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An enhancer element lies 3′ to the human $^{A}\gamma$ globin gene

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We have surveyed 22 kb of DNA from the region surrounding the human fetal (γ) globin genes and have identified one fragment that meets all of the criteria for a non-tissue specific enhancer element. The enhancer-containing fragment starts approximately 400 bp 3′ to the polyadenylation signal of the $^{A}\gamma$ gene and is less than 750 bp in length. Addition of this fragment to plasmids containing a 'γ-CAT' hybrid gene [consisting of the γ globin gene promoter fused to the chloramphenicol acetyl transferase (CAT) gene] increases CAT expression 6–23-fold in K562 erythroleukemia cells, depending upon the method of transfection. The increase in expression is essentially independent of the orientation or position of the fragment with respect to the γ-CAT hybrid gene. The 3′ γ enhancer activates heterologous promoters in erythroleukemia cells, and is also active in non-erythroid cell lines. The enhancer acts by increasing the number of transcripts initiated from the normal γ globin gene transcription initiation site. The enhancer region contains two DNase I hypersensitive sites in erythroleukemia cells but none in non-erythroid human leukemia cell lines. The 3′ γ globin gene enhancer contains a unique element that is similar to sequences found in an enhancer 3′ to the chicken β globin gene, suggesting that this conserved element may have a role in enhancer function.

Key words: enhancer element/globin gene expression/CAT assay/K562 cells

Introduction

At least three different *cis*-acting DNA elements have been shown to play a role in the transcription of eukaryotic genes. These include promoters (McKnight and Kingsbury, 1982; Dierks *et al.*, 1983; Charnay *et al.*, 1985; Myers *et al.*, 1986), responsive elements (Walker *et al.*, 1983; Lee *et al.*, 1981), and enhancer elements (for a review see Khoury and Gruss, 1983). Enhancer elements are DNA sequences that increase transcription from a linked promoter. The increase in transcription is relatively independent of the position or orientation of the enhancer with regard to the promoter (Khoury and Gruss, 1983). Enhancers can increase transcription from promoters other than the one with which they are normally associated (Banerji *et al.*, 1981), but most enhancer elements show some degree of tissue specificity (Laimins *et al.*, 1982; Gillies *et al.*, 1983; Edlund *et al.*, 1985).

Enhancer elements have been identified in several viruses (Benoist and Chambon, 1981; Mosthaf *et al.*, 1985) and in the U3 region of many retroviral long terminal repeats (LTRs; Levinson *et al.*, 1982; Gorman *et al.*, 1982a; Luciw *et al.*, 1983), including that of the Friend Murine Leukemia Virus (FrMuLV), which displays specificity for erythroid cells (Bosze *et al.*, 1986). In eukaryotic genomes, enhancer elements have been shown to be associated with several genes, including the immunoglobulin (Gillies *et al.*, 1983; Banerji *et al.*, 1983; Neuberger, 1983; Queen and Baltimore, 1983); insulin (Edlund *et al.*, 1985), and c-fos genes (Deschamps *et al.*, 1985; Triesman, 1985).

Although the human β-like globin genes have been shown to require the SV40 enhancer element for high levels of properly initiated transcripts in heterologous cells (Humphries *et al.*, 1982; Treisman *et al.*, 1983), there has been no direct evidence to date that enhancer elements exist within the human β-like gene cluster. However, Hesse *et al.* (1986) and Choi and Engel (1986) have recently shown that an enhancer-like element exists about 400–600 bp 3′ to the chicken adult β globin gene poly(A) addition site. This element possesses developmental and tissue specificity, enhancing the expression of the chicken β globin gene promoter when transfected into 9 or 12 day erythrocytes but not 5 day erythrocytes, chick fibroblasts, or mouse L cells (Hesse *et al.*, 1986).

We tested 22 kb of the human β globin gene cluster for enhancer activity, using an enhancer-dependent hybrid gene consisting of the $^{G}\gamma$ promoter fused to the CAT gene (γ-CAT). Using this strategy, we have characterized a unique enhancer element that extends from 400–1150 bp 3′ to the polyadenylation signal for the human $^{A}\gamma$ globin gene.

Results

Experimental strategy

The strategy used to test fragments of DNA for enhancer activity is shown in Figure 1. The γ-CAT hybrid gene was constructed by fusing a short $^{G}\gamma$ promoter (−384→+36) to the coding sequence of the CAT gene and by then adding *Sal*I linkers. Seventeen *Eco*RI or *Pst*I fragments were cloned into the appropriate site in the polylinker of pUC9. The subclones were digested with *Sal*I for the insertion of the γ-CAT hybrid gene cartridge. This strategy generates two orientations of γ-CAT for each subclone. These plasmids were individually transfected into the human erythroid cell line K562 (Lozzio and Lozzio, 1975) which constitutively expresses all of the human globin genes except the adult β globin gene (Dean *et al.*, 1983). After 48 h in culture, the transfected cells were lysed and analyzed for CAT activity.

γCAT is enhancer responsive in K562 cells

Figure 2 shows the CAT activity of equal amounts of soluble protein from whole cell extracts of K562 cells transfected with a variety of plasmids. Mock transfected K562 cells (lane 2) have no endogenous CAT activity. γ-CAT^{1} (γ-CAT in the same orientation as the Lac Z gene in pUC9; lane 3) and γ-CAT^{2} (γ-CAT in the opposite orientation as the Lac Z gene in pUC9; lane 4) convert ~0.2% of the unacetylated chloramphenicol (CAP) to the acetylated forms (acCAP), with a small difference in the level of expression depending on the orientation of γ-CAT in pUC9 (see also Table I). The addition of the 3.0 kb *Eco*RI fragment containing the SV40 enhancer region derived from pLTN3B

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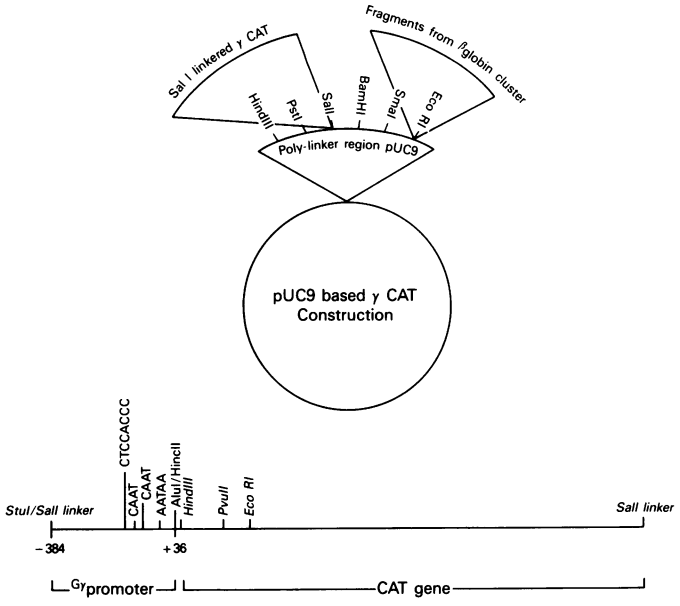

