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Transcription of the HS2 Enhancer toward a *cis*-Linked Gene Is Independent of the Orientation, Position, and Distance of the Enhancer Relative to the Gene

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The locus control region (LCR) regulates transcription of the downstream β-like globin genes 10 to 50 kb away. Among hypersensitive sites HS4, -3, -2, and -1, which define the LCR in erythroid cells, HS2 possesses prominent enhancer function. The mechanism by which the HS2 enhancer and other functional components of the LCR act over the distance is not clear. We have used reverse transcription-PCR and RNase protection assays to analyze the transcriptional statuses of both the endogenous and the transfected HS2 enhancer in erythroid K562 cells. A novel pattern of HS2 enhancer transcription was observed. The endogenous HS2 enhancer was transcribed predominantly in the direction toward the downstream globin genes. The HS2 enhancer in transfected recombinant chloramphenicol acetyltransferase (CAT) plasmids was also transcribed predominantly toward the CAT gene, regardless of whether the enhancer was placed (i) in the genomic or reverse genomic orientation, (ii) in a position 5′ or 3′ to the gene, or (iii) at various distances up to 6 kb from the gene. The orientation, position, and distance independence in gene-tropic transcription of the HS2 enhancer correlates with the observed orientation, position, and distance independence of HS2 enhancer function and suggests that enhancer transcription may play a role in enhancer function.

The locus control region (LCR) of the human β-globin gene domain is defined by four erythroid-cell-specific DNase I-hypersensitive (HS) sites, HS1, -2, -3, and -4, located 50 to 70 kb upstream of the β-globin gene in a transcriptional direction: 5′ HS4-HS3-HS2-HS1-//-ε-^{G}γ-^{A}γ-δ-β 3′ (17, 20, 37, 50). The LCR has been shown by studies of natural deletions of γδβ-thalassemia (8, 11, 24) and by gene transfer experiments (14, 18, 20, 51) to be indispensable for erythroid-cell-specific and high-level transcription of *cis*-linked globin genes and transgenes. The mechanism by which the LCR regulates transcription of the distant embryonic ε-, fetal γ-, and adult β-globin genes at the respective developmental stages is not fully understood.

In previous studies examining the mechanism of LCR function, a number of laboratories have investigated the ability of the individual HS sites to activate the transcription of *cis*-linked genes in cell lines and transgenic mice. Among the LCR HS sites, HS2 has been found to possess developmental-stage-independent enhancer function (52). It is capable of stimulating the transcription of embryonic ε-, fetal γ-, and adult β-globin genes in erythroid cells at the corresponding developmental stages (9, 28, 34, 42, 45, 47) and may therefore constitute a major functional component of the LCR. However, a recent targeted deletion study shows that deletion of the HS2 enhancer from the murine LCR generated only mild effects on the expression of the β-like globin genes (15), suggesting that HS2 is not essential for LCR function and that LCR function may be due to the contribution of the other HS sites. Surprisingly, similar targeted deletion of HS3 also did not cause significant changes in the expression of the β-like globin genes (22). These findings suggest that LCR function is due not to the contribution of any specific HS site but to a series of interactions among its functional components, including the enhancer elements underlying the HS sites.

In the above-described targeted deletion studies, deleting the HS2 or the HS3 sequence from the endogenous murine LCR and inserting in its place an independent transcription unit—a neomycin-resistant gene driven by a strong promoter—has been found to disrupt LCR function and cause severe anemia in and the deaths of homozygous transgenic mice (15, 21). In the human β-LCR, inserting an independent transcription unit at a location between the HS2 enhancer and the downstream globin genes also disrupted LCR function (23) and caused the distantly downstream β-globin gene to be transcriptionally turned off. The mechanism by which the transcription of a foreign gene within the LCR might disrupt LCR function is not clearly understood (21).

In an attempt to delineate the functional mechanism of the HS2 enhancer and ultimately of the LCR, we have previously analyzed the transcriptional status of the HS2 enhancer in transfected recombinant chloramphenicol acetyltransferase (CAT) plasmids (53). This earlier study showed that the transfected HS2 enhancer is itself transcribed in erythroid cells. Both HS2 enhancer transcription and HS2 enhancer function depend on the integrity of a 26-bp enhancer core containing two tandem Ap-1 sites located at the 5′ end of the HS2 sequence. Splitting the enhancer core between the Ap-1 sites, thereby preventing assembly of the intact enhancer core complex, diminishes both enhancer transcription at the enhancer site and enhancer function as measured by enhancement of CAT mRNA synthesis at the promoter site (53). This study suggests that enhancer transcription and enhancer function may be coordinately regulated.

In the present study, we further analyze this new class of

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enhancer RNAs and characterize their mode of synthesis. Using reverse transcription-PCR (RT-PCR), we found that the endogenous HS2 enhancer was transcribed predominantly in a direction toward the downstream ε-globin gene in erythroid K562 cells but not in nonerythroid retinoblastoma (RB) cells. Using RNase protection assays, we found that the HS2 enhancer in integrated plasmids in K562 cells was also transcribed predominantly toward the *cis*-linked CAT gene, independent of the orientation, position, and distance of the HS2 enhancer relative to the gene. As revealed by CAT enzymatic assays, the HS2 sequence exhibited enhancer activity which was also independent of the orientation, position, and distance of the HS2 sequence relative to the gene. Both gene-tropic enhancer transcription and enhancer function were observed regardless of whether the transfected plasmids were integrated in tandem or single copies in the K562 genome as determined by Southern blot hybridization. The correlation between the mode of enhancer transcription and enhancer function suggests that a transcription mechanism may constitute a basic component of HS2 enhancer function.

MATERIALS AND METHODS

Purification of RNA. Cellular RNAs from K562 and RB cells (Y79 cells from the American Type Culture Collection) were purified as described previously (53).

RT-PCR. Total cellular RNAs from either K562 or RB cells (1 μg) were used as the templates in RT reactions. Incubation for the RT step was at 42°C for 60 min. The PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 1.5 min, 62°C for 1 min, and 72°C for 3 min and a final cycle of 72°C for 15 min. $MgCl_2$ (2 mM) was present in both the RT and the PCR steps. PCR samples (25-μl aliquots) were analyzed in 2% agarose gels. The 5′ → 3′ sequences of the primers (27) were ATGCCTGAGACAGAATGTGAC for primer 1, TATGTG AGCATGTGTCCTCT for primer 2, CTGTTAGAGGACACATGCTCA for primer 3, AAGCTTTGAGTACTTCCTATAGCC for primer 4, GTTCTCTTC CAGTTTGCAGGTCTTC for primer 5, and GCTTTCTCTCAAGGCCAAGC CCAGT for primer 6. The β-actin primer pair was obtained from Stratagene.

