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A Silencer Element from the α-Globin Gene Inhibits Expression of β-Like Genes

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We have studied the *cis* and *trans* interactions of the α- and β-globin genes in a transient expression system. We found that the α-globin gene inhibited β-globin expression in *cis* but not in *trans*. The silencer element responsible for this inhibition was localized to a 259-base-pair fragment at the 5′ end of the α-globin gene.

The human α- and β-globin genes are coordinately regulated in vivo to result in the production of approximately equivalent amounts of the two subunits of adult hemoglobin A (α2 and β2) (7). The α-like genes (ζ, α2, α1, θ1) are present on chromosome 16, while the β-like genes (ε, $^{G}\gamma$, $^{A}\gamma$, δ, β) are present on chromosome 11 (7). It has been suggested, based on expression studies in vitro, that the α- and β-globin genes are under different types of regulation in vivo (8). The cloned α-globin gene is expressed efficiently when introduced into a variety of heterologous cells by DNA-mediated gene transfer regardless of whether a linked viral enhancer is present (10, 14). The β-globin gene, however, is not expressed efficiently in such cells unless it is linked to a viral enhancer (10, 16). The reason for this discrepant in vitro behavior remains unclear. One possible explanation for these observations is that the α-globin gene contains an endogenous enhancer which activates its own promoter and obviates the need for a linked viral enhancer. Humphries et al. (10) and Treisman et al. (16) examined this hypothesis by linking an α-globin gene to a β-globin gene on "enhancerless" expression vectors and assaying for the expression of both genes in COS cells and HeLa cells, respectively. Both groups observed efficient expression of the α-globin gene in the transfected cells, while the β-globin gene was not expressed (10, 16). Thus, using this approach, they could not demonstrate the presence of an endogenous enhancer in the α-globin gene capable of activating the promoter of a linked β-globin gene. Both groups, however, appropriately concluded that an α-globin gene enhancer may have been masked by position effects in the vectors they used (10, 16). More recently, an enhancer element has been identified 3′ to the human β-globin gene which activates its expression in a tissue-specific and stage-specific manner in transgenic mice (2, 4, 18). Another enhancer element has been described 3′ to the $^{A}\gamma$-globin gene (5). No such element has been described in the α-globin gene cluster.

We have designed a number of experiments to shed some light on the mechanism of regulation of the α-globin gene. We used a transient expression system in which a miniplasmid vector (πVX) (15) containing a simian virus 40 (SV40) enhancer (πSVplac) (17) was used to introduce the genes of interest into HeLa cells by DNA-mediated gene transfer exactly as described earlier (3). We used established recombinant DNA techniques (restriction digestion, gel purification of DNA, ligation, linker modification, and bacterial transformation) (12) to make the different constructs used in this study. Gene expression in the transfected cells was quantitated by measuring the steady-state levels of the mRNA of interest 36 h after transfection by S1 nuclease mapping (3). The different DNA probes used for S1 mapping were all 3′-end labeled (20) at the *Nco*I site of the translation initiation codon and extended to the *Hin*dIII site of the second exon of the α-globin gene (391 nucleotides [nt]) or the *Bam*HI sites of the second exons of the β-globin gene (420 nt) and γ-globin gene (232 nt). The level of expression of the different genes was quantitated by counting the radioactivity in the protected band after excising it from the polyacrylamide gel. Each transfection and S1 nuclease analysis experiment was repeated at least three times. In each experiment, one of the three globin genes (α, β, or γ) that was not present on the experimental constructs was cotransfected into the same cells to serve as a control for the efficiency of transfection.

