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β-Globin gene promoter generates 5' truncated transcripts in the embryonic/fetal erythroid environment

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ABSTRACT

We report here the localisation of sequences responsible for the faulty expression of human β-globin gene in Putko and K562 cells. Complete β-globin gene introduced into these cells produces transcripts with abnormal 5' ends, while cotransfected mouse H2 gene is expressed correctly. Using hybrid constructs of these two genes we demonstrate that aberrant activity is conferred by sequences 5' of the β-globin gene. Thus β-globin promoter attached to the H2 coding sequence produces H2 transcripts with truncated 5' ends. By introducing a series of deletions in the β-globin promoter we restrict these sequences to the -77 / +28 base pair region spanning the CAAT element to the translation initiation site. These results are consistent with the lack of recognition of the β-globin gene major cap site in Putko and K562 cells. We suggest that inactivity of the adult globin gene in the embryonic/fetal environment is at least in part conferred by sequences within the β-globin gene promoter.

INTRODUCTION

Globin genes become activated in the later stages of erythroid differentiation during transition of the proerythroblast to erythroblast. In humans three different types of β-like genes (ε, γ, β) are clustered together in the short arm of chromosome 11 in the sequencial order of their activation (for review see 1). Regulation of expression of these genes is largely determined by interaction of transacting factors with sequences within or flanking the genes (2,3,4,5,6,7).

Introduction of cloned β-globin gene into mammalian cell lines has revealed three distinct upstream sequences required for efficient transcription from the cap site of this gene: the ATA, CCAAT and CCA/TCACCCT elements located at -30, -75 and -90 base pairs from the mRNA capping site (8). These sequences which are responsible for constitutive expression are also present in the promoters of the embryonic and foetal globin genes. The mouse erythroleukemic cell line (MEL)(9) has been used as a model system to investigate developmental specific regulation of the adult globin gene expression. Studies with cloned hybrid human genes in this system have identified at least two additional controlling ele-

ments. One is located 5' to the gene and the other downstream from the mRNA capping site. It has been suggested that negative or positive interaction of *trans*-acting factors with these elements is responsible for the developmental regulation of the β-globin gene. Interestingly the upstream element responds to induction when attached 5' to the mouse H2 gene but not when 5' to the human α-globin gene (10,11). B

A complementary system to the MEL cells is provided by the K562 cell line and its derivatives Putko and Sutko (13). These lines in contrast to MEL cells express the embryonic and foetal but not the adult globin genes (14,15, 16). We showed earlier that the differential expression of the globin genes is regulated in *trans* in K562 cells. We also showed that aberrant β-globin transcripts can be detected in tranfected cells, but were unable to define the 5' end and therefore the origin of these transcripts (17). Other independant studies have reported complete absence of β-globin transcripts in K562 cells transfected with cloned β-globin genes (18). We report here that β-globin transcripts in Putko cells have shorter 5' ends than expected. Using hybrid constructs with the mouse H2 gene attached to the human β-globin promoter we show that upstream regulatory elements are responsible for the abnormal transcription. Sequential truncation of the upstream β-globin sequences limited these sequences to a region of 77 base pairs overlapping the constitutive promoter.

MATERIALS AND METHODS

Transfection of cells

Putko cells (13) were maintained in RPMI medium supplemented by 10% fetal calf serum. Cells were transfected with electroporation. 10^7 cells were resuspended in 0.5 ml PBS together with 25 μg DNA tagged with AGPT gene which confers resistance to the antibiotic G418. The mixture was placed in a Sarsted disposable cuvette 0.5x1.0 cm, which had been adapted with two 1.0 cm aluminium electrodes. The cells were subjected two electric shocks at 2 KV, using an ISCO electrophoresis power supply (Model 494). Shocked cells were kept at 4°C for 30 minutes and then plated out in the normal growth medium. 24 hours after transfection G418 selection was applied at concentrations of 500 μg/ml of medium. Resistant cells were plated in soft agar (0.3%) in the same medium. Individual clones were picked and grown further in the presence of G418.

K562 cells (12) were maintained in αMEM medium supplemented with 10% fetal calf serum. Cells were transfected using CaPO4 as described before (10). Individual G418 resistant clones were picked from 0.3% agar and grown further in the presence of G418.

Purification of RNA

10^7 cells were lysed with 1.0 ml 6 M guanidium chloride and phenol extracted. Nucleic

acid was ethanol precipitated, dissolved in 10 mM Tris (pH 7.5), 10 mM MgCl, 50 mM NaCl, and treated with RNase-free DNase. RNA was finally phenol extracted and ethanol precipitated.