Construction of recombinant CAT plasmids. Constructs 1, 2, and 3 (see Fig. 2) have been described previously (52). Construct 4 (HS2-5.8-εp-CAT) was made from a HS2-HS1-5.8-εp-CAT clone which contained the following: HS2 between the *Hin*dIII and *Bgl*II sites, with the *Hin*dIII site changed to a *Bam*HI site by linker addition; HS1 between the *Bcl*I and *Hin*dIII sites; the 5.8 kb of intervening DNA between *Hin*dIII and *Bam*HI; and the 200 bp of the ε-globin promoter (εp) between *Bam*HI and *Pvu*II (see Fig. 2) spliced into the pA10CAT2 vector, which had been previously digested with *Bgl*II and *Stu*I to remove the simian virus 40 (SV40) promoter. The HS1 site was removed from this clone by *Stu*I and *Sma*I digestions followed by recircularization of the plasmid to produce construct 4. Construct 5 (HS2-εp-CAT) was made from the 1.2-HS2-εp-CAT clone (52) by removing 1.2 kb of DNA with *Bgl*II, which cleaved between the 1.2-kb DNA and HS2, and with *Sal*I, which cleaved 95 bp in the vector immediately 5′ of the 1.2-kb DNA, followed by recircularization of the plasmid DNA. Construct 6 (HS2-1.2-εp-CAT) was made by the following steps. The 0.74-kb HS2 with 5′ *Bam*HI and 3′ *Bgl*II ends was spliced into the *Bam*HI site in the polycloning sites of pUC19. The HS2 DNA with 5′ *Bam*HI and 3′ *Sal*I ends was excised from pUC19 and spliced in reverse orientation into HS2-1.2-εp-CAT (construct 3) from which HS2 had been removed by digestions with *Bgl*II, which cleaved between HS2 and the 1.2-kb DNA (see Fig. 1), and with *Sal*I, which cleaved 95 bp upstream of HS2 in the vector. Construct 7 (1.2-εp-CAT) was made by recircularization of the above-described HS2-1.2-εp-CAT vector following removal of HS2 by *Bgl*II and *Sal*I digestions. Construct 8 (5.8-εp-CAT) was created by digestion of the HS2-HS1-5.8-εp-CAT plasmid with *Hin*dIII to excise the *Hin*dIII fragment containing the 5.8 kb of intervening DNA and the ε-globin promoter, which was then inserted into the *Hin*dIII-digested promoterless pSV0 CAT vector plasmid (6). Constructs 9 and 10 (CAT-HS2 and CAT-HS2) were made by inserting the 0.74-kb HS2 fragment with 5′ *Bam*HI and 3′ *Bgl*II ends in either the genomic or the reverse genomic orientation into the *Bam*HI site downstream of the CAT gene in construct 1.

Creation of K562 cell lines harboring integrated CAT plasmids. Cell lines were created by electroporation or calcium phosphate precipitation according to previously described protocols (53). Before transfection, the CAT plasmids were linearized at a unique *Pst*I site in the Amp^r gene to yield the linear plasmids as depicted in Fig. 3. To avoid integration of tandemly integrated plasmids, such that the HS2 enhancer spliced upstream of a CAT gene within a plasmid might be located downstream of a CAT gene in a neighboring plasmid, a modified calcium phosphate precipitation method was also used. In the modified protocol, the linearized plasmids (10 μg) were mixed and cotransfected with a stuffer DNA (20 μg of human genomic DNA digested with *Pst*I and *Hgi*AI to average sizes of around 10 kb). This was done to intersperse the plasmid DNA molecules with fragments of the genomic DNA in the calcium phosphate precipitate so that the transfecting plasmids would not be transfected into host cells in tandem copies. After transfection, G418 at 400 μg/ml was added to the culture medium. For cells transfected with enhancerless constructs 1, 7, and 8, the G418 concentration was lowered to 200 μg/ml after the 12th day. Resistant colonies on a culture dish were selected or pooled and expanded.

CAT assays. The CAT assays were carried out as described previously (52).

Southern blot hybridization. Five micrograms of purified genomic DNA digested with appropriate single or combinations of two restriction enzymes were blotted and hybridized as described previously (50).

Syntheses of ^{32}P-labeled RNA probes. (i) Syntheses of the P1 and P3 probes have been described previously (53). The P1 and P3 probes of 1,300 nucleotides (nt) contained 95 bases of vector sequence. (ii) The DNA template for the P4 probe was excised from the HS2-εp-CAT plasmid by digestions with *Sal*I, which cleaved 5′ of the HS2 sequence, and *Eco*RI, which cleaved the CAT gene at base 271, and inserted into the pGEM 3 vector (from Promega), which had been previously digested with *Sal*I and *Eco*RI. The P4 probe of 1,206 nt was synthesized by T7 polymerase from *Sal*I-linearized template plasmid. P4 did not contain 95 bases of vector DNA. (iii) The template plasmid for the P5 and P6 probes was excised from εp-CAT-HS2 by double digestions with *Sty*I, which cleaved at a site downstream of the splice sites, and *Bam*HI, which cleaved between the HS2 enhancer and the vector sequence. The excised template DNA with the *Sty*I end blunted was inserted into the pGEM 3 vector between the *Sma*I and *Bam*HI sites. The sense P5 probe was synthesized from *Bam*HI-linearized plasmid with T7 polymerase and the antisense P6 probe from the *Eco*RI-linearized plasmid with Sp6 polymerase. (iv) The template plasmid for syntheses of the P7 and P8 probes was excised from the template construct for the P1 probe by digestions with *Stu*I, which cleaved 40 bp upstream of the *Bgl*II site at the 3′ border of HS2, and *Eco*RI, which cleaved at base 271 of the CAT gene to remove the εp-CAT sequence, followed by recircularization of the plasmid. The P7 and P8 probes were synthesized with T7 or Sp6 polymerase from the *Sal*I- or *Eco*RI-linearized template plasmid. The P7 and P8 probes contained 95 bases of vector DNA. Probes were labeled to a specific activity of 1×10^9 to 2×10^9 cpm/μg.

