The human β-globin gene contains a downstream developmental specific enhancer

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ABSTRACT

        The human β-globin gene is part of a multigene family and is expressed
specifically in adult human erythroid tissue (for review, 1). When the human
β-globin is introduced into fertilized mouse eggs, it is first activated in
foetal liver and remains expressed in adult erythroid tissues (2,3,4). It
therefore mimicks the pattern of expression of its murine counterpart. It has
previously been shown in tissue culture (5) and transgenic mice (4) that
sequences downstream from the β-globin promoter are involved in this
regulation. We now show that at least part of these sequences are located
0.5-1.2kb downstream from the polyA addition site and constitute a
transcriptional enhancer element that is erythroid and developmental specific.

INTRODUCTION

        Cellular differentiation in eukaryotes can be directly attributed to
mechanisms that regulate gene expression. One of these mechanisms is at the
level of gene transcription. Recent studies have shown the existence of two
classes of cis acting transcriptional control elements, i.e. promoters and
enhancers. Enhancer sequences were originally identified in animal viruses and
characterized by a number of properties (for review, 6). They stimulate
transcription from homologous and some heterologous promoter elements in cis
in an orientation, position and distance independent manner. Cellular
enhancers have been described for a number of eukaryotic genes, including the
immunoglobulin (7), insulin (8), interferon (9), α-fetoprotein (10) and the
major histocompatibility genes (11). The best characterized of these is the
immunoglobulin heavy (IgH) chain gene enhancer. It is located downstream from
the promoter (inside the transcriptional unit in an intron) and together with
the IgH promoter directs B-cell specific expression of the IgH gene (14). When
linked to heterologous promoters, the IgH enhancer is bidirectional, distance
dependent and is also capable of stimulating transcription in non lymphoid
cells (15). Our previous results indicate that a similar situation might be
found in the human β-globin gene, i.e a specific promoter (5) and a specific

downstream element (4,5). The latter was shown to be developmental specific
and located downstream from the second exon (4). An enhancing element has
been reported downstream from the chicken globin gene (12,13), which probably
has similar properties, although it has only been tested in cultured cells. In
this paper we describe the localization and properties of the downstream human
β-globin element using the human γ-globin gene as the reference gene.

METHODS

1.   Production of transgenic mice

       The recombinant genes were purified from plasmid vector sequences by
gel electrophoresis, were diluted to concentrations of 1-2 µg/ml in 10mM Tris
pH 7.4, 0.1mM EDTA and microinjected into mouse zygotes as described
previously (4,23). Transgenic mice were identified by Southern blot
hybridization (24) of DNA isolated either from tail or placental tissue (see
text). Approximate copy numbers of integrated transgenes were estimated by
comparisons to a single copy control sample.

2.   Gene expression analysis

       RNA extraction from mouse tissues and S1 nuclease analysis were
performed as described elsewhere (4).

RESULTS

The β-globin enhancer is located downstream of the gene

       A γ-globin transgene is regulated as an embryonic mouse globin gene. It
is expressed in embryonic yolk sac, but not in foetal liver or any adult ery-
throid tissue (4,16). It can therefore be used as a reference gene to test the
effects of parts of the β-globin gene on its expression pattern. There are two
ways to assess the level of adult type erythroid expression of the globin
transgene. Firstly, by measuring RNA levels in adult erythroid tissues, which
do not contain detectable amounts of embryonic globin mRNA. Alternatively, it
can be assessed at an earlier stage by a comparison of the ratios of embryonic
to foetal/adult globin mRNA levels at 12.5 days of gestation. At that stage,
the switch in globin gene expression from embryonic yolk sac to foetal liver
is characterized by a decrease in embryonic globin mRNA levels and a strong
increase in foetal/adult globin mRNA (e.g. βh1 and βmaj globin mRNA, Fig. 3).
An increase in γ-globin transgene RNA levels therefore shows a foetal/adult
type of expression, while a decrease shows a purely embryonic type of expres-
sion. Positive embryonic transcription at this early stage serves as a control
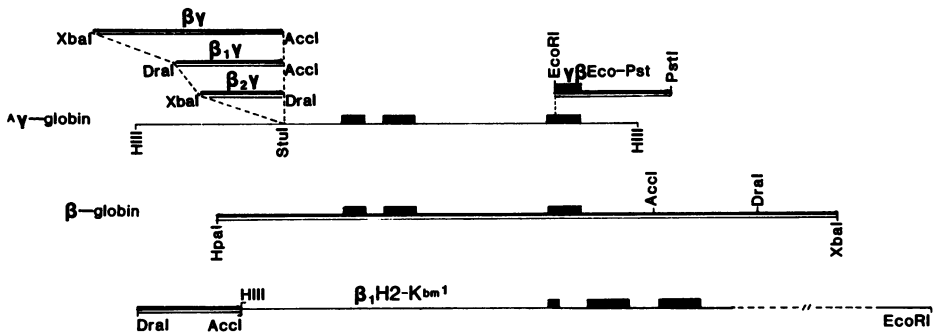that inactivity of a given gene at the adult stage is not the result of a