For the preparation of poly A RNA, 10^7 cells were washed with PBS and resuspended in 2.5 ml of 100 mM NaCl, 20 mM Tris-Cl (pH 7.4), and 10mM EDTA. Proteinase K was added to 200 mg/ml, and SDS to 0.5%. The mixture was homogenised with a Sorvall omnimixer at 3/4 full speed for 30 seconds. The solution was then incubated at 37°C for 20 minutes. 0.5 M NaCl and 50 mg oligo (dT) cellulose was added and the slurry was shaken for 6 hours a room temperature. The oligo (dT) was washed with 100 mM NaCl, 10 mM Tris-Cl (pH 7.4), 1 mM EDTA and 0.2% SDS. Poly A RNA was eluted with 1 mM Tris-Cl (pH 7.8), 1 mM EDTA and 0.2% SDS. 20 μg carrier yeast RNA was added and the total was ethanol precipitated.

Northern blotting

Poly A RNA from 10^7 cells was resuspended in 2 mM MOPS (pH 7.0), 0.2 mM EDTA, 0.5 mM NaAc, 50% Formamide, 7.4% Formaldehyde heated to 60° C for 5 minutes, immediately chilled on ice and loaded onto a 0.7% agarose gel containing 20 mM MOPS (pH 7.0), 1 mM EDTA, 5 mM NaAc and 2.2 M formaldehyde. Electrophoresis buffer was of the same composition. After electrophoresis the RNA was blotted onto nitrocellulose and hybridised with nick translated DNA probe.

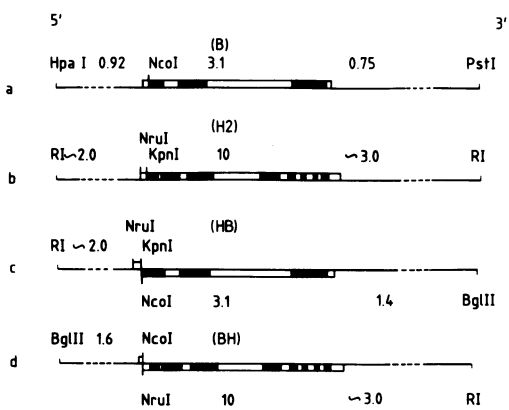


Figure 1: Structure of the human β-globin, mouse H2Kbm1 gene and hybrids of these two genes. The construction of the hybrid genes was described in (10). Filled boxes represent exons, and open boxes introns. Sizes in kb of the flanking genomic sequences and of the genes are shown. a)β-globin gene b)H2Kbm1 gene c)H2β hybrid gene d) βH2 hybrid gene.

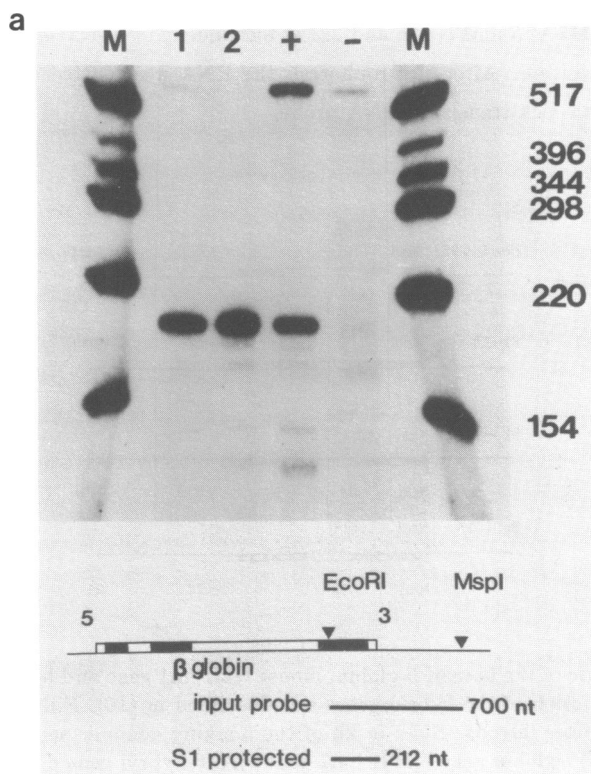
S1 nuclease analysis

Levels of mRNA were measured by S1 muclease analysis as described before (10). DNA probes were end-labeled using T4 polynucleotide kinase or reverse transcriptase to a specific activity of greater than 10^7 cpm/μg. Labeled probe was hybridized to 10-50 μg samples of total cellular RNA in 30 μl 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% recrystallized formamide for 8-12 hours. Hybridization temperature was 52°C. After hybridization, samples were digested for 2 hours with 3000 units S1 nuclease (Boehringer) in 300 μl 200 mM Nacl, 30 mM NaAc (pH 4.8), 2 mM ZnSO4 at 20°C. S1 protected DNA was ethanol precipitated and electrophoresed on a 7 M urea 7% acrylamide gel.