In the first experiment, we transfected HeLa cells with constructs that contained the β-globin gene 3.7-kilobase (kb) *Bgl*II-*Pst*I fragment (πSVplacβ) or the α-globin gene 1.5-kb *Pst*I fragment (πSVplacα) either separately or simultaneously by cotransfection as shown in Fig. 1. Both the α- and β-globin genes were expressed efficiently when introduced separately into HeLa cells. This was expected, since both constructs contained an SV40 enhancer. When both genes were cotransfected in the same cells, the level of expression of each gene was comparable to its level of expression when introduced into HeLa cells separately. In other words, there was no demonstrable *trans* interaction between the α- and β-globin genes.

In the second experiment, we linked the α- and β-globin genes on a vector which contained an SV40 enhancer. The purpose of this experiment was to determine whether *cis*-acting position effects from the α-globin gene interfered with the enhancement of the β-globin gene by the linked SV40 enhancer. The results of this experiment are shown in Fig. 2. When the α-globin gene was placed 5′ to the β-globin gene in the same transcriptional orientation [πSV-β5′α(+)] or in the opposite orientation [πSV-β5′α(−)], the α-globin gene was expressed efficiently, whereas the expression of the β-globin gene was reduced by a factor of 5 to 10 in different experiments compared with its expression when present alone in πSVplacβ. This wide range of suppression was due to the very profound decrease in β-globin gene expression on constructs which contained a linked α-globin gene to levels that were very close to background. Thus, very minor changes in this denominator have a big impact on the estimation of the suppression factor. This same negative

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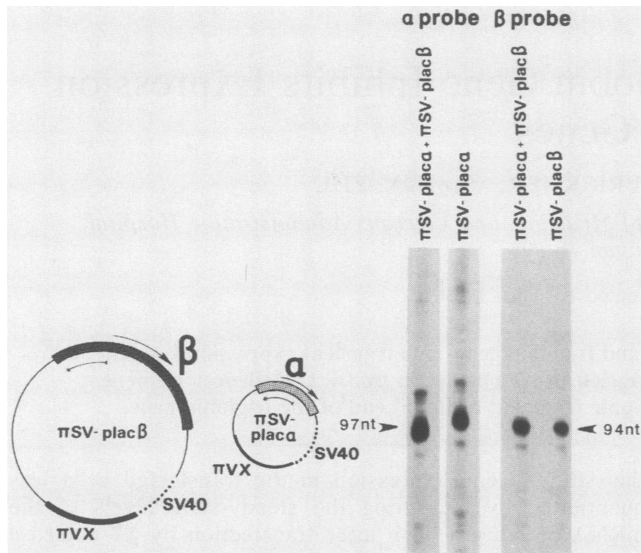

FIG. 1. Study of the *trans* interactions of the α- and β-globin genes. The structure of πSV-placα and πSV-placβ, which were used to introduce the α- and β-globin genes into HeLa cells, is shown. The location of the probes used in S1 analysis to measure the concentration of the globin mRNA is shown on the inside of the schematic vectors, where the asterisk marks the radioactive label at the 3′ end. The shaded boxes represent the transcribed sequences of the genes, and the arrows indicate the direction of transcription. In the first and third lanes of the autoradiograph, RNA from cells cotransfected with πSV-placα and πSV-placβ was studied by S1 analysis with the α and β probes, respectively. In the second lane, RNA from cells transfected with πSV-placα was analyzed with an α probe, and in the fourth lane, RNA from cells transfected with πSV-placβ was analyzed with a β probe. The accumulation of globin mRNAs in cells transfected with a single globin gene (second and fourth lanes) was similar to their accumulation in cells that were cotransfected with two unlinked genes (first and third lanes).

effect on β-globin expression was noted when the α-globin gene was cloned 3′ to the β-globin gene in either orientation [πSV-β3′α(−) and πSV-β3′α(+)], as shown in Fig. 3.