Fig. 1. Plasmids used to test DNA fragments for enhancer activity. The positions of the *Sal*I linkered γ-CAT hybrid gene and the *Eco*RI fragments in pUC9 are shown above. A partial restriction map of the γ-CAT hybrid gene appears below. The positions of regulatory consensus sequences within the γ promoter are shown. Plasmid nomenclature is described in Materials and methods

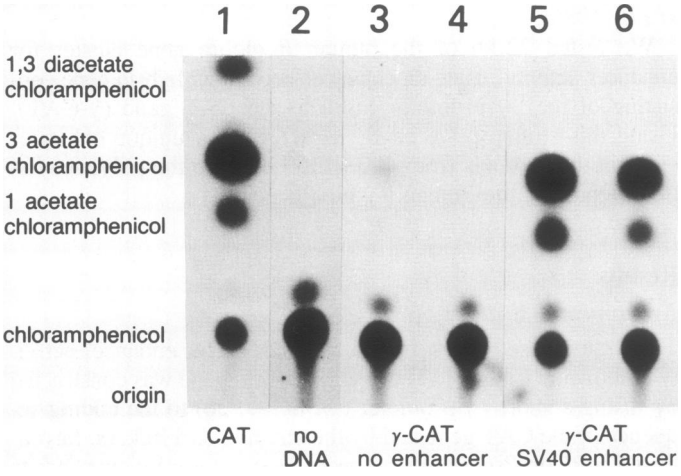

Fig. 2. Enhancer responsiveness of γ-CAT. Equal amounts of soluble protein from K562 cells transfected with the constructions listed below were analyzed for CAT activity. **Lane 1** (CAT), 0.5 units purified CAT enzyme (Pharmacia); **lane 2** (no DNA), lysate from mock transfected K562 cells; **lanes 3, 4** (γ-CAT), the γ-CAT hybrid gene in pUC9 in the same (pγ-CAT[1]) or opposite (pγ-CAT[2]) orientation with respect to the pUC9 Lac Z gene; **lanes 5, 6** (γ-CAT with enhancer), pγ-CAT[2] with the SV40 enhancer in the same (pSVγ-CAT[1]) or opposite (pSVγ-CAT[2]) orientation with respect to the pUC9 Lac Z gene.

(Humphries *et al.*, 1982) to γ-CAT[2] in either orientation (pSVγ-CAT; lane 5, or pSVγ-CAT[2]; lane 6) increased γ-CAT activity 80- to 100-fold. This strong effect of the SV40 enhancer on β-globin gene promoters has been observed in other systems (Banerji *et al.*, 1981; Humphries *et al.*, 1982; Treisman *et al.*, 1983).

Identification of an enhancer in the β globin gene cluster

Since the γ-CAT hybrid gene proved to be enhancer responsive in K562 cells, a series of 32 constructs were generated with γ-CAT in both orientations and both positions with regard to various restriction fragments from the cosmid cosHG25 (Grosveld *et al.*, 1982). These constructs were individually transfected into K562 cells and cultured for 48 h, after which the cell lysates were analyzed for CAT activity. Figure 3 shows the results of a survey with *Eco*RI fragments (in some cases the fragments were subdivided with *Bam*HI) derived from this region. Representative CAT assays for each fragment are shown above the schematic representation of the 5′ end of the β globin gene cluster. The average conversion of CAP to acCAP ($n > 3$) for each construct is shown in Table I. Of the 11 *Eco*RI or *Eco*RI/*Bam*HI fragments tested, and an additional 6 *Pst*I fragments tested (data not shown), only fragment 11, a 2.3 kb *Eco*RI fragment located 3′ to the $^{A}\gamma$ gene, demonstrated enhancer activity. When this fragment was added to plasmids containing γ-CAT, the average conversion of CAP to acCAP is 4.8% (Table I), a 23-fold increase over that of γ-CAT alone. Expression of γ-CAT was increased 18- to 27-fold in three positions and orientations of fragment 11 that were tested.

Table I. The average CAT activity in K562 cells transfected with the plasmid constructions depicted in Figure 3

Test plasmid (# of constructions tested)	Average % CAT conversion	Relative conversion
pγ-CAT[1]	0.3	1.0
p-CAT[2]	0.1	–
p1γ-CAT (1)	0.1	0.5
p2γ-CAT (2)	0.0	–
p3γ-CAT (1)	0.1	0.5
p4γ-CAT (2)	0.1	0.5
p5γ-CAT (2)	0.2	1.0
p6γ-CAT (3)	0.33	1.6
p7γ-CAT (2)	0.3	1.5
p8γ-CAT (2)	0.0	–
p9γ-CAT (2)	0.2	1.0
p10γ-CAT (2)	0.4	2.0
p11γ-CATa	4.8	24.0
p11γ-CATb	3.6	18.0
p11γ-CATc	5.4	27.0
p12γ-CAT (2)	0.2	1.0
pRSVcat	9.7	48.5

The percent conversion is defined as P/S+P × 100, where P is the counts per minutes (c.p.m.) of acetylated chloramphenicol, and S is the c.p.m. of unacetylated chloramphenicol. All transfections and assays were performed at least three times. The fold increase was determined by dividing the average percent conversion for each fragment by the average percent conversion of γ-CAT[1] and [2].

Further experiments determined that the CAT assay was not linear with respect to time nor amount of lysate when the conversion rate was less than 1% (data not shown). For this reason it was necessary to increase the efficiency of transfection so that the CAT activity of the enhancerless plasmids could be measured accurately. Using electroporation to increase the efficiency of DNA transfer (Potter *et al.*, 1984), equimolar amounts of γ-CAT[1], γ-CAT[2], p11γ-CATa and p11γ-CATc (terminology outlined in Materials and methods) were introduced into K562 cells. Table II compares the results of transfection by electroporation to those generated by transfection with DNA−$CaPO_4$ coprecipitation. The enhancer effect of fragment 11 was also seen in cells transfected by electroporation (with CAT assays that were linear with respect to time and amount of lysate), indicating that the increased CAT activity was not an artifact of $CaPO_4$-mediated transfection. The CAT activity generated by p11γ-CATa or