RESULTS

Human β – globin gene introduced into Putko and K562 cells is expressed aberrantly.

We studied the expression of the human β-globin gene introduced into Putko and K562 cells. The former was a 3.1 kb HpaI-PstI fragment containing the entire human β



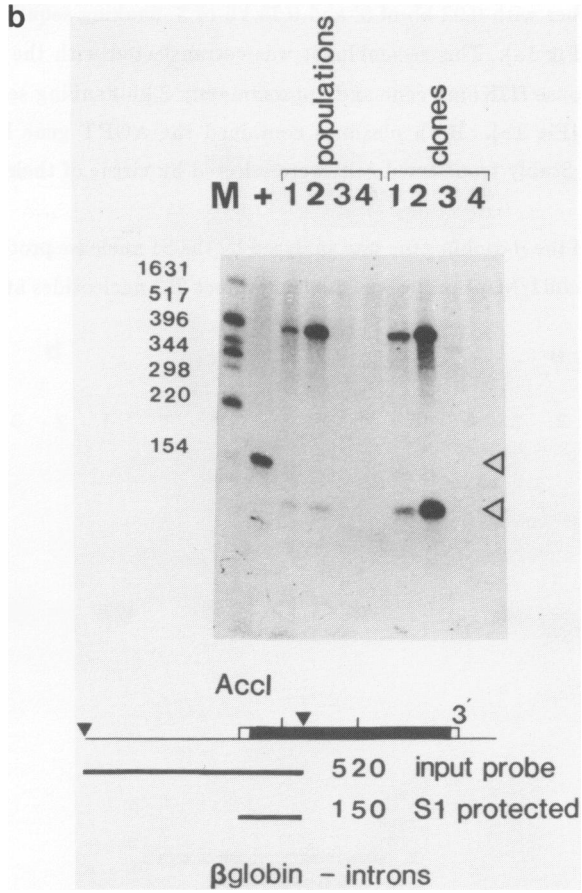


Figure 2 : S1 nuclease protection analysis of human β-globin aberrant transcripts in transfected Putko cells.
a) Expanded clones of transfected Putko cells expressing β-globin were harvested in late log phase of growth. Poly A RNA corresponding to $2x10^7$ cells was analysed by S1 nuclease protection assay using the illustrated 3' end labelled probe. Lanes 1 and 2 are two isolates of Putko cells expressing β-globin. 0.1 ng of human sickle polyA RNA was used for positive control (+). RNA from untransfected Putko cells was used for negative control (-). End labeled PBR322 DNA predigested with HinfI was used as molecular weight markers (M).
b) 40 μg total cellular Putko RNA was analysed by S1 nuclease protection assay using the illustrated 5' end labelled probe. Lanes 1 and 2 correspond to those in Figure 2a. Lanes marked 3 and 4 correspond to RNA from Putko cells that contain transfected copies of β-globin gene but do not show any detectable expression with the 3' end labelled probe . 0.1 ng of human sickle polyA RNA was used for positive control (+). Lane (M) is molecular weight markers. Arrows point to S1 protected fragments resulting from the hybridization of probe to the 5' end of control β-globin RNA and β transcripts in Putko or K562 cells. The structure of an intron-less β-globin gene used for the isolation of S1 probes is shown.

globin gene together with 0.92 kb of 5' and 0.75 kb of 3' flanking sequences cloned in the βPTM plasmid (Fig 1a). This recombinant was cotransfected with the H2PTM plasmid containing the mouse H2Kbm1 gene and approximately 2 kb flanking sequences on either end of the gene (Fig 1b). Both plasmids contained the AGPT gene linked to the TK promoter (10,19) Stably transfected cells were selected by virtue of their resistance to the antibiotic G418.