To determine whether this negative effect on β-globin expression was a specific effect of the α-globin gene or a nonspecific effect related to the presence of any other gene on the same vector, we cloned a 3.4-kb *Hin*dIII fragment containing the $^{A}\gamma$-globin gene in the place of the α-globin gene in both orientations [πSV-β5′γ(+) and πSV-β5′γ(−)]. Figure 4 shows that the level of β-globin gene expression in cells transfected with πSVplacβ, πSV-β5′γ(+), or πSV-β5′γ(−) was approximately the same. In other words, expression of the β-globin gene was not affected by the presence of the γ-globin gene on the same vector. In a separate experiment, the expression of the β-globin gene was also shown not to be affected by the presence of the δ-globin gene on the same vector (data not shown). The same negative effect of a linked α-globin gene was also seen when the α- and γ-globin genes were linked on a similar vector. Figure 5 shows a profound decrease in the expression of the γ-globin gene on πSV-γ3′α(+) compared with its expression on πSVplacγ.

Having established that the α-globin gene exerted a negative effect on a linked β- or γ-globin gene in *cis* but not in *trans* and in a position- and orientation-independent manner (i.e., a "silencer") (6, 11), we proceeded to localize the region of the gene responsible for this effect. We first deleted part of the 5′ end of the α-globin gene from a vector which contained linked α- and β-globin genes (πSV-β3′αΔP). The deleted region extended from the *Sma*I site at position −236 to the *Sma*I site at position +159 of the α-globin gene. Figure 3 shows that deletion of this *Sma*I fragment from the 5′ end of the α-globin gene restored the expression of the linked β-globin gene to the same level seen in πSVplacβ. Thus, we concluded that the silencer element must be in the 5′ end of the α-globin gene.

To localize the exact sequences of the α-globin gene sufficient to reproduce this effect, we cloned several overlapping fragments from the 5′ end of the α-globin gene on the 3′ side of the β-globin gene in πSVplacβ. Those fragments

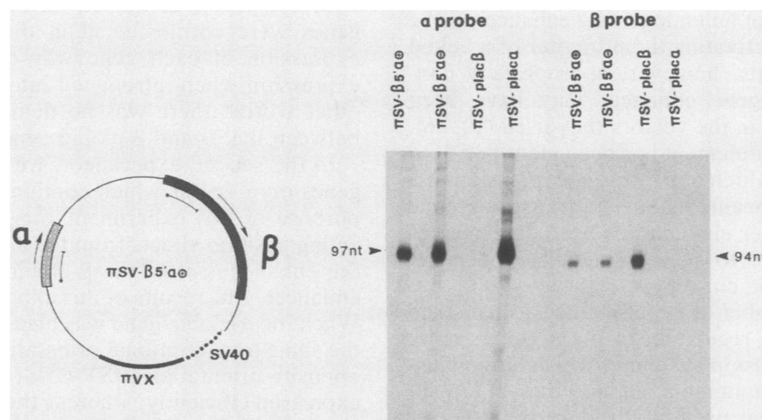

FIG. 2. Study of the *cis* interactions of the α- and β-globin genes. The organization of the linked α- and β-globin genes on πSV-β5′α(+) is shown in the schematic on the left. πSV-β5′α(−) is identical to πSV-β5′α(+) except for the orientation of the α-globin gene. The first four lanes show that α-globin mRNA accumulated to a high level in cells transfected with πSV-β5′α(+) (first lane), πSV-β5′α(−) (second lane), and πSV-placα (fourth lane), whereas no α-globin mRNA was seen in cells transfected with πSVplacβ alone (third lane). The higher level of α-globin mRNA in the third lane is a result of the smaller size of πSV-placα, which resulted in a higher molar ration of transfected α genes when the same amount of plasmid DNA was used in the transfections. The last four lanes show that β-globin mRNA accumulation was markedly decreased in cells transfected with πSV-β5′α(+) and πSV-β5′α(−) compared with that in cells transfected with πSV-placβ. As expected, cells transfected with πSV-placα showed no accumulation of β-globin mRNA.