Fig. 1: Structure of recombinant βγ-globin and β-globin/H2-K^{bm1} genes microinjected into (CBAxC57B1/10) F2 hybrid mouse zygotes. The 1.2kb AccI-XbaI, 680nt AccI-DraI, and 530nt DraI-XbaI fragments from the 3' flanking region of the human β-globin gene (1) were inserted in the opposite orientation into the -400 region (StuI) of a HindIII fragment containing the human ^{A}γ-globin gene (1) (βγ, β1γ and β2γ, respectively). The 770nt EcoRI-PstI fragment containing the third exon and 550nt of 3' flanking sequences from the human β-globin gene were ligated to replace the homologous EcoRI-HindIII sequences from the 3' part of the human ^{A}γ-globin HindIII fragment (γβ Eco-Pst construct). The 680nt AccI-DraI fragment from the 3' flanking region of the human β-globin gene was ligated with linkers, to the 5' HindIII site of a 10kb EcoRI fragment containing the H2-K^{bm1} gene.

position effect. We have previously shown that the 3' half of the downstream region of the β-globin gene contains a DNA sequence that confers a "β-type" expression pattern on the γ-globin gene (4). We therefore constructed a 5'-γ-3'β-globin gene (γβ EcoPst, Fig. 1) to test whether the third exon and 550bp past the polyA site confer the adult type specificity of expression. Injection of the hybrid gene fragment into mouse zygotes resulted in four transgenic mice that carried two to fifty copies of the integrated gene (not shown). None of the γβ Eco-Pst animals expressed the hybrid gene in adult blood (Fig. 2, lanes 6-9) or foetal liver (not shown). Analysis of embryonic gene expression revealed a low, but detectable expression of the γβ Eco-Pst gene in embryonic yolk sac of one of the transgenic lines bred for this purpose (Fig. 2, lane 5). We therefore conclude that the γβ Eco-Pst construct lacks the "β-type" activator sequence. On the basis of this result and the previously published data on β-globin gene expression in transgenic mice (2, 3,4). it was most likely that an adult specific sequence was located between the PstI and the XbaI site (950bp and 1680bp downstream from the polyA Fig. 1). It should be noted that a second regulatory site might be located in the gene (Antoniou et al., unpublished) that results in the induction of the β-globin gene in MEL cells and would be disrupted by an EcoRI cleavage in the
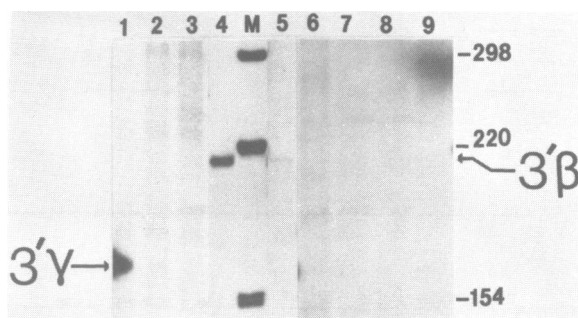
Fig. 2:Expression of recombinant β2γ and γβEco-Pst genes in transgenic mouse
tissues.
    Two β2γ transgenic mice were identified carrying 5 and 20 copies of the
transgene. Both were negative for γ-globin expression in all developmental
stages (see text). Further microinjection of mouse zygotes resulted in three
12.5 day transgenic embryos carrying 2, 5 and 50 copies of the β2γ transgene.
Foetal liver RNA was negative for γ-globin expression. The S1 nuclease
analysis of total RNA extracted from 12.5 day yolk sacs of the 5 and 50 copies
transgenic mice is shown (lanes 2 and 3, respectively). Fifty micrograms of
this RNA was hybridized to a 546nt 3' end-labelled EcoRI-HindIII probe derived
from the 3' end of the human ^{A}γ-globin gene. Normal transcription termination
of the ^{A}γ-globin gene produces mRNA that protects 168nt of the probe from S1
nuclease digestion. One microgram of RNA from an ^{A}γ-globin producing cell line
(PUTKO, 25,26) was used as a positive control (lane 1).
    Four γβEco-Pst transgenic mice were identified carrying approximately
1,8,50 and 100 copies of the transgene. Total RNA was extracted from all four
adult blood samples (lanes 6-9) and from 12.5 day yolk sac of the mouse which
contained 50 copies of the gene (lane 5). The RNA was hybridized to a 700nt 3'
end-labelled EcoRI-MspI probe derived from the 3' end of the human β-globin
gene. Normal transcription termination of the γβ hybrid gene produces an mRNA
that protects 212nt of the probe from S1 nuclease digestion. Ten micrograms
of β-globin containing RNA was used as a positive control (lane 4).
    The products of the S1 digestion assay were run on 6%
polyacrylamide-urea gels and autoradiography was for 6 days for lanes 1-3,
overnight for lanes 4 and M, 10 days for lane 5 and 2 weeks for lanes 6-9.
Sizes were estimated with an end-labelled HinfI digest of pBR322 DNA (lane M).