Expression of the β-globin gene was analysed by the S1 nuclease protection assay. A 3' labelled 700 bp EcoRI/MspI probe was used to protect 212 nucleotides at the 3' end of the

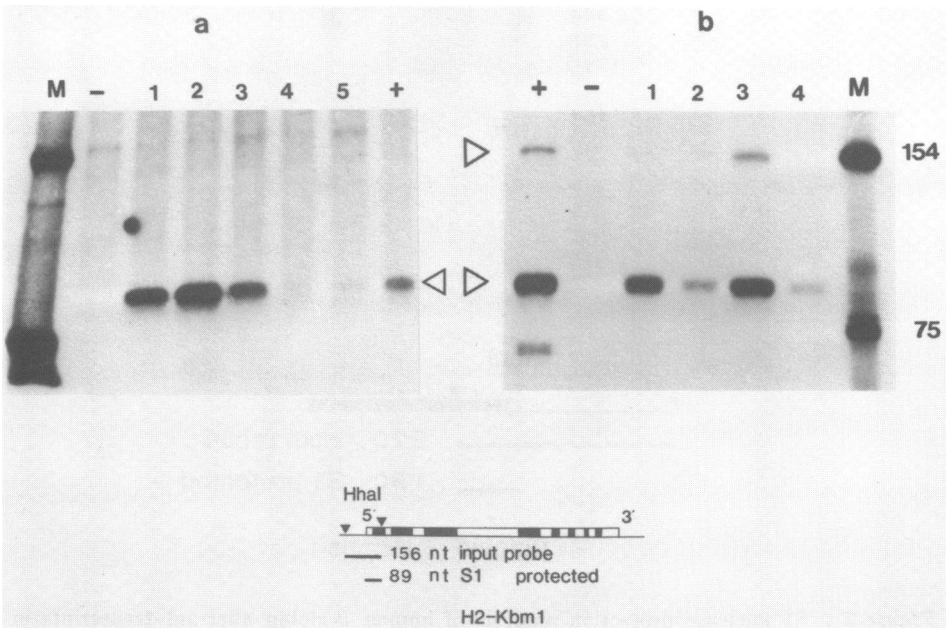


Figure 3 : S1 nuclease analysis of mouse H2Kbm1 gene transcripts in transfected human Putko or K562 cells.
a) Putko populations were cotransfected with cloned mouse H2Kbm1 gene and human β-globin gene. 10 μg samples of total cellular RNA from the transfected populations (lanes 1-5) were analysed by S1 nuclease protection assay using the 5' end labeled mouse H2Kbm1 probe illustrated. 5 μg of total cellular RNA from 16 day old C57 BL/10 mouse embryos was used as positive control (+). 10 μg of RNA from untransfected Putko cells was used for negative control (-). The arrow points to S1 protected fragments. Lane (M) is molecular weight markers.
b) K562 cells were transfected with cloned mouse H2Kbm1 gene. 10 μg samples of total cellular RNA was analysed by S1 protection assay as above. RNA from untransfected K562 cells was used for negative control (-). Positive control (+) and markers are as above. Arrows point to S1 protected fragments resulting from self hybridization of input probe and from hybridization of probe to the complementary 5' end of H2 RNA respectively.

β-globin mRNA while the entire first and 10 bp of the second exons were detected by a 5' labelled 520 bp AccI-AccI probe. Expression of the β-globin gene was detected in only two from a total of twenty independently transfected G418 resistent Putko populations. These were seeded in soft agar in order to obtain individual clones. Fig 2a shows 3' S1 analysis of the β-globin transcripts in the isolated clones. The level of β-globin mRNA in the expressing Putko clones was 0.5-1 transcript per cell as compared to the human sickle cell RNA control. Surprisingly clones from both the β-globin expressing populations showed 5' protected fragments 30-40bp shorter than expected, suggesting abnormal initiation of transcription (Fig 2b). Consistent with this were the results obtained using the 460bp RsaI-BamHI probe which protected fragments shorter than the expected 340bp at the 5' of the β-globin mRNA (results not shown). Southern blot analysis showed that both expressing clones contained the intact 5' flanking and coding regions of the introduced β-globin gene (results not shown). In addition no β-globin transcripts were detected in 10 independent K562 populations transfected with the human β-globin gene.

In contrast to β-globin the cotransfected mouse H2Kbm1 gene was normally expressed in all the G418 resistant populations analysed. This was revealed by S1 nuclease analysis using a 5' labelled 156bp HhaI-HhaI probe, that produced an 89 bp protected fragment (Fig 3). RNA from untransfected Putko or K562 cells does not protect this probe. The H2 Kbm1 gene is a mouse class I major histocompatibility antigen and is expressed in a non-tissue specific manner.

