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Chromatin interaction mechanism of transcriptional control *in vivo*

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We have used a kinetic analysis to distinguish possible mechanisms of activation of transcription of the different genes in the human β globin locus. Based on *in situ* studies at the single-cell level we have previously suggested a dynamic mechanism of single genes alternately interacting with the locus control region (LCR) to activate transcription. However, those steady-state experiments did not allow a direct measurement of the dynamics of the mechanism and the presence of loci with *in situ* primary transcript signals from two β-like genes *in cis* has left open the possibility that multiple genes in the locus could initiate transcription simultaneously. Kinetic assays involving removal of a block to transcription elongation in conjunction with RNA FISH show that multiple β gene primary transcript signals *in cis* represent a transition between alternating transcriptional periods of single genes, supporting a dynamic interaction mechanism.

Keywords: α globin/β globin/locus control region/single gene activation/transcription

Introduction

Several models have been proposed for the activation of gene transcription after chromatin activation of a locus. In one model, the genes would simply be accessible to binding of transcription factors and be transcribed in a stochastic fashion (Groudine and Weintraub, 1982; Martin *et al*., 1996). In such a model the dimensional aspects of the locus would not play an important role. In a second model regulatory sequences could be the entry site of (part of) the transcriptional machinery which would scan the DNA for genes to be transcribed (Herendeen *et al*., 1992; Tuan *et al*., 1992). Such a model is basically linear and predicts that the order of the genes relative to the regulator is an important parameter. In a third model it has been proposed that gene regulatory elements participate in direct chromatin interactions with regulatory elements at a large distance as a prerequisite to transcriptional activation (Ptashne, 1988; Mueller-Storm *et al*., 1989; Bickel and Pirotta, 1990; Foley and Engel, 1992; Wijgerde *et al*., 1995; Dillon *et al*., 1997). Such a looping model is three dimensional and predicts that the relative distance of the genes from the regulator is important. All these models have been put forward to explain the role of the human β globin locus control region (LCR) in the developmental regulation of transcription of the β gene cluster (Tuan *et al*., 1992; Wijgerde *et al*., 1995; Martin *et al*., 1996; Dillon *et al*., 1997).

The β globin system has long been a prototypic system for the study of transcription in vertebrates (reviewed in Grosveld *et al*., 1993). The locus consists of five active genes that are activated and silenced at different stages of erythroid development (Figure 1A). The expression of all of these genes is dependent on the presence of the LCR which is located 15 kb upstream of the ε gene (Grosveld *et al*., 1987). The ε gene is expressed first in the embryonic yolk sac followed by a gradual switch to expression of the γ genes between weeks 6 and 10 of gestation. Expression of the γ genes predominates during the fetal liver stage. In the later fetal liver and neonatal stages, there is a second transition to expression of the β gene and the γ genes are almost completely silenced during adult life. When the entire human β locus is incorporated in transgenic mice a similar expression pattern is observed, although the γ genes are expressed early in the embryo and are switched off at day 16 of development in the fetal liver (Strouboulis *et al*., 1992; Peterson *et al*., 1993). The analysis of mutated loci found in patients and the use of single ε, γ and β genes in transgenic mice have shown that the ε and γ genes are suppressed autonomously through sequences directly flanking the genes (Raich *et al*., 1990; Dillon and Grosveld, 1991). However, the β globin gene when present in the whole locus is (at least in large part) silenced during early development in a non-autonomous manner. This β globin gene suppression can be explained by a scanning mechanism because genes closer to the regulatory sequences would have a natural advantage over distal genes due to proximity. This would also be the case in a looping mechanism; proximal genes would have a higher frequency of interaction with the regulatory sequences and thus have a competitive advantage over distal genes (Giglioni *et al*., 1984; Enver *et al*., 1990; Hanscombe *et al*., 1991; Peterson and Stamatoyannopoulos, 1993; Dillon *et al*., 1997). However, this would not be the case in an accessibility model and hence an extra parameter was postulated to explain the silencing of the distal β gene in early development, namely a process of interference of the proximal genes with the distal genes via some topological constraint (Martin *et al*., 1996).

Recent analysis of primary transcription in single cells (Wijgerde *et al*., 1995, 1996) and the results obtained by placing a second β globin gene at different positions in the locus (Dillon *et al*., 1997) support a dynamic looping mechanism with single genes alternately interacting with the LCR. However, the presence of a minority of loci which display two gene signals *in cis* (Wijgerde *et al*., 1995) could be interpreted as evidence in support of