transgenic γβ EcoPst construct. To prove the localization of a regulatory

element between the PstI and XbaI sites and to show the enhancer properties

(orientation and position independence) of the "activating" sequence, we

isolated an AccI-XbaI fragment (polyA +470nt to +1680nt) and placed it in the

opposite orientation 400bp upstream (StuI site) of the γ-globin gene (βγ

construct, Fig. 1). Microinjection of the βγ-gene resulted in six transgenic

animals as determined by Southern blot analysis of tail DNA. Four of the six

animals expressed human γ-globin mRNA in adult blood as determined by S1

nuclease protection analysis. The levels of expression of the γ-globin in each

mouse were variable, but not related to the number of integrated copies of the

transgene (not shown). One of the transgenic mice was bred and analyzed for embryonic and foetal stage expression. As expected, the $\beta\gamma$-gene is expressed in yolk sac and foetal liver at ratios comparable to endogenous globin expression (Fig. 3, lanes 2 and 3), indicating that the AccI-XbaI fragment contains an enhancer-like element, which specifically activates expression in foetal/adult erythroid tissue. To map the sequences further, the AccI-XbaI fragment was subdivided into two smaller fragments, AccI-DraI (polyA +470nt and +1180nt) and DraI-XbaI (polyA +1180nt and +1680nt) and recloned 400nt upstream of the γ-globin gene again in the opposite orientation ($\beta_1\gamma$ and $\beta_2\gamma$, respectively, Fig. 1). Microinjection of $\beta_1\gamma$ and $\beta_2\gamma$ resulted in two trans-genic mice in both cases. The two $\beta_2\gamma$ mice were negative for expression in adult blood, one of them was bred and also shown to be negative in embryonic yolk sac. A further set of eggs was injected with the $\beta_2\gamma$ gene and the embryos were dissected at 12.5 days of gestation. Three transgenic embryos were identified by Southern blots of placental DNA and one animal expressed the gene in embryonic yolk sac at very low levels (Fig. 2, lane 2). Expression of the $\beta_2\gamma$ gene in 12.5 day foetal liver was not detectable. In contrast, analysis of the two $\beta_1\gamma$ mice showed expression in adult blood and one was bred to analyze the 12.5 day embryonic and foetal expression (Fig. 3, lanes 4 and 5). Since the usual increase in expression from yolk sac to foetal liver was observed (4), we concluded that (at least part of) the adult specific enhancer element is located between 470 and 1180 nucleotides downstream from the β-globin gene polyA site (see Discussion).

The β-globin enhancer weakly stimulates a heterologous promoter at large distance.