Our results suggest that the human β-globin gene promoter is inactive in Putko and K562 cells. Low level expression of this gene in Putko cells is due to aberrant transcription initiation events. The mouse H2Kbm1 gene is accurately transcribed in both cell lines.

Expression of hybrid genes containing the H2Kbm1 promoter and human β – globin coding sequences in Putko and K562 cells.

In order to localize the sequences responsible for inactivity of the β-globin gene, we used hybrid constructs containing the H2Kbm1 promoter joined to the human β-globin coding and 3' flanking sequences (Fig 1c) (10). This construct was stably introduced in Putko and K562 cells by electroporation. Transcripts were analysed using a 280 bp AvaII-AvaII 5' labelled probe. Two out of five Putko and four out of ten K562 transfected populations expressed the Hβ hybrid gene. Expression of this construct was accurate in both Putko and K562 cells (Fig 4). Thus the β-globin coding or 3' sequences are not responsible for the observed aberrant transcription. This suggests that sequences 5' of the β-globin gene prevent normal initiation of transcription in these cells. Our results can not eliminate the possible existence of independent down regulatory elements 3' of the β globin gene cap site that do not interfere with correct initiation of transcription.

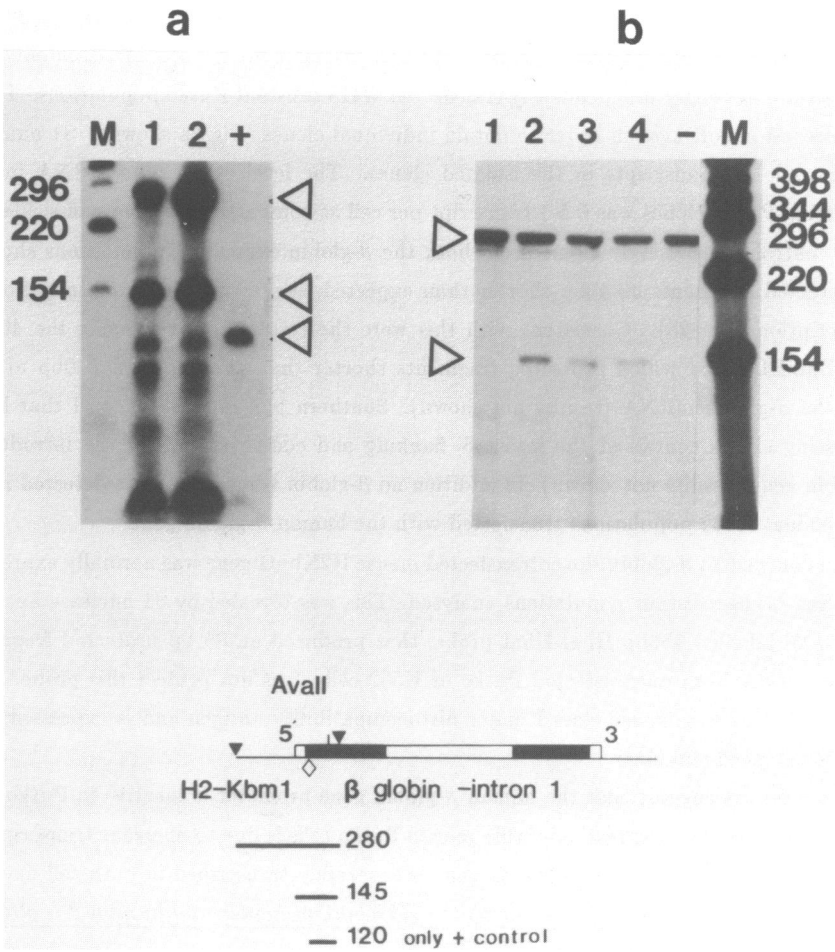


Figure 4 : S1 nuclease protection analysis of hybrid mouse H2 human β-globin gene transcripts in transfected Putko or K562 cells.

a) Expanded clones of transfected Putko cells expressing Hβ hybrid transcripts were harvested . 30 μg samples of total cellular RNA was analysed by S1 nuclease protection assay using the 5' end labeled probe illustrated. 0.1 ng of poly A human sickle RNA was used as positive control (+). Only 120 bp of the probe is protected by the sickle RNA which lacks the 5'H2 sequences. The expected protected fragment for normal Hβ transcripts is 145 bp. Arrows point to the S1 protected fragments resulting from protection of the probe by self hybridization or by hybridization to RNA respectively.

b) RNA from K562 populations transfected with hybrid Hβ gene was analysed by S1 nuclease protection assay. RNA from untransfected K562 cells was used for negative control (-). Arrows point to input probe and the RNA protected probe fragment. The structure of an Hβ hybrid gene lacking the first intron, used for preparing S1 probes is shown. The diamond symbol shows the junction of the two genes.