RPAs. RNase protection assays (RPAs) were carried out as described previously (53). Briefly, 50 μg of RNA samples or baker's yeast tRNA controls were hybridized to RNA probes (2×10^6 cpm). RNase concentrations of 25 to 40 μg/ml and digestion temperatures of 25 to 37°C were used, since under these conditions the nonspecific hybrids formed between yeast tRNA and the probes were completely degraded. Each experiment was repeated two to four times. A probe lane containing 2,000 cpm of undigested probe was included in each gel to serve as a gauge for the exposure times of the autoradiograms. ^{32}P-labeled, *Hae*III-digested φX174 DNA served as size markers; the apparent lengths of RNAs may therefore be 3 to 5% longer than actual lengths.

RESULTS

The endogenous HS2 enhancer of K562 cells is transcribed toward the ε-globin gene. RT-PCR was used to detect the endogenous HS2 enhancer transcripts in cellular RNAs isolated from erythroid K562 and nonerythroid RB cells. The following PCR primer pairs were synthesized: primers 1 and 2, which amplified a DNA fragment of 380 bp spanning the 3′ half of the 0.74-kb HS2 enhancer, and primers 3 and 4, which amplified an overlapping DNA of 1,230 bp spanning the DNA immediately downstream of HS2 (Fig. 1a). To detect transcripts of the ε-globin gene located 10 kb further downstream of HS2, primer pair 5 and 6, which spanned 84 bp of the 3′ untranslated region of the ε-globin gene, was synthesized (Fig. 1a). An additional β-actin primer pair was used to produce an amplified band of 660 bp from the ubiquitous β-actin mRNA, which served as an internal quantitative control for comparing the intensities of RT-PCR bands from K562 and RB RNAs. To detect sense enhancer RNAs synthesized in a direction toward the ε-globin gene, primer 2 or 4 was used in the RT step for cDNA synthesis, but to detect antisense enhancer RNAs, primer 1 or 3 was used in the RT step (Fig. 1a). The cDNAs of the sense and antisense transcripts from the same region were subsequently amplified in PCRs with an identical primer pair, i.e., primer pair 1 and 2 or 3 and 4.

In K562 RNA, the sense transcripts of the HS2 enhancer and of the immediate downstream DNA produced the expected amplification bands of 380 and 1,230 bp, respectively (Fig. 1b, lanes 1 and 3). The antisense transcripts of HS2 and of the immediate downstream DNA were apparently not

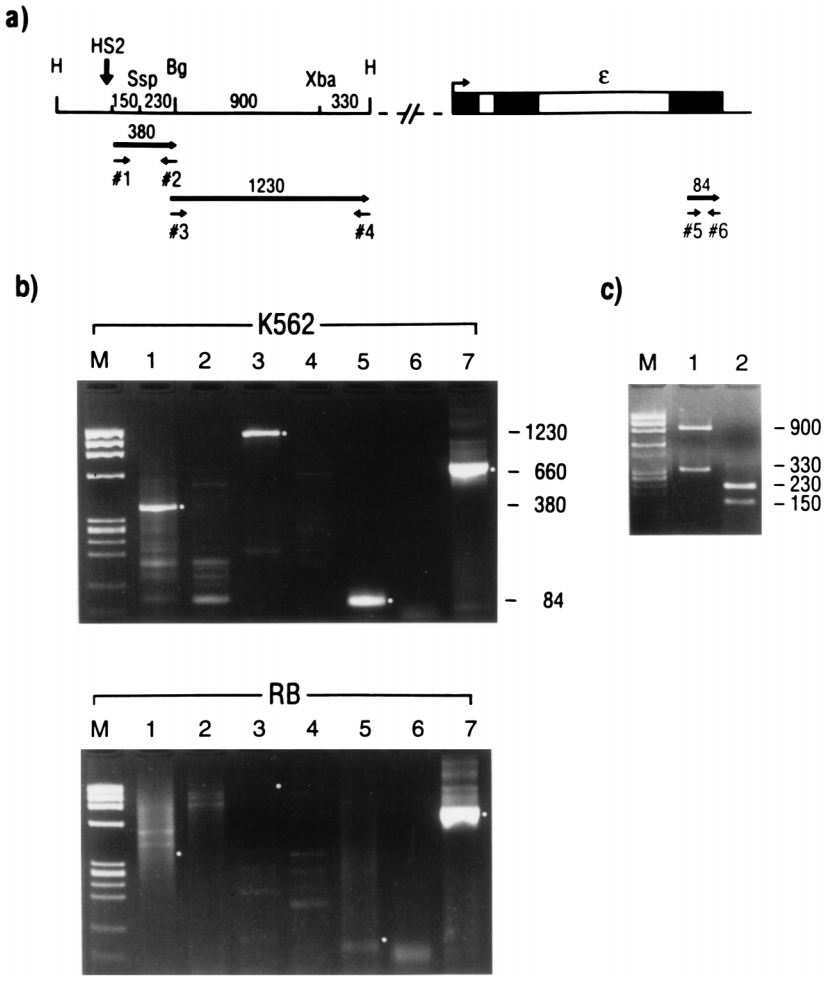

FIG. 1. Mapping endogenous HS2 transcripts in K562 and RB cells by RT-PCR. (a) Genomic DNA map of the HS2 enhancer, the intervening DNA, and the downstream ε-globin gene. Vertical bars indicate locations of restriction sites as follows: H, *Hin*dIII; Ssp, *Ssp*I; Bg, *Bgl*II; and Xba, *Xba*I. Numbers indicate the sizes in base pairs of the restriction fragments. Thick vertical arrow, location of the HS2 enhancer between the *Hin*dIII and the *Bgl*II sites; filled boxes, exons of the ε-globin gene; angled arrow, cap site of the ε-globin gene; dotted line, DNA far downstream of HS2 (not drawn to scale); thick horizontal arrows, segments of the sense transcripts of the HS2 enhancer, the DNA immediately downstream of HS2, and the ε-globin gene amplified by primer pairs 1 and 2, 3 and 4, and 5 and 6, respectively; directions of the arrows, 5′ → 3′ direction of the transcripts or the primers; vertical bar 5′ of the *Ssp*I site, 5′ end of primer 1; numbers above the horizontal arrows, predicted sizes in base pairs of the amplified DNA. (b) Analysis of the sizes of DNA fragments amplified from K562 RNA (top gel) and RB RNA (bottom gel). Primer pairs used to amplify the sense and antisense transcripts in each lane are listed below; the first primer in a pair designates the primer used for cDNA synthesis in the RT step. Lanes 1, primers 2 and 1; lanes 2, primers 1 and 2; lanes 3, primers 4 and 3; lanes 4, primers 3 and 4; lanes 5, primers 6 and 5; lanes 6, β-actin primer pair, (the RT step was carried out in the absence of reverse transcriptase); lanes 7, β-actin primer pair; lanes M, *Hae*III-digested ϕX size marker bands of 1,353, 1,078, 872, 603, 310, 281 plus 271, 234, 194, 118, and 72 bp. White dots, positions of the expected amplication bands; numbers in the right margin, sizes in base pairs of amplified bands. (c) Restriction enzyme digestion of DNA amplified from K562 RNA. Lane M, ϕX size markers; lane 1, *Xba*I digestion of the 1,230-bp DNA; lane 2, *Ssp*I digestion of the 380-bp DNA.

