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Two closely spaced promoters are equally activated by a remote enhancer: evidence against a scanning model for enhancer action

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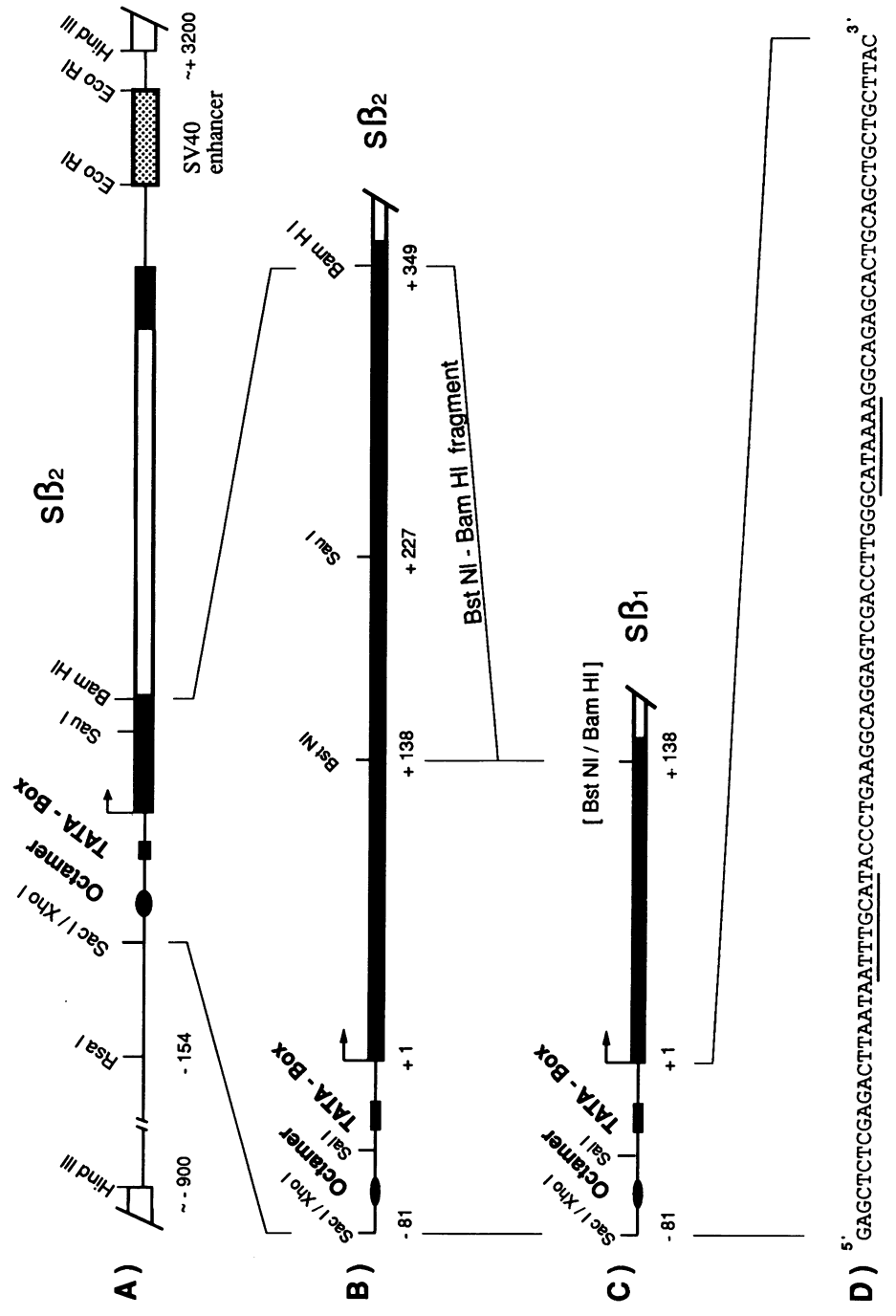
ABSTRACT

To explain the activation of transcription by a remote enhancer, two models are most often considered, namely looping and scanning. A scanning model, also referred to as 'polymerase entry site' model predicts that for two adjacent promoters the one proximal to an enhancer would be preferentially activated. Preferential activation of the proximal promoter in a tandem promoter arrangement has been found before in several laboratories, including our own, but for technical reasons the data were inconclusive with regards to the enhancer mechanism. In the work presented here, we readdress the question of preferential promoter activation by an enhancer using a more clearly defined system. Two identical promoters were kept closeby in a divergent, or directly repeated orientation. The SV40 enhancer was placed at a great distance on one or the other side of the two promoters, to see whether the enhancer position influenced the relative efficiency of the two promoters in transfected cells. Our finding that the promoter usage is virtually unaffected by the enhancer position does not favor a scanning model, but is compatible with a looping model of enhancer action.

INTRODUCTION

Enhancers are operationally defined by the following features: (i) they strongly activate transcription of a linked gene in cis; (ii) they function in either orientation over considerable distances, up to many thousand base pairs and largely independently of position, i.e. they can also be located within the transcribed region or even downstream; (iii) in many cases they confer cell type specificity or inducibility to the gene they control. Enhancers stimulate initiation of transcription by increasing the RNA polymerase II density over the linked gene (1, 2). Enhancers can be dissected into several modules (sequence motifs; elements;), which are known to bind transcription factors (3−7). In many cases, single enhancer modules can exert an enhancer effect when oligomerized (8−11). Hence, it is likely that many if not all of the enhancer binding transcription factors act via a common mechanism, involving, for example, negatively charged residues in the protein domain that activates transcription (12−14).

Numerous models have been proposed to explain the enhancer mechanism (3,4,5,15−22). Today, the two principal models most often considered are the scanning model and the looping model. The looping model proposes that proteins bound to promoter and enhancer sequences associate to form a complex and thereby facilitate initiation of transcription. As a consequence of protein complex formation, the intervening DNA is looped out. Solid evidence for DNA looping came from investigations in prokaryotes concerning gene regulation by cooperative repressor binding over a distance, site-specific recombination


A)
Hind III
Eco RI
Eco RI
SV40 enhancer
~+ 3200
Hind III
~ - 900
Rsa I
- 154
Sac I / Xho I
Octamer
TATA - Box
Sau I
Bam HI
Sβ2
B)
Sac I / Xho I
Octamer
TATA - Box
Sal I
- 81
+ 1
Bst NI
+ 138
Sau I
+ 227
Bam H I
+ 349
Sβ2
Bst NI - Bam HI fragment
C)
Sac I / Xho I
Octamer
TATA - Box
Sal I
- 81
+ 1
[Bst NI / Bam HI]
+ 138
Sβ1
D) 5' GAGCTCTCGAGACTTAATAATTTGCATACCCTGAAGGCAGGAGTCGACCTTGGGCATAAAAGGCAGAGCACTGCAGCTGCTGCTTAC 3'

and DNA replication (19,23−33). In eukaryotes however, evidence for a looping mechanism is rather sparse. In several cases, evidence for cooperative action or binding of transcription factors over a distance has been observed (34−37). Takahashi et al. (38) found that insertion of an odd number of half helix turns between the SV40 early promoter upstream elements and the TATA box decreased transcription *in vivo* drastically. These experiments can be taken as evidence for DNA looping. However, they addressed only short range interactions, and in many cases the efficiency of eukaryotic transcription is not affected by the insertion of half helix turns (39−43).

The scanning, or entry site model is based on the assumption that enhancer or upstream promoter elements are 'entry sites' with a high affinity for RNA polymerase II (or another transcription factor(s)), which after binding scans bidirectionally along the DNA until it encounters a promoter (15). This idea was supported by experiments in which a physical obstacle, such as a bacterial repressor bound to its operator site, or chemically modified DNA bases, was placed between enhancer and promoter, thus reducing transcription of the linked gene (44,17).