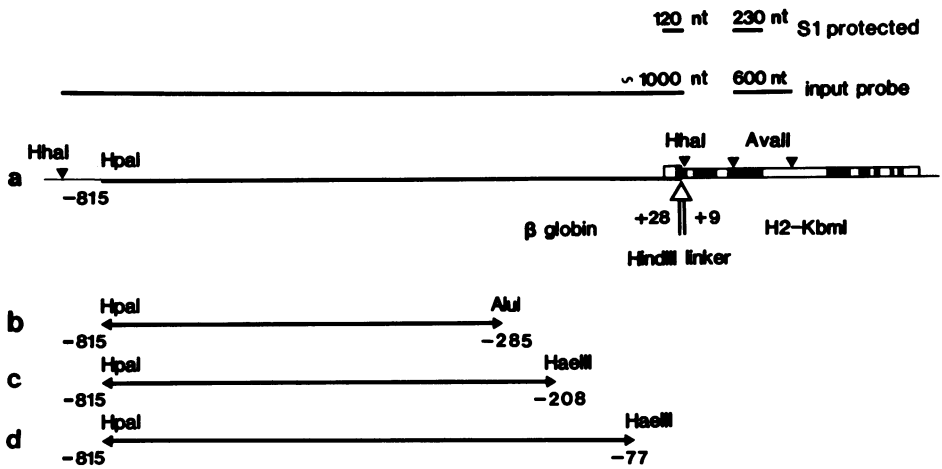


Figure 5 : Structure of human β-globin mouse H2Kbm1 gene recombinants with truncations in the upstream sequences. Deletions within the 5' region of the human β-globin gene promoter were as shown. Hha1 restriction cuts producing a 1 kb 5' βH hybrid probe are shown.

Aberrant transcription in Putko cells of gene hybrids containing β – globin 5' and H2Kbm1 3' sequences.

Since expression of human β-globin in Putko cells directed by the H2 promoter is accurate,sequences 5' of the β are likely to be responsible for aberrant transcription. To examine this suggestion a construct containing the β-globin promoter attached to the H2Kbm1 coding sequences was introduced into Putko and K562 cells. This chimeric gene has been previously shown to be inducible in differentiating MEL cells (10). Additional hybrids were made by sequencially deleting sequences down to -815, -295, or -77 bp of the β-globin promoter (Fig 5).

Transcription of βH mRNA was studied by S1 nuclease analysis using a 600 bp AvaII-AvaII internal probe which spans the junction between the third exon and intron of the H2Kbm1 gene and normally detects a 230 bp protected fragment. Expression of βH transcripts was detected from all constructs used. The detection of βH mRNA was not related to rearrangement or deletion of the introduced sequences upon integration into the host chromosomes. On the other hand the presence of intact promoter did not always result in expression of the hybrid gene. Intensities of S1 nuclease resistant bands were comparable for the various deletions and varied by up to five fold in the populations carrying the same deletion (Fig 6a).

Correct initiation of transcription was examined by S1 nuclease analysis using a 1kb