present, since the respective amplification bands were not detectable (Fig. 1b, lanes 2 and 4). In K562 cells, the ε-globin gene is expressed (3, 7, 10); accordingly, the sense transcripts of the gene produced the expected amplification band of 84 bp (Fig. 1b, lane 5).

In nonerythroid RB cells of the retinal-neural lineage, neither the HS2 enhancer nor the DNA immediately downstream of HS2 was detectably transcribed in either the sense or the antisense direction; hence, neither the amplification band of 380 bp nor the band of 1,230 bp was generated (Fig. 1b, RB lanes 1 to 4). Consistent with the absence of enhancer transcription, the ε-globin gene was not transcribed and the amplification band of 84 bp was not detected (Fig. 1b, RB lane 5). We verified that similar amounts of RB and K562 RNAs were used in the RT-PCRs: the ubiquitous β-actin mRNA present in both RNAs generated amplified bands of similar intensities (Fig. 1b, lanes 7). To ensure that the RNAs were not contaminated with DNA, we carried out a control RT-PCR in which the reverse transcriptase was omitted in the RT step so that no cDNA synthesis took place; subsequent PCR amplification of the sample by the β-actin primer pair then failed to produce any amplified DNA band from either K562 or RB RNAs (Fig. 1b, lanes 6).

To confirm that the observed 380-bp and 1,230-bp bands (Fig. 1b, K562 lanes 1 and 3) expected to be amplified from the gene-tropic HS2 enhancer transcripts were not produced by RNAs transcribed from elsewhere in the K562 genome, the identities of the bands were examined by restriction enzyme digestions. *Ssp*I digestion of the 380-bp band produced two expected subfragments of 150 and 230 bp, and *Xba*I digestion of the 1,230-bp band produced two expected subfragments of 900 and 330 bp (Fig. 1a and c) as predicted from the DNA

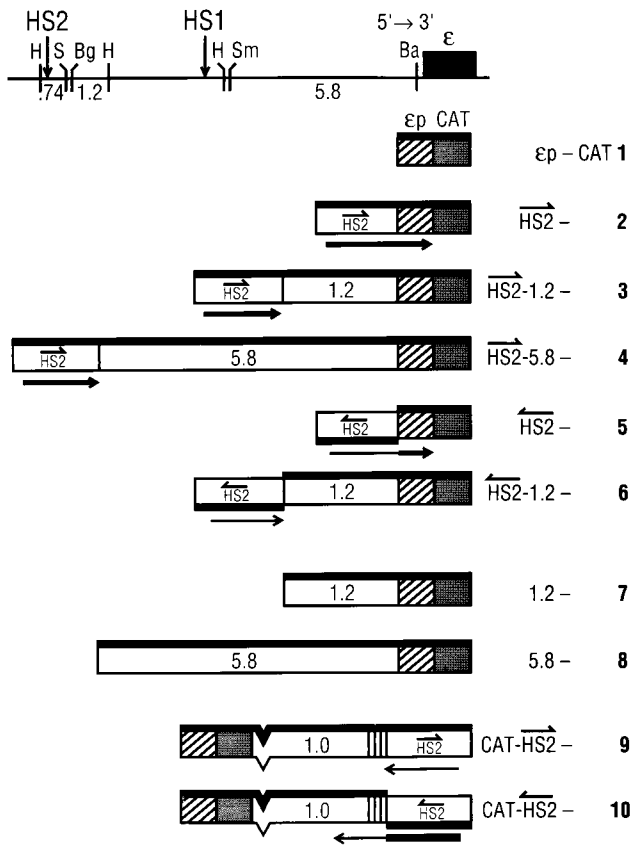

FIG. 2. Maps of recombinant CAT constructs. The top diagram is a partial restriction enzyme map of genomic DNA between the HS2 enhancer and the ε-globin gene used in plasmid constructions. S, *Stu*I; Sm, *Sma*I; Ba, *Bam*HI. Other restriction enzyme abbreviations are as described in the legend to Fig. 1a. Vertical arrows, locations of HS2 and HS1 DNase I hypersensitive sites; numbers below the line, sizes in kilobases of HS2 and segments of the intervening DNA; filled box, the ε-globin gene; stippled boxes, the CAT gene; hatched boxes, the ε-globin promoter (εp); open boxes, the 0.74-kb HS2 enhancer or the intervening DNAs of 1, 1.2, or 5.8 kb; $\overrightarrow{HS2}$ or $\overleftarrow{HS2}$, genomic or reverse genomic orientation of the enhancer, respectively. In constructs 2 to 4 and 9 containing $\overrightarrow{HS2}$, the upper thick horizontal lines and the lower thin horizontal lines denote, respectively, the sense and antisense strands of DNA; in constructs 5, 6, and 10 containing $\overleftarrow{HS2}$, the directions of the thick and thin strands of HS2 are reversed. Horizontal arrows beneath the constructs indicate gene-tropic RNAs transcribed from the corresponding plasmids detected in Fig. 4 to 6. Each individual arrow represents contiguous RNA sequence detected by a single RPA probe.

sequence of this region (27). The identities of the amplified bands were further confirmed by Southern blot hybridization experiments (data not shown). The above-described RT-PCR results indicate that the endogenous HS2 enhancer is transcribed predominantly in a direction toward the downstream globin genes.

