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The human β-globin gene contains multiple regulatory regions: identification of one promoter and two downstream enhancers

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We have introduced into murine erythroleukaemia (MEL) cells several series of deletion mutants of hybrid genes between the human β-globin gene and the murine H-2K^b gene. S1 nuclease and transcriptional run-off analysis showed that the human β-globin gene contains at least three globin specific transcriptional control elements. One is a promoter element and is located 160 bp upstream from the transcription initiation site; it increases the efficiency of the promoter after MEL cells are induced to differentiate. The other two elements are tissue-specific transcriptional enhancers and are located downstream from the transcription initiation site, the first in the structural β-globin gene and the second in the 3′ flanking sequences.

Key words: human β-globin gene/regulating elements/ erythroid cells.

Introduction

The human β-globin gene family contains five transcriptionally active genes, which are regulated in a tissue and developmental specific manner (for review, see Collins and Weissman, 1984). The embryonic ε-globin gene is expressed in the yolk sac, while the human γ-globin genes are expressed in the fetal liver. The human β-globin gene is only expressed in erythroid cells in the adult. To determine how this tissue and developmental regulation takes place, we have introduced human globin gene constructs in an erythroid cellular test system. Murine erythroleukaemia (MEL) cells are Friend virus transformed and arrested at the pro-erythroblast stage (Marks and Rifkind, 1978). They can be triggered to undergo erythroid maturation that closely resembles normal erythroid differentiation, resulting in high levels of murine globin mRNA. It has previously been shown that regulated transcription of the human β-globin gene can be obtained in these cells after DNA mediated gene transfer (Chao *et al.*, 1983; Wright *et al.*, 1983). Interestingly, subsequent experiments showed that the sequences involved in this regulation are located both upstream and downstream of the 5′ end of the gene (Wright *et al.*, 1984; Charnay *et al.*, 1984). The presence of the downstream sequences, also shown in the chicken β-globin gene using transfection experiments (Hesse *et al.*, 1986), have been confirmed for the human gene by experiments in transgenic mice. Correct tissue and developmental specific expression of the genes was still obtained by using promoter deletions (Townes *et al.*, 1985) and by using γ−β hybrid genes (Kollias *et al.*, 1986). The latter result in particular provided a direct link to the normal *in vivo* function of these sequences because it closely mimicked the situation in Hb Kenya (Ojwang *et al.*, 1983). Patients with this condition exhibit elevated levels of fetal haemoglobin in the adult due to a deletion−fusion event between the γ- and β-globin genes. At least part of the downstream regulatory sequences have been characterized further by showing that a fragment downstream of the β-globin gene can act as a developmental specific enhancer when placed upstream of the γ-globin gene (Kollias *et al.*, 1987). It was however not clear from these experiments whether the enhancer is also tissue-specific. Moreover, the results obtained in transgenic mice do not provide any insight as to the presence (or importance) of 5′ and flanking sequences in the globin gene. The only experiments carried out with promoter deletions still carried the downstream elements and were active (Townes *et al.*, 1985). When the downstream enhancer sequences were removed, the β-globin promoter was inactive (Kollias *et al.*, 1987). The only evidence for the importance of the upstream sequences has been obtained by stable transfection experiments in MEL cells (Wright *et al.*, 1984). This was possible by separation of the upstream from the downstream region in a hybrid gene construct containing the β-globin gene promoter linked to a non-inducible mouse H-2K gene. (The complementary hybrid gene containing the H-2K promoter and the β-globin gene showed the importance of the downstream region.) It has also been shown that the 5′ sequences prevent expression of the β-globin gene in embryonic/fetal stage K562 cells (Antoniou *et al.*, 1987; Lin *et al.*, 1987), but that manipulation of the 5′ end sequences can restore expression of the β-globin gene in K562 cells (M.Antoniou *et al.*, unpublished results). In this paper we describe the further characterization of the upstream and downstream regulatory regions of the β-globin gene by using MEL cells and hybrid β-globin/H-2K genes. In particular, we show the localization of an induction specific regulatory element in the 5′ end promoter and two separate downstream tissue-specific regulatory elements, one element in the 3′ flanking region as well as one in the β-globin gene.

Results

The promoter region

In order to map the 5′ end regulatory region we continued the approach described by Wright *et al.* (1984). The complete (or deleted) 5′ end flanking region of the human β-globin gene from −815 to +50 (*Hpa*I−*Nco*I) was ligated onto the murine H-2K^{bm1} gene at position +9 (*Nru*I) using a *Hin*dIII linker and cloned in the G418 selection vector pTM (Grosveld *et al.*, 1982a) (Figure 1, panel C). We have previously shown that the expression of the H-2K^{bm1} gene does not change upon differentiation of the MEL cells (Wright *et al.*, 1984; Figure 6). The constructs were linearized with *Pvu*I (in the pBR moiety) and introduced into C88 MEL cells