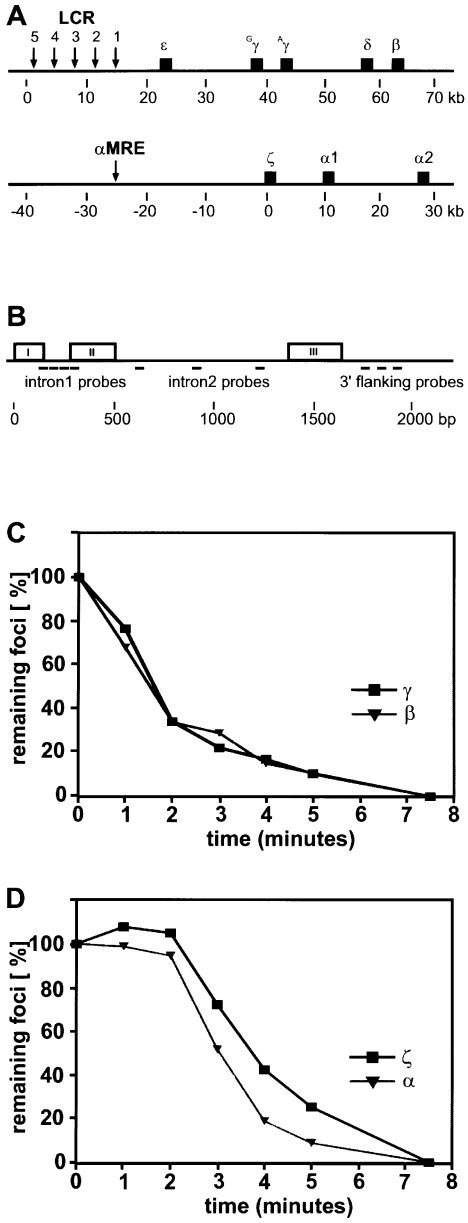


Fig. 1. Lifetime of the human β and mouse α globin primary transcript signals *in situ*. (**A**) Schematic diagrams of the human β globin locus and mouse α globin locus. Black boxes represent genes and vertical arrows represent the hypersensitive sites of the β globin LCR and the α globin major regulatory element (αMRE). (**B**) Intron/exon structure of a β-like globin gene with localization of probes used for *in situ* hybridizations. (**C**) Decay of the γ and β primary transcripts *in situ* signals in day 11.5 fetal liver cells treated with 5 μg/ml actinomycin-D. The percentage of remaining intron 2 signals are shown as a function of time. (**D**) As (C) for the mouse ζ and α genes in day 10.5 embryonic blood cells.

the scanning or the accessibility model of transcription initiation (Martin *et al*., 1996). Thus a crucial difference between the looping model and the others is single versus multiple gene activation at any moment in a single locus. We have therefore used a novel kinetic analysis utilizing inhibition of transcription elongation and release in conjunction with RNA fluorescence *in situ* hybridization (FISH) to show that multiple β gene primary transcript signals *in cis* represent a transition between alternating transcriptional periods of single genes, rather than the co-initiation of transcription of multiple genes in the locus.

Results

The lifetime of primary transcript in situ hybridization signals

A key parameter in a kinetic analysis of the transcription process *in vivo* using *in situ* hybridization is the time required for a signal to decay below the level of detection. To enable the detection of short-lived events we probed for the presence of intronic RNA, as these sequences are rapidly cleaved from the primary transcript and degraded. We used actinomycin-D to measure the detection lifetime of the γ and β primary transcript signals at days 11.5 (Figure 1C) and 12.5 (not shown), and ζ and α primary transcript signals at day 10.5 (Figure 1D) of development. On both day 11.5 and 12.5 the intron signals of the γ and β genes, as well as ζ and α intron signals at day 10.5, disappear below detection level 7.5 min after the addition of actinomycin-D. Interestingly the intron signals of the ζ and α genes do not decrease immediately when compared with the β-like genes. This could indicate a possible difference between the two loci (see below), but could also be due to experimental parameters such as a higher sensitivity of the α-like probes. Unfortunately, actinomycin-D inhibition is irreversible and hence cannot be used in reactivation experiments.

We therefore used 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) to reversibly inhibit pol-II transcriptional elongation in single-copy human β globin transgenic mouse (Strouboulis *et al*., 1992) fetal liver cells. Previous studies have shown that DRB does not effect initiation of transcription (Fraser *et al*., 1978; Marshall and Price, 1992) but prematurely aborts elongating transcripts ~400–600 bp from the initiation site (Chodosh *et al*., 1989; Marshall *et al*., 1996) by inhibiting the activity of the P-TEFb kinase which phosphorylates the C-terminal domain (CTD) of pol II (Marshall *et al*., 1996; Peng *et al*., 1998). The effect of DRB treatment on globin gene transcription in mouse erythroleukemia cells has been reported previously (Tweeten and Molloy, 1981). The results demonstrate that DRB causes premature termination without affecting initiation of transcription. We tested this in transgenic mouse fetal liver cells using *in situ* hybridization with probes that hybridize at different distances relative to the site of initiation of the β globin primary transcript (Figures 1B and 2). *In situ* signals with probes that hybridize to intron 1, located in the first 300 bases of the β globin primary transcript, are still visible in 85% of the erythroid cells after 15 min of DRB treatment (Figure 2A and C) when compared with the untreated control. Probes that hybridize to intron 2, 600–1200 bases 3′ of the initiation site (Figure 1B), have completely disappeared after 7.5 min of DRB treatment (Figure 2B and C). The fact that intron 1 signals are not affected by DRB confirms earlier reports which indicated that the process of transcription initiation is not disturbed and only elongation is affected, resulting in short, prematurely aborted transcripts. Since initiation continues and the balance between γ and β is maintained we conclude that whichever mechanism (scanning, accessibility or looping) is responsible for the activation of the genes, it