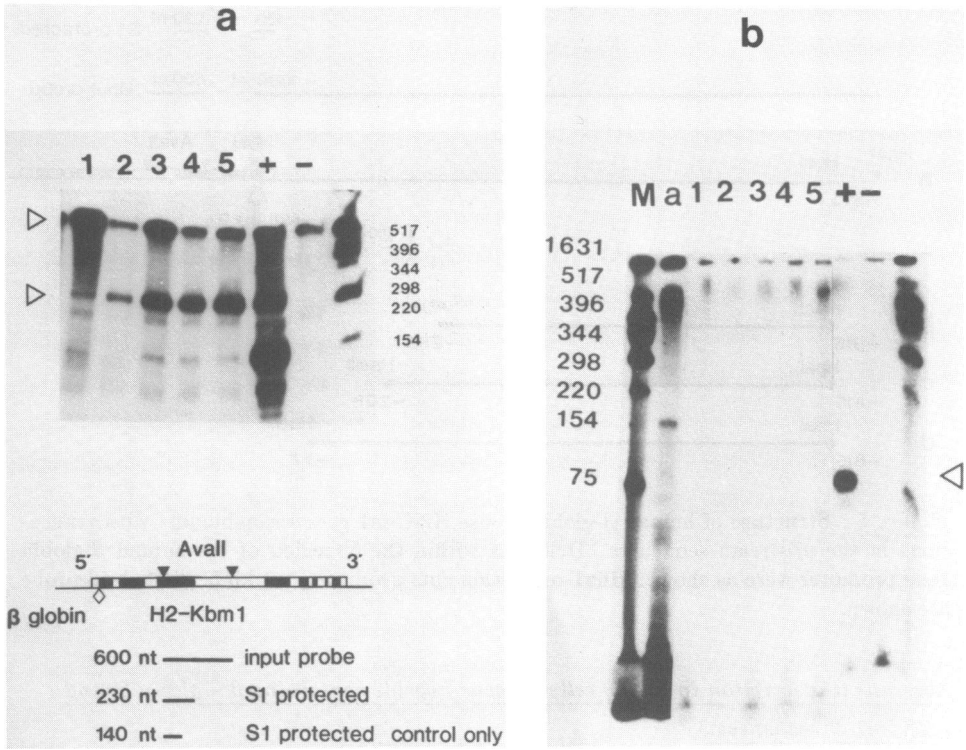


Figure 6: S1 nuclease analysis of human β-globin mouse H2Kbm1 hybrid gene transcripts in Putko cells transfected with truncated β-globin promoter recombinants.
a) 10 μg samples of total cellular RNA from five Putko populations transfected with βH hybrid gene having only 77 bp of β-globin promoter region (see Fig 5d) was analysed by S1 nuclease protection assay (lanes 1-5). An internal 3' labeled probe, as illustrated , was used. Positive control was 5μg RNA from 16 day old C57 BL/10 mouse embryos (+). RNA from these embryos produce two different protected fragments with the AvaII probe (230 and 140 nucleotides) due to a single base pair mismatch with the probe used (see 10). RNA from untransfected cells was used for negative control (-). Lane (M) is molecular weight markers. Arrows point to the input fragment and the RNA protected probe fragment.
b) 30 μg samples of total cellular RNA from the same five Putko populatons as above was analysed by S1 nuclease protection assay (lanes1-5) using a 1 kb HhaI 5' labeled probe (see Fig 5). Transcripts from a transient vector in which the expression of βH was driven by an SV40 enhancer produced a 120 bp protected fragment (lane a), while 5μg RNA from 16 day old C57 BL/10 mouse embryos produced a 75 bp protected fragment (+). Untransfected Putko cells were used as negative control (-). Lane (M) is molecular weight markers. Arrows points to the S1 protected fragment resulting from the hybridization of probe to the complementary 5' end of mouse H2 RNA (+).

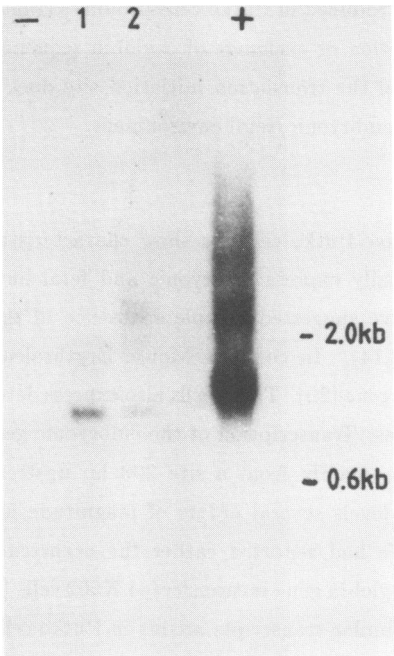


Figure 7 : Northern blot hybridization analysis of βH hybrid transcripts from expressing Putko cells. The RNA blot was hybridized to a labeled mouse H2 Ava probe as illustrated in Figure 6a. Lanes 1 and 2 are RNA from 10^7 Putko cells stably transfected with βH hybrid gene. These two lanes correspond to lanes 3 and 4 in Figure 5a. Positive control was poly A RNA from $5x10^7$ C57 BL/10 mouse embryo cells (+). This RNA was expected to be 45 bp shorter than the hybrid βH RNA. Poly A RNA from 10^7 untransfected Putko cells was used as negative control (-).