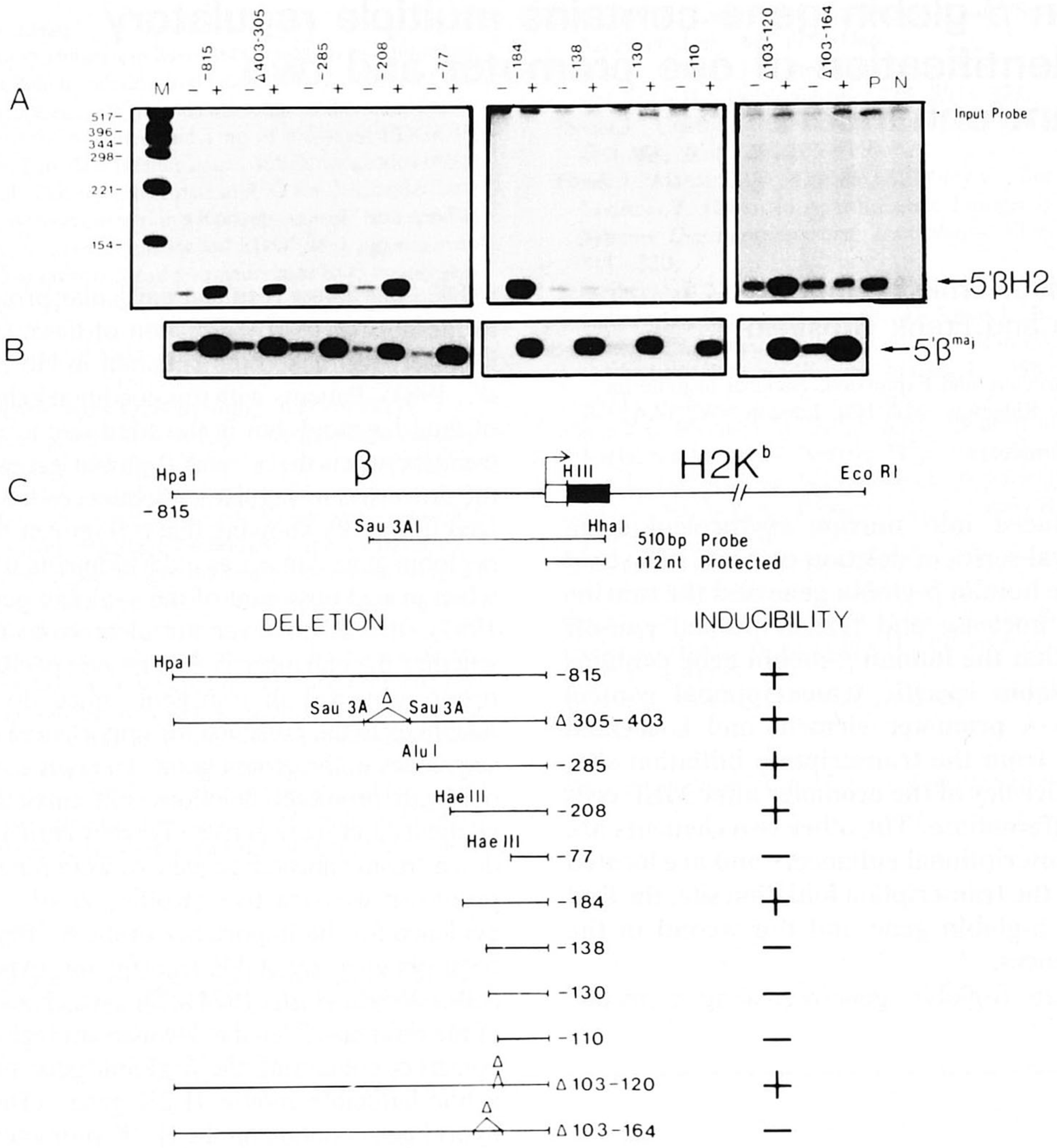

Fig. 1. β-Globin promoter deletions. **Panel A**: the β-globin gene promoter region up to +50 bp (*Nco*I site) and its various deletion mutants (see panel C) were linked to the H-2K^b gene at its *Nru*I site (+9) via a *Hin*dIII linker. These constructs were transfected into C88 MEL cells by electroporation (Smithies *et al.*, 1985). At least three populations were isolated after selection in G418 and analysed before and after induction in 2% DMSO for 4 days. 50 μg of RNA from each population was hybridized to a 512 bp βH2 probe from the 5′ end of the gene (panel C). Correctly initiated RNA protects a 112 nucleotide fragment from degradation by S1 nuclease (5′βH2). The numbers indicate the position of the 5′ deletion, − and + are RNA samples before and after induction. The marker was pBR322 × *Hin*fI. The positive (P) and negative (N) controls are a transient expression of the (−815) β-H2K^b gene driven by pBSV (Grosveld *et al.*, 1982b) in HeLa cells and RNA from untransfected MEL cells respectively. **Panel B**: shows the expression levels of the endogenous mouse β major gene (β maj) in 5 μg RNA samples before and after induction, as determined by S1 nuclease analysis using the 5′ half of the second exon (Kollias *et al.*, 1986). **Panel C**: a schematic representation of the β-globin deletion constructs.

by electroporation (Smithies *et al.*, 1985). Each experiment was carried out as a minimum of three independent populations, which were selected and expanded in G418 containing medium. Each population was split and half was induced to differentiate for 3−4 days by the addition of 2% DMSO. mRNA levels were measured before and after differentiation by S1 nuclease protection analysis. A first set of 5′ deletion mutants using existing restriction sites showed that mutants up to −208 were fully inducible, compared with the full promoter construct (Figure 1). Deletion to −77 resulted in low mRNA levels as expected, because the CAAT box sequence and the −100 region are affected (Grosveld *et al.*, 1982b; Dierks *et al.*, 1981). Most importantly, however, the level of mRNA does not change upon differentiation of the cells, suggesting that an additional regulatory region had been deleted between 208 and 77 bp from the β-globin cap site. We therefore constructed a further set of deletions by *Bal*31 digestion from (a linkered *Xba*I site at) −400 (Figure 1, panel C). Analysis of this set shows that inducibility after differentiation is lost between position −184 and −138 (Figure 1, panel A). To obtain a 3′ border on this region, a series of deletions was created by *Bal*31 deletion in the opposite direction, creating internal deletions (*Cla*I linkered) in the promoter (Figure 1, panel C). The first two of these −103 to −120 (inducible) and −103 to −164 (non-inducible) immediately define the inducible sequence as between −120 and −164 (Figure 1, panel A). When the 5′ and 3′ deletions are combined it is clear that the regulatory sequence is located over or between positions −164 to −138. A sequence comparison of this region with other globin genes reveals one very strong consensus sequence only found in the adult β-globin genes (Figure 2).

The downstream region

From our previous results using the rabbit β-globin gene (Wright *et al.*, 1984) we had determined that at least some

human β	C T C C T A A G C C A G T G C C
mouse β^{maj}	G T C C T A A G C C A G T G A G
goat β^{A}	G T C T A A A G T C A G T G C C
rabbit $\beta 1$	C T C C T A A G C C A T T G C C

Fig. 2. Comparison of adult β-globin promoter sequences. The underlined nucleotides are mismatches with the human sequence.