An important prediction from the scanning mechanism is that from two adjacent promoters the proximal one should be preferentially activated by an enhancer. Some experiments support this interpretation (45−47), while others do not (48,49). However, in all the former cases a looping mechanism cannot be excluded, since it is also possible that modifications of DNA could interfere with loop formation. Furthermore, one of the nonidentical promoters was always considerably closer to the enhancer than the other and chain statistics predict that a smaller DNA loop is energetically favored (32).

In the work presented here we readdress the question of preferential promoter activation by an enhancer using a more clearly defined system. We constructed plasmids containing two different rabbit β-globin reporter genes with identical, synthetic promoters arranged in divergent or tandem orientation. The promoters, which should be of equal intrinsic strength, were kept close to each other. The SV40 enhancer (72 bp repeat) however, was located on either side of the combined transcription units at a relatively great distance, thus excluding or at least reducing short range distance effects (50) which could also be explained by a preferential loop formation between closely spaced enhancer and promoter sequences (as discussed above). After transfection into HeLa and J558L myeloma cells, the relative transcript levels of each gene were quantified.

Our results yield no evidence for a promoter preference, regardless of the enhancer position. Thus they are compatible with a looping model, rather than a scanning model.

Figure 1. Description of the plasmids sβ1 and sβ2
A) The constructs sβ2 with a β-globin gene, in which the first intron was removed, as in all derivatives of the OVEC reporter genes (51) is shown. A 200 bp Eco RI fragment containing the SV40 enhancer (from wild type position +99 to +294) is inserted into the unique Eco RI site at positions +1767 in sβ2 and +1554 in sβ1, respectively. The SV40 enhancer is active in both B- and non B-cells. The β-globin gene sequences (in the case of sβ2 extending about 900 bp 5′ and 3200 bp 3′ of the transcription start site) are embedded in pUC18 sequence (thin lined open box). Solid box, exon sequences; heavy lined open box, intron sequence; solid line, β globin 5′ and 3′ sequence; B) shows an enlarged section of sβ2 in comparison to sβ1 (C) which lacks the 211 bp Bst NI−Bam HI fragment. D) Upstream sequence from −81 to +1 with the 'TATA' box and the 'octamer' sequence ATTTGCAT (underlined). This promoter region is common to all plasmids used except for the reference gene OVEC REF (51).
The figures are not drawn to scale.

RESULTS

Construction of two different β-globin genes that can be easily distinguished by RNase mapping.

In order to set up a test system in which we would be able to distinguish the transcriptional response of two genes with identical promoters on one plasmid, we constructed sβ1 and sβ2 (single β-globin; Fig. 1A, B). These are derivatives of the OVEC reporter gene system (51). sβ1 and sβ2 have a promoter containing a single 'octamer' sequence (ATGCAAAT) and a TATA box (Fig. 1D). This promoter is common to all plasmids used except the β-globin reference gene OVEC REF (51), which was cotransfected in all experiments to

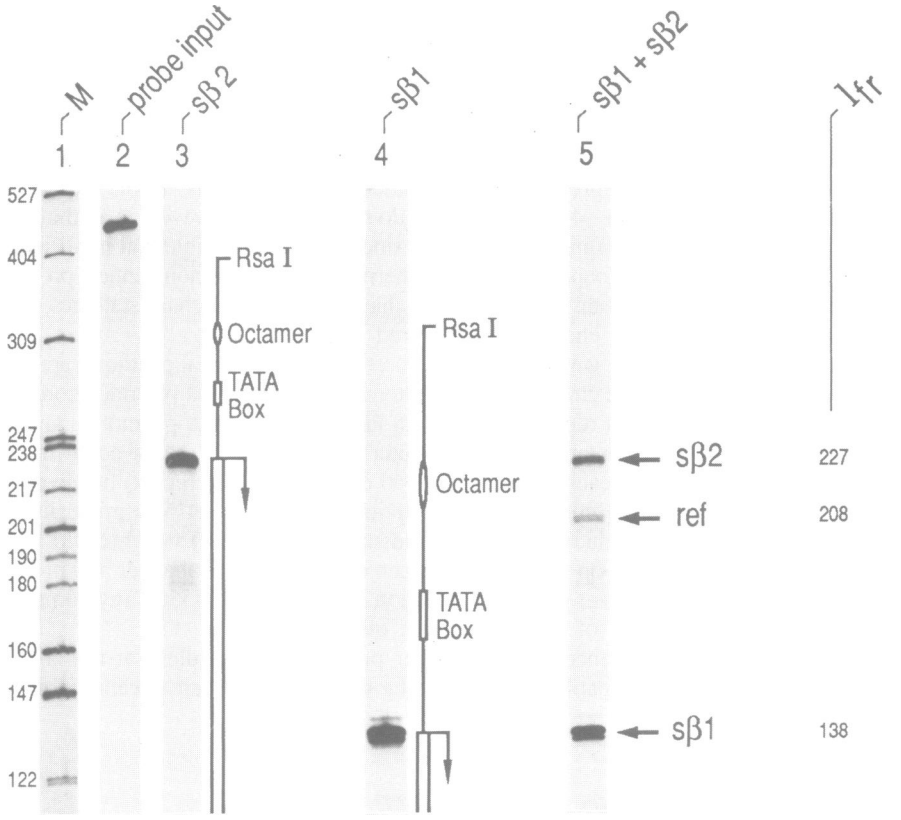


Figure 2. RNase mapping of sβ1, sβ2 and OVEC REF.

18 μg of sβ1 (lane 3) or sβ2 (lanes 4) or 9 μg each of plasmid DNA sβ1,sβ2 and 0.4 μg of OVEC REF DNA (lane 5) were transfected into HeLa cells. The RNA from the transfections was processed as described in Materials & Methods, and the results of the RNA analysis on a 5% polyacrylamide/urea gel are shown here. The theoretical ratio of radioactivity present in the sβ1 and sβ2 signals is β1 : β2 = 0.556 (see Materials & Methods). The same type of analysis was done to obtain the data presented in Figures 4, 5, 6, and 8. The correction value of 0.556 was also used for Tables 1–4. Lane 1: pBR322 Hpa II digested size marker; lane 2: input probe; lane 3: sβ2 with corresponding schematic diagram; lane 4: sβ1 with corresponding schematic diagram; lane 5: cotransfection of sβ1, sβ2 and OVEC REF; the length of the Hpa II digested restriction fragments of the size marker is given in nucleotides; arrows indicate the signals corresponding to correctly initiated transcripts (sβ1, OVEC REF, sβ2); l_{fr}, length of the protected fragments as expected from the plasmid sequence.

compare transfection efficiencies. sβ1 is distinguished from sβ2 by a deletion in the coding body of the β-globin gene (Fig. 1B,C). For RNase-mapping, a homologous probe of 445 nucleotides was inserted into the polylinker of a Bluescript vector (see Materials & Methods). With this probe the sβ1, sβ2 and reference gene transcripts could be mapped simultaneously.

To confirm that we could identify the transcript from each of these identical promoters, HeLa cells were transfected with the reference gene (OVEC REF.) plus either sβ1 or sβ2, or a mixture. The signals from sβ1 (Fig. 2, lanes 4,5) and sβ2 (Fig. 2, lanes 3,5), indicated by arrows, correspond to the correct transcription initiation site. None of the additional weak signals overlaps the correct transcriptional start sites of sβ1, sβ2 and the reference gene OVEC REF (Fig. 2, lane 5).