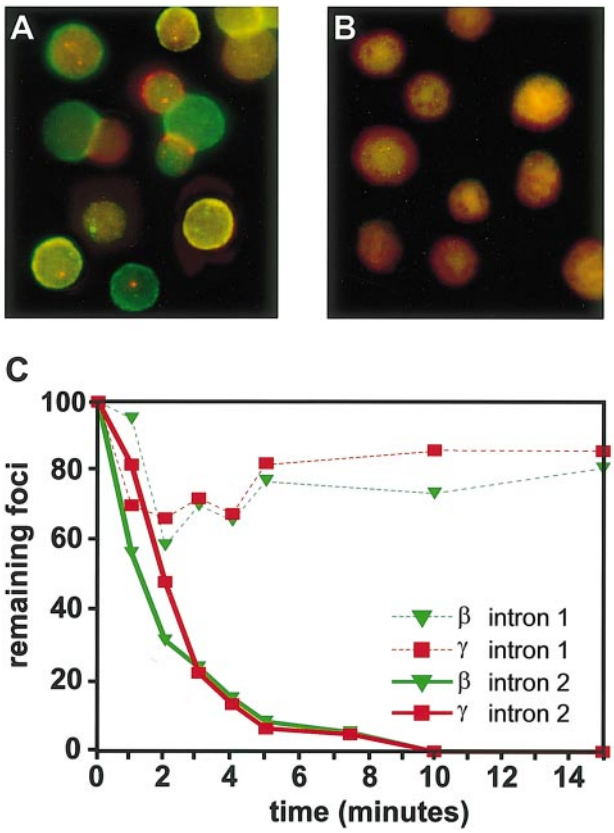


Fig. 2. The effect of DRB on transcriptional elongation *in vivo*. (**A**) *In situ* hybridization with intron-1-specific probes on homozygous transgenic day 11.5 fetal liver cells treated for 15 min with 100 μM DRB. γ globin signals are shown in red (Texas red), and β globin signals are green (FITC). (**B**) *In situ* hybridization with intron-2-specific probes on homozygous transgenic 11.5 day fetal liver cells treated for 7 min with 100 μM DRB. γ globin signals are red (Texas red), and β globin signals are green (FITC). (**C**) Decay of γ and β intron 1 and intron 2 *in situ* signals plotted as a function of time after the addition of DRB.

is not disturbed by the addition of DRB. The results also show that after inhibition of transcription by DRB the time required to decay the existing β globin intron 2 primary transcript signal via splicing, to levels beyond the limit of detection, is in good agreement with the actinomycin-D results (Figure 1C). The same is found for the α-like genes (not shown).

Kinetic analysis of single and double primary transcript signals

Primary transcript *in situ* hybridization with gene-specific intron probes for human γ and β globin in transgenic 11.5 day fetal liver cells containing a single copy of the complete human β globin locus show single gene transcription signals in ~85% of the human globin loci (Wijgerde *et al*., 1995). A small percentage of loci (~15%, e.g. Figure 3A, bottom right) contain signals for both γ and β globin genes *in cis*, and it is these signals that make a distinction between the different mechanisms difficult. If double signals are due to simultaneous initiation of the γ and β genes according to the scanning and (on a random basis) accessibility models, then release of the DRB block should result in the reappearance of double signals (using intron 2 probes) at the same rate as single signals. A lag in the reappearance of double signals compared with single gene signals would be indicative of alternating single gene initiation. This model predicts that double signals would result from the overlap between decaying primary transcripts (7.5 min, see above) from a recently active gene and the nascent transcription of an active gene.

We treated transgenic 11.5 day homozygous fetal liver cells with DRB for 15 min to block elongation of globin primary transcripts (Figure 3). The cells were then released from the DRB block by washing with phosphate-buffered saline (PBS), and aliquots of cells were fixed onto slides at various intervals and prepared for *in situ* hybridization as described previously (Wijgerde *et al*., 1995). The cells were probed with intron 2-specific probes for human γ (probes detect both $^{G}\gamma$ and $^{A}\gamma$ primary transcripts) and β globin primary transcripts. No transcription signals are visible at the zero time point immediately after the wash (Figure 3B). Five minutes after washing out the DRB (Figure 3C) primary transcript signals are again detectable in a high proportion of cells and continue to rise to the level observed before addition of DRB and in the non-DRB treated controls. Single (γ or β) and double (γ and β *in cis*) gene signals were counted and the averaged results of four separate experiments from two transgenic lines, line 72 and line 2 (Strouboulis *et al*., 1992), are presented in Figure 4. The results show that the reappearance of double gene signals significantly lags behind the reappearance of single gene signals in the population. A lag in the reappearance of double signals is also consistently observed when the two γ genes are compared with gene-specific probes for $^{G}\gamma$ and $^{A}\gamma$ (data not shown) and for the genes in the endogenous mouse β-globin locus (T.Trimborn, J.Gribnau, F.Grosveld, M.Wijgerde and P.Fraser, submitted).

To determine whether there is an intrinsic bias against the reappearance of double signals, we counted the reappearance of two signals, *in trans* (i.e. a signal on each chromosome) as an internal control. Their reappearance (Figure 4; loci trans) closely approximates to the single signal curves. We also calculated a theoretical curve that would be expected when the two loci are activated independently *in trans* (not shown). This curve coincides with the curve found for the appearance of two signals *in trans*. We therefore conclude that the two allelic globin loci behave independently of each other in terms of transcription (showing a stochastic reappearance of two signals without a lag), whereas the genes within a locus on one chromosome do not behave independently (double signal reappearance is non-random with a lag).