inducible sequences might be located between −58 and the *Bgl*II site at 380 bp downstream from the rabbit gene poly(A) addition site. Because the human inducible promoter element is at −160 and because the rabbit and human β-globin genes are very homologous over this entire distance [up to 700 bp past the poly(A) addition site with the exception of the intervening sequences], we concluded that the inducible sequences would very likely be located upstream from the *Pst*I site [poly(A) addition site +569 bp] in the human β-globin gene. On the other hand, the data obtained in transgenic mice indicated that additional regulatory sequences were located outside the *Pst*I fragment (Chada *et al.*, 1985; Townes *et al.*, 1985; Kollias *et al.*, 1986). The suggestion that multiple 3′ regulatory regions might be present also agreed with the data of Groudine *et al.* (1983) who showed that DNase I hypersensitive sites occur both in the third exon of the gene and the 3′ flanking region downstream of *Pst*I. To determine whether the β-globin gene has multiple regulatory regions in the downstream area we used a similar approach as before (Wright *et al.*, 1984). The H-2K^b gene promoter and part of the first exon (up to *Kpn* at +30) which are not induced in MEL cells (Figure 6) were placed upstream of the β-globin gene from the *Nco*I site (+50) and the 3′ flanking region to *Bgl*II [+2000 from the poly(A) addition site, Figure 3, H2β−*Bgl*]. As we had shown previously, the overall level of expression of such constructs is position dependent, but they are clearly inducible (Figure 4A; Wright *et al.*, 1984). A similar control H-2K^b−γ-globin hybrid gene is not induced upon differentiation in these cells (Figure 4B, H2γ). Deletion of the β-globin sequences downstream of the *Bam*HI site at the end of the second exon and replacement with the same region of the γ-globin gene leads to the loss of inducibility (Figure 4B, H2$\beta\gamma$). We therefore conclude that the inducible sequences are not located in the 5′ half of the body of the gene. This is confirmed by deletion of the first intron from the H2β−*Bgl* construct (ΔIVS1) which shows no loss of inducibility. A similar conclusion can be made for the second intron. A construct lacking this intron (ΔIVS2) shows very similar inducibility to the H2β−*Bgl* construct. We therefore (tentatively) concluded that the inducible sequences were located between the end of IVS2 and the downstream *Bgl*II site. To determine whether any regulatory regions were located in the gene and immediate 3′ flanking region, two constructs were tested which had deleted the sequences downstream from *Pst*I or *Mnl*I. This resulted in H2β−*Pst* and H2β−*Mnl* which still contain the 3′ end of the gene plus either +560 or +45 bp of the poly(A) addition site respectively (Figure 3). Both of these constructs are still inducible (Figure 4C, H2β−*Pst* and H2β−*Mnl*) indicating that the regulatory sequences are within the gene. However, since these S1 analyses represent steady state levels of mRNA, it is possible that the increase is caused by a stabilization of the globin RNA sequences rather than an increase in the rate of transcription. We therefore carried out transcriptional run-off experiments using the MEL nuclei carrying the H2β−*Mnl* construct. The result (Figure 5) shows a 4- to 5-fold increase in the signal of labelled RNA hybridization to a large intron probe and a 3′ end specific probe from the human β-globin gene (β^H-BE and 3′ β^H) whereas the control histone H4 and total RNA signals increased to 1.2 and decreased to 0.8 respectively. The steady state levels of human globin mRNA from the same cells were increased by the same amount (not shown). This suggests that the rise in H2β mRNA accumulation is almost totally due to an increase in the rate of transcription. The mouse run-off signal, on the other hand, increased (15- to 20-fold) while its steady state level rose more than 40-fold, indicating that the 5′ end of globin mRNA is involved in its stabilization. Because the precursor mRNA levels are increased significantly (β^H-BE) we conclude that the H2β−*Mnl* gene is transcriptionally induced and that the body of the gene contains a transcriptional enhancer capable of acting on a heterologous promoter (H-2K^b) in erythroid cells. This is confirmed by the fact that the control vector (marked pBR) also shows an increase, resulting from a rise in tk−neo vector transcription.

To determine whether there is indeed a second regulatory region downstream from the *Pst*I site we cloned the *Acc*I−*Xba*I fragment spanning this region in two orientations upstream of the H-2K^{bm1} gene (Figure 6). The fragment was placed 2000 bp from the cap site, i.e. at a similar distance as it normally occurs downstream of the β-globin cap site. Stable transformation with these constructs and induction of the MEL cells leads to an increase in the levels of H-2K^{bm1} RNA (Figure 6). Because this can only be due to an increase

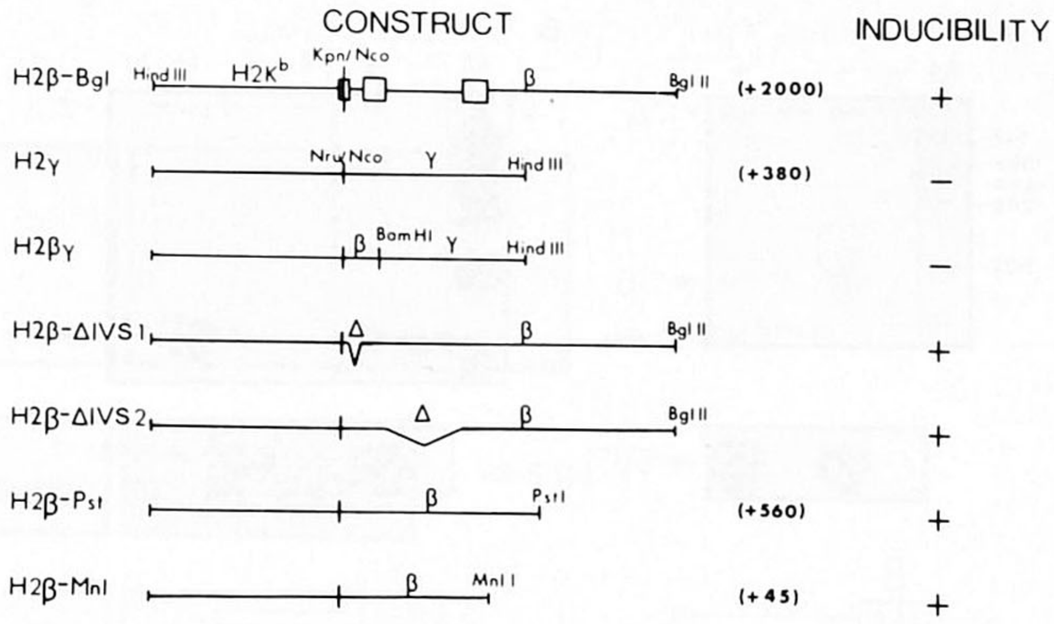

Fig. 3. H-2K^b promoter β-globin hybrid gene constructs. The construct H2β−*Bgl* has been previously described (Wright *et al.*, 1984). It consists of the H-2K^b promoter up to the *Kpn*I site at +30 linked to the *Nco*I site at +50 within the first exon of the human β-globin gene. H2β−*Bgl* formed the starting point for the other constructs as follows: (i) removal of either of the two β-globin introns gave the variants ΔIVS1 and ΔIVS2; (ii) replacement of the β-globin sequences downstream of the second exon *Bam*HI site with the equivalent human γ-globin region gave rise to H2$\beta\gamma$; deletion of the 3′ flanking sequences to the first *Pst*I and *Mnl*I sites past the poly(A) addition site gave H2β−*Pst* and H2β−*Mnl* respectively. The H2γ construct consisted of the H2 promoter to the *Nru*I site (at +9) linked to the *Nco*I site (at +50) of the human γ-globin gene via a *Hin*dIII linker. The H2β−*Bgl*, −ΔIVS1 and −ΔIVS2 constructs were cloned within a plasmid vector possessing the gene conferring resistance to hygromycin (see Materials and methods). All other constructs were cloned in the G418 resistance cosmid vector pTM (Grosveld *et al.*, 1982a). The numbers in brackets refer to the number of base pairs of 3′ flanking sequences which are present past the poly(A) addition site. The boxes in H2β−*Bgl* mark the positions of the exons which are present in the same positions in all the other constructs. The inducibility of the constructs in MEL cells is deduced from the experiments illustrated in Figure 4.