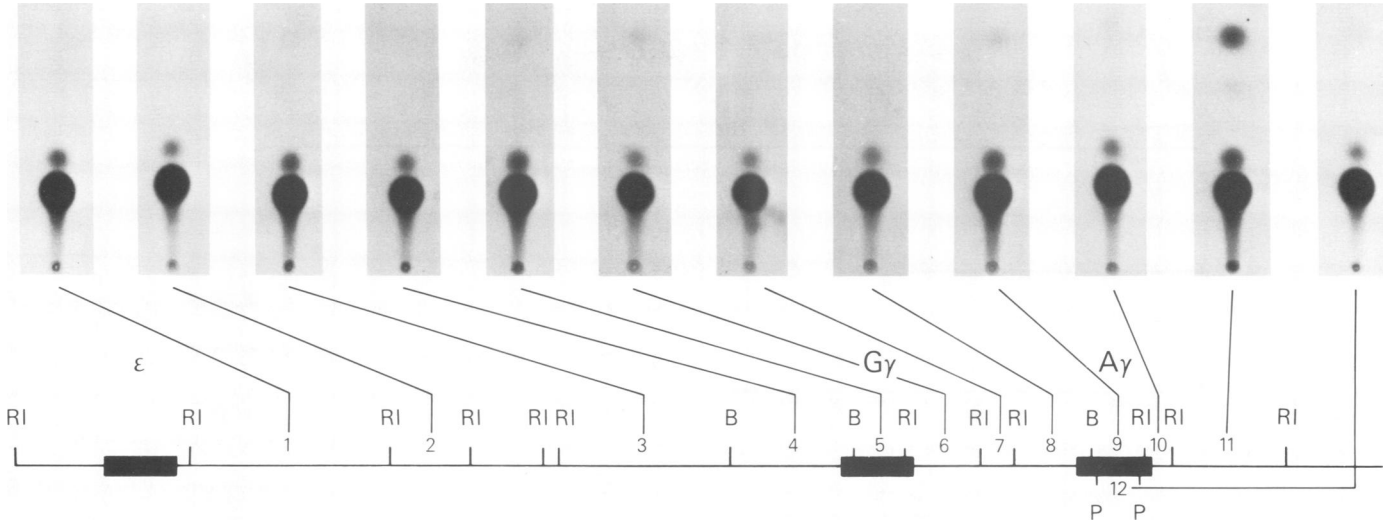

Fig. 3. Survey of the β globin gene cluster for enhancer elements. A representative CAT assay for each fragment tested is shown above a schematic representation of the 5′ end of the β globin gene cluster. RI, *Eco*RI, B, *Bam*HI, P, *Pst*I. Regions with at least 7 of 8 bp homologous to the enhancer core sequence GTGG$\frac{AAA}{TTT}$G (Khoury and Gruss, 1983) are found in Fragments 1 (14 cores), 2 (5 cores), 3 (5 cores), 4 (7 cores), 5 (2 cores), 6 (4 cores), 7 (4 cores), 8 (4 cores), 9 (2 cores), 10 (3 cores), 11 (6 cores), and 12 (2 cores). The presence of enhancer cores clearly does not confer enhancer activity on these fragments.

p11γ-CATc was 6-fold greater than that of the enhancerless plasmids.

To define the region containing the enhancer activity more precisely, γ-CAT was inserted into a series of overlapping subclones of fragment 11. These constructions were transfected into K562 cells by DNA−$CaPO_4$ coprecipitation. Figure 4 shows the results of this survey. Below the restriction map of fragment 11, the fragments tested with γ-CAT are indicated, with representative CAT assays (one for each orientation of the fragment) shown at the right. The enhancer activity is contained within a 750 bp *Eco*RI/*Hin*dIII fragment at the 5′ end of fragment 11. A fragment containing the first 1.4 kb of fragment 11 also demonstrates the enhancer activity, while the other fragments do not produce any CAT activity in K562 cells.

The 3′ γ enhancer activates heterologous promoters and is active in non-erythroid cells

To test the ability of the 3′ γ enhancer to increase transcription from nonglobin promoters, fragment 11 was inserted into vectors containing two different hybrid genes: tk-CAT (a 595 bp HSV tk promoter from −540 to +54, fused to the CAT gene), and DHFR-CAT (a 126 bp human DHFR promoter from −73 to +53, fused to the CAT gene — a gift of T.Shimada). A variety of cell lines were transfected with pγ-CAT, p11γ-CAT, ptk-CAT, p11tk-CAT, pDHFR-CAT, p11DHFR-CAT, or pRSVcat by DNA−$CaPO_4$ coprecipitation. At 48 h post-transfection, the cells were assayed for CAT activity (Table III). In K562 cells and a second human erythroleukemia cell line, HEL (Martin and Papayannopoulou, 1982), fragment 11 significantly increases the expression of γ-CAT as well as that of tk-CAT or DHFR-CAT. In HeLa cells, fragment 11 strongly enhances γ-CAT, but tk-CAT expression is increased only 3-fold, and DHFR-CAT expression is not affected. In 293 cells (cells that constitutively express adenovirus EIA protein, Treisman *et al.*, 1983), γ-CAT, tk-CAT and DHFR-CAT are all nearly as active as RSV-CAT. Fragment 11 increases γ-CAT expression 2-fold in 293 cells, but strongly represses HSVtk-CAT and DHFR-CAT. This observation is similar to previous studies that have demonstrated strong repression of some viral and cellular enhancers by EIA protein (see Discussion).

Table II. A comparison of CAT activity in K562 cells transfected with pγ-CAT[1] and [2], or p11γ-CATa and c, by DNA−$CaPO_4$ coprecipitation versus electroporation

Test plasmid	DNA−CaPO coprecipitation		Electroporation	
	% CAT conversion	Relative conversion	% CAT conversion	Relative conversion
(1) pγ-CAT[1]	0.3	1.0	8.4	1.0
(2) pγ-CAT[2]	0.1		7.2	
(3) p11γ-CATa	4.8	24	48.6	6.2
(4) p11γ-CATc	5.4	27	45.0	5.8

The γ enhancer acts by increasing the number of transcripts initiated from the γ gene cap site

To determine whether the CAT activity detected in transfected cells was due to the presence of γ-CAT transcripts initiated at the γ globin gene transcription initiation site, we analyzed RNA from transfected cells utilizing an S1 nuclease protection assay. 30 pmol of pγ-CAT, p11γ-CAT, or pSVγ-CAT were transfected into HeLa cells by DNA−$CaPO_4$ coprecipitation, or into K562 cells by electroporation. At 24 h post-transfection, the cells were harvested and analyzed for CAT activity, and RNA was prepared. Of note, the total amount of CAT activity per microgram of HeLa whole cell lysate was ~100-fold greater than that of K562 cells transfected with the same plasmid. This result probably reflects a higher efficiency of HeLa cell transfection, and may explain the large difference between the number of γ-CAT transcripts in HeLa versus K562 cells (Figure 5).