Weak competition versus strong competition

The γ genes are being expressed in embryonic cells in conjunction with ε and in the early fetal liver cells in competition with β. This provides a unique opportunity to investigate the interdependence of genes within a locus by examining the effects of weak versus strong gene competition during development independent of a DRB treatment. The ε gene, which is a weak competitor (Wijgerde *et al*., 1995), increases in expression from 9.5 to 11.5 days in the embryonic blood and γ gene expression decreases reciprocally (Strouboulis *et al*., 1992; Table I). The ε-globin gene is not expressed in the early fetal liver erythroid cells, but γ expression is further decreased due

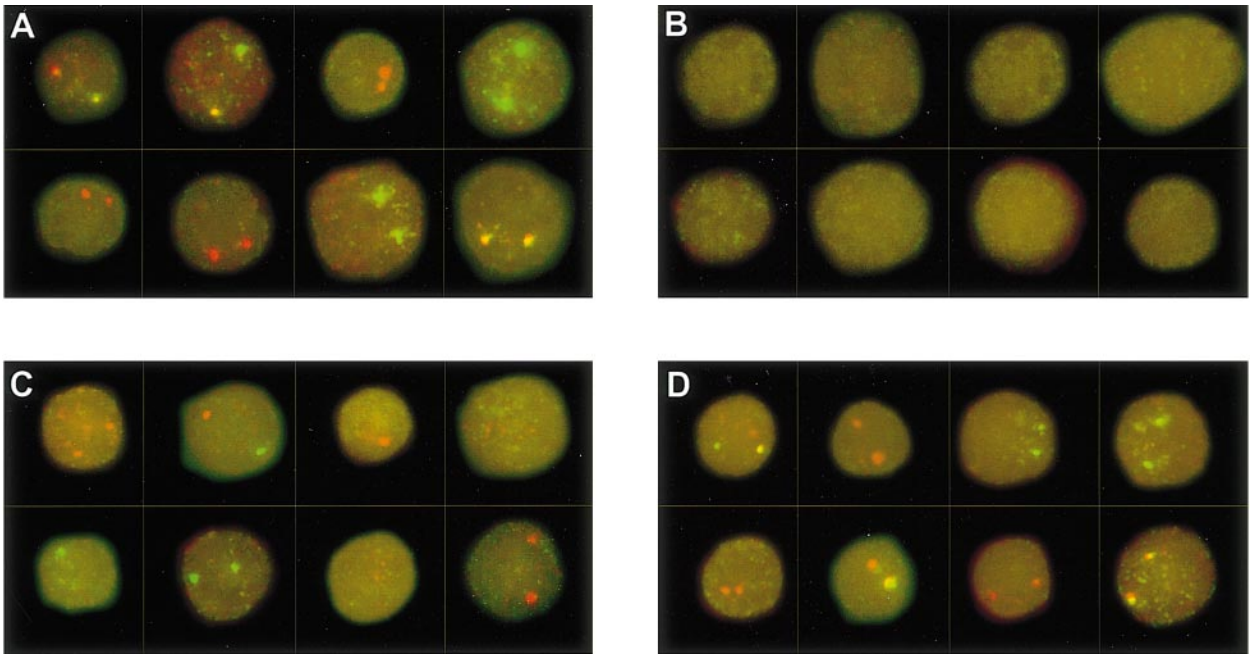


Fig. 3. Reversible inhibition of transcription elongation on homozygous 11.5 day transgenic fetal liver cells. (A–D) Primary transcript *in situ* hybridizations using γ and β intron 2 probes; γ signals are red (Texas red) and β signals are green (FITC). (**A**) γ and β primary transcript signals prior to DRB treatment. (**B**) γ and β primary transcript signals after 15 min treatment with 100 μM DRB. (**C**) Five minutes after release of the transcriptional elongation block by washing out DRB. Note most cells show single γ or β transcription signals *in cis*, while many have more than one signal *in trans*. (**D**) Twenty minutes after release from the elongation block, the distribution of loci having single γ or β and double signals (γ and β *in cis*) are back to control levels. Representative cells are shown for each time point.

to strong competition from the highly expressed β gene (Wijgerde *et al*., 1995, 1996). If co-initiation of transcription occurs one would predict that the percentage of $^{G}\gamma$–$^{A}\gamma$ double signals in the γ-expressing cells would change very little. In contrast, if alternating transcription of the γ genes and the other globin genes (ε or β) occurs then addition of a third gene into the competition should affect the percentage of double γ signals due to the resulting three-way alternation. When the percentage of $^{G}\gamma$–$^{A}\gamma$ double signals is measured in the γ-expressing cells during development, it changes from 83% at day 9.5 when ε expression is low, to 56% at day 11.5 in embryonic blood when ε expression is at its maximum (Table I). In the fetal liver where the γ genes are expressed with high levels of β expression, the percentage of double $^{G}\gamma$–$^{A}\gamma$ signals decreases further to 29% (Table I). Thus when γ gene expression is accompanied by relatively low-level expression of the ε gene in embryonic cells or high-level expression of the β gene in fetal liver cells, the percentage of $^{G}\gamma$–$^{A}\gamma$ double signals decreases accordingly. This result is most easily explained by an alternating single gene mechanism and is difficult to explain if one assumes that the γ genes are co-initiated.

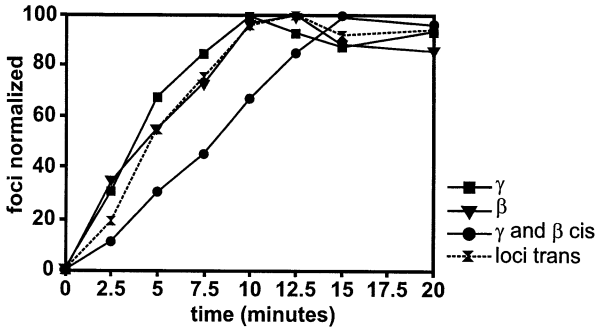


Fig. 4. Kinetics of reappearance of single versus double gene signals. Primary transcript *in situ* signals for γ and β (see Figure 3) were scored after release of the DRB block to transcription elongation in 11.5 day fetal liver cells from lines 72 and 2 (Strouboulis *et al*., 1992) and plotted versus time. The curves were normalized to their maximum values and the average of four experiments is shown. All experiments show the early reappearance of single γ or β signals and a clear lag in reappearance of double (γ and β *in cis*) signals. Cells in which both homologues display signals for either γ or β are plotted as loci trans showing the independent reappearance of two signals in the same cell.