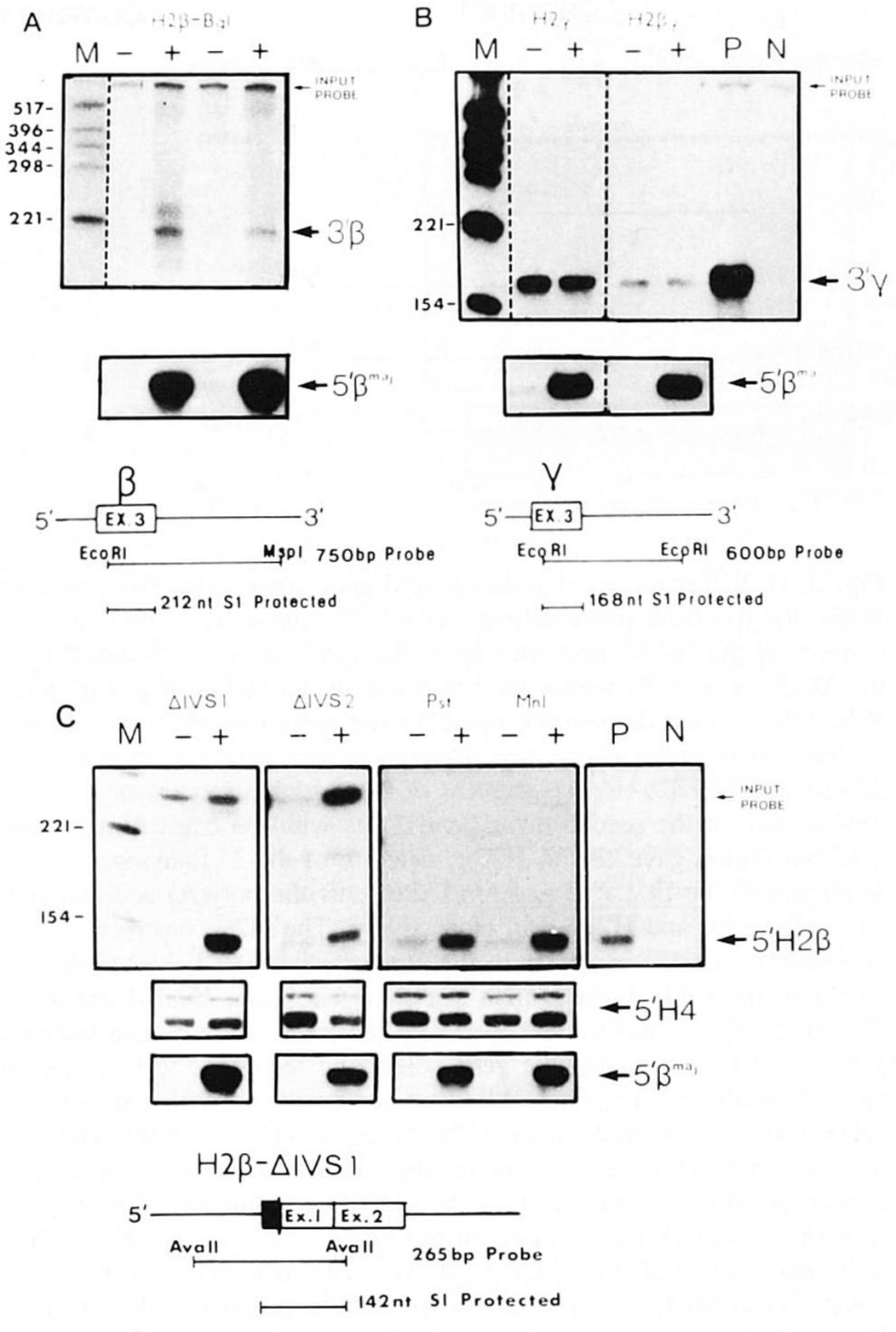

Fig. 4. Analysis of H2β hybrid genes in MEL cells. The H2K^b promoter globin hybrid genes illustrated in Figure 3 were transfected into C88 MEL cells by electroporation, selected for drug resistance and induced to differentiate as described in Materials and methods. **Panels A** and **B**: 50 μg of RNA from populations of cells before (−) and after (+) differentiation was analysed by S1 nuclease protection using 3′β or 3′γ end-labelled DNA probes. The positive control RNAs (P) were from the MEL-human hybrid cell line Hu-11 (Zavodny *et al.*, 1983) or K562 (Anderson *et al.*, 1979). Negative (N) control RNA is from untransfected C88 MEL cells. The marker (M) is end-labelled DNA from a *Hin*fI digest of pBR322. The extent of differentiation of each transfected population of MEL cells was monitored by S1 protection analysis of β^{maj}-globin gene expression as described in Figure 1. **Panel C**: S1 protection analysis on 50 μg of RNA was carried out as described in panels A and B, but using a 5′H2β probe. In addition, one internal control was included by probing for the non-inducible endogenous transcript of the histone H4 gene. These samples were run on a separate gel to avoid co-migration of cross-hybridizing partially protected fragments of the H4 probe. The probe for H4 was a 460 bp 5′ *Eco*RI−*Tth*III I fragment, which gives S1 protected fragments of 240 and 180 nucleotides (Rosenthal *et al.*, 1985).

in the rate of transcription, we conclude that this fragment also contains an enhancer fragment which can act on a heterologous promoter in an orientation independent manner. To determine whether this fragment is also capable of enhancing H-2K expression in non-erythroid cells, we carried out transient transformations of HeLa cells with these same constructs. Figure 7 shows little or no difference in the levels of mRNA between the β-globin enhancer containing constructs and the control. Each of the pools of transfectants contains the same amount of the correctly initiated transcript after correction for the control human β-globin signal. The unusually high signal at the 150 nucleotide position is due