HhaI 5' labelled probe (see Fig 5). This probe detects 120bp of the β-H2 transcript in HeLa cells transiently expressing the gene from an SV40 enhancer. None of the cells expressing βH hybrid RNA showed visible protected fragment with this probe (Figure 6b). Similarly RNA from ten independently transfected K562 populations did not show protection of this probe. These results were confirmed using an independent 235 bp DdeI-DdeI probe . We conclude that the level of transcripts with intact 5' ends is below 0.1 per cell, which is the limit of detection by this method. To estimate the size of the expressed βH hybrid RNA, Northern blot analysis was performed. This revealed transcripts approximately 100bp shorter than control H2 mRNA (Fig 7). The presence of intact βH DNA in these cells was confirmed by Southern hybridisation. These results are consistent with our finding that the

complete β-globin gene introduced in Putko cells produces transcripts with shorter 5' ends than expected. Thus deletion or exchange of β-globin sequences upstream of the CAAT box and/or downstream of the translation initiation site does not improve transcription from this promoter in an embryonic/fetal environment.

DISCUSSION

K562 and its derivative Putko cell line show characteristics of embryonic erythroid cells since they preferentially express embryonic and fetal but not adult human globin gene. Several reports have suggested complete absence of the adult globin protein or mRNA in K562 cells (18,14). In contrast Mouse Erythroleukemic Cells preferentially express the adult β-globin gene (20). These cells also express detectable levels of introduced cloned γ and ε globin genes. Transcription of the embryonic genes in MEL cells is clearly inappropriate as it occurs mainly from a site 200 bp upstream of the major cap site and reaches steady state levels several orders of magnitude less than that of the adult gene transcripts (21). We had reported earlier the occurrence of aberrant transcripts containing the 3' end of β-globin gene in transfected K562 cells (17). In this paper we have shown that the 5' end of similar transcripts arising in Putko cells is shorter than expected by approximately 40 bp. Furthermore a hybrid gene containing β globin promoter and mouse H2 gene 3' sequences is transcribed from approximately 100 bp downstream of the major cap site. Thus genes that are suppressed/not expressed in a developmental specific manner are still capable of aberrant transcriptional activity when cloned and introduced into cells representing the nonpermissive stage. Since we only detect abnormal expression with introduced rather than endogenous genes we suggest that *trans*-regulation may not be sufficient for efficient regulation of gene activity. It is possible that the sequential expression of individual members of a gene family is more dependant on *trans*- than *cis*-regulation. This may explain the low level expression of globin genes observed in erythroid precursor cells during normal blood cell differentiation (22).

We have attempted to define mechanisms involved in trans-regulation of the β-globin gene in the Putko/K562 environment. To this aim we replaced β-globin sequences with those of a constitutively expressed gene, that of the mouse H2 transplantation antigen. The choice of sequences that are to replace the adult globin DNA is important. For example α-globin downstream sequences interfere with 'induction' of transcription from the β-globin promoter in differentiating MEL cells (11). This interference is not present in hybrid genes containing β-globin promoter and mouse H2 coding sequences (10). Using such hybrid β-H2 constructs we have shown that adult specific 'inducible' sequences which had been

previously localised downstream of the mRNA capping site (10) are not responsible or sufficient for suppression of activity of the β-globin gene in the embryonic/fetal erythroid environment. The nature of aberrant transcripts arising from the β-globin promoter allows us to speculate on the mechanism of promoter inactivity. It is interesting that in both cases of the complete β-globin gene and of the β-H2 chimeras the detected transcripts appear to have shorter 5' ends. However the site of initiation of transcription is probably further downstream of the mRNA capping site in the hybrid than in the complete gene. One possibility is that transcriptional factors bind to the inactive β-globin promoter but are prevented from recognising the major cap site. These factors then proceed to initiate transcription from putative downstream sequences. This conclusion is supported by our observation that sequences overlapping the constitutive promoter elements are responsible for the inactivity of the β-globin gene.

The nature of *trans*-regulation involved in the differential expression of the globin genes remains to be defined. Sequences downstream of the β-globin mRNA capping site have been shown to be involved in the developmental specific regulation of this gene (10,11). Our results suggest a similar role for the β-globin gene promoter. Clearly the major mRNA capping site of this gene is not recognised in an embryonic/fetal environment. We have shown that this is an inherent property of the isolated β-globin promoter sequences. Interestingly β-globin promoter elements that are required for constitutive expression of this gene are also present in the promoters of the embryonic and fetal globin genes. Obvious differences are duplication of the CCAAT motif in the γ and the CCA/TCACAT motif in the β promoters (1). However neither of these duplications appear to play a role in the developmental specific regulation of globin genes (22 and our results). Our results suggest that differences in the sequences of promoters of globin genes within 77 bp spanning the CCAAT motif to the translation initiation signal are at least in part responsible for the developmental specific expression of the globin genes.

Acknowledgments

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