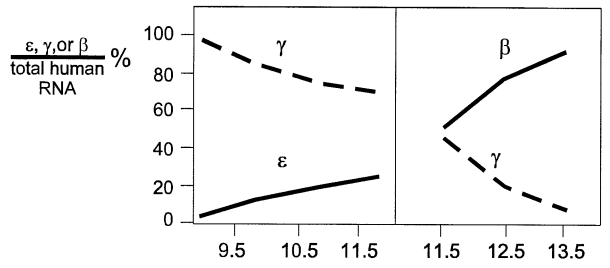
Transcription initiation of the α-like globin genes

The DRB analysis above does not exclude the possibility that treatment with the drug could somehow artifactually cause a delay in the reappearance of double signals *in cis*, even though this is clearly not the case for the reappearance of double signals *in trans* (Figure 4). There is indirect evidence that the α-globin genes may be regulated differently from the β-like genes (Craddock *et al*., 1995). Instead of the five hypersensitive regions present in the β globin LCR, only a single hypersensitive site has been identified 40 or 26 kb upstream of the human and mouse α genes, respectively (Gourdon *et al*., 1994, 1995). When this site is present as part of the human α locus in transgenic mice, the expression of the α genes is suppressed as development proceeds (Sharpe *et al*., 1993; Gourdon *et al*., 1994), indicating that additional sequences are required for full expression.

We examined the primary transcription of the mouse α-like genes in embryonic red cells in which all three α genes are expressed. Primary transcript *in situ* analysis shows a high percentage of double ζ and α signals *in cis* (80%) versus single ζ or α signals (Figure 5A), while the

lifetime of the signal is similar to that observed for the β signals (Figure 1D) in the presence of actinomycin-D as well as DRB (data not shown). This could mean that the genes are co-initiated in many loci or that frequently alternating initiation takes place. If alternating initiation occurs well within the lifetime of the signal, a large proportion of double signals *in cis* would result. We then measured the reappearance of single and double signals after the addition and removal of DRB (Figure 5B–D). Plotting the reappearance of the signals as a function of time (Figure 6) shows that the double signals appear at the same rate as the single signals and that there is no measurable conversion of single signals into double signals. On the basis of these data it is tempting to suggest that the α-like genes are co-initiated, but rapidly alternating initiation of transcription would give a similar result and hence cannot be excluded. Importantly, the result shows that the treatment with DRB does not artificially result in a lag in the appearance of double signals *in cis*.

Table I. Developmental expression and transcription of the human γ genes



	% in situ primary γ RNA signals			% total γ RNA	
	Gγ	Gγ/Aγ	Aγ	Gγ	Aγ
day 9.5 blood	5	83	12	39	61
day 10.5 blood	8	78	14	31	69
day 11.5 blood	7	56	37	27	73
day 11.5 liver	23	29	48	26	74

The top panel shows the relative expression of human ε, γ and β genes during development in transgenic mice (Strouboulis *et al*., 1994). Shown below are the percentages of loci with single $^{G}\gamma$ or $^{A}\gamma$ signals and double $^{G}\gamma$–$^{A}\gamma$ primary transcript *in situ* signals during development. The bottom right shows the percentage of $^{G}\gamma$ and $^{A}\gamma$ mRNA of total γ mRNA as determined by primer extension (data not shown). Note that changes in steady-state RNA levels (accumulated mRNA) lag behind changes in transcription as detected by primary transcript *in situ* hybridization.

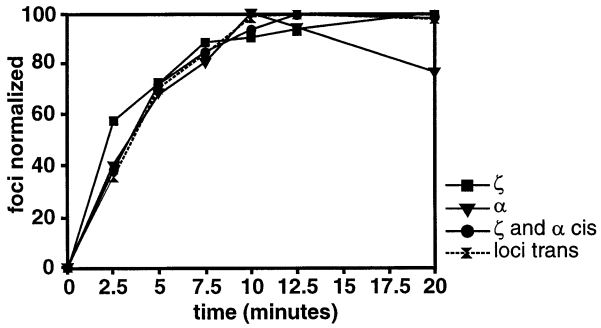


Fig. 6. Kinetics of reappearance of single versus double ζ and α primary transcript signals. *In situ* signals as shown in Figure 5 were scored after release of the transcriptional elongation block in 10.5 day embryonic blood cells and normalized to their maximum values for single (ζ or α) and double (ζ and α *in cis*) signals. Cells in which both homologues display signals for either ζ or α are plotted as loci trans showing the independent reappearance of two signals in the same cell.