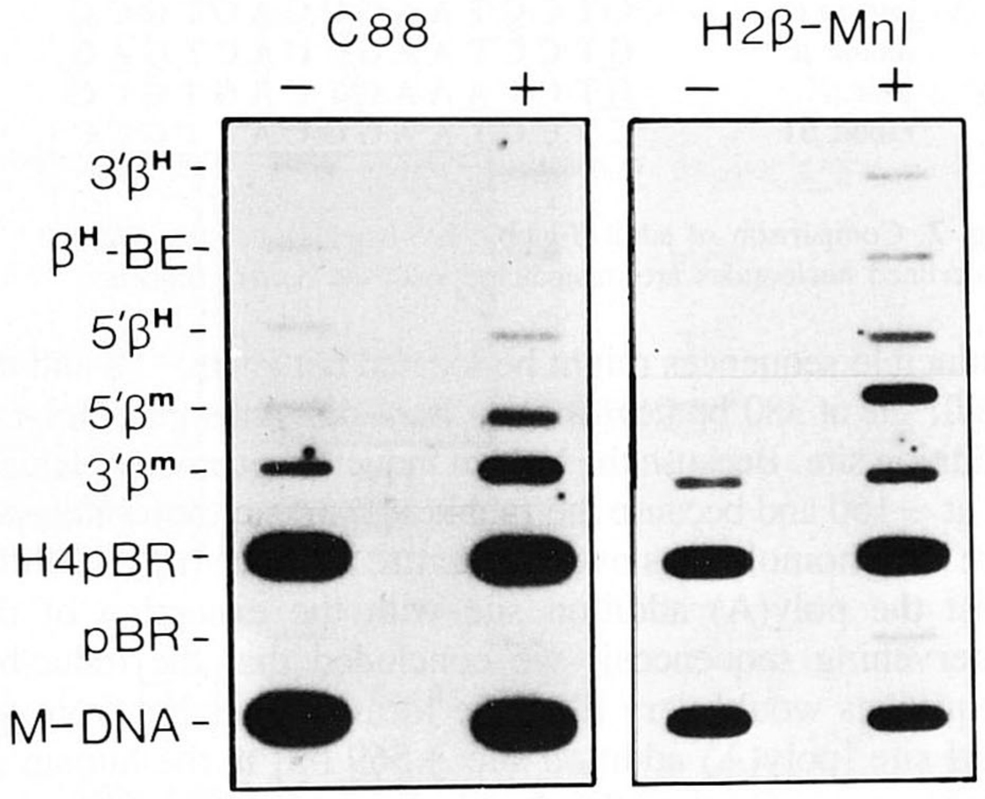

Fig. 5. Nuclear 'run-off' analysis of H2β−*Mnl*. Nuclei were isolated from uninduced (−) and 3-day induced (+) C88 MEL cells and used in a nuclear 'run-off' transcription assay (see Materials and methods). The ^{32}P-labelled RNA from the nuclei was hybridized to identical strips of nitrocellulose filter paper possessing the following denatured DNA probes: 3′β^H, human β-globin *Eco*RI−*Pst*I fragment from the third exon; β^H-BE human β-globin *Bam*HI−*Eco*RI fragment spanning IVS2; 5′β^H, human β-globin *Nco*I−*Bam*HI fragment covering a region from the middle of exon I to the end of exon II (including IVS1); 5′β^M, mouse β^{maj}-globin *Hin*dIII−*Bam*HI fragment which includes the 5′ half of the gene; 3′β^m including the 3′ half of the B^{maj} gene; H4, mouse histone H4 gene cloned in pBR322 and digested with *Eco*RI; pBR, pBR322 DNA and m-DNA, total mouse DNA. The panel on the left (C88) shows the result with nuclei obtained from untransfected C88 MEL cells. The right hand panel (H2β−*Mnl*) shows the result from a population of cells transfected with the H2β−*Mnl* hybrid gene (Figure 4C). The β^H-BE and 3′β^H probes are specific for human β-globin sequences. 5′β^H shows extensive cross-hybridization with mouse β-globin RNA. The 5′β^{maj} probe shows a 16-fold increase in β^{maj}-globin gene transcription. The histone H4 probe and total mouse DNA act as control sequences which do not alter or slightly decrease upon MEL differentiation. It should be noted that the transfected cells contain a low level of pBR transcripts which increases after induction.

to renatured (high GC) input probe (see lanes N and P). Since the levels of transcription are not significantly different, we conclude that this enhancer acts in a tissue-specific (erythroid) manner.

Discussion

In this paper we describe the use of MEL cells to assess the functional role of the promoter, internal and 3′ flanking sequences of the human β-globin gene. Each of these regions was combined with the complementary region of the murine H-2K^b gene. The results show that each of these regions contains a sequence element that increases the rate of transcription after the induction of (erythroid) differentiation of the MEL cells.

The β-globin gene promoter has previously been shown to contain a number of separate promoter elements (Grosveld *et al.*, 1982b; Dierks *et al.*, 1983). Of these the TATA and CAAT box elements have been studied in detail (for a number of genes) and proved to be general transcription elements. The −100 CAC-imperfect repeat sequence has been studied in less detail. The results obtained in this study indicate that these CAC-imperfect repeat sequences around −100 are not involved in the increase of globin gene transcription after induction, although it is interesting to note