The HS2 sequence in transfected plasmids exhibits enhancer activity, regardless of its orientation, position, and distance relative to the *cis*-linked CAT gene and of whether the transfected plasmids are integrated in tandem or single copies. In order to study the effects of the orientation, position, and distance of the HS2 sequence relative to the CAT gene on enhancer function and transcription, seven test plasmids and three control plasmids were constructed (Fig. 2). In the test plasmids, the HS2 enhancer was spliced in the genomic orientation immediately 5′ of the CAT gene (construct 2) or at a distance of 1.2 and 5.8 kb 5′ of the gene (constructs 3 and 4, respectively), in the reverse genomic orientation immediately 5′ of the CAT gene (construct 5) or at a distance of 1.2 kb 5′ of the gene (construct 6), and in the genomic or reverse genomic orientation at a distance of 1 kb 3′ of the CAT gene (constructs 9 and 10, respectively). The three enhancerless control plasmids were construct 1, which contained the ε-globin promoter spliced 5′ of the CAT gene, and constructs 7 and 8, which contained, respectively, the 1.2-kb intervening DNA of constructs 3 and 6 and the 5.8-kb intervening DNA of construct 4 spliced 5′ of the ε-globin promoter and the CAT gene. In all seven test constructs, the HS2 enhancer activated the production of the CAT enzyme to levels 2,500- to 4,000-fold over that of the enhancerless εp-CAT plasmid regardless of the orientation, position, and distance of the HS2 enhancer relative to the CAT gene (Table 1).

The majority of the plasmids were introduced into K562 cells by the calcium phosphate precipitation method which introduces plasmids in multiple tandem copies. Because of the concerns that the HS2 enhancer may be active only when it is integrated in multiple tandem copies (13) and that the subsequently detected HS2 transcripts are therefore not RNA transcribed from the HS2 in each individual plasmid but are nonspecific readthrough transcripts of the tandemly integrated upstream plasmids, we also transfected a number of the plasmids by a modified calcium phosphate method in an attempt to integrate the plasmids in single copies into K562 cells (see Materials and Methods). For purpose of comparison, a number of plasmids were also transfected by the electroporation method.

To determine the modes of integration and the copy numbers of the integrated plasmids transfected by the different methods, the Southern blot hybridization technique was employed. Genomic DNAs purified from the transfected K562 cells were digested with restriction enzymes (*Hin*dIII or *Bam*HI), which cleaved only once in the plasmid DNA; after being blotted, the digested DNAs were hybridized to an appropriate CAT or ampicillin gene probe (see Fig. 3a). In the Southern blots, plasmids that integrated tandemly in a head-to-tail fashion should produce a single, intense band with a size

TABLE 1. Enhancer function of the HS2 sequence in recombinant CAT plasmids as measured by CAT enzymatic activities

Construct	Plasmid[a]	Plasmid/cell[b]	CAT enzymatic activity[c]
1	εp-CAT	300	1
2	$\overrightarrow{HS2}$(1)*	1.4	2,650 ± 300
	$\overrightarrow{HS2}$	10	3,400 ± 280
	$\overrightarrow{HS2}^{+}$	14	3,250 ± 120
3	$\overrightarrow{HS2}$-1.2	11	3,060 ± 60
4	$\overrightarrow{HS2}$-5.8	8	3,940 ± 30
5	$\overleftarrow{HS2}$	12	2,840 ± 400
6	$\overleftarrow{HS2}$-1.2	13	2,470 ± 75
7	1.2	6	200 ± 50
8	5.8	150	200 ± 20
9	CAT-$\overrightarrow{HS2}$*	3	3,660 ± 150
10	CAT-$\overleftarrow{HS2}$*	4	3,250 ± 370

[a] Recombinant CAT plasmids integrated into K562 cells. Plasmid designations are the same as described for Fig. 2. Plasmids marked with * were introduced into cells by electroporation, those marked with $^{+}$ were introduced by a modified calcium phosphate precipitation protocol, and the other plasmids were introduced by calcium phosphate precipitation.

[b] Average copy numbers of integrated plasmids per cell as determined by Southern blot hybridization (Fig. 3 and data not shown).

[c] Relative CAT enzymatic activities per copy of integrated plasmid, with the enhancerless εp-CAT plasmid as the reference, the CAT value of which was set at 1.

corresponding to that of the linearized plasmid because of the identical locations of the single restriction enzyme site in the neighboring plasmids; the intensity of the band should be proportional to the copy numbers of the integrated plasmid. On the other hand, plasmids integrated in single copies into multiple sites in the host genome should produce multiple bands whose sizes vary according to the location of the next *Hin*dIII or *Bam*HI site in the contiguous host genome; the intensities of these bands should be relatively uniform and correspond to the intensity generated by a single-copy reference plasmid. Indeed, plasmids transfected by the calcium phosphate precipitation method produced single, strong bands with sizes corresponding to the respective, linearized plasmids and the intensities of the bands proportional to the copy numbers of the integrated plasmids (Fig. 3b, lanes 1 to 7, and Table 1). This indicates that these plasmids were integrated in the K562 genome in multiple tandem copies. On the other hand, plasmids transfected by the electroporation method produced two to four uniformly weak bands of various sizes, whose intensities were comparable to the intensity of the single-copy reference standard (Fig. 3b, lanes 8 to 10). This result indicates that these plasmids were integrated in single copies in a limited number of two to four host sites per cell. Finally, the plasmid that was transfected by the modified calcium phosphate precipitation method produced many more weak bands of various sizes (Fig. 3b, lane 11). This result indicates that the plasmid was integrated in single or double copies into multiple sites in the host genome.

The results of CAT enzymatic assays indicate that regardless of whether the plasmids were integrated in tandem or single copies, the HS2 sequence in the integrated plasmids activated the CAT gene and exhibited prominent enhancer activities (Table 1).

The HS2 enhancer spliced in either genomic or reverse genomic orientation 5′ of the CAT gene is transcribed predominantly in a direction toward the gene. To study the effect of enhancer orientation on enhancer transcription, we analyzed the transcriptional status of two integrated test plasmids, $\overrightarrow{HS2}$-εp-CAT and $\overleftarrow{HS2}$-εp-CAT (Fig. 4a, top diagrams), in which the HS2 enhancer was spliced in either the genomic or reverse genomic orientation 5′ of the ε-globin promoter and the CAT gene. For the $\overrightarrow{HS2}$-εp-CAT plasmid, two populations of transfected K562 cells were selected for study: one contained an average of 1 to 2 plasmids per cell, and the other contained an average of 10 tandem plasmids per cell [$\overrightarrow{HS2}$ (1) and $\overrightarrow{HS2}$, respectively] (Table 1). For the $\overleftarrow{HS2}$-εp-CAT plasmid, the transfected K562 cell population contained an average of 12 tandem plasmids per cell ($\overleftarrow{HS2}$) (Table 1).