Two divergently oriented, identical promoters are equally activated by the SV40 enhancer

We used a new approach to readdress the question of preferential promoter activation by an enhancer: (i) we tested two inverted transcription units with identical promoters; (ii) the promoters were kept close to each other, whereas the enhancer was placed at a relatively large distance to the combined promoters. An inverted gene arrangement is useful because it excludes some problems which might occur with tandem promoters such as promoter occlusion or RNA instability effects (see below). We reasoned that locating the enhancer

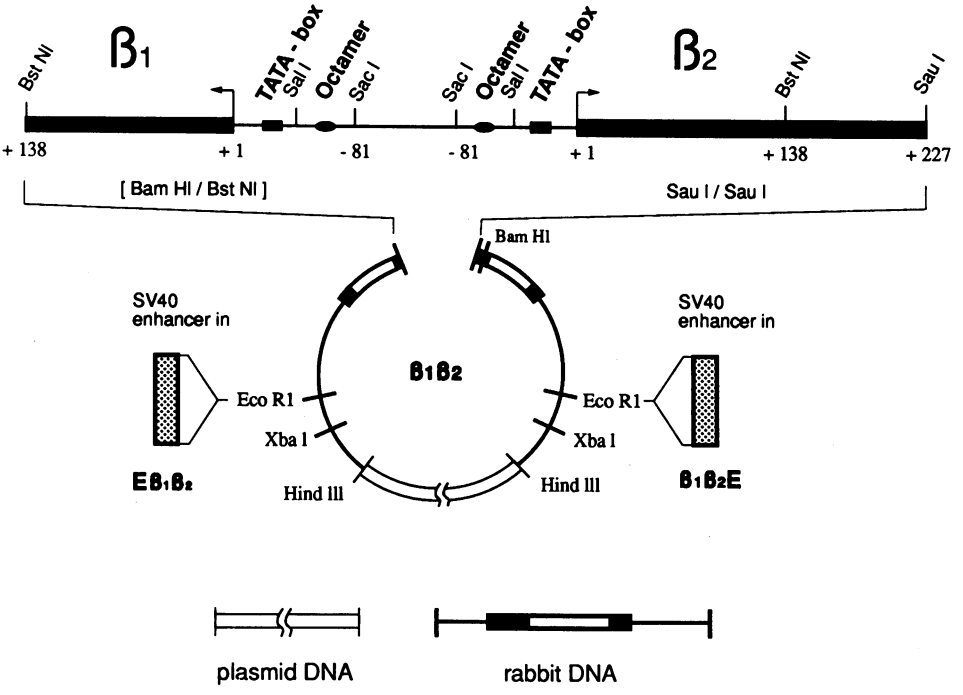
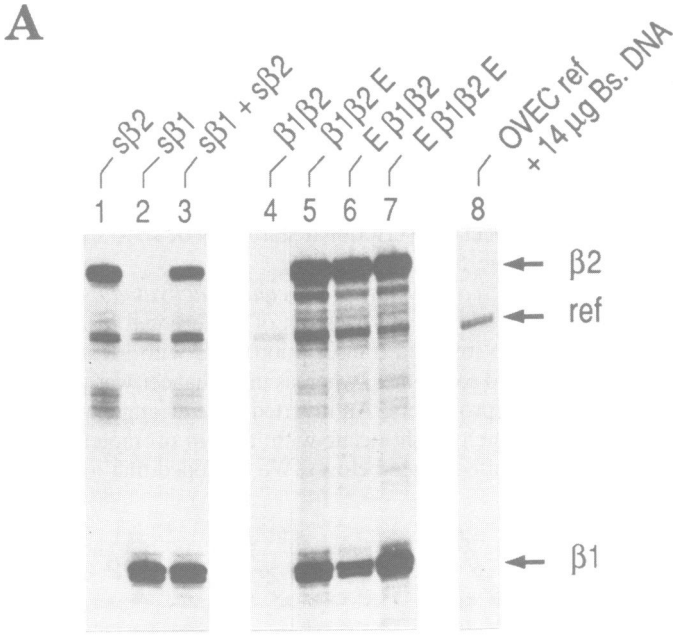


Figure 3. Schematic representation of recombinant plasmids with divergent β-globin genes
A) A 200 bp SacI-SacI spacer DNA was cloned between two Sac I–Hind III fragments derived from the plasmids sβ1 and sβ2. The vector DNA was derived from either pUC18 or a 10 kb pCRI derivative, as indicated in the text. An SV40 enhancer is inserted into the Eco RI site either on one side (β1β2 E, or E β1β2;) or on both sides (E β1β2 E) of the divergent transcription units. The distances between the enhancers and the center point between the two promoters is 1735 bp and 1948 bp for E β1β2 and β1β2 E respectively. The distance between the two promoters is 200 bp. The figures are not drawn to scale.


A
sβ2
sβ1
sβ1 + sβ2
β1β2
β1β2 E
E β1β2
E β1β2 E
OVEC ref
+ 14 µg Bs. DNA
1 2 3 4 5 6 7 8
β2
ref
β1

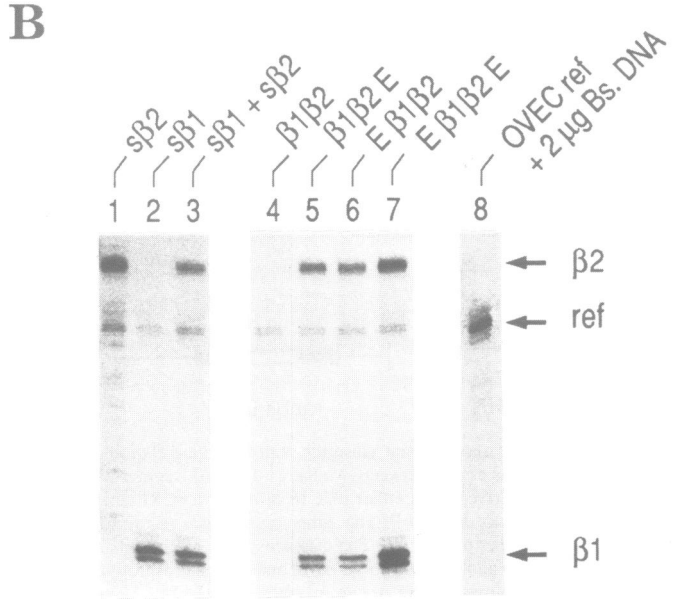

B
sβ2
sβ1
sβ1 + sβ2
β1β2
β1β2 E
E β1β2
E β1β2 E
OVEC ref
+ 2 µg Bs. DNA
1 2 3 4 5 6 7 8
β2
ref
β1

Table 1. Ratios of transcript levels for divergent β globin genes

Clone	HeLa β_1 / β_2	J558L β_1 / β_2
$s\beta_1$ + $s\beta_2$	4.44	4.02
$\beta_1\beta_2$	3.01	–
E $\beta_1\beta_2$	1.14	3.82
$\beta_1\beta_2$ E	0.77	3.38
E $\beta_1\beta_2$ E	1.67	5.13

Gel slices corresponding to the main signals of β1, β2 shown in Figure 4 were cut out, quantified by Cerenkov counting and expressed as a ratio (see Materials & Methods). The same type of analysis was done to obtain the data presented in all Tables. A minus sign (−) indicates that the counts obtained were lower than twice the background.

on either or both sides of the two β-globin genes, could reveal a preference in promoter usage, if one existed. Since a promoter preference would be most easily explained by a scanning mechanism, one should be able to provide evidence favoring or disfavoring this model of enhancer activation.