20 μg of total cellular RNA was analyzed with a γ-CAT probe end-labeled at the *Eco*RI site located 250 bp from the 5′ end of the CAT gene. Transcripts initiated from the γ globin gene Cap site are expected to protect a probe fragment of 301 nucleotides (nt) from S1 nuclease digestion (Figure 5, bottom panel). A faint band of this length is generated by HeLa cell RNA transfected with pγ-CAT (Figure 5, lane 8), but a much stronger signal is present in HeLa cells transfected with p11γ-CAT (lane 7). pSVγ-CAT transfected into HeLa cells (lane 9) yields a very strong

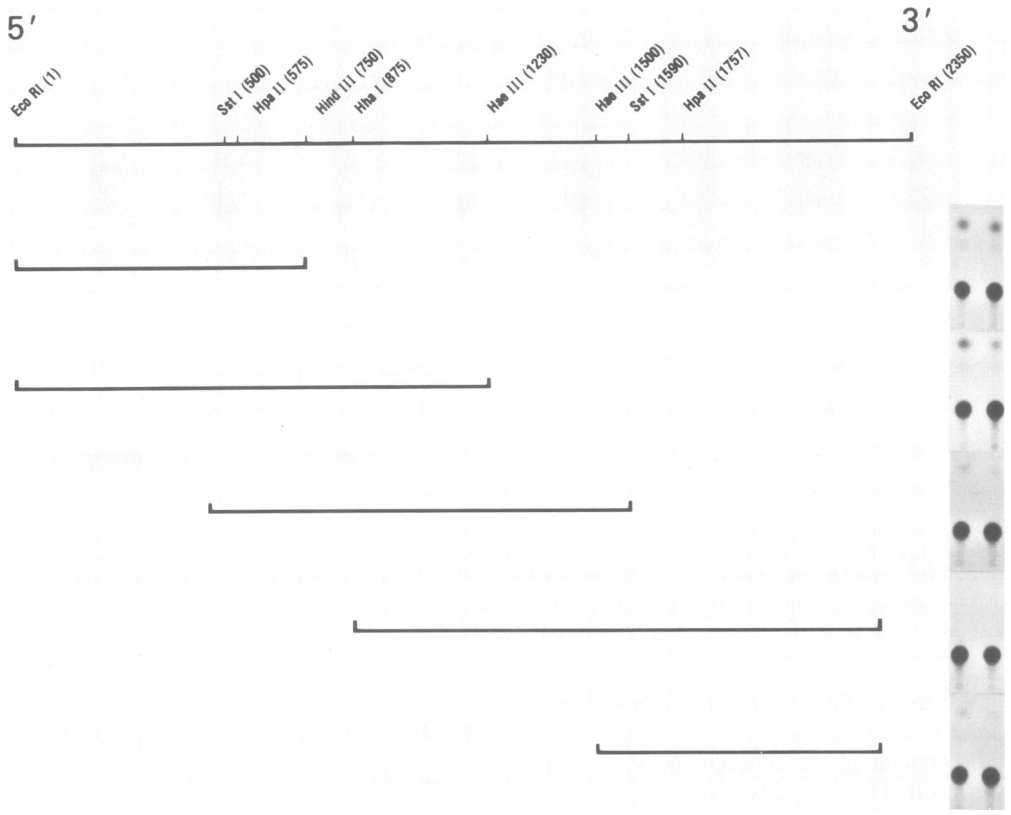

Fig. 4. Localization of enhancer activity in Fragment 11. The γ-CAT hybrid gene was inserted into plasmids containing the indicated restriction fragments. Representative CAT assays for each orientation of the fragment appear at the right. The enhancing activity is located in the first 750 bp of this fragment.

Table III. The effects of Fragment 11 on various promoters in erythroid and non-erythroid cell lines

Plasmid construction	CAT activity (relative to pRSVcat)			
	K562	HEL	HeLa	293
pRSVcat	1.0	1.0	1.0	1.0
pγ-CAT[1]	0.03	0.03	0.005	0.66
p11γ-CATc	0.52 (+17.3X)	0.35 (+11.7X)	0.112 (+22X)	1.2 (+1.8X)
ptk-CAT[1]	0.15	0.08	0.12	0.77
p11tk-CATc	0.57 (+3.8X)	0.91 (+11X)	0.36 (+3.0X)	0.03 (−25X)
pDHFR-CAT[1]	0.23	0.03	0.19	0.77
pDHFR-CATc	1.2 (+5.2X)	0.44 (+14.7X)	0.21 (+1.1X)	0.03 (−25X)

All transfections and assays were performed at least twice.

signal at 301 nt, but several other start sites in the 5′ γ flanking sequence and within the CAT gene itself are also utilized with this construction. No signal was obtained from non-transfected K562 (lane 2) or HeLa cells (lane 6). As predicted from the protein data mentioned above, no correctly initiated γ-CAT transcripts were detected in K562 cells electroporated with the same constructions (lanes 3−5). Five μg of RNA from all samples protected approximately equal amounts of a c-myc exon 2 probe from S1 nuclease digestion (Figure 5, middle panel). The reduced amounts of steady-state c-myc mRNA in lanes 3−5 may reflect reduced rates of K562 cell growth after electroporation.

The 3′ γ enhancer contains two tissue-specific DNase I hypersensitive sites

To determine whether the region 3′ to the $^{A}\gamma$ globin gene was in an active chromatin conformation in erythroleukemia cells, we assayed this region for DNase I hypersenstiive sites. Nuclei from uninduced K562 cells, HL60 promyelocytic cells, or U937 monocytoid cells were prepared and digested with increasing amounts of DNase I. Purified DNA was restricted to completion with *Pst*I, electrophoresed, blotted, and hybridized to a nick-translated probe consisting of the 0.50 *Pst*I−*Hin*dIII fragment from the 3′ end of the $^{A}\gamma$ globin gene (see Figure 6). This probe hybridizes with the 3′ end of the $^{G}\gamma$ globin gene, producing a band of 4.9 kb, and the 3′ end of the $^{A}\gamma$ gene, yielding a 2.7 kb band. Increasing digestion with DNase I leads to the production of 4.3, 1.8, 1.1, and 0.7 kb sub-bands in uninduced K562 cells. An identical set of bands was generated from K562 cells induced for two days with 20 μM hemin, but no sub-bands were generated in DNase I curves obtained from HL60 (Figure 6, right panel) or U937 cells (data not shown).

The putative locations of the DNase I cutting sites denoted by A, B, C, and D are designated on the diagram below Figure 6. Sub-band A is clearly due to the presence of a strong DNase