To test whether the β-globin gene enhancer has the same adult/erythroid effect when linked to a completely heterologous (non erythroid) promoter, we cloned the AccI-DraI fragment in the opposite orientation into the flanking region of the $H2$-K^{bm1} major histocompatibility gene (18) at a similarly long distance (1.5kb) from the TATA box as it normally occurs in the β-globin gene (2.4kb, see Fig. 1). This was done by insertion of the β-globin fragment into a HindIII site of a 10kb EcoRI fragment containing the $H2$-K^{bm1} gene (18). It has been shown that the H_2K gene is activated in early embryonic development (19) and that the H2-K EcoRI fragment is expressed appropriately in all tissues when introduced into transgenic mice, with the possible exception of liver which shows a slight suppression of the transgene (20). Microinjection of the β_1 $H2$-K^{bm1} EcoRI fragment resulted in nine transgenic 13.5 day embryos as determined by Southern blot analysis of placental DNA, of which five mice
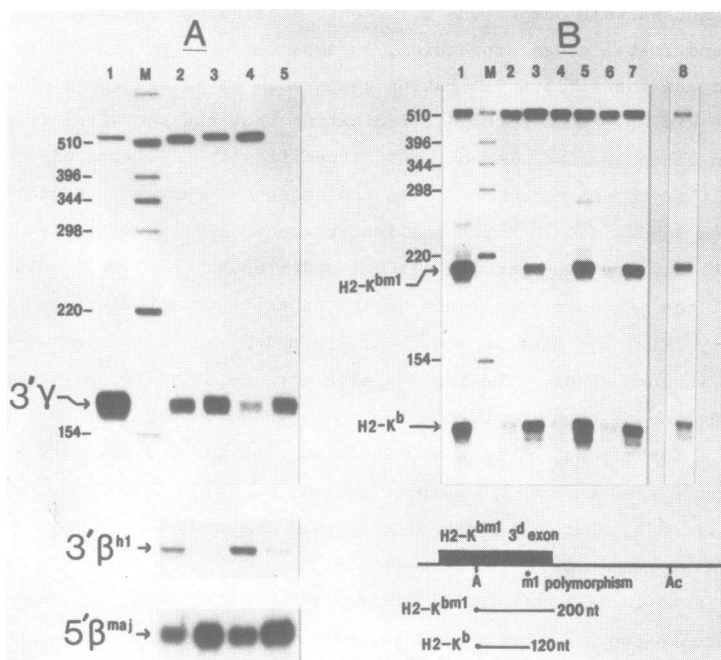
Fig. 3: Localization of human β-globin enhancer sequences by expression of recombinant βγ, β1γ and β1H2-K^{bm1} genes in transgenic mouse tissues.

Panel A:

Six βγ and two β1γ transgenic mice were identified carrying approximately 2 to 100 copies (βγ) and 30 and 100 copies (β1γ) of the transgene. Shown is the S1 nuclease analysis of total RNA extracted from yolk sac (βγ lane 2; β1γ lane 4) and liver (βγ lane 3; β1γ lane 5) of 12.5 days of gestation transgenic mice. Twenty micrograms of this RNA was hybridized to a 546nt 3' end-labelled EcoRI-HindIII probe derived from the 3' end of the human ^{A}γ-globin gene. Normal transcription termination of the γ-globin gene produces an mRNA that protects 168nt of the probe from S1 nuclease digestion. Five micrograms of RNA from ^{A}γ-globin producing cell line (PUTKO 25,26) was used as a positive control (lane 1).

The two insets at the bottom show the autoradiographic signals obtained from S1 nuclease protection assays of probes specific for the mouse embryonic βh1 and mouse adult β^{maj} mRNAs using 2μg of the same total RNA samples (lanes 2-5). The βh1 probe was a 255nt HinfI fragment derived from the third exon of the βh1 gene, and the β^{maj} probe was a 700nt HindIII-NcoI fragment derived from the second exon of the β^{maj} gene. Specific activities of the two probes and time of exposure of the autoradiograph were equalized to allow comparisons.

Panel B:

Two transgenic mouse lines are shown with typical patterns of expression of the exogenous β1-H2-K^{bm1} gene (see text). Total RNA samples from 13.5 days of gestation normal F1 (CBAxC57B1/10) mouse muscle and liver (lanes 2 and 3); β1 H2-K^{bm1}-1 transgenic muscle and liver (lanes 4 and 5) and β1 H2-K^{bm1}-2 transgenic muscle and liver (lanes 6 and 7), was hybridized to a 500nt 3' end-labelled AvaII-AccI probe derived from the third exon of the mouse H2-K^{bm1} gene. This probe detects a 120nt band for the endogenous H2-K^b

mRNA by cleavage at a mismatch with H2-K^{bm1} and a 200nt band for the third exon boundary of the H2-K^{bm1} gene. RNA from a MEL cell line stably trans-fected with an H2-K^{bm1} gene served as a positive control (lane 1). Lane 8 is a shorter exposure of lane 5. The exogenous signal obtained for normal mouse liver is due to incomplete S1 cleavage (see text for correction of background).