The complete sβ1 and sβ2 genes (starting at position −81) were combined in one plasmid (Fig. 3). The divergently arranged promoters are separated by 200 bp of spacer DNA comprising the upstream sequence of the wild type rabbit β-globin gene between positions −1250 and −1050.

Since the transcripts were not identical even though transcribed from identical promoters, we could not expect them to be present in an exact 1:1 ratio. However an unequal representation of the two transcripts could indicate preferential promoter utilisation. To distinguish between these possibilities, the enhancer was placed either far upstream or far downstream of the two divergent promoters. We reasoned that the given ratio of transcripts would not depend on the position of the enhancer unless there was indeed a preferential activation of the proximal promoter due to a scanning mechanism.

The plasmids Eβ1β2, β1β2E, Eβ1β2E, sβ1 and sβ2 were cotransfected with OVEC REF into HeLa and J558L myeloma cells. We used two different cell lines, firstly, to show that the observations are not restricted to a specific cell line and secondly to test the effects under conditions of strong and weak transcription, since the octamer/TATA promoter is relatively strong in J558L cells compared to HeLa cells. The RNA analysis is shown in Figure 4. The transcript ratios (β1/β2) for the four test constructs range between 0.7 and 1.7 for HeLa cells and between 3.4 and 5.1 for J558L myeloma cells (Table 1). These slight variations might be due to long range distance effects (compare individual ratios in Table 1 and enhancer/promoter distance data in the legend to Fig. 3). Most importantly, there was no clearcut preference in promoter usage depending on the enhancer position. The β1/β2 ratios of the transcripts obtained with sβ1 and sβ2 in HeLa and J558L

Figure 4. No preferential promoter usage is obtained with divergently arranged β-globin constructs.
A) HeLa cells were transfected with 14 μg of the indicated test plasmid and 0.4 μg OVEC REF DNA (lanes 1,2,4 to 7), or with 7 μg sβ1 + 7 μg sβ2 DNA and 0.4 μg OVEC REF DNA (lane 3), or with 0.4 μg OVEC REF DNA and 14 μg of Bluescript DNA (Bs. DNA) as carrier DNA (lane 8). B) J558L myeloma cells were transfected with 2 μg of the indicated test plasmid and 0.2 μg OVEC REF DNA (lanes 1,2,4 to 7), or with 1 μg sβ1 + 1 μg sβ2 DNA and 0.2 μg OVEC REF DNA (lane 3), or with 0.2 μg OVEC REF DNA and 2 μg of Bluescript DNA (Bs. DNA) as carrier DNA (lane 8).

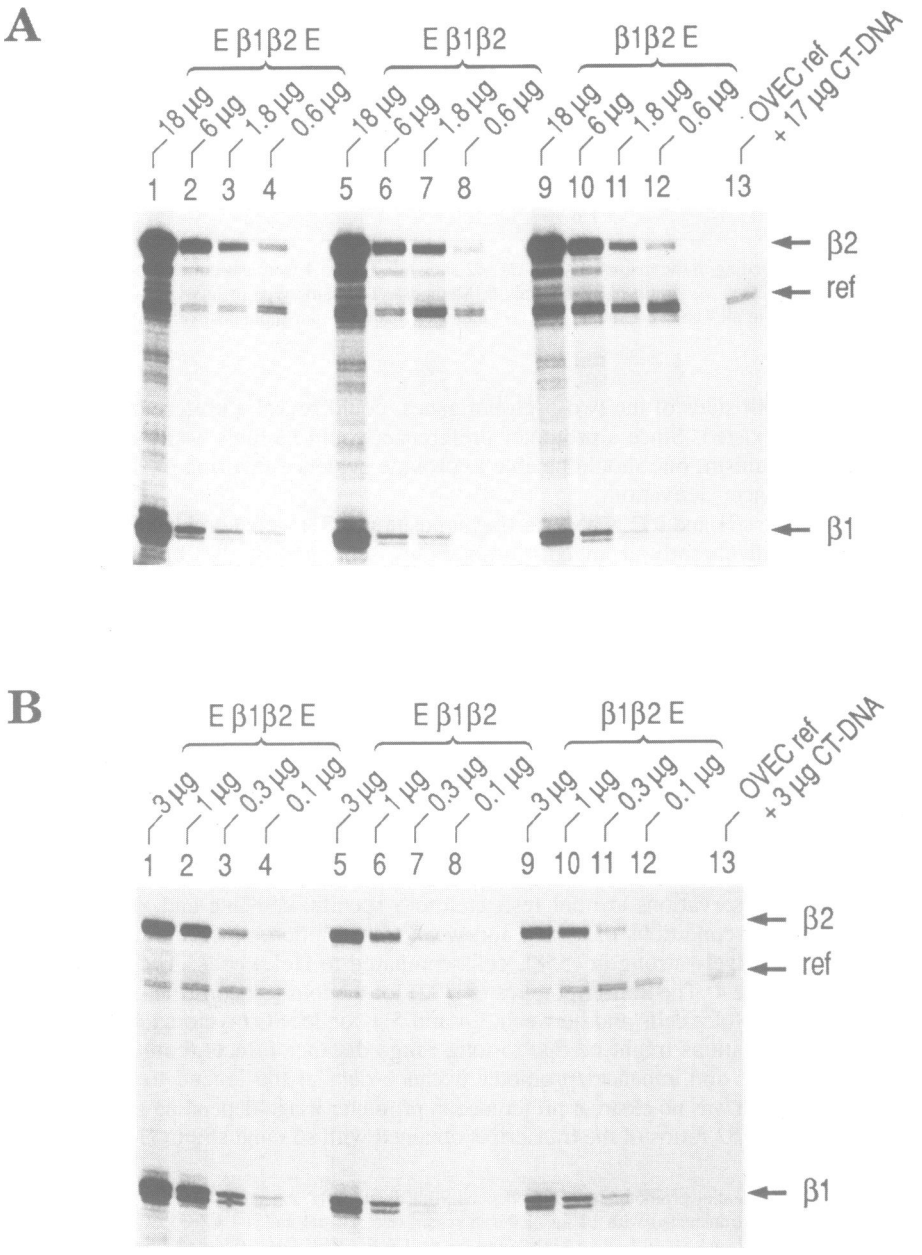


Figure 5. Plasmid dilution experiment of divergent constructs
A) HeLa cells; B) J558L myeloma cells. The amounts of transfected test DNAs are given in the Figure. 0.4 µg [0.1 µg] OVEC REF was cotransfected in HeLa [J558L myeloma] cells. Calf thymus (c.t.) DNA was added as carrier DNA to achieve equal amounts of DNA in each transfection.

Table 2. Ratios of transcript levels for the plasmid dilution experiment

Clone / Dilut.	HeLa Plasmid DNA	HeLa β_1 / β_2	J558L Plasmid DNA	J558L β_1 / β_2
E $\beta_1\beta_2$	18.0 μg	0.71	3.0 μg	2.53
/ 3 ×	6.0 μg	0.69	1.0 μg	1.91
/ 10 ×	1.8 μg	0.83	0.3 μg	2.76
/ 30 ×	0.6 μg	0.40	0.1 μg	2.99
mean value		0.66 ± 0.18		2.55 ± 0.47
$\beta_1\beta_2$ E	18.0 μg	0.46	3.0 μg	2.47
/ 3 ×	6.0 μg	0.59	1.0 μg	1.93
/ 10 ×	1.8 μg	0.84	0.3 μg	2.48
/ 30 ×	0.6 μg	0.73	0.1 μg	3.31
mean value		0.66 ± 0.17		2.55 ± 0.57

Gel slices corresponding to the main signals of β1, β2 shown in Figure 5 were cut out and quantified by Cerenkov counting. For the dilution series of each construct the mean value and the standard deviation is indicated.

myeloma cells and for the divergent constructs in J558L myeloma cells are quite similar, with values of around 4 (Table 1). For unknown reasons however the β1/β2 ratios of the transcripts obtained from the divergent constructs in HeLa cells are significantly lower (Table 1). The latter finding, which was not further analysed, does not influence the conclusions about relative promoter usage depending on the enhancer position.