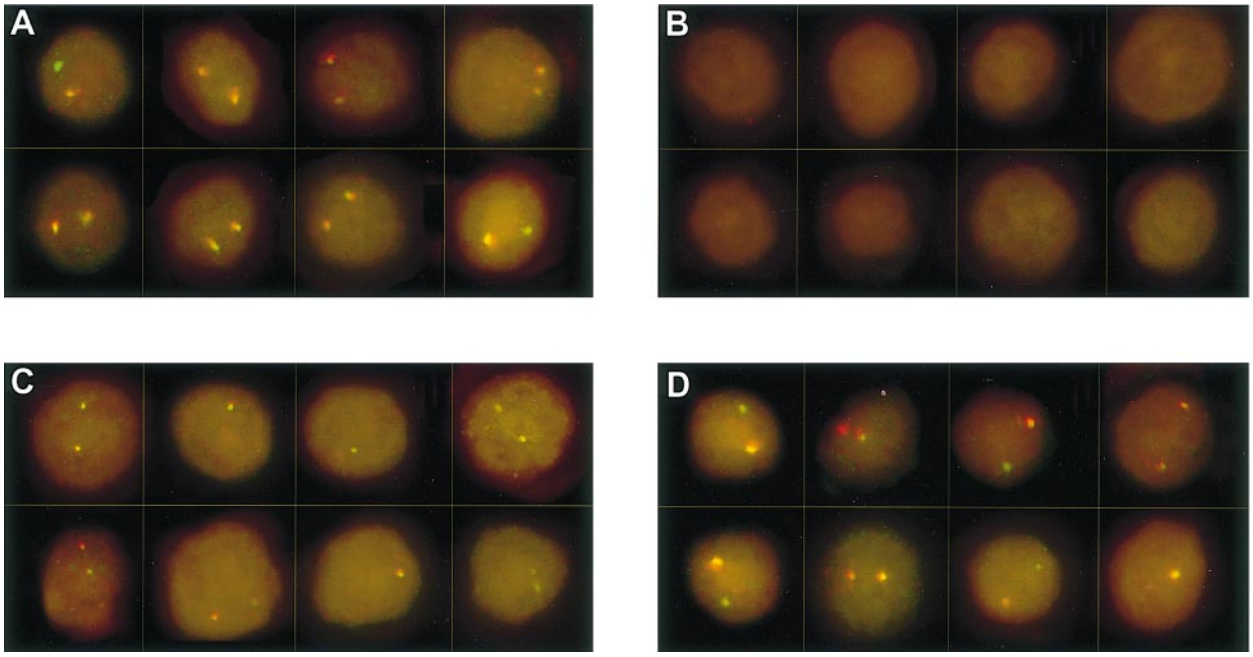


Fig. 5. Reversible inhibition of transcription elongation on day 10.5 blood cells. (A–D) Primary transcript *in situ* hybridizations using mouse ζ and α intron probes; ζ signals are shown in red (Texas red) and α signals in green (FITC). (**A**) ζ and α signals prior to DRB treatment. (**B**) Fifteen minutes after treatment with 100 μM DRB. (**C**) Five minutes after release of the transcriptional elongation block by washing out DRB. Note that many loci show double ζ- and α-globin transcription signals *in cis*. (**D**) Twenty minutes after release of elongation block, the distribution of loci having single ζ or α and double signals (ζ and α *in cis*) are back to control levels. Representative cells are shown for each time point.

Discussion

The results obtained previously with primary transcript *in situ* analysis suggested that the human β globin genes are regulated via a dynamic process of alternating initiation of transcription of the different genes. That proposal appeared to contradict existing data. It had been known for many years that γ and β mRNA and proteins are found in the same cell during the switchover from γ to β, even in heterozygotes, and this appeared to be confirmed by single-cell PCR data (Furukawa *et al*., 1994). These data, taken together with the primary transcript *in situ* results which revealed the presence of a minority of loci with double primary transcription signals *in cis*, were interpreted to mean that the γ and β globin genes within a single locus were or could be co-initiated (Bresnick and Felsenfeld, 1994; Furukawa *et al*., 1994; Martin *et al*., 1996).

Three models have been proposed to explain transcriptional regulation by distant regulatory sequences. The accessibility model proposes that after the activation of the chromatin, the genes bind transcription factors and are activated in a stochastic fashion (Groudine and Weintraub, 1982; Martin *et al*., 1996). The scanning model suggests that regulatory sequences act as a nucleation site for (part of) the transcription machinery, which subsequently scans the DNA for transcriptionally competent genes (Herendeen *et al*., 1992; Tuan *et al*., 1992). Finally the looping model postulates that the distant regulatory sequences interact directly with the gene to initiate transcription (Ptashne, 1988; Wijgerde *et al*., 1995 and references therein). Both the accessibility and the scanning models allow co-initiation of multiple genes *in cis*, whereas the looping model predicts initiation of only one gene at a time. It is therefore important in understanding the process of transcriptional initiation *in vivo* involving distant regulatory sequences to distinguish whether alternating single or multiple co-initiation takes place.

We have shown that in the presence of DRB *in vivo*, initiation of transcription is unaffected whereas transcriptional elongation is reversibly blocked. Inhibition studies with both DRB and actinomycin-D show that the globin primary transcript signals have a maximum lifetime of ~7 min, confirming that we are detecting actively transcribed genes or genes transcribed within the last 7 min. By reversibly inhibiting elongation with DRB for 15 min we have allowed intron 2 primary transcript signals to decay via splicing and degradation. Removal of the block theoretically permits the immediate detection of only actively transcribed genes. Obviously, not all loci cross the detection threshold simultaneously, introducing a small but measurable degree of asynchrony to the experiment, and therefore allowing the possibility of the reappearance of some double signals in the earliest time point. By analyzing large populations of cells at various time points after removal of the elongation block we have been able to demonstrate a clear and consistent difference in the amount of time required to detect single versus double signals. If we compare the $t_{1/2max}$ (the time required to re-establish 50% of the maximum number of single or double foci) for single (γ or β) and double (γ and β) signals, we find 4 and 8 min, respectively. This shows that on average double signals take twice as long to reappear as single signals, indicating that double signals result from sequential periods of initiation of single genes. The fact that the same result is obtained for the human β globin locus in two different transgenic lines and that it is essentially the same as observed for the endogenous murine β-globin locus (T.Trimborn, J.Gribnau, F.Grosveld, M.Wijgerde and P.Fraser, submitted) shows that the results are not due to a 'transgenesis' artefact. Interestingly, although the human β-globin locus in line 72 is integrated in a euchromatic environment, while in line 2 it is integrated in a heterochromatic environment close to the centromere, this appears to make no difference in terms of mechanism.