Total cellular RNAs isolated from the transfected K562 cells were analyzed by RPAs (29). To map the sense RNAs transcribed in a direction colinear with that of CAT mRNA from the $\overrightarrow{HS2}$-εp-CAT plasmid, the antisense P1 probe was synthesized from this plasmid. The P1 probe contained the sequence of the antisense strand of the entire HS2 enhancer (735 nt), the ε-globin promoter (200 nt), and the first 271 bases of the CAT gene in this plasmid (Fig. 4a). To map the antisense RNAs transcribed from this plasmid, the sense P3 probe, which contained the corresponding sense strand of the $\overrightarrow{HS2}$-εp-CAT plasmid, was synthesized (Fig. 4b). To map the sense RNAs transcribed from the $\overleftarrow{HS2}$-εp-CAT plasmid, the antisense P4 probe was synthesized from this plasmid. The P4 probe contained at the 5′ end the same εp-CAT sequence of 471 nt as the P1 probe. However, because of the reverse genomic orientation of HS2 in this plasmid, the HS2 sequence in the P4 probe was not identical but complementary to that in the P1 probe (see the depictions of the P1 and P4 probes in Fig. 4a and c).

The sense transcripts of the $\overrightarrow{HS2}$-εp-CAT and $\overleftarrow{HS2}$-εp-CAT plasmids hybridized to the antisense P1 probe and generated many prominent P1 bands (Fig. 4d, lanes $\overrightarrow{HS2}$ and $\overleftarrow{HS2}$). This indicates that these plasmids were transcribed in a sense direction colinear with the direction of transcription of CAT mRNA. The $\overrightarrow{HS2}$-εp-CAT plasmid generated long enhancer

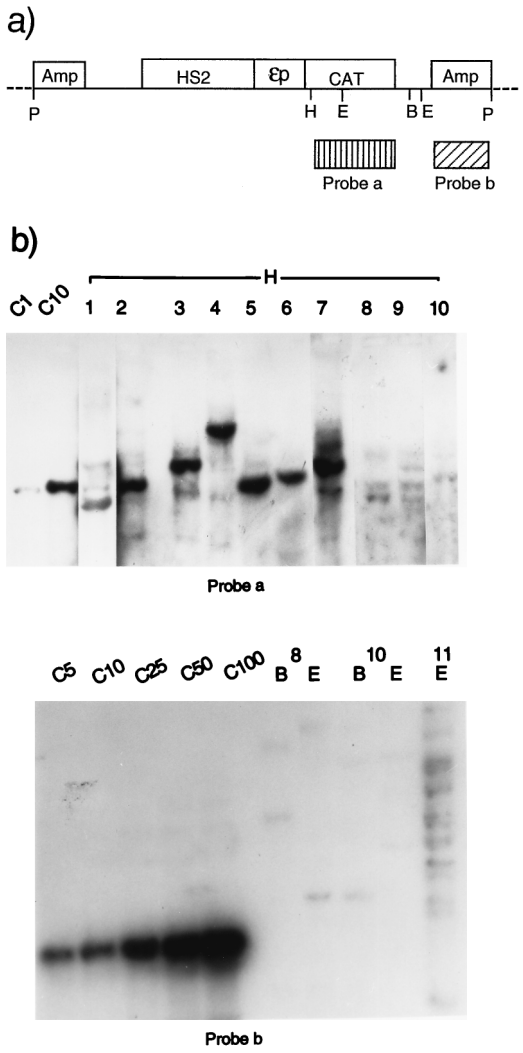

FIG. 3. Determination of copy numbers and mode of integration of the recombinant plasmids by Southern blot hybridization. (a) Representative map of an integrated plasmid. Amp, the ampicillin resistence gene; P, *Pst*I, the enzyme used to linearize the plasmids before they were transfected into the K562 cells. *Hin*dIII (H), *Eco*RI (E), and *Bam*HI (B) were the enzymes used to cleave the genomic DNAs for Southern blots. Probes a and b are hybridization probes containing, respectively, CAT and Amp gene sequences. (b) Southern blots. The top blot shows *Hin*dIII (H)-cleaved genomic DNA hybridized to probe a. Lanes 1 to 7 contain DNAs purified from pooled populations of K562 cells harboring, respectively, constructs 1, 2, 3, 4, 5, 7, and 6 as depicted in Fig. 2; plasmids were transfected by calcium phosphate precipitation (Table 1). Lanes 8 to 10 contain DNAs purified from pure clones of K562 cells harboring, respectively, constructs 9, 10, and 2 as depicted in Fig. 2; plasmids were transfected by electroporation (Table 1). Lanes C1 and C10 contain copy number standards calculated to contain 1 copy and 10 copies, respectively, of the reference εp-CAT plasmid per cell. The exposure time for lane 1 was 20 min, and that for all other lanes was 70 h. The bottom blot shows DNAs hybridized to probe b. Lanes 8 and 10 contain the same DNA samples as in lanes 8 and 10, respectively, in the top blot, but the samples were cleaved with either *Bam*HI or *Eco*RI. Lane 11 contains *Eco*RI-digested genomic DNA purified from a pure clone of K562 cells harboring construct 2 transfected by the modified calcium phosphate precipitation method (Table 1). Lanes C5 to C100 contain copy number standards calculated to contain, respectively, 5 to 100 copies of εp-CAT plasmid per cell.