No promoter preference is found at any template DNA concentrations.

Promoter preference could possibly be obscured by template saturation effects. In our experiments this might have meant that in a single cell nucleus with many copies of a particular plasmid, only one of the two identical promoters on any one plasmid is functionally saturated with the necessary transcription factors, and hence active. Therefore we performed the following dilution experiment. HeLa cells and J558L myeloma cells were transfected with various amounts of plasmid DNA (Fig. 5; Table 2). The constructs tested were β1β2E, Eβ1β2, Eβ1β2E. As seen in Figure 5, the β1 and β2 signals steadily decrease in intensity within each individual dilution series. The mean values for the β1/β2 ratios of the individual dilution series remain essentially unchanged, with standard deviations between 20 and 30% (Table 2). Thus, within the range of the dilution series, the same results are obtained at high or low template concentrations i.e. we do not obtain evidence for a preferential promoter usage.

No promoter preference is detected even with a 10 kb vector sequence instead of the 2.6 kb pUC18 sequence

Since all our experiments are done with circular template DNA, one might argue within the framework of a scanning model that each promoter could be activated from either side through the vector DNA. To exclude this possibility we took advantage of an observation made in several laboratories, namely that the activating effect of an enhancer is inhibited or even totally blocked if the enhancer is flanked on both sides by long stretches of prokaryotic DNA (17,52, 53). Whatever the cause of the blocking effect, a very long stretch of prokaryotic DNA separating enhancer from the promoters only on one side, but not on the other should ensure that the enhancer effect is directed only to one side (53). It might have been that the plasmid DNA used in our experiments was too short to prevent a bilateral enhancer/promoter interaction since the inhibitory effect described, for example, by Banerji et al. (52) and Schreiber and Schaffner (53) was observed with slightly longer

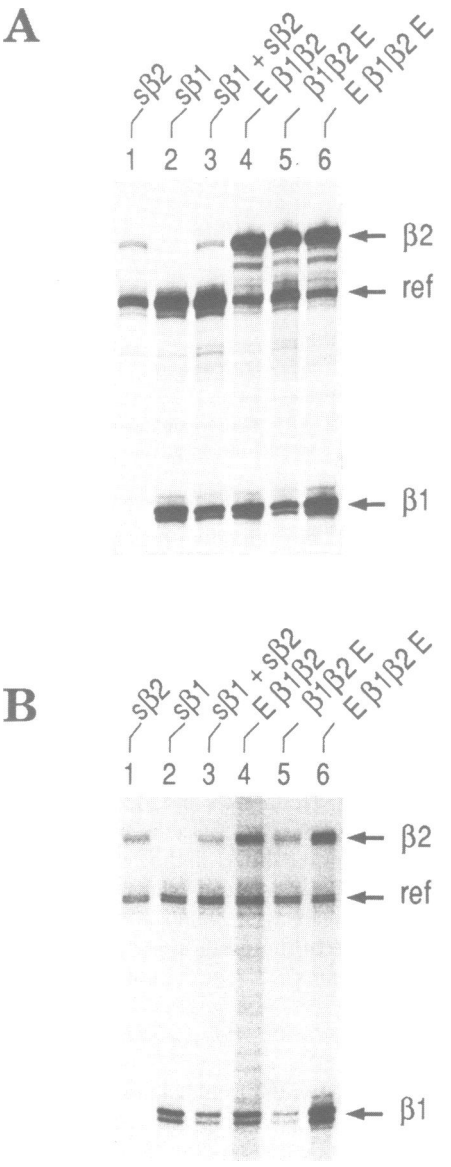


Figure 6. No preference in promoter usage with divergent β-globin gene constructs in plasmids where the 2.6 kb pUC18 sequence is replaced by a 10 kb pCR1Δ vector sequence.
A) Transfection of HeLa cells with 28 μg of test plasmid and 0.4 μg OVEC REF DNA (lanes 1, 2, 4, 5, 6), or 14 μg sβ1 + 14 μg sβ2 DNA and 0.4 μg OVEC REF DNA (lane3). B) Transfection of J558L myeloma cells with 4 μg of test plasmid and 0.2 μg OVEC REF DNA (lanes 1, 2, 4, 5, 6), or 2 μg sβ1 + 2 μg sβ2 DNA and 0.2 μg OVEC REF DNA (lane3). Transfection mixes for HeLa [J558L myeloma] cells in the experiments shown in Figure 4 were supplemented with 14 μg [2 μg] Bluescript (Bs.) DNA as carrier DNA to achieve, firstly, equal amounts of transfected DNA and, secondly, equimolar amounts of plasmids when using the 2.6 kb pUC18 sequence in case of the 10 kb pCR1Δ sequence as vector DNA in this experiment.

Table 3. Ratios of radioactivity for constructs with divergent β globin genes, where the 2.6 kb pUC18 vector sequence was replaced by a 10 kb pCRIΔ vector sequence

Clone	HeLa β_1 / β_2	J558L β_1 / β
$s\beta_2$ + $s\beta_1$	8.48	10.31
E $\beta_1\beta_2$	1.06	4.07
$\beta_1\beta_2$ E	0.75	2.62
E $\beta_1\beta_2$ E	1.54	5.95

Gel slices corresponding to the main signals of β1, β2 shown in Figure 6 were cut out and quantified by Cerenkov counting.

vector sequences than the 2.6 kb pUC18 used in our constructs, namely a 3.7 kb pJC-1 sequence or a 3.3 kb pBR327 sequence. Therefore the pUC18 fragment was replaced by the 10 kb pCRIΔ (see Materials & Methods) in sβ1, sβ2 and in the $\beta1\beta2$ constructs (E$\beta1\beta2$,