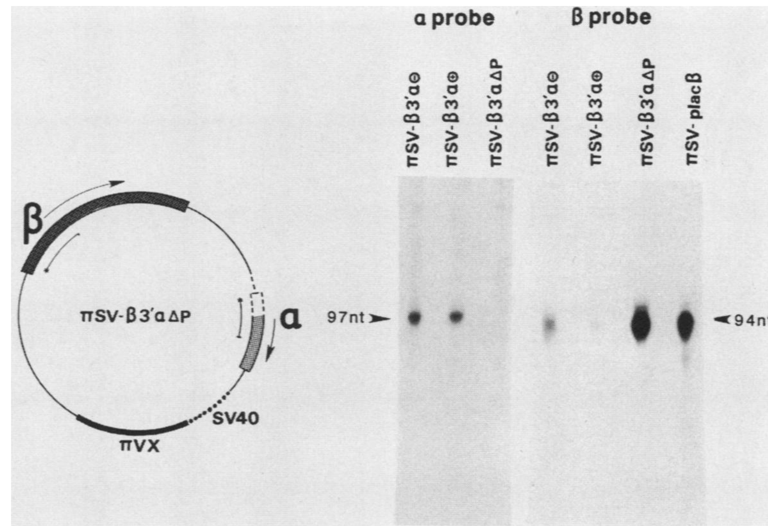

FIG. 3. Effect of deletion of the 5′ end of the α-globin gene on the expression of a linked β-globin gene. The schematic on the left shows the organization of πSV-β3′αΔP, in which the interrupted lines represent the deleted α-globin promoter and surrounding sequences. πSV-β3′α(+) is the original construct before the α-globin promoter was deleted, and πSV-β3′α(−) is identical to πSV-β3′α(+) except for the orientation of the α-globin gene. The first three lanes show the accumulation of α-globin mRNA in cells transfected with πSV-β3′α(−) and πSV-β3′α(+) and the absence of α-globin mRNA in cells transfected with πSV-β3′αΔP. The next two lanes show a marked decrease in the accumulation of β-globin mRNA in cells transfected with πSV-β3′α(−) and πSV-β3′α(+) compared with that in cells transfected with πSV-placβ (last lane). However, the deletion in πSV-β3′αΔP restored the level of accumulated β-globin mRNA (sixth lane) to the same level seen in cells transfected with πSV-placβ (last lane).

are shown in Fig. 6. The *Sma*I fragment that was deleted in the previous experiment was not sufficient to inhibit the expression of the β-globin gene (πSVβ3′αSS), nor was another fragment which overlapped the 5′ half of the *Sma*I fragment and extended 336 nt in the 5′ direction (πS Vβ3′αPN). However, a third fragment which overlapped the 3′ half of the *Sma*I fragment and extended another 135 nt in the 3′ direction (πSVβ3′αNT) was sufficient to inhibit the expression of the linked β-globin gene by a factor of 5 to 10 in different experiments. Therefore, this 259-nt fragment, which extended from the *Nco*I site at the translation initiation codon to the *Taq*I site at position 44 of exon 2, was sufficient to reproduce the silencer effect of the entire α-globin gene.

Thus, we have demonstrated the existence of a silencer element in the α-globin gene which inhibits the SV40-enhanced expression of a linked β- or γ-globin gene. This observation supports the hypothesis that an endogenous α-globin globin enhancer may have been masked by this silencer in the experiments of Humphries et al. (10) and Treisman et al. (16) described earlier, in which the α- and β-globin genes were linked. Therefore, the question whether