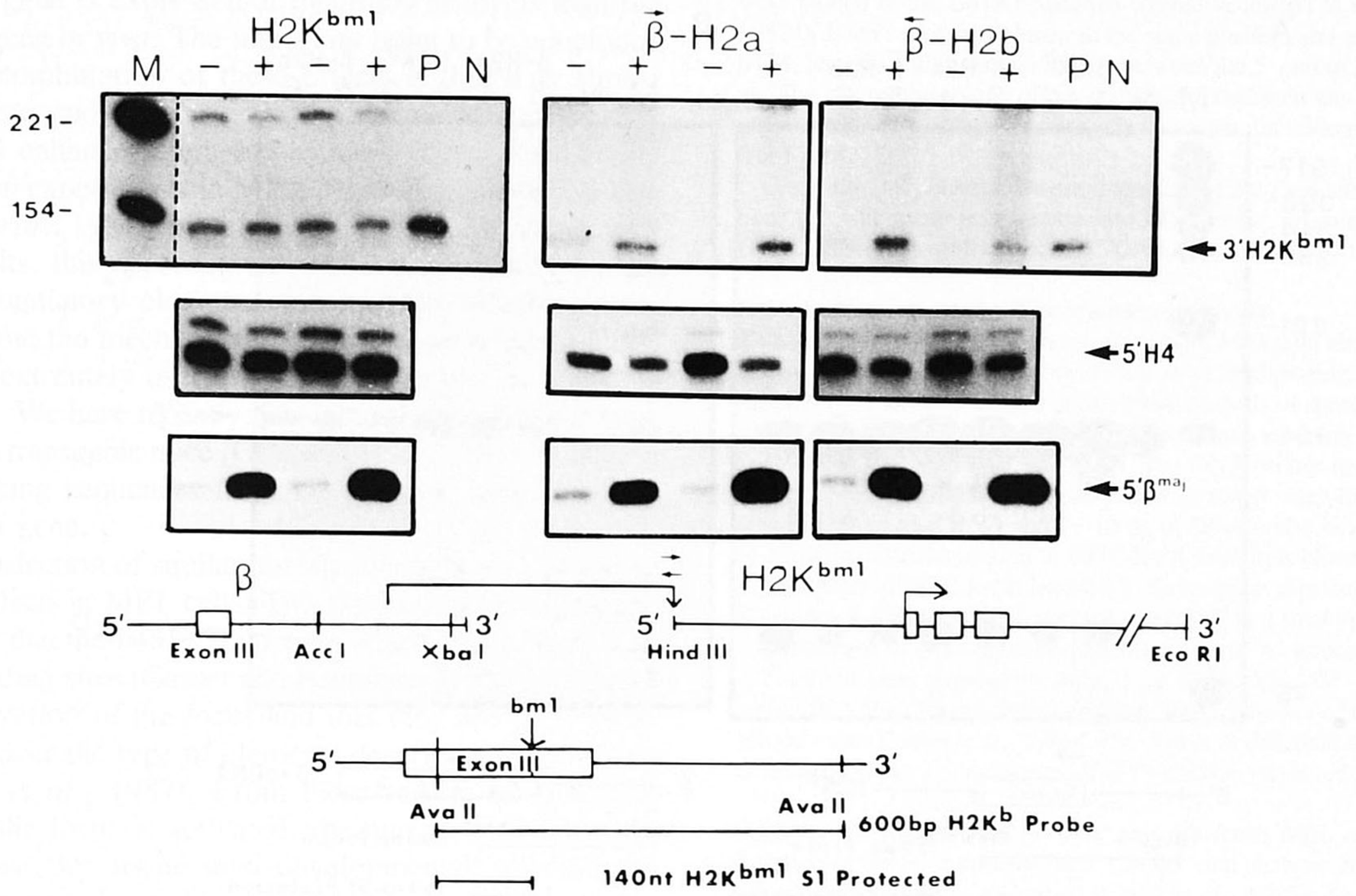

Fig. 6. Analysis of the human β-globin 3′ flanking region for enhancer function. A 1210 bp *Acc*I−*Xba*I fragment from the 3′ flanking region of human β-globin [β, +466 to +1680, past poly(A) addition], was cloned in either orientation (β-H2a and β-H2b) at a *Hin*dIII site at −2000 of the mouse $H2K^{bm1}$ gene (middle panel). These constructs, in addition to unmodified $H2K^{bm1}$, were used to transfect C88 MEL cells as described (see Materials and methods). The plasmid vector employed in all cases was the G418 resistance cosmid pTM. Drug selected populations were induced to differentiate for 4 days. Analysis of RNA (50 μg) from cells before (−) and after (+) differentiation was by S1 nuclease protection assay. The 3′ end-labelled probe employed was a 600 bp fragment from the third exon−fourth intron of $H2K^b$, with hybridization at 52°C and S1 nuclease digestion at 37°C for 2 h. This gives a 140 nucleotide S1 nuclease protected fragment upon hybridization due to mismatch at the site of the bm1 polymorphism present in the transfected gene (Wright *et al.*, 1984). These transcripts are initiated at the same position as the endogenous $H\text{-}2K^b$ gene as determined by a 5′ end protection analysis (not shown). The positive (P) control is RNA from K562 cells stably transfected with the mouse $H2K^{bm1}$ gene. The negative (N) control is RNA from untransfected C88 MEL cells. The inset shows S1 protection analysis of mouse histone H4 and β^{maj}-globin gene expression as a measure of MEL cell induction as described in Figure 4. The marker (M) is pBR322 × *Hin*fI as in the earlier figures.

that one complete and one partial repeat of the CCACACC are present in the third exon. The newly identified −160 promoter element however (Figure 2) is necessary for the induction of transcription upon differentiation. It is clear that this element acts as a positive transcription (promoter) element, rather than by the release of repression upon differentiation, because deletion of this sequence does not lead to an increase of transcription before induction. It should be noted that the γ-globin promoter is also inducible in C88 MEL cells (data not shown; N.P.Anagnou, personal communication), although this must take place via a different element or mechanism for two reasons. First, the γ-globin gene does not contain the conserved β-globin promoter sequence (Figure 2) and secondly, the γ-globin promoter is not inducible in other MEL cells (Wright *et al.*, 1984; N.P.Anagnou, personal communication). The latter indicates differences in MEL cell lines, possibly resulting in a lack of suppression of the γ-globin gene promoter in some MEL cells, which show high pre-induction levels of γ-globin mRNA (Wright *et al.*, 1984; M.Antoniou, unpublished data). It is interesting to note that the conserved sequence from −160 to −147 (Figure 2) in the β-globin promoter is immediately preceded and partially overlapped by the sequence TGAAGTCCAACTCCT (−171 to −157) (Figure 8) which is also present in both the downstream elements. A homologous sequence is protected in footprints of the chicken β-globin enhancer (Emerson *et al.*, 1987). There are a number of other homologies between each of the enhancer elements, but only one other sequence is common to all three (Figure 8) and it is interesting to note that the equivalent region of the human γ-globin gene has undergone a number of base changes, despite the fact that these are also protein coding sequences.

It has been shown that the downstream regulatory sequences can act as developmental specific elements to activate erythroid β or γ-globin promoters (Kollias *et al.*, 1987; Behringer *et al.*, 1987). It was, however, not clear whether these enhancers can stimulate a non-erythroid promoter and whether they are erythroid cell specific. Our data show that both enhancers can stimulate the transcription of a heterologous promoter in MEL cells (H2β−*Mnl* and β-H2, Figures 4−6). In addition, no enhancing activity was observed in HeLa cells (Figure 7), indicating that at least the downstream enhancer shows tissue specificity.