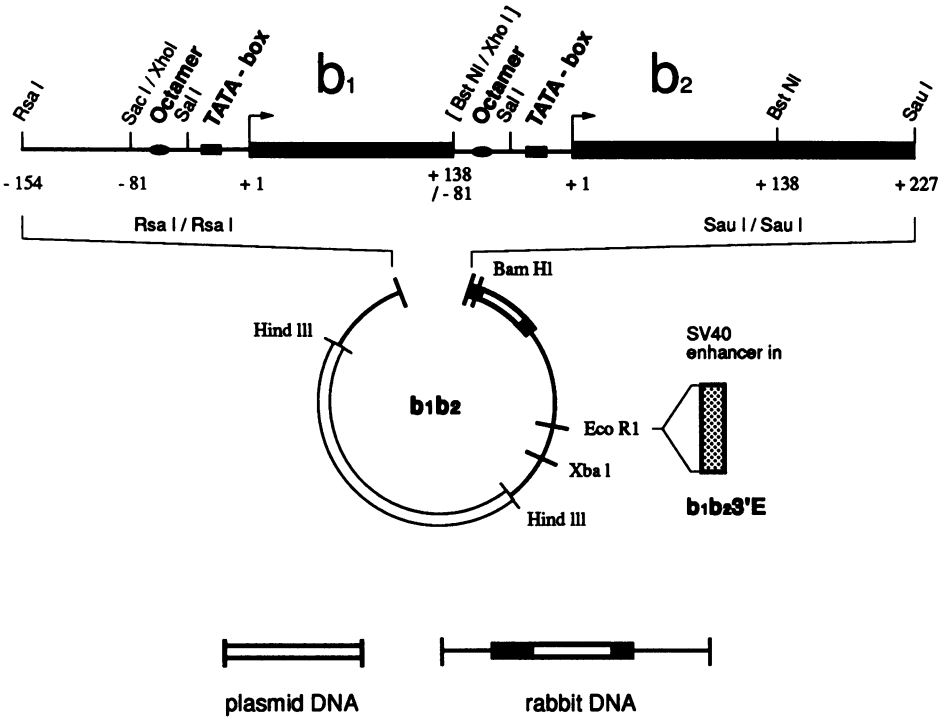


Figure 7. Structure of plasmids with tandem transcription units
Enlarged section of tandem β-globin genes (b1b2) showing the 3′ truncated b1 gene and a part of the b2-globin gene. If the SV40 enhancer is inserted 3′ of the two β-globin genes, the plasmid is designated b1b2 3′E. In order to test the enhancer in a 5′ position, a 2350 bp Bam HI- Xba I fragment from sβ2 was inserted into the remaining Xho I -site of b1b2, thus yielding the plasmid 5′ Eb1b2 (not shown). The distances between the enhancer upstream of the b1 gene or downstream of the b2 gene and the center of the their promoters is 1606 bp and 1875 bp respectively. The distance between the two promoters is 219 nts. We also constructed the plasmid 5′E b_1b_2 3′E which has an enhancer 5′ and 3′ of both β-globin genes as an additional control. Figures are not drawn to scale.

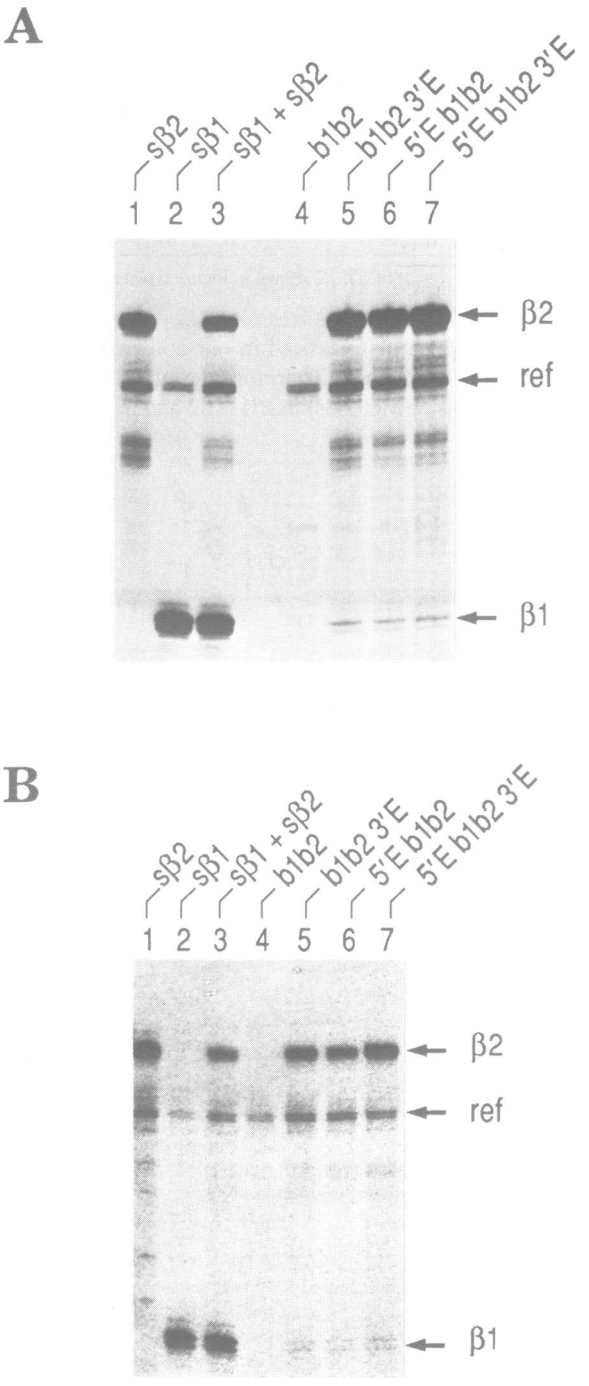

A
sβ2
sβ1
sβ1 + sβ2
b1b2
b1b2 3'E
5'E b1b2
5'E b1b2 3'E
1 2 3 4 5 6 7
β2
ref
β1
B
sβ2
sβ1
sβ1 + sβ2
b1b2
b1b2 3'E
5'E b1b2
5'E b1b2 3'E
1 2 3 4 5 6 7
β2
ref
β1

Table 4. Ratios of transcript levels for tandem β-globin genes

Clone	HeLa b_1 / b_2	J558L b_1 / b_2
$s\beta_2$ + $s\beta_1$	4.44	4.02
b_1b_2	2.05	–
b_1b_1 3E	0.19	0.56
5'E b_1b_2	0.14	0.47
5'E b_1b_2 3'E	0.11	0.37

Gel slices corresponding to the main signals of β1, β2 shown in Figure 8 were cut out and quantified by Cerenkov counting. A minus sign (−) indicates that the counts obtained were lower than twice the background.

β1β2E, Eβ1β2E). After transfection in HeLa and J558L myeloma cells, these constructs gave results very similar to the pUC18 containing plasmids (compare Fig. 6, Table 3 with Fig. 4, Table 1). Thus it is appears that the pUC18 plasmid and the pCRIΔ plasmid are equally effective in preventing activation of the second promoter 'around the circle' (through the vector DNA).

Transcriptional activation of directly repeated, identical promoters is also independent of the enhancer position.

Finally, we wanted to confirm the results obtained with divergent promoters using constructs with a tandem arrangement of promoters (Fig. 7). The distance between the two directly repeated promoters was kept small, whereas the enhancer was located at a relatively great distance either 5′ or 3′ of the two transcription units (see legend to Fig. 7).

The test genes b1b2, 5′E b1b2, b1b2 3′E, 5′E b1b2 3′E and sβ1 and sβ2 were transfected into HeLa and J558L myeloma cells. The results for the steady state transcript levels are given in Figure 8 and Table 4. The b1 signals of the test constructs (Fig. 8, lanes 5 to 7) are much weaker compared to sβ1 which contains the complete β1 gene. Unfortunately, the b1 RNA is considerably less stable than the b2 RNA (data not shown), most probably because its transcription unit lacks a polyadenylation signal and runs directly into the downstream b2 promoter. The b2 signals of the test constructs have about the same intensity as the sβ2 signal (Fig. 8, lane 1). The enhancerless tandem construct b1b2 gives almost no detectable signals, as expected (Fig. 8, lane 4).

Although the signals produced by the tandem b1 globin genes are weak, the results confirm those obtained with divergent promoters. The ratios of relative transcript levels remain essentially unchanged, irrespective of the enhancer position.