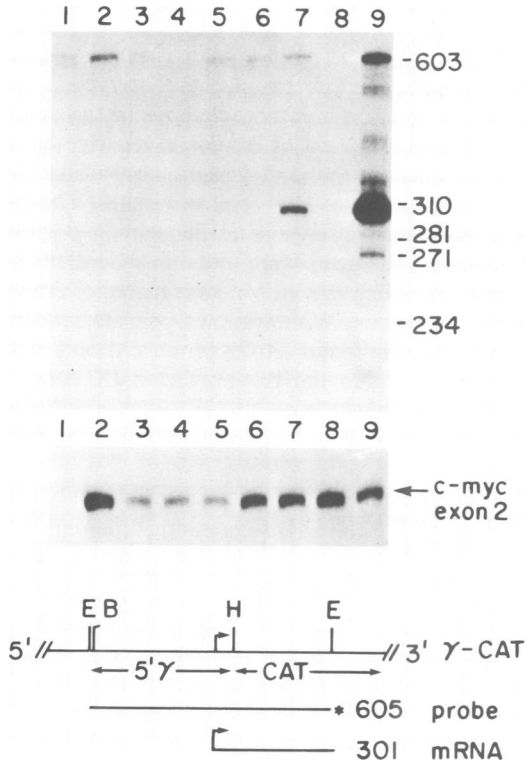

Fig. 5. S1 nuclease analysis of γ-CAT transcripts in transfected cells. The diagram at the bottom of the figure represents the 5′ end of a hybrid γ-CAT gene. γ-CAT consists of the $^{A}\gamma$ *Alu*I fragment from −299 to +36 subcloned into the *Hin*cII sites of pUC9, and subsequently fused to the CAT gene at *Hin*dIII (H) site of pUC9. This plasmid was cleaved with *Eco*RI (E), phosphatased, and end-labeled with [^{32}P]γ-ATP and polynucleotide kinase. To generate an antisense end-labeled probe, the *Eco*RI fragment was cleaved with *Bam*HI (B) and gel purified. mRNA correctly initiated at the γ globin gene CAP site should protect a probe fragment of 301 nucleotides from S1 nuclease digestion. **Lane 1** contains no RNA. **Lanes 2–9** contain 20 μg of total cellular RNA from the following sources: **lane 2**, non-transfected K562 cells; **lane 3**, K562 cells electroporated with pγ-CAT; **lane 4**, K562 cells electroporated with p11γ-CAT; **lane 5**, K562 cells electroporated with pSVγ-CAT; **lane 6**, non-transfected HeLa cells; **lane 7**, HeLa cells transfected with p11γ-CAT; **lane 8**, HeLa cells transfected with pγ-CAT; **lane 9**, HeLa cells transfected with pSVγ-CAT. The positions of simultaneously run molecular weight markers are shown at the right. **Middle panel**: S1 nuclease analysis of the same RNA samples hybridized with a uniformly labeled probe complementary to c-myc mRNA. The 0.35 kb *Pst*I−*Sst*I c-myc fragment (Bernard *et al.*, 1983) spanning the exon 2−intron 2 splice junction was ligated into M13mp11 and used as a template for probe synthesis. The position of correctly processed c-myc mRNA is shown. All analyses were performed in probe excess.

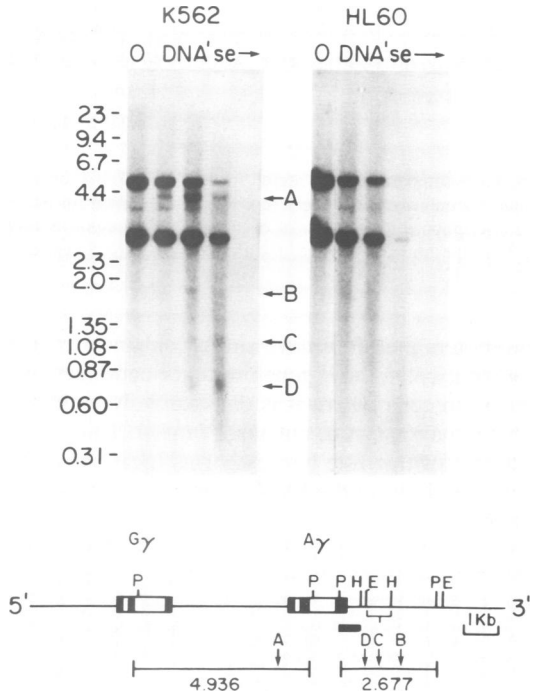

Fig. 6. DNase I analysis of chromatin structure near the $^{G}\gamma$ and $^{A}\gamma$ globin genes. The diagram below the figure represents the organization of the $^{G}\gamma$ and $^{A}\gamma$ globin genes. Flanking regions are represented by thin lines, coding regions by solid blocks, and intervening sequences by open blocks. The letters represent a partial restriction map of the region; P, *Pst*I, H, *Hin*dIII, and E, *Eco*RI. Brackets 3′ to the $^{A}\gamma$ globin gene represent the γ enhancer. The solid block below the $^{A}\gamma$ gene represents the 0.50 kb *Pst*I−*Hin*dIII probe used in these studies. The left hand panel displays K562 cell analysis; HL60 cell analysis is shown at the right. Nuclei were digested with no DNase I (**lane 1**) or with increasing amounts of DNase I. The positions of sub-bands generated by DNase I digestion are indicated, and are correlated with cutting sites on the diagram below. The sizes of molecular weight markers (in kb) are shown on the left. The DNase I digestion curves employed in these studies were not designed to compare overall DNase I sensitivity of the γ globin gene region in K562 versus HL60 cells. Higher concentrations of DNase I were used in the HL60 samples (right panel) to demonstrate that no DNase I hypersensitive sites appear near the γ genes of these cells, even when extensive DNase I digestion is clearly occurring.

I hypersensitive site 100−200 bp 5′ to the $^{A}\gamma$ globin gene CAP site (Lachman and Mears, 1983; Groudine *et al.*, 1983; Tuan *et al.*, 1985). Sub-bands B, C, and D are less intense, but are all present in the same lane as sub-band A, and therefore are almost certainly due to DNase I cleavage 3′ to the $^{A}\gamma$ gene, not the $^{G}\gamma$ gene. Hypersensitive sites D and C lie ~650 and 1150 bp 3′ to the $^{A}\gamma$ poly(A) site, respectively, and lie within the enhancer. Sub-band B appears to be due to cleavage occurring about 1.6 kb 3′ to the $^{A}\gamma$ poly(A) site, in a region not clearly associated with enhancer activity (Figure 4).

Discussion

Several lines of evidence suggest that the 3′ γ enhancer may have a role in the normal regulation of human globin genes. First, the enhancer dependence of β globin genes in heterologous cells (Banerji *et al.*, 1981; Humphries *et al.*, 1982; Treisman *et al.*, 1983) suggested that the expression of these genes might be influenced by enhancer elements located within the β globin gene cluster itself. The region from 400−1150 bp 3′ to the $^{A}\gamma$ gene poly(A) addition site fulfills the requirements for an enhancer element (Khoury and Gruss, 1983). Addition of this fragment to plasmids containing γ-CAT increased CAT expression 6−23-fold (depending on the method of transfection) by increasing the number of γ-CAT transcripts initiated at the γ gene CAP site. This increase in transcription is relatively independent of the orientation or position of the fragment with respect to the γ-CAT gene.