In contrast, in the α locus the $t_{1/2max}$ of the single and double signals are essentially the same (~3 min) suggesting that the α genes are either co-initiated or that they alternate at a frequency which is shorter than the time required to reach the detection threshold. There is indirect evidence for competition in the mouse α locus which may be indicative of an alternating transcriptional mechanism. Insertional mutation of the ζ gene with a PGK-Neo cassette results in decreased expression of the α genes in definitive erythroid cells when ζ would normally be silenced (Leder *et al*., 1997). Although the DRB results alone do not allow us to make firm conclusions regarding the mechanism of multiple α gene expression they do show a clear difference compared with the γ and β genes and exclude an artificial lag of reappearing double signals *in cis* as a consequence of the DRB treatment.

Restriction digestion of sites in the ^{G}γ and ^{A}γ promoter regions in isolated K562 nuclei indicated that both promoters were accessible to digestion *in cis* in ~50% of the loci (Bresnick and Felsenfeld, 1994). Co-accessibility of the promoters was inferred to be synonymous with simultaneous nuclease hypersensitivity of the two gene promoters. Although these links have not been established formally, it was suggested that this was an indication of co-initiation of transcription of the genes. Our DRB results in conjunction with the developmental transcription analysis indicate that the two γ genes are alternately transcribed. The high percentage of double ^{G}γ–^{A}γ signals in comparison with the low percentage of double γ–β signals suggests that alternations occur at a higher frequency between the two γ genes than between the γ genes and the β gene.

Thus the data presented show that the double signals in the β locus are not generated as predicted by a co-initiation mechanism, but represent a transition between alternating transcriptional periods of single genes. A number of additional *in vivo* observations support this conclusion. A single regulatory region (the LCR) is required by all of the genes in the β globin locus for activation (reviewed in Fraser *et al*., 1998) and the genes compete with each other for this function (Giglioni *et al*., 1984; Behringer *et al*., 1990; Enver *et al*., 1990; Hanscombe *et al*., 1991; Dillon *et al*., 1997), with LCR proximal genes having a competitive advantage over distal genes (Hanscombe *et al*., 1991; Peterson and Stamatoyannopoulos, 1993; Dillon *et al*., 1997). During the period of switching from γ to β expression nearly all erythroid cells have both γ and β mRNA in the cytoplasm, yet the overwhelming majority of loci have only γ or β transcription signals (Wijgerde *et al*., 1995). In addition, individual loci within the same cell can respond differently

to the same *trans*-acting factor environment with γ transcription on one homologue and β on the other. The balance of expression between the γ and β genes can be tipped in either direction by mutations in the γ promoter which prevent normal γ gene silencing or alterations in the level of EKLF, which is required for β gene transcription (Wijgerde *et al*., 1996; Tewari *et al*., 1998). In each case modulation of the expression of one gene leads to reciprocal changes in the expression of the other. A dynamic interaction between the LCR and the genes via looping explains all of the basic properties. It explains competition, because it predicts that the time taken up by LCR-driven transcription of one gene takes time away from another gene. In this model the competitive advantage of a gene is the result of increased frequency of LCR–gene interactions, which are dependent on distance (see Dillon *et al*., 1997).

Looping and direct contact between regulatory regions is therefore the most simple mechanistic explanation for the observed results and is supported by previous experiments with other systems (Dunaway and Dröge, 1989; Mueller-Storm *et al*., 1989; Bickel and Pirotta, 1990). It implies that direct chromatin interactions between the LCR and a single gene are required for initiation of transcription and suggests that continued loading of polymerases or re-initiation of that gene would require continuous LCR contact (Wijgerde *et al*., 1995; Milot *et al*., 1996; Dillon *et al*., 1997). In the context of this mechanism the data suggest that chromatin *in vivo* is highly dynamic or diffusible, allowing the LCR–gene complex to change rapidly to bring about co-expression of multiple genes.

Materials and methods

Reversible inhibition of transcription elongation with DRB

Homozygous transgenic mice containing a single integrated copy of the complete human β globin locus were bred to obtain embryos. Peripheral blood and fetal livers from the indicated developmental time points were dissected out in PBS. Fetal livers were gently disrupted by repeated pipetting. Actinomycin-D was used at a final concentration of 5 μg/ml. DRB (Sigma) was added to cell suspensions to a final concentration of 100 μM and incubated at 37°C for 15 min. Five volumes of ice cold PBS were added and the cells were immediately pelleted by centrifugation for 2 min at 1500 r.p.m. in an Eppendorf centrifuge. Cells were washed twice more with 1.5 ml ice-cold PBS and resuspended in 250 μl of PBS at 25°C. Aliquots were taken at the designated intervals and fixed onto poly-L-lysine coated slides (Sigma) for *in situ* hybridization.