DISCUSSION

To explain the activation of transcription by a remote enhancer, two models are most often considered, namely looping and scanning. A scanning model, also referred to as polymerase entry site model, would predict that in an experimental situation with two adjacent promoters the one closer to the enhancer is preferentially activated. Although this sounds like a very straightforward experiment, there are numerous technical problems. Firstly, the promoters

Figure 8. mRNA analysis of β-globin tandem constructs
A, HeLa cells were transfected with 14 μg of the indicated test plasmid and 0.4 μg OVEC REF DNA (lanes 1,2,4 to 7), or with 7 μg sβ1 + 7 μg sβ2 DNA and 0.4 μg OVEC REF DNA (lane 3). B, J558L myeloma cells were transfected with 2 μg of the indicated test plasmid and 0.2 μg OVEC REF DNA (lanes 1,2 4 to 7), or with 1 μg sβ1 + 1 μg sβ2 DNA and 0.2 μg OVEC REF DNA (lane 3).

should be identical, yet their transcripts must be distinguishable by some difference in the coding sequence. This, however, almost invariably results in a difference in transcript stability and non-identical representation of transcripts. To overcome this problem, we have placed an enhancer on either side of two identical promoters (Fig. 1D, Fig. 3, Fig. 7), to see whether the enhancer position shifts the transcript ratio, i. e. the promoter usage. A distinction between scanning and looping might be obscured when the enhancer is much closer to one of the two promoters, since looping might be favored with a short intervening DNA as was theoretically predicted (32). Therefore, we have placed the enhancer far away from the two promoters, which were kept at a short distance from each other. The latter was achieved by constructing two divergent transcription units (Fig. 3), and also by creating two directly repeated tandem promoters (Fig. 7). The transcript levels of divergent and tandem promoters were similar within each construction series tested, irrespective of the enhancer position. Even in the case of directly repeated promoters, where the transcript from the first promoter is always weak, the same overall conclusion emerges.

At present we cannot exclude the possibility that the findings of this paper reflect a peculiarity of octamer-containing promoters. However we considered this unlikely, in particular since additional octamer binding factors are present in B cells (J558L) versus non-B cells (HeLa).

Since circular plasmids were used in our transient expression assays, we also had to exclude an activation from both sides 'around the clock'. A spacer DNA of prokaryotic origin inserted between enhancer and promoter is known to prevent transmission of the enhancer effect (17,52,53). Therefore, we tested two segments of prokaryotic DNA of different length separating the enhancer from the promoters on one side. The same results were obtained with both vectors.

While our data do not yield any evidence for scanning, experiments with ribosomal RNA genes give some support for a scanning mechanism: In Xenopus, RNA polymerase I can apparently enter the DNA far upstream of the 18S/28S transcription unit, at the enhancer/pseudopromoter sequences. From there, it proceeds by synthesizing short-lived RNA from the 'non-transcribed' spacer (54). Also, in experiments by Brent and Ptashne (44) with yeast, insertion of a transcriptional terminator sequence between the upstream promoter elements and the TATA box reduced the frequency of initiation further downstream. Thus it is possible that RNA polymerase II synthesizes on its way to the promoter, short RNA segments which are rapidly degraded. However, experiments in our laboratory to detect such short-lived transcripts between enhancer and promoter, using the 'nuclear run-on' technique, were negative (P. Matthias, unpublished observations).

In our laboratory we have tested the effect of the lac repressor bound between the SV40 enhancer and the rabbit β-globin gene. No reduction of correctly initiated *in vitro* transcripts was detected (55). Taken together, we have not found any conclusive evidence for a scanning mechanism with RNA polymerase II in mammalian cells, rather the opposite: Evidence for a looping model, rather than a scanning model was provided by Müller et al. (22). There we demonstrate *in vitro* that the enhancer effect is efficiently transmitted to a promoter even when enhancer and promoter are noncovalently linked via the protein streptavidin. Such a transcriptional activation *in trans* is hardly compatible with a scanning mechanism, unless unconventional combinations of scanning with small leaps are considered (big leaps can however be excluded because enhancers do not act in bonafide trans situations). It is also possible, although unlikely, that at any given promoter only a minor fraction of

polymerases initiates transcription, while the majority of polymerase molecules continues the scanning process.

Our data with carefully designed template DNAs help to explain some seemingly contradictory results regarding a possible preference for the enhancer-proximal promoter. Our experiments provide further evidence against scanning and are compatible with a looping mechanism for enhancer action.

MATERIALS & METHODS

All DNA constructs were made by standard recombinant DNA techniques (56).

Construction of plasmids

sβ1: The 6510 bp [Bam HI] (blunt ended by T4 DNA polymerase)−Sac I fragment from sβ2 (KAPOVEC 2S; M. M. Müller, unpublished) was ligated to the 219 bp Sac I−[Bst NI] (treated with mung bean nuclease) fragment.

Constructs with divergent β-globin genes, β1β2, Eβ1β2, β1β2 E, E β1β2 E: The inverted plasmids were constructed by ligation of the following fragments: the 3060 bp enhancerless or 3260 bp enhancer containing Sac I−Hind III fragment from sβ1 to the 200 bp Sac I−Sac I fragment (positions −1250 to −1050 of the wild type β-globin gene) from p18 Globin (P. Matthias, unpublished;) to the 3280 bp enhancerless or 3480 bp enhancer containing Sac I−Hind III fragment from sβ2 and to the 2640 pUC18 vector fragment.

pCRI derivative pCRIΔ: PCRIΔ was created by inserting the 52 bp Eco RI−Asp 718 polylinker fragment from BLUESCRIPT (Stratagene®) into the Eco RI, Asp 718 (partial) cut pCRI (57,58) vector. pCRIΔ is thus 2 kb shorter than its parental pCRI vector.

Inverted constructs with the pCRIΔ sequence instead of pUC18 vector sequence, β1β2, E β1β2, β1β2 E, E β1β2 E: The Hind III−Hind III fragments containing the appropriate inverted β-globin genes were inserted into Hind III (partial) digested pCRIΔ. pCRIΔ has two Hind III sites, one in the polylinker and one in the gene for kanamycin resistance.

b1b2: The 6470 bp Bam HI−Sac I fragment from the enhancerless F1 OVEC (P. Matthias, unpublished) was ligated to the 219 bp Sac I−[Bst NI] (treated with mung bean nuclease) fragment and to the 423 bp [Xho I]−Bam HI (treated with T4-DNA polymerase) fragment, derived from sβ2.

b1b2 3'E: The 5396 bp Asp 718−Pvu II (partial) fragment from b1b2 was ligated to the 1930 bp Pvu II (partial)−Asp 718 fragment from sβ2.

5'E b1b2: The 2090 bp [Bam HI] (treated with T4-DNA polymerase)−[Xba I] (T4-DNA pol.) fragment from sβ2 was inserted into the [Xho I] (treated with T4-DNA polymerase) cut plasmid. b_1b_2:.

5'E b1b2 3'E: The 3050 bp Bam HI−Asp 718 fragment from sβ2 was inserted into the 6460 bp Asp 718−Bam HI cut 5'E b1b2.

Rsa I−Sau I T7, a Bluescript derivative to prepare radioactive transcripts for RNAse mapping: The 500 bp Rsa I−Bam HI fragment from sβ2 was inserted into the Bam HI, Eco RV cut Bluescript (KS, Stratagene®) vector. This subclone was cut with [Sac II] (treated with mung bean nuclease), [Sau I] (treated withT4-DNA polymerase) and religated to reconstitute the Sau I site. The resulting Rsa-Sau I fragment comprises about 380 bp; the entire transcript with the additional polylinker comprises about 445 nts. The radioactive probe was prepared according to Stratagene's Bluescript user manual.

Cell culture: HeLa cells were grown in DMEM (GIBCO) supplemented with 2.5% fetal calf serum (Boehringer), 2.5% calf serum (GIBCO) and 100 U/ml streptomycin. J558L

myeloma cells were grown in RPMI (GIBCO) supplemented with 10% fetal calf serum (Boehringer), 100 U/ml streptomycin and 2 mM glutamine. Transfections were done using either the DEAE dextran proceedure according to de Villiers and Schaffner (45) for J558L myeloma cells or the calcium phosphate co-prepitation method according to de Villiers and Schaffner (45) for HeLa cells. DMSO boost was carried out for 1 to 3' at room temperature with 25% dimethylsulfoxide (3ml for a 10 cm ϕ dish) in TBS (tris-buffered saline).The cells were washed and incubated with appropriate medium for 40 hrs at 37°C and 5% CO_2 atmosphere. During this time cells undergo about two cell doublings. Harvesting of tissue culture cells and extraction of cytoplasmic RNA was done according to de Villiers and Schaffner (45). After the residual input plasmid DNA was removed by treatment with RNAse-free DNAse I, RNA was analysed with a T7 polymerase-generated radioactive RNA probe from the Bluescript derivative RsaI-Bam HI-T7 described above.