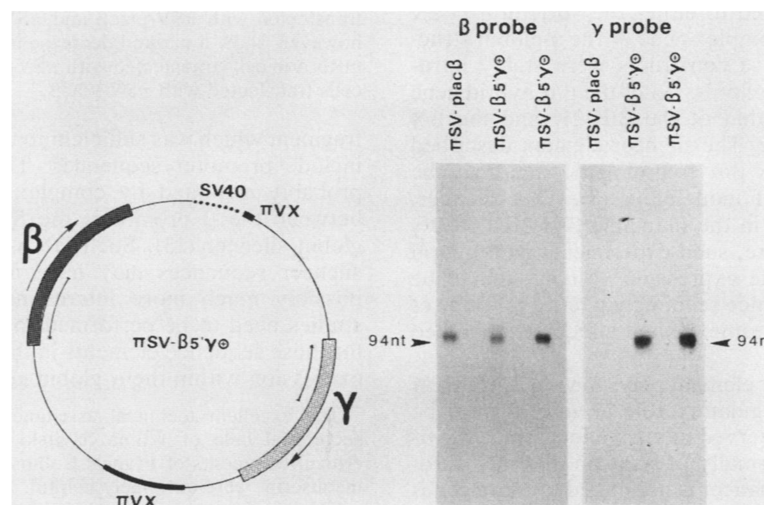

FIG. 4. Study of the *cis* interactions of the β- and γ-globin genes. The organization of the linked β- and γ-globin genes on πSV-β5′γ(+) is shown in the schematic on the left. πSV-β5′γ(−) is identical to πSV-β5′γ(+) except for the orientation of the γ-globin gene. The first three lanes show that the level of accumulated β-globin mRNA was approximately the same in cells transfected with πSV-placβ, πSV-β5′γ(+), and πSV-β5′γ(−). The fourth lane shows no γ-globin mRNA in cells transfected with πSV-placβ, while significant accumulation of γ-globin mRNA was seen in cells transfected with πSV-β5′γ(+) (fifth lane) and πSV-β5′γ(−) (sixth lane).

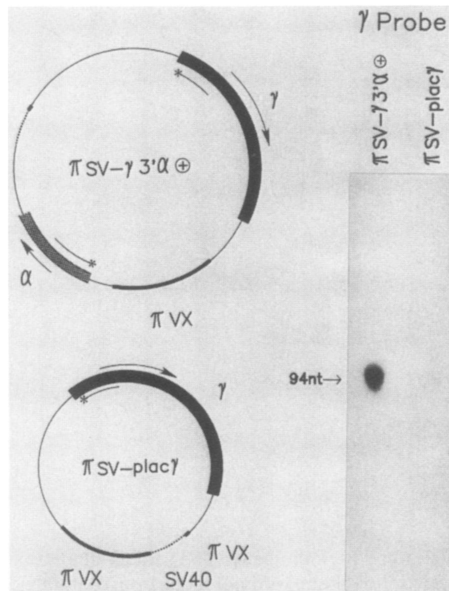

FIG. 5. Effect of a linked α-globin gene on γ-globin expression. The organization of the α- and γ-globin genes on πSV-placγ and πSV-γ3'α(+) is shown in the schematic on the left. The autoradiograph on the left represents an S1 nuclease study, which shows the high level of γ-globin mRNA accumulation in cells transfected with πSV-placγ (first lane). A very profound suppression of γ-globin gene expression to near undetectable levels occurred when an α-globin gene was linked to the γ gene on πSV-γ3'(+), as seen in the second lane.