The $^{G}\gamma$ globin gene promoter element used in this study is enhancer-dependent when transiently expressed in HeLa cells or erythroleukemia cells, a result that has been previously described for the β globin gene promoter in HeLa or COS cells. The enhancer requirement of the $^{G}\gamma$ promoter is relieved in 293 cells, which constitutively express adenovirus EIA protein; similarly, the β globin gene promoter is enhancer-independent when transiently expressed in these cells, and is not further activated by the addition of an SV40 enhancer in *cis* to the β globin gene (Treisman *et al.*, 1983; Green *et al.*, 1983). The γ and β

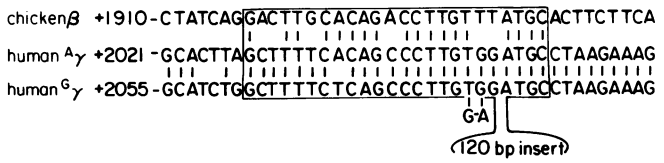

Fig. 7. Sequence comparison of a region in the 3′ chicken β enhancer and the 3′ human $^{A}\gamma$ enhancer. Numbers represent the distance (in nucleotides) from the transcription initiation site of each gene. This region is highly conserved 3′ to the human $^{G}\gamma$ gene, but the homologous sequence is interrupted by a 120 bp insert near its 3′ end.

globin promoters therefore have similar enhancer requirements in heterologous cells; the γ gene promoter continues to respond to enhancers in cells where it is constitutively expressed, suggesting that it may in fact be enhanced *in vivo*. The 3′ γ enhancer is a candidate for this role because of its location, and because it functions similarly to the SV40 enhancer in transient expression systems.

Surprisingly, the γ enhancer also activates HSVtk-CAT and DHFR-CAT in erythroleukemia cells, but has a lesser effect on these promoters in the HeLa cell environment. In contrast to some cellular enhancers, the γ enhancer therefore does not appear to be promoter- or tissue-specific with the constructs and cell lines tested thus far. In addition, the 3′ $^{A}\gamma$ enhancer markedly *inhibits* the activity of HSVtk-CAT and DHFR-CAT in 293 cells. This result is similar to the previously described repressor effect of EIA on the SV40, polyoma virus, adenovirus EIA, or immunoglobin heavy chain enhancers (Borrelli *et al.*, 1984; Velcich and Ziff, 1985; Hen *et al.*, 1985). We do not understand why the γ and β globin gene promoters, when linked to enhancers, fail to be down-regulated by EIA protein in 293 cells, while a variety of other viral and cellular enhancers and promoters are negatively regulated by this protein. This result underlies the complexity of promoter-enhancer interactions in heterologous cells.

The 3′ γ globin gene enhancer lies in a DNase I sensitive domain in K562 cells, and contains at least two DNase I hypersensitive sites. The DNase I hypersensitive sites do not change with hemin-induction, and are not found in HL60 or U937 cells, which do not express the β-like globin genes. Groudine *et al.* (1983) previously described a DNase I hypersensitive site located just 3′ to the $^{A}\gamma$ globin gene in K562 cells (probably the same as D in Figure 6). This site appears to the strongest of the sites 3′ to the $^{A}\gamma$ gene, and lies ~600−700 bp 3′ to the poly(A) addition site of the $^{A}\gamma$ globin gene within the 5′ end of the enhancer. Previous studies of cytosine methylation in *Hpa*II sites located in the γ enhancer [at positions +981 and +2164 with respect to the $^{A}\gamma$ poly(A) site] revealed hypomethylation of one site (+981) specifically in fetal or adult hematopoeitic tissues; the other site (+2164) was hypomethylated only in fetal and adult erythroid tissues (Van der Ploeg and Flavel, 1980; Mavilio *et al.*, 1983). Cytosine residues in *Hpa*II sites just 5′ and 3′ to the γ enhancer were heavily methylated in most tissues and cell lines tested. Collectively, these results show that chromatin located 3′ to the $^{A}\gamma$ gene is in an active conformation in erythroid cells, consistent with previous studies of chromatin conformation near other active cellular enhancers. This region may therefore be accessable to regulatory *trans*-acting factors, similar to those that interact with an immunoglobulin enhancer in B cells (Sen and Baltimore, 1986a and b).

A comparison of sequences found in the 3′ chicken β globin gene enhancer (Hesse *et al.*, 1986; Choi and Engel, 1986) and the 3′ $^{A}\gamma$ globin gene enhancer has revealed one small area with striking homology (Figure 7). This region, beginning about 410 bp 3′ to the chicken β^{A} poly(A) addition site, or about 480 bp 3′ to the $^{A}\gamma$ gene poly(A) site, is matched at 17 of 21 positions. These sequences are highly conserved 3′ to the $^{G}\gamma$ gene, but a 120 bp insert interrupts the homologous element near its 3′ end. The interruption of the conserved element may explain why sequences 3′ to the $^{G}\gamma$ gene do not enhance the γ promoter, nor contain DNase I hypersensitive sites. The conserved element is not found elsewhere in the human β globin gene cluster; no similar elements were found in a GenBank search of known viral (including FrMuLV), mammalian, vertebrate and invertebrate sequences. Within known primate sequences, the conserved element is found (20/23 bp match) only near the 3′ end of intron 6 within the human factor IX gene (position 29498−29520, Yoshitake *et al.*, 1985). Appropriate experiments to test the importance of this sequence for enhancer function are in progress.

Finally, the study of deletions that cause hereditary persistance of Fetal Hemoglobin (HPFH) suggests that the region 3′ to the $^{A}\gamma$ gene may somehow be involved in normal γ gene regulation. HPFH deletions remove large (>100 kb) regions from the β globin gene cluster, and result in persistent expression of the γ globin genes on the same chromosome (reviewed in Bunn and Forget, 1986; Stamatoyannopoulos and Nienhuis, 1987). Examination of the described deletions reveals that the 3′ γ enhancer fragment is preserved in all four HPFH deletions, HPFH-1 (USA), HPFH-2 (Ghana), HPFH-3 (Indian), and HPFH-4 (Italian, Saglio *et al.*, 1986). However, many deletions that remove large fragments from the 3′ end of the β-globin gene cluster do not result in persistent γ gene expression in adult erythroblasts (δβ thalassemia). Some of these deletions remove all or part of the $^{A}\gamma$ enhancer, but at least one preserves it (Japanese $^{G}\gamma$ $^{A}\gamma$ $(\delta\beta)^{\circ}$ thalassemia, Matsunaga *et al.*, 1985). Collectively, these data suggest that sequences near the 3′ end of the $^{A}\gamma$ gene may act as a positive regulatory element in adult erythroblasts, but only when dominant *cis*-acting sequences 3′ to this element are removed. No simple model explains all of these observations, however, and further study is required to understand γ gene regulation in these syndromes.

We have designed a simple system that has allowed us to identify an enhancer element within the human β globin gene cluster. The role of this element for normal function of the β-like globin genes is not yet clear, but is amenable to study by a variety of techniques. The effect of the 3′ γ enhancer on the expression of globin genes in transgenic mice and in stably transformed cell lines containing exogenous human globin genes will be tested. We are also initiating attempts to define soluble proteins that may be capable of interacting with this element to alter its structure and/or function in erythroid cells.

