors, the principal ones being Sp1 and TEF-2. A similar-sized core site 3 element also has at least three binding sites for NF-E1 [Talbot et al. 1990]. In addition, this site 3 region contains a sequence homologous to the Moloney and Friend viral enhancers and also a dimer-binding site at -103, with a synthetic oligonucleotide bearing the described changes (Fig. 1). The βH-2K constructs were cloned between the ClaI and KpnI sites of the DCR microlocus cassette [Collis et al. 1990]. Variants of the DCR [see Fig. 4] were prepared by replacing the full four-site AavII–ClaI region with the corresponding deletion mutant [see Collis et al. 1990].

All constructs were linearized at the unique PvuII site within the vector before transfection into MEL cells.

Maintenance and transfection of MEL cells

All manipulations of MEL cells, regarding maintenance, transfection to produce G418-resistance populations, and induced erythroid differentiation, were exactly as described previously [Collis et al. 1990].

Extraction and analysis of RNA

Extraction of RNA from MEL cells and its analysis by S1 nuclease protection using end-labeled DNA probes is also as described previously [Antoniou et al. 1988; Collis et al. 1990].

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