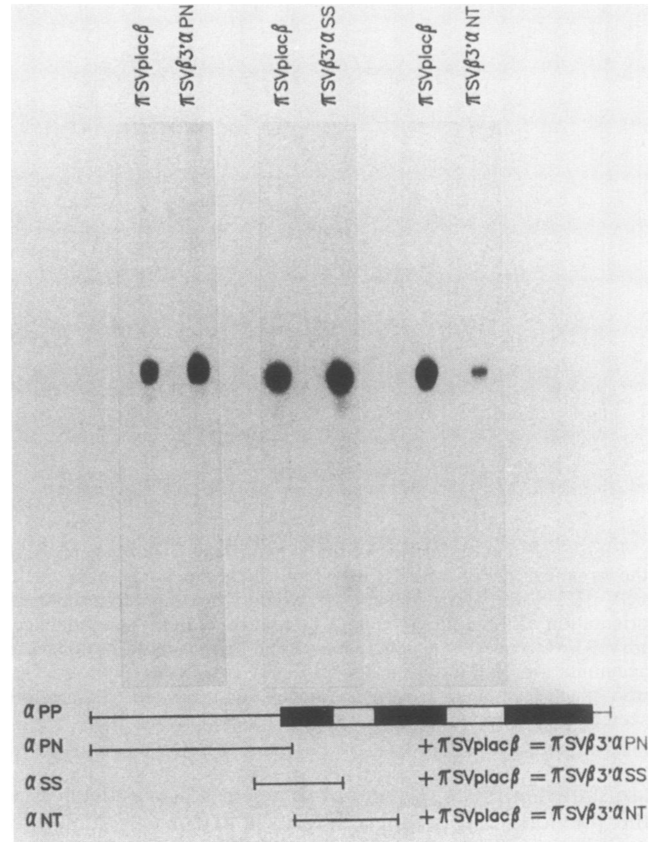

FIG. 6. Localization of the silencer sequences of the α-globin gene. In the schematic map of the α-globin gene below the autoradiograph, filled boxes represent exons and empty boxes represent introns. αPN, αSS, and αNT are the different restriction fragments (P, *Pst*I; N, *Nco*I; S, *Sma*I; T, *Taq*I) from the 5' end of the α-globin gene that were linked to the 3' end of the β-globin gene on πSV-placβ to generate πSVβ3'αPN, πSVβ3'αSS, and πSVβ3'αNT, respectively. The first two lanes of the autoradiograph demonstrate that β-globin mRNA accumulated to similar levels in cells transfected with πSV-placβ and πSV-β3'αPN. The third and fourth lanes show that β-globin mRNA also accumulated to similar levels in cells transfected with πSV-placβ and πSV-β3'αSS. The last two lanes, however, show a marked decrease in the accumulation of β-globin mRNA in cells transfected with πSV-β3'αNT compared with that in cells transfected with πSV-placβ.

the α-globin gene contains an endogenous enhancer or whether its promoter is truly enhancer independent remains unanswered, and a thorough search for such an enhancer by a different approach is warranted. This observation may also have implications for other experiments in which hybrid genes were introduced into mouse erythroleukemia (MEL) cells in order to localize the region of the β-globin gene responsible for its regulated expression. These hybrid genes consisted of the β promoter linked to the α structural gene (8) or the β promoter linked to either the structural H-2K major histocompatibility complex gene or the γ-globin structural gene (19). When these constructs were stably introduced into MEL cells, the expression of the β/α hybrid gene was not inducible, while that of the β/H-2K and the β/γ hybrid genes was inducible. The silencer element described here was included in the β/α hybrid gene and may be responsible for its lack of inducibility (8). This silencer, however, was not present in the inducible β/H-2K and β/γ hybrid genes (19). Therefore, such experiments with hybrid genes in transient or stable expression systems should be interpreted with caution, since seemingly neutral portions of a particular gene may exert unexpected effects when linked to other genes.

The role that this silencer element plays in vivo is not clear at this stage. If it has a regulatory role in vivo, it must be quite different from the observed in vitro effect, since the α- and β-globin genes are normally present on different chromosomes and the SV40 enhancer is not linked to those genes in vivo. Although the mechanism by which the silencer exerts its effect has not been worked out, it is clearly different from the promoter occlusion model (1) and the transcriptional interference model (9), since this effect was position and orientation independent and since the 259-nt fragment which was sufficient to reproduce this effect did not include promoter sequences. This silencer effect is most probably mediated by complex DNA-protein interactions between the β promoter, the SV40 enhancer, and the α-globin silencer (13). Such DNA-protein interactions in the silencer sequences may have a completely different and possibly much more interesting function in vivo. More studies need to be performed to investigate a possible role for these sequence elements in the in vivo regulation of gene expression within the α-globin gene cluster.

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