It is however, not clear from these results, or those obtained in mice (Kollias *et al.*, 1987), whether the enhancer activates a homologous erythroid promoter more efficiently than a heterologous promoter (H2K). This is caused by the fact that the quantitations of stable transfection experiments cannot be compared. Each population of cells consists of a fairly small number of individual clones (approximately ten) and as a result the levels of expression are subject to

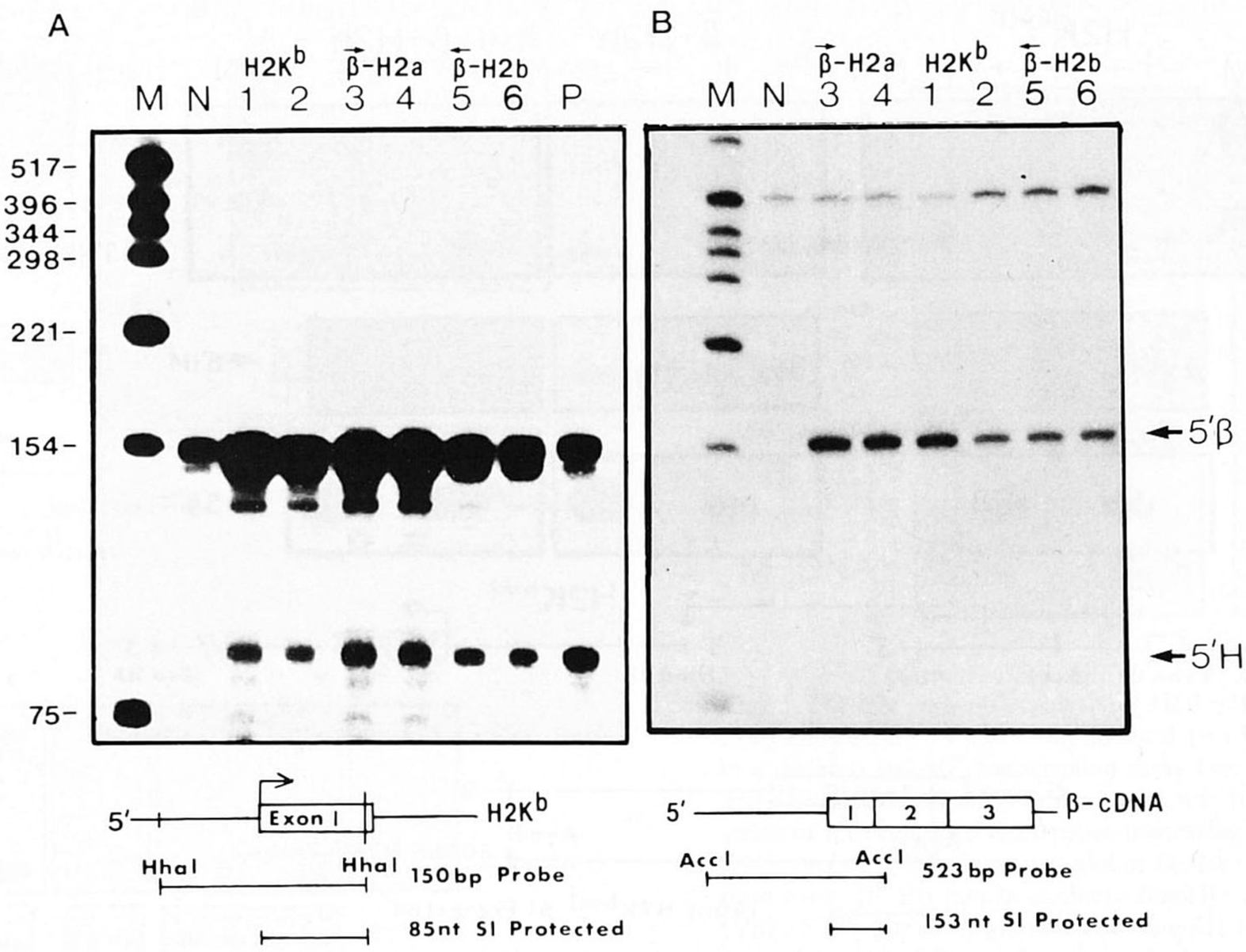

Fig. 7. Analysis of β-globin enhancer function in HeLa cells. The constructs described in Figure 6 ($H2K^{bm1}$, β-H2a and β-H2b), consisting of the human β-globin 3′ flanking region cloned upstream of the mouse $H2K^{bm1}$ promoter, were used in a transient expression assay in HeLa cells (see Materials and methods), using human βpBSV as a co-transfecting standard. **Panel A**: RNA (50 μg) from duplicate cultures was analysed by S1 nuclease protection. The 5′ end-labelled DNA probe used was a 150 bp *Hha*I fragment from the first exon of the $H2K^b$ gene. This gives an S1-protected fragment of 85 nucleotides. **Panel B**: the efficiency of transfection in each case was monitored by analysis of expression of the co-transfected human β-globin gene (pBSV) using a 5′ β probe. The hybridizations with the 5′ β probe contained 5 μg RNA and were run on a separate gel because the $H2K^b$ and the β-globin signal co-migrate. The positive (P) control was 25 μg RNA from K562 cells stably transfected with the mouse $H2K^{bm1}$ gene. The negative (N) control was RNA from untransfected HeLa cells. The marker (M) is pBR322 × *Hin*fI as in earlier figures.

position effects which are particularly evident when individual clones or very small populations (circular plasmid electroporation) are studied (not shown). Two types of effects are observed. First, constructs with the β-globin promoter (Figure 1) show position dependent expression levels before induction, but the increase of expression upon differentiation is proportional to that observed for the endogenous mouse gene. Secondly, constructs with the β-globin enhancer elements also show similar position effects before induction but, in addition, the increase upon differentiation also varies when compared with the endogenous mouse β-globin gene (Figures 4−6). The higher the expression of the gene before induction, the lower the amount of induction after differentiation. This is particularly clear in some individual clones or very small populations.