Quantification of steady state transcript levels: The fragments corresponding to the signals of β_1, β_2, b_1 and b_2 were excised from the gel and quantified by Cerenkov counting. The values obtained were corrected for a blank (slice of gel with no detectable radioactivity after film exposure). The ratios of transcription were obtained according to the following formula: β_1 / β_2 or b_1 / b_2 = value for β_1 [or b_1] signal) : (value for β_2 [or b_2] signal$\times$0.556), where 0.556 is the correction factor for the β_2 [b_2] fragment due to its longer homology to the radioactive probe compared to β_1 [b_1].

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REFERENCES

1. Weber,F., and Schaffner,W. (1985) Nature **315**, 75–77.
2. Treisman,R., and Maniatis,T. (1985) Nature **315**, 72–75.
3. Serfling,E., Jasin,M., and Schaffner,W. (1985) Trends Genet. **1**, 224–230.
4. Maniatis,T., Goodbourn,S., and Fisher,J.F. (1987) Science **236**, 1237–1245.
5. Müller,M.M., Gerster,T., and Schaffner,W. (1988) Eur. J. Biochem. **176**, 485–495.
6. Ondek,B., Gloss,L., and Herr,W. (1988) Nature **333**, 40–45.
7. Dynyn,W.S. (1989) Cell **58**, 1–4.
8. Veldman,G., Lupton,S., and Kamen,R. (1985) Mol. Cell. Biol. **5**, 649–658.
9. Schirm,S., Jiricny,J., and Schaffner,W. (1987) Genes & Dev. **1**, 65–74.
10. Ondek,B., Shepard,A., and Herr,W. (1987) EMBO J. **6**, 1017–1025.
11. Pierce,J., Lenardo,M., and Baltimore,D. (1988) Proc. Natl. Acad. Sci. USA **85**, 1482–1486.
12. Hope,I.A., and Struhl,K. (1986) Cell **46**, 885–894.
13. Ma,J., and Ptashne,M. (1987) Cell **48**, 847–853.
14. Ptashne,M. (1988) Nature **335**, 683–689.
15. Moreau,P., Hen,R., Wasylyk,B., Everett,R., Gaub,M.P., and Chambon, P. (1981) Nucl. Ac. Res. **9**, 6047–6068.
16. Picard,D. (1985) Oxf. Surv. Eukaryotic Genes **2**, 24–28.
17. Courey,A.J., Plon,S.E., and Wang ,J.C. (1986) Cell **45**, 567–574.
18. Plon,S.E., and Wang,J.C. (1986) Cell **45**, 575–580.

19. Ptashne,M. (1986) Nature **322**, 697–701.
20. Gasser,S.M., and Laemmli,U.K. (1987) Trends Genet. **3**, 16–22.
21. Schleif,R. (1987) Nature **327**, 369–370.
22. Müller,H.P., Sogo,J.M., and Schaffner,W. (1989) Cell, **58**, 767–777
23. Dunn,T.M., Hahn,S., Ogden,S., and Schleif,R.F. (1984) Proc. Natl. Acad Sci. USA **81**, 5017–5020.
24. Hahn,S., Hendrickson,W., and Schleif,R. (1986) J. Molec. Biol. **188**, 355–367.
25. Griffith,J., Hochschild,A., and Ptashne,M. (1986) Nature **322**, 750–752.
26. Hochschild,A., and Ptashne,M. (1986) Cell **44**, 681–687.
27. Martin,K., Huo,L., and Schleif,R. (1986) Proc. Natl. Acad. Sci. USA **83**, 3654–3658.
28. Krämer,H., Niemöller,M., Amouyal,M., Revet,B., von Wilcken–Bergmann,B., and Müller–Hill,B. (1987) EMBO J. **6**, 1481–1491.
29. Gellert,M., and Nash,H. (1987) Nature **325**, 401–404.
30. Mukherjee,S., Erickson,H., and Bastia,D. (1988) Proc. Natl. Acad. Sci. USA **85**, 6287–6291.
31. Schleif,R. (1988) Science **240**, 127–128.
32. Wang,J.C., and Giaever,G.N. (1988) Science **240**, 300–304.
33. Gralla,J.D. (1989) Cell **57**, 193–195.
34. Theveny,B., Bailly,A., Rauch,C., Rauch,M., Delain,E., and Milgrom,E. (1987) Nature **329**, 79–81.
35. Schüle,R., Muller,M., Otsuka–Murakami,H., and Renkawitz, R. (1988) Nature **332**, 87–90.
36. Cohen,R.S., and Meselson,M. (1988) Nature **332**, 856–858.
37. Schüle,R.,Muller,M., Kaltschmidt,Ch., and Renkawitz,R. (1989) Science **242**, 1418–1420.
38. Takahashi,K., Vigneron,M., Matthes,H., Wildeman,A., Zenke,M., and Chambon,P. (1986) Nature **319**, 121–126.
39. Chodosh,L.A., Carthew,R.W., Morgan,J.G., Crabtree,G.R., and Sharp,P.A. (1987) Science **238**, 684–688.
40. Wirth,Th., Staudt,L., and Baltimore,D. (1987) Nature **329**, 174–178.
41. Ruden,D.M., Ma,J., and Ptashne,M. (1988) Proc. Natl. Acad. Sci. USA **85**, 4262–4266.
42. Wu,L., and Berk,A., (1988) Genes & Dev. **2**, 403–411.
43. Matthias,P., Künzler,P., and Schaffner,W., unpublished observation.
44. Brent,R., and Ptashne,M. (1984) Nature **312**, 612–615.
45. De Villiers,J., Olson,L., Banerji,J., and Schaffner,W. (1983) Cold Spring Harbor Symp. Quant. Biol. **47**, 911–919.
46. Wasylyk,B., Wasylyk,C., Augereau,P., and Chambon,P. (1983) Cell **32**, 503–514.
47. Kadesch,T., and Berg,P. (1986) Mol. & Cell. Biol. **6**, 2593–2601.
48. Wang,X.-F., and Calame,K. (1985) Cell **43**, 659–665.
49. Atchison,M. L., and Perry,R.P. (1986) Cell **46**, 253–262.
50. Wasylyk,B., Wasylyk,C., and Chambon,P. (1984) Nucleic Acids Res. **12**, 5589–5608.
51. Westin,G., Gerster,T., Müller,M.M., Schaffner,G., and Schaffner,W. (1987) Nucleic Acids Res. **17**, 6787–6798.
52. Banerji,J., Rusconi,S., and Schaffner,W. (1981) Cell **27**, 299–308.
53. Schreiber,E., and Schaffner,W., (1989) Somatic Cell and Mol. Genetics, in press.
54. Baker,S.M., and Platt,T., (1986) Cell **47**, 839–840.
55. Müller,H.P., and Schaffner,W. (in preparation).
56. Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) Cold Spring Harbor New York: A Laboratory Manual, Cold Spring Harbor Laboratory.
57. Armstrong,K.A., Hershfield,V., and Helinski,D.R. (1972) Science **196**, 172.
58. Covey,C., Richardson,D., and Corbon,J. (1976) Mol. Gen. Genet. **145**, 155.

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