Materials and methods

Plasmid constructions

The $^{G}\gamma$ promoter was isolated as a *Stu*I (−384 relative to the Cap site) partial *Alu*I (+36) fragment. This 410 bp fragment was cloned into the *Hin*cII site of pUC9, and the orientation of the fragment was determined by digestion with appropriate restriction enzymes. Plasmids containing the $^{G}\gamma$ promoter in the opposite orientation of the plasmid's Lac Z gene were digested with *Bam*HI and *Hin*dIII. This 427 bp fragment was used in a triple ligation with a 1.7 kb *Hin*dIII/*Bam*HI fragment containing the CAT cartridge from pRSVcat (Gorman *et al.*, 1982), and *Bam*HI cleaved pUC9. Recombinant colonies were isolated and analyzed by restriction enzyme digestions. *Sal*I linkers were added by standard techniques. The other hybrid gene, HSVtk-CAT, was constructed using the same strategy.

The cosmid cosHG25 (a gift of Frank Grosveld) was digested with *Eco*RI or *Pst*I, and restriction fragments were isolated and cloned into pUC9. The orienta-

tion of each fragment was determined by digestion with appropriate restriction enzymes. Since there are no *SalI* sites in this region of the β globin gene cluster (Collins and Weissman, 1984), each subclone was digested with *SalI*, the ends were treated with calf intestinal phosphatase (Pharmacia), and γ-CAT (released from the parent plasmid by *SalI* digestion) was cloned into the *SalI* site. By using both orientations of each test fragment, all four possible combinations of orientation and position of the fragments relative to γ-CAT were generated. The orientation and positions of γ-CAT and the fragments in the vector are denoted as: 'a', 5'-test fragment-3'→5'-γ-CAT-3'; 'b', 5'-test fragment-3'→3'-γ-CAT-5'; 'c', 5'-γ-CAT-3'→5'-test fragment-3'; 'd', 3'-γ-CAT-5'→5'-test fragment-3'.

Transfection and analysis of CAT activity

All cell types used in this study were grown in IMEM (Biofluids) supplemented with 10% fetal calf serum (Biofluids), 50 mg/l gentamicin sulfate (Gibco), and 2.5 mg/l fungizone (Gibco). Best results were obtained with cells passaged no more than 10 times from frozen aliquots.

For transfection, cells were grown to mid-log phase and plated in 10 ml on 100 cm^2 dishes in the following amounts: K562, 6×10^5; HEL, 7×10^5; HeLa, 5×10^5; and 293, 5×10^5. Twenty four hours after plating, the cells were transfected with 12 pmol (the equivalent of 20 μg of pRSVcat) of the various constructs by DNA−$CaPO_4$ coprecipitation (Graham and Van der Eb, 1973; Wigler *et al.*, 1978). The cells were then incubated 24 h before replacement of the DNA−$CaPO_4$-containing medium with fresh medium.

Forty eight hours after transfection, cells were harvested, lysed by three cycles of freeze−thaw, and the protein concentration of the cleared lysate was determined by the method of Lowry *et al.* (1951). Equal amounts of protein from each cell type (~50 μg for K562 or HEL cells, and ~5 μg for HeLa or 293 cells) were analyzed for CAT activity by the method of Gorman *et al.* (1982b). The CAT activity was quantified by scintillation counting of spots cut from the thin layer chromatography sheet representing chloramphenicol or acetylated chloramphenicol.

Electroporation was performed as described by Potter *et al.* (1984), using 2×10^7 K562 cells and 30 pmol of supercoiled plasmid DNA. The cells were then cultured for 48 h and analyzed as described above.

S_1 nuclease analysis of RNA

For the S_1 studies, 3×10^7 HeLa cells were transfected with 70 pmol of plasmid DNA, or 4×10^7 K562 cells were electroporated with 60 pmol of plasmid DNA as described above. Total RNA was isolated 24 h after transfection by the procedure of Kantor *et al.* (1980). 20 μg of total cellular RNA was hybridized to 1×10^5 c.p.m. (~40 ng) of a ^{32}P end-labeled γ-CAT probe (for a description see Figure 6) in a previously described hybridization cocktail (Favalaro *et al.*, 1980) at 50°C overnight. Hybrids were digested with 200 units of S1 nuclease (BRL) at 37°C for 30 min, ethanol precipitated, and electrophoresed on 8% sequencing gels with subsequent autoradiography. 5 μg of total cellular RNA derived from the same samples was hybridized to 1×10^5 c.p.m. (~5 ng) of a uniformly labeled DNA probe complimentary to c-*myc* mRNA made in an M13 phage vector as previously described (Ley *et al.*, 1982). Analysis was identical to that described above.

Chromatin studies

K562, HL60, or U937 cells in late log phase growth were harvested and washed in 1 × Hebs buffer. 2×10^8 cells were incubated for 15 min in hypotonic lysis buffer (10 mM KCl, 10 mM Hepes pH 8.0, and 1.5 mM $MgCl_2$) at 4°C for 15 min. Cells were lysed with 20 strokes of a mechanical Dounce homogenizer, and nuclei were harvested by low speed centrifugation and washing. Nuclei were resuspended at 5×10^7 nuclei per ml of DNase I digestion buffer (15 mM Tris, pH 7.5, 60 mM KCl, 15 mM NaCl, 3 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.25 M sucrose), DNase I (final concentration 0−10 μg/ml) was added, and nuclei were incubated at 37°C for 10 min. Reactions were stopped by adding an equal volume of 1% SDS, 600 mM NaCl, 20 mM Tris pH 7.5, 20 mM EDTA, and 400 μg/ml proteinase K. The lysates were incubated overnight at 37°C, and DNA was purified. 10 μg of total cellular DNA was digested to completion with *PstI*, purified, electrophoresed on 1.2% agarose gels, and transferred to Gene Screen Plus (Amersham) with the alkaline transfer method (Reed and Mann, 1985). Filters were hybridized to ^{32}P-labeled, nick-translated DNA probes (sp. act. $>2 \times 10^8$ c.p.m./μg) using conditions recommended by Amersham for Gene Screen Plus.