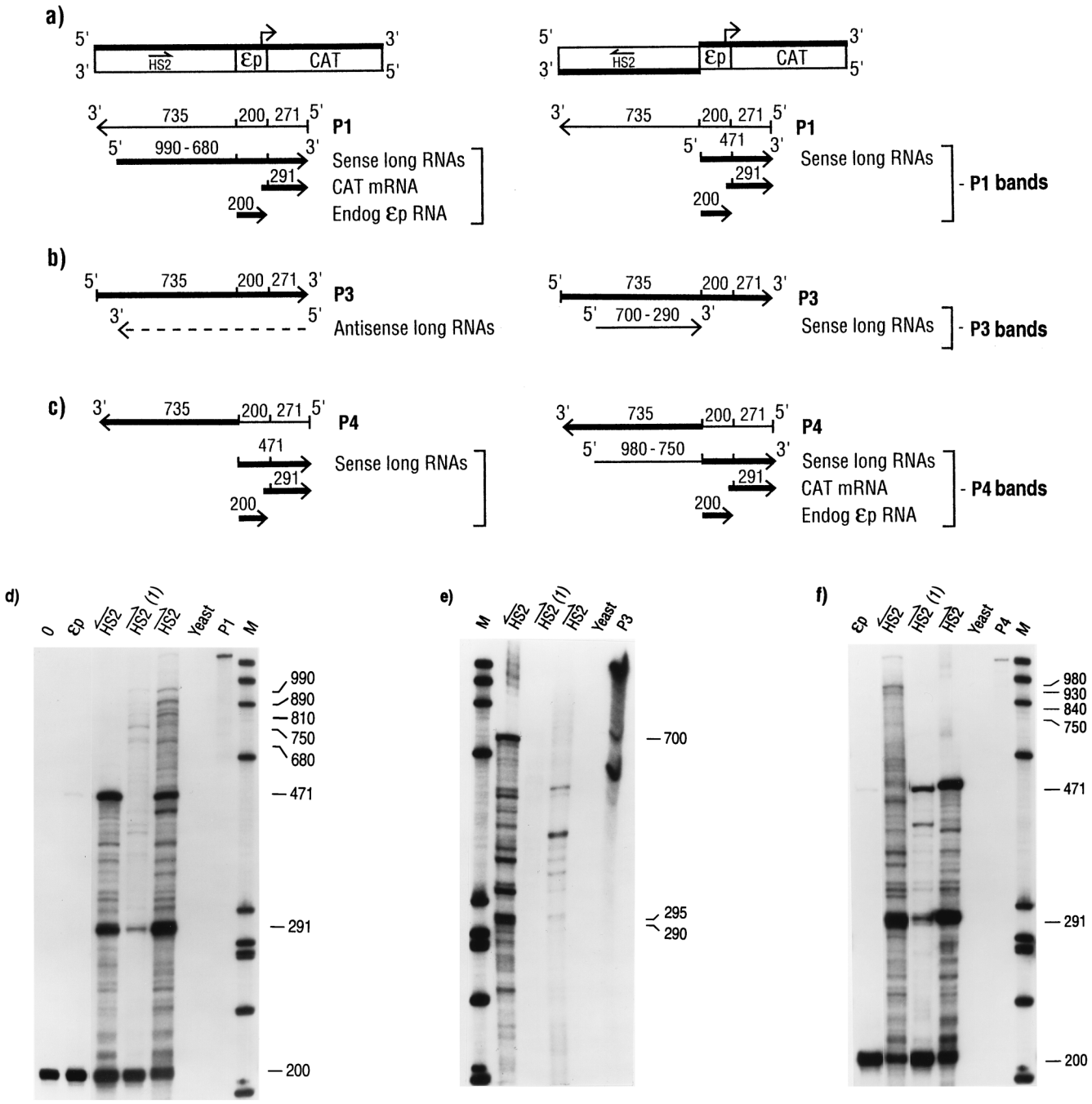

FIG. 4. RPAs of sense and antisense RNAs transcribed from the $\overrightarrow{HS2}$-εp-CAT and the $\overleftarrow{HS2}$-εp-CAT plasmids. (a) The left and right diagrams are plasmid maps of $\overrightarrow{HS2}$-εp-CAT (left) and $\overleftarrow{HS2}$-εp-CAT (right) along with depictions of the antisense P1 probe and the protected P1 bands generated by sense RNAs of the respective plasmids. The upper thick and lower thin lines of the plasmids indicate the sense and antisense DNA strands in the plasmids. Note that in $\overleftarrow{HS2}$-εp-CAT (right diagram), because of the reverse genomic orienttion of HS2, the sense and antisense directions of the thick and thin strands of HS2 are reversed. Angled arrow, the cap site of CAT mRNA; thin right-to-left arrow, the antisense P1 probe, aligned with the same strand in the plasmid map; thick left-to-right arrows, protected P1 bands generated by multiple sense long RNAs, CAT mRNA, and sense RNAs of the endogenous ε-globin promoter of K562 cells (Endog. εp RNA) (d). The HS2, ε-globin promoter, and CAT regions in the sense RNAs are aligned with the complementary regions in the P1 probe. Numbers indicate the sizes in nucleotides of the HS2, ε-globin promoter, and CAT gene regions in the P1 probe or of the protected P1 bands. (b) Depiction of the P3 probe and protected P3 bands generated by $\overrightarrow{HS2}$-εp-CAT (left diagram) and $\overleftarrow{HS2}$-εp-CAT (right diagram). Thick left-to-right arrow, sense P3 probe, aligned with the complementary P1 probe; dotted arrow, faint P3 bands generated by low levels of antisense long RNAs from $\overrightarrow{HS2}$-εp-CAT (panel e, lane $\overrightarrow{HS2}$); thin arrow, P3 bands generated by the HS2 sequences in sense long RNAs from $\overleftarrow{HS2}$-εp-CAT (panel e, lane $\overleftarrow{HS2}$) (the arrow is aligned with the complementary HS2 sequence in P3 probe). (c) Depiction of the P4 probe and protected P4 bands generated by $\overrightarrow{HS2}$-εp-CAT (left diagram) and $\overleftarrow{HS2}$-εp-CAT (right diagram). Thin and thick right-to-left arrow, the antisense P4 probe aligned with the same strand in the $\overleftarrow{HS2}$-εp-CAT plasmid; thin and thick left-to-right arrow, protected P4 bands generated by sense long RNAs from $\overleftarrow{HS2}$-εp-CAT. Other designations are the same as described for panel a. (d, e, and f) Autoradiograms of protected P1, P3, and P4 bands, respectively, produced by RNAs from nontransfected K562 cells (lane 0) and K562 cells transfected by enhancerless εp-CAT (lanes εp), $\overrightarrow{HS2}$-εp-CAT [lanes $\overrightarrow{HS2}$ (1) and $\overrightarrow{HS2}$], and $\overleftarrow{HS2}$-εp-CAT (lanes $\overleftarrow{HS2}$). Lanes Yeast, control yeast tRNA hybridized to the respective probes; lanes P1, P3, and P4, 2,000 cpm of the respective undigested probes; lanes M, ϕX size marker bands. Numbers in the right margins are sizes in nucleotides of protected bands. Exposure times were as follows: 12 h (d), 56 h (e), and 8 h (f).