The products of the S1 digestion assay were run on 6% polyacrylamide-urea gels and autoradiographed for visualization of bands. Sizes were estimated with an end-labelled HinfI digest of pBR322 DNA (lane M).

expressed adequate levels of H2-K^{bm1} mRNA. S1 nuclease protection analysis with a third exon probe from the H2-K^{bm1} gene, detects a 120nt band for the endogenous H2-K^b RNA by cleavage at a mismatch with H2-K^{bm1} and a 200nt band for the third exon boundary of the H2-K^{bm1} gene (Fig. 3). Incomplete S1 cleavage at the mismatch adds endogenous signal to the exogenous protected fragment (see normal mouse control, Fig. 3, lanes 2 and 3). After densitometer scanning and subtraction of the background, two types of expression were observed in five transgenic mice that were analyzed in detail. Either the ratio of exogenous H2-K^{bm1} to endogenous H$_2$K^b was increased (2-3-fold in two mice) in foetal liver when compared to muscle (Fig. 3, lanes 4 and 5), or the ratio was the same (three mice, Fig. 3, lanes 6 and 7); a decrease was not observed.

DISCUSSION

We have shown that the sequences located 0.5-1.2kb downstream from the polyA addition site of the human β-globin gene constitute a transcriptional enhancer element that is erythroid and developmental specific. It is worth noting, however, that by using transgenic mice it is not possible to determine the exact boundaries of the enhancer element, primarily due to integration-position effects on the level of expression. It is entirely possible that the complete, fully active element is larger than this fragment and consists of several different motifs as proposed by the cassette model for enhancer function (6). We might be missing sequences which bind " ubiquitous" factors or even negative regulatory elements as observed for other cellular enhancers (15,17). Such elements could be inhibitory in non adult erythroid tissue and might explain the strikingly low levels of γ-globin gene expression in the γβ-EcoPst and β$_2$γ transgenic mice (Fig. 2). On the other hand, the HindIII γ-globin fragment we used does not contain the homologous region to the β-globin enhancer region (Fig. 1) and might therefore be missing activating elements resulting in low expression in a small percentage of the mice (4).

We have also tested whether the β-globin gene enhancer has the same

adult/erythroid effect when linked to a completely heterologous (non erythroid promoter). Our results indicate that the β-globin gene enhancer has some, albeit weak, effect on the expression of the H2-K gene in foetal liver and implies that it has a much greater effect on an erythroid (γ and β-globin) than a non erythroid promoter, at least when situated at a large distance. This is also observed in similar experiments with the IgH enhancer, which displays a distance dependence with heterologous promoters (15). The choice of the "heterologous" H-2K gene was based on results in MEL cells, where the β-globin downstream sequences stimulate the H2-K promoter when they are placed at a similarly long distance (5). This difference might be explained by the fact that the DNA transfected into cells is always incorporated into an active chromatin structure either transiently or stably by selection. In contrast, in mice the transgene is present throughout differentiation and the transcript- ionally active status might be achieved by a more complex process. Since the H2-K EcoRI fragment we used, contains all the necessary information to achieve the appropriately active state (11,20,22), the effect of the addition of an erythroid specific enhancer might be minimized, especially if the β-globin gene enhancer is required for the activation rather than the maintenance of transcription, as is the case for the Ig (27) and SV40 enhancers (29).

It is interesting that the DNA sequences located 0.5-1.2kb downstream from the human β-globin gene (Fig. 1) do function as an erythroid and develop- mental specific enhancer in the mouse. This implies that the trans-acting factors and the enhancer sequences in mouse and man are homologous. However, a comparison of the human β-globin enhancer fragment with the mouse β-maj globin gene (28) and its flanking regions (D. Kuebbing, personal communication) does not reveal any strong homology such as that observed when the human β-globin and rabbit β1 globin 3' flanking regions are compared (21). Among several short homologies which occur in scrambled positions in the different genes, the best is between the human sequence TTGATATTCACTACTGT which is located 670bp downstream from the polyA addition site and a sequence located at 580 and 500bp in the rabbit and mouse genes, respectively. Interestingly, the homologous sequence TT(A)ATA(G)T(T)ACTACT(A)T is also present in the chicken β-globin enhancer (12,13) in the opposite orientation.

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