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References

Anagnou,N.P., Karlsson,S., Moulton,A.D., Keller,G., Nienhuis,A.W. (1986) *EMBO J.*, **5**, 121−126.
Banerji,J., Rusconi,S. and Schaffner,W. (1981) *Cell*, **27**, 299−308.
Banerji,J., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729−740.
Benoist,C. and Chambon,P. (1981) *Nature*, **290**, 304−310.
Bernard,O., Cory,S., Gerondakis,S., Webb,E. and Adams,J.M. (1983) *EMBO J.*, **2**, 2375−2383.
Borrelli,E., Hen,R. and Chambon,P. (1984) *Nature*, **312**, 608−612.
Bosze,Z., Thiesen,H.-J. and Charnay,P. (1986) *EMBO J.*, **5**, 1615−1623.
Bunn,H.F. and Forget,B.G. (1986) In *Hemoglobin: Molecular, Genetic, and Clinical Aspects*. WB Saunders, Philadelphia, USA.
Charnay,P., Mellon,P. and Maniatis,T. (1985) *Mol. Cell. Biol.*, **5**, 1498−1511.
Choi,O.-R. and Engel,J.D. (1986) *Nature*, **323**, 731−734.
Collins,F.S. and Weissman,S.M. (1984) *Prog. Nucleic Acids Res. Mol. Biol.*, **31**, 315−438.
Dean,A., Ley,T.J., Humphries,R.K., Fordis,M. and Schechter,A.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5515−5519.
Deschamps,J., Meiglink,F. and Verma,I.M. (1985) *Science*, **230**, 1174−1177.
Dierks,P., van Ooyan,A., Cochran,M.D., Dobkin,C., Reiser,J. and Weissman,C. (1983) *Cell*, **32**, 695−706.
Edlund,T., Walker,M.D., Barr,P.J. and Rutter,W.J. (1985) *Science*, **230**, 912−916.
Favalaro,J., Treisman,R. and Kamen,R. (1980) *Methods Enzymol.*, **65**, 618−749.
Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) *Cell*, **33**, 717−728.
Gillies,S.D., Folsom,V. and Tonegawa,S. (1984) *Nature*, **310**, 594−597.
Gorman,C.M., Merlino,G.T., Willingham,M.C., Pastan,I. and Howard,B.H. (1982a) *Proc. Natl. Acad. Sci. USA*, **79**, 6777−6781.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982b) *Mol. Cell. Biol.*, **2**, 1099−1051.
Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456−467.
Green,M., Treisman,R. and Maniatis,T. (1983) *Cell*, **35**, 137−148.
Grosveld,F.G., Lund,T., Murray,E.J., Mellor,A.L., Dahl,H.H.M. and Flavell,R.A. (1982) *Nucleic Acids Res.*, **10**, 6715−6732.
Groudine,M., Kohwi-Shigematsu,T., Gelinas,R., Papayannopoulou,Th. and Stamatoyannopoulos,G. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7551−7555.
Gruss,P., Dhar,R. and Khoury,G. (1981) *Proc. Natl. Acad. Sci. USA*, **83**, 4312−4316.
Hen,R., Borrelli,E. and Chambon,P. (1985) *Science*, **230**, 1391−1394.
Hesse,J.E., Nichol,M.D., Lieber,M.R. and Felsenfeld,G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4312−4316.
Humphries,R.K., Ley,T., Turner,P., Moulton,A.D. and Nienhuis,A.W. (1982) *Cell*, **30**, 173−183.
Kantor,J.A., Turner,P. and Nienhuis,A.W. (1980) *Cell*, **21**, 149−157.
Khoury,G. and Gruss,P. (1983) *Cell*, **33**, 313−314.
Lachman,H.M. and Mears,J.G. (1983) *Nucleic Acids Res.*, **11**, 6065−6077.
Laimins,L.A., Khoury,G., Gorman,C., Howard,B. and Gruss,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6453−6457.
Lee,F., Mulligan,R., Berg,P. and Ringold,G. (1981) *Nature*, **294**, 228−232.
Levinson,B., Khoury,G., Van de Woude,G. and Gruss,P. (1982) *Nature*, **295**, 568−572.
Ley,T.J., Anagnou,N.P., Pepe,G. and Nienhuis,A.W. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4775−4779.
Lowry,O.H., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265−275.
Lozzio,C.B. and Lozzio,B.B. (1975) *Blood*, **45**, 3212−3234.
Luciw,P.A., Bishoo,J.M., Varmas,H.E. and Capecchi,M.R. (1983) *Cell*, **33**, 705−716.
Martin,P. and Papayannopoulou,Th. (1982) *Science*, **216**, 1233−1235.
Matsunaga,E., Kimura,A., Yamada,H., Fukumaki,Y. and Tagaki,Y. (1985) *Biochem. Biophys. Res. Commun.*, **126**, 185.
Mavilio,F., Giampaolo,A., Care,A., Migliaccio,G., Calandrini,M., Russo,G., Pagliardi,G.L., Mastroberadino,G., Marinucci,M. and Peschle,C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6907−6911.
McKnight,S.L. and Kingsbury,R. (1982) *Science*, **217**, 316−324.
Mellon,P., Parker,V., Gluzman,Y. and Maniatis,T. (1981) *Cell*, **27**, 279−288.
Mosthaf,L., Pawlita,M. and Gross,P. (1985) *Science*, **232**, 613−618.
Myers,R., Tilley,K. and Maniatis,T. (1986) *Science*, **232**, 613−618.
Neuberger,M.S. (1983) *EMBO J.*, **2**, 1373−1378.
Papayannopoulou,Th., Brice,M. and Stamatoyannopoulos,G. (1986) *Cell*, **46**, 469−476.
Potter,H., Weir,L. and Leder,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7161−7165.
Queen,C. and Baltimore,D. (1983) *Cell*, **33**, 741−748.
Reed,K.C. and Mann,D.A. (1985) *Nucleic Acids Res.*, **13**, 7207−7221.

Saglio,G., Camaschella,C., Serra,A., Bertero,T., Rege Cambrin,G., Guerrasio,A., Mazza,V., Izzo,P., Terrangi,F., Giglioni,B., Comi,P. and Ottolenghi,S. (1986) *Blood*, **68**, 646–651.
Sen,R. and Baltimore,D. (1986a) *Cell*, **46**, 705–716.
Sen,R. and Baltimore,D. (1986b) *Cell*, **47**, 921–928.
Stamatoyannopoulos,G. and Nienhuis,A.W. (1987) In Stamatoyannopoulous,G., Nienhuis,A.W., Leder,P. and Majerus,P. (eds), *Molecular Basis of Blood Diseases*. WB Saunders, Philadelphia, USA.
Treisman,R. (1985) *Cell*, **42**, 889–902.
Treisman,R., Green,M.R. and Maniatis,T. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7428–7432.
Tuan,D., Solomon,W., Li,Q. and London,I.M. (1985) *Proc. Natl. Acad. Sci. USA*, **83**, 6384–6388.
van der Ploeg,L.H.T. and Flavell,R.A. (1980) *Cell*, **19**, 947–958.
Velcich,A. and Ziff,E. (1985) *Cell*, **40**, 705–716.
Walker,M.D., Edlund,T., Boulet,A.M. and Rutter,W.J. (1983) *Nature*,**306**, 557–561.
Weaver,R.F. and Weissman,C. (1979) *Nucleic Acids Res.*, **7**, 1175–1193.
Wigler,M., Pellicer,A., Silverstein,A. and Axel,R. (1978) *Cell*, **14**, 725–731.
Yoshitake,S., Schach,B.G., Foster,D.C., Davie,E.W. and Kurachi,K. (1985) *Biochemistry*, **24**, 3736–3750.

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