Probe sequences and in situ hybridization analysis

The following probes were used for the *in situ* hybridization analysis:

Human β intron 1 probes:

5′-CTGTCTCCACATGCCCAGTTTCTATGGTCTCCTTAAACCTG-TCTTGTAA-3′
5′-GGGTGGGAAAATAGACCAAAGGCAGAGAGAGTCAGTGCC-TATCAGAAAC-3′
5′-AGGGCAGTAACGGCAGACTCTCCTCAGGAGTCAGGT-3′
5′-ATAACAGCATCAGGAGGGACAGATCCCCAAAGGACTCA-3′

Human β intron 2 probes:

5′-TTCCACACTGATGCAATCATTCGTCGTTTCCCATTCTAACT-GTACCCT-3′
5′-CTGATTTGGTCAATATGTGTACACAATTAAAACATTACACT-TTAACCCA-3′
5′-GGTAGCTGGATTGTAGCTGCTATAGCAATATGAAACCTCTT-ACATCAGT3′

Human γ intron1probes:

5′-AGGCACAGGGTCCTTCCTTCCCTCCCTTGTCCTGGTCAC-3′
5′-TGACAAGAACAGTTTGACAGTCAGAAGGTGCCACAATCCT-GAGAAGCGA-3′
5′-AGGCTTGTGATAGTAGCCTTGTCCTCCTCTGTGAAATGA-CCCA-3′
5′-AGAGCCTACCTTCCCAGGGTTTCTCCTCCAGCATCTTCCA-CATT-3′

Human γ intron 2 probes:

5′-GCAGTTTCTTCACTCCCAACCCCAGATCTTCAAACAGCTCA-CACCCGC-3′
5′-CCTTCTGCCTGCATCTTTTTAACGACCAACTTGTCCTGCCT-CCAGAAG-3′
5′-ACAGAGCTGACTTTCAAAATCTACCCAGCCCAAATGTTTC-AATTGTCC-3′

Human ^{A}γ 3′ flanking region probes:

5′-TCATATAAAAATAAATGAGGAGCATGCACACACCACAAACA-CAAACAGGC-3′
5′-CAGAACTCCCGTGTACAAGTGTCTTTACTGCTTTTAT-3′
5′-TTCATTAAGAACCATCCTTGCTACTAGCTGCAATCAATCCA-GCCCCCA-3′
5′-ATTTCACTTTCTTAGGCATCCACAAGGCTGTGAAAAGCTAA-GTGCCAT-3′

Human ^{G}γ 3′ flanking region probes:

5′-AAAAAAGTGTGGAGTGTGCACATGACACAAACACACATAG-CCATGTATAA-3′
5′-TGCAGACGCTCCCATGTATAAGTTTCTTTATTGCCTAGTTCT-TTTATTTG-3′
5′-ACGTAAACAAAAAAGTGTGGAGTGGCACATGACACAAACA-CACATAG-3′
5′-GCAGACGCTCCCATGTATAAGTTCTTTATTGCCTAGTTCTTTT-ATTT-3′

Mouse α intron probes:

5′-CACAGAAAAGCATAGTTAGAAGCGCCCACTGAGCGAGTGC-CAGGTCC-3′
5′-AGCCCTTCCTAGGGGCCCAGATGCCGCCTGCCAGGTCCC-3′
5′-GCTCCCCTTCCTGGGACCACTATGTCCCTGCCTTGGGCACG-AGGACCC-3′

Mouse ζ intron probes:

5′-CCTTCTCAGTGGCTTCTCCTCACAACTGCTCTTTGTCACTTC-TGTCTC-3′
5′-ATGGAAGACTCTGGTGAGCTCTGGAATGCCAGCCCACCTC-CTTTAGTA-3′
5′-ACAACCCCAAGAGTGATGTTACTATTGCTGTTGCACAAGGG-TCTACA-3′
5′-AAGGGGATTTGATGCCTCCAGCCCCAATGGCACCCATGCCT-GCGCTCG-3′

The two γ genes are highly homologous in both intron and exon sequence making the use of gene-specific intron probes extremely difficult. Transcriptional termination of γ gene transcription is known to occur 1–2 kb downstream of the polyadenylation site (Ashe *et al*., 1997). We therefore used probes (3′ flanking region probes) which hybridize to regions 300–500 bp downstream of the polyadenylation sites of the γ genes to detect the ^{G}γ and ^{A}γ gene primary transcripts separately.

Cells were fixed onto poly-L-lysine coated slides in 4% formaldehyde/5% acetic acid for 18 min at room temperature. The cells were subsequently washed three times for 5 min in PBS and stored in 70% ethanol at –20°C. The slides were pretreated for hybridization by a 0.01% pepsin digestion (5 min, 37°C) in 0.01 M HCl, followed by a short wash in water and a 5 min fixation in 3.7% formaldehyde at room temperature. The slides were washed in PBS, dehydrated in 70, 90 and 100% ethanol steps and air dried. The hybridization mixture was applied (12 μl per 24 × 24 mm coverslip) and incubated at 37°C in a humidified chamber for 12 h. The hybridization mixture contained 1 ng/μl of each oligonucleotide probe haptenized with either digoxygenin or biotin side chains in the middle and on the 5′ and 3′ ends of the oligonucleotide (Eurogentec, Belgium) in 25% formamide, 2× SSC, 200 μg/ml salmon sperm DNA, 5× Denhardt's, 1 mM EDTA and 50 mM sodium phosphate pH 7.0. The coverslip was removed by dipping in 2× SSC and the cells were washed three times for 10 min in 2× SSC at 37°C, followed by a 5 min wash in 0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20 at room

temperature. Antibody detection of the labels was essentially as described by Dirks *et al*. (1993), with three or four amplification steps. Mounting was in DAPI/DABCO:Vectashield (1:1) in glycerol (90%) and stored at 4°C in the dark. Fluorescence was detected by epifluorescence microscopy and photographs recorded with an CCD camera.

The graphical results represent the average of three separate experiments. In all cases >1000 cells were counted per data point using a dictaphone to record the results.

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