These two (independent) phenomena show several important points about stable transfection experiments in general and the globin regulatory sequences in particular. The different levels of expression before (MEL cell) induction with any of the constructs in different populations is a reflection of the transcriptional activity of the integration site of the incoming gene. This phenomenon has been observed and discussed before and represents a classical position effect (Wright *et al.*, 1984; Smithies *et al.*, 1985). The constant increase (after correction for mouse β-globin induction) observed for promoter constructs shows that the 5′ promoter element at −160 always increases the efficiency of transcription of the promoter by a similar percentage. Saturation of the promoter which would result in a lower increase is never

A	
human β-globin promoter	T G A A G T C C A A C T C C T A A G C C A G
human β-globin 3rd exon	T A A A G T C C A A C T A C T
human β-globin 3′ flanking region	G G A A G T C C C A T T C T C
B	
human β-globin 3rd exon	T G C T G G C C C A
human γ-globin 3rd exon	T T T T G G C A A T
human β-globin 3′ flanking region	T G C T G G C T C C
chicken β-globin 3′ flanking region	T G C T A G C C C A

Fig. 8. Comparison of human β-globin sequences.

observed because the expression is always considerably below that observed *in vivo*. The variable increase observed with the downstream enhancer elements indicates that the enhancer acts to establish an efficient transcription complex, maybe in a similar fashion to the Ig gene enhancer in B cells (Atchison and Perry, 1987). The action of the globin enhancers is most apparent when low efficiency complexes are present before induction. When transcription is already at a high level due to an endogenous enhancer (at the integration position) the additional action of a second enhancer (the β-globin enhancers) is minimized. In addition, the maximal efficiency of the H-2K promoter might be reached, because

the H-2K gene is expressed at much lower levels than the β-globin gene *in vivo*. The important point to be concluded from the combination of these effects is that it is almost impossible to study or observe co-operation between the promoter and enhancer elements in these or previous stable transfection experiments in MEL cells (Wright *et al.*, 1984; Charnay *et al.*, 1984). Nevertheless, as we have shown with these results, this approach can be used to identify the individual regulatory elements and provide valuable information about the mechanism of globin gene regulation and would be extremely useful if the position effects could be eliminated. We have recently demonstrated that this is at least possible in transgenic mice (Grosveld *et al.*, 1987) by linking large flanking sequences from the β-globin gene locus to a β-globin gene.

The transfection of similar constructs might also eliminate position effects in MEL cells. The experiments in transgenic mice show that the border sequences which might be nuclear matrix binding sites (Gasser and Laemmli, 1986) are crucial to the activation of the locus and that they are completely dominant over the type of elements described in this paper (Grosveld *et al.*, 1987). From these results we postulate that once the locus is activated, the human β-globin gene uses at least two tissue (and developmental) specific enhancer sequences in a co-operative fashion to form an active transcription complex at the β-globin gene promoter. The promoter also contains at least one upstream element that specifically increases the efficiency of the promoter in erythroid cells and the combination of upstream and downstream elements is similar to that observed for the Ig gene family (for review, see Calame, 1985). An additional negatively acting promoter element (M.Antoniou, unpublished results) and/or suppressing *trans*-acting factors (Barberis *et al.*, 1987) would suppress the action of the promoter (and prevent leakiness of transcription) in embryonic/fetal erythroid cells.

Materials and methods

Tissue culture and cell transfections

Stocks of the APRT$^-$, tetraploid MEL cell line C88 (Deisseroth and Hendrick, 1975) were maintained in αMEM supplemented with 10% fetal calf serum and 50 μg/ml diaminopurine. Transfection of MEL cells was by electroporation as described by Smithies *et al.* (1985). Routinely 1−3 × 10^8 cells were used per experiment and a concentration of 25 μg/ml of transfecting DNA. Plasmid DNA was linearized before use. The cells were transferred to medium without diaminopurine, but containing either 800 μg/ml G418 or 200 μg/ml hygromycin 2−3 days after electroshock. At the time of electroporation, the cells were divided in order to give rise to at least three independent transfected populations. Populations of transfected cells (0.5−1 × 10^8 cells) were ready for further use 2 weeks after the application of drug selection. At this stage, each population was further sub-divided, with one half maintained in normal selection medium. The other half was induced to differentiate by culturing for 3−4 days in the presence of 2% (v/v) DMSO. Optimal differentiation was obtained with log phase growing cultures at a cell density of 2−5 × 10^5 cells/ml at the time of induction.

HeLa cells were grown in αMEM plus 10% fetal calf serum. Transfection was by DNA−$CaPO_4$ precipitation followed by 'shock' with 15% (v/v) glycerol as described (Parker and Stark, 1979). Cells were harvested 48 h after transfection for transient expression analysis of the introduced genes.

Hybrid gene constructs

The construct H2β−*Bgl* (Wright *et al.*, 1984) and its ΔIVS1 and ΔIVS2 derivatives (Figure 3) were modified to contain the gene conferring resistance to the drug hygromycin. The TK-hygro gene was obtained as a *Sal*I−*Nru*I fragment from pHyg (Sugden *et al.*, 1985) and was cloned downstream of the H2β constructs in a reverse transcriptional orientation with respect to the H2 promoter. All other constructs described in Figures 1, 3 and 6 were cloned in the G418 resistance cosmid vector pTM (Grosveld *et al.*, 1982a). Each series was cloned in the same position and orientation within pTM, namely within the *Eco*RI site between the 5′ end of the TK-neo gene and the *cos* region. None of the constructs possessed any other functional gene or enhancer except for the bacterial ampicillin resistance gene derived from pBR322.

Generally, all constructs were linearized at the *Pvu*I site within the *amp* gene prior to stable transfection into MEL cells. Transient expression in HeLa cells employed supercoiled DNA.

RNA extraction and S1 protection analysis

Total cellular RNA was extracted from both MEL and HeLa cells by lysis in 6 M guanidinium hydrochloride followed by digestion with DNase I as previously described (Wright *et al.*, 1984). Levels of specific RNA species were determined by S1 nuclease protection analysis using ^{32}P end-labelled DNA probes (Antoniou *et al.*, 1986). The DNA probes used are illustrated in the appropriate figures. Unless otherwise stated, each hybridization consisted of 50 μg total RNA and 5−10 ng of DNA probe in a reaction volume of 20 μl. After denaturation at 90°C for 5 min, hybridization was allowed to take place at 52°C for at least 16 h. Subsequent digestion with 100 units S1 nuclease (Boehringer) was for 2 h at 25°C in a final volume of 270 μl.

In the case of MEL cells, differentiation was monitored by analysis of β^{maj}-globin gene expression using 5 μg RNA. The β^{maj} probe used was a *Hin*dIII−*Nco*I fragment that protects 90 nucleotides of the 5′ half of the second exon (Kollias *et al.*, 1986). The degree of differentiation was assumed to be acceptable if the amount of β^{maj} mRNA increased at least 20-fold.

Isolation of nuclei and nuclear run-off from MEL cells

Isolation of nuclei, transcriptional 'run-off' and analysis of the ^{32}P-labelled RNA products was as described (Linial *et al.*, 1985). Each reaction contained 10^8 nuclei from uninduced and 3-day induced MEL cells and 400 μCi [^{32}P]UTP (NEN). Identical nitrocellulose filters possessing 1−5 μg of immobilized DNA probes were prepared using a 'slot-blot' manifold (S and S).

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