RNAs that were apparently initiated from multiple sites in the HS2 enhancer and elongated through the promoter into and past base 271 of the CAT gene; these enhancer RNAs produced an array of long P1 bands of 1,000 to 471 nt, including the five distinct bands of 990, 890, 810, 750, and 680 nt at the top of the RPA gel [Fig. 4d, lanes $\overrightarrow{HS2}$ (1) and $\overrightarrow{HS2}$]. RNAs initiated from the junction of HS2 and the ε-globin promoter and elongated into the CAT gene produced the strong band of 471 nt (Fig. 4d, lane $\overrightarrow{HS2}$); this strong band was, however, not consistently detected in the other cell populations [Fig. 4d, lane $\overrightarrow{HS2}$ (1)] or in cell clones (53) that harbor the $\overrightarrow{HS2}$-εp-CAT plasmid. Shorter sense RNAs initiated from sites in the ε-globin promoter and extended past base 271 of the CAT gene generated the multiple bands shorter than 471 nt. In addition, sense RNAs initiated more upstream from sites in the enhancer but prematurely terminated before reaching the CAT gene also generated these shorter P1 bands. CAT mRNA, which was initiated from the proper cap site in the ε-globin promoter (2) of these plasmids, generated the prominent band of 291 nt (Fig. 4a, d, and f).

Sense RNAs transcribed from the endogenous ε-globin promoter in the genome of K562 cells produced the strong P1 band of 200 nt which was present in comparable intensities in transfected as well as nontransfected K562 cells [Fig. 4d, lanes 0, εp, $\overleftarrow{HS2}$, $\overrightarrow{HS2}$ (1), and $\overrightarrow{HS2}$]. These transcripts were apparently initiated from sites upstream of the endogenous ε-globin promoter and elongated through the promoter into the ε-globin gene. They hybridized to the antisense ε-globin promoter sequence of 200 nt in the P1 probe and produced the prominent band of 200 nt. Confirming the origin of this band, antisense RPA probes lacking the ε-globin promoter sequence did not generate the strong 200 nt band (data not shown).

In contrast to the sense transcripts of the endogenous ε-globin promoter, the sense HS2 enhancer RNAs transcribed from the endogenous HS2 sequence 10 kb more upstream (Fig. 2) were apparently present at a level below the optimal detection limit of RPA, so none of the anticipated P1 bands was readily discernible in the autoradiogram (Fig. 4d, lane 0). However, these endogenous HS2 enhancer transcripts were detectable by RT-PCR (Fig. 1).

With the intensities of the 200-nt band generated by the sense transcripts of the endogenous ε-globin promoter as an internal quantitative reference, a comparison of the intensities of the 291-nt bands generated by CAT mRNA indicates that the level of CAT mRNA synthesized from the $\overrightarrow{HS2}$-εp-CAT plasmid integrated tandemly at an average of 10 copies per cell was proportionally higher than that from the same plasmid integrated singly at an average of one to two copies per cell [Fig. 4d, lanes $\overrightarrow{HS2}$ (1) and $\overrightarrow{HS2}$]. This suggests that each copy of the HS2 sequence activated the *cis*-linked CAT gene to approximately equivalent levels. Thus, HS2 in different cell populations possesses equivalent enhancer activity regardless of whether the plasmid was integrated in single or tandem copies (Table 1).

The prominent P1 bands generated by the integrated plasmids were produced only by plasmids containing the HS2 enhancer but not by the enhancerless εp-CAT plasmid (Fig. 4d, compare lane εp with lanes $\overrightarrow{HS2}$ and $\overleftarrow{HS2}$), even though the enhancerless plasmid contained the ε-globin promoter sequence with potential transcriptional initiation sites and was integrated at an average of 300 tandem copies per cell (Table 1). This observation suggests that the HS2 enhancer was the driving force for the transcription of both the long and the short sense RNAs, including CAT mRNA.

The above observation also suggests that both the long and the short sense RNAs were initiated from sites within the enhancer and the ε-globin promoter of the integrated plasmids and were not produced by readthrough transcripts initiated from sites more upstream in the host genome, which were subsequently degraded by the RNases used in the RPAs. Consistent with this, we observed that reproducible RPA bands were produced by the sense RNAs digested with different concentrations of RNase in the RPAs (see Materials and Methods) and also by different plasmids integrated in either tandem or single copies into different host sites [Fig. 4d, lanes $\overrightarrow{HS2}$ (1) and $\overrightarrow{HS2}$].

In the $\overleftarrow{HS2}$-εp-CAT plasmid in which HS2 was spliced in the reverse genomic orientation, sense RNAs were also initiated from multiple sites in the HS2 enhancer and elongated past base 271 of the CAT gene. However, these long sense RNAs were complementary to the P1 probe not in the HS2 region but only in the ε-globin promoter and CAT gene regions of 471 nt (see the depictions of the P1 probe and the sense strand of the $\overleftarrow{HS2}$-εp-CAT plasmid in Fig. 4a). Therefore, the multiple, long enhancer RNAs from this plasmid protected the P1 probe only in the εp-CAT regions and generated a strong band of 471 nt (Fig. 4d, lane $\overleftarrow{HS2}$). In agreement with the RPA result obtained with the P1 probe, these sense enhancer RNAs hybridized to the P4 probe (Fig. 4c) and generated multiple P4 bands of sizes longer than 471 nt (Fig. 4f, lane $\overleftarrow{HS2}$).

In contrast to the sense RNAs, the antisense RNAs from the $\overrightarrow{HS2}$-εp-CAT plasmid were present at much lower levels. Hence, these RNAs hybridized to the sense P3 probe and generated in the autoradiogram after prolonged exposure only faint P3 bands [Fig. 4b and e, lanes $\overleftarrow{HS2}$ (1) and $\overleftarrow{HS2}$]. The prominent P3 bands generated by the $\overleftarrow{HS2}$-εp-CAT plasmid which contained HS2 spliced in the reverse orientation (Fig. 4e, lane $\overleftarrow{HS2}$) were produced apparently not by the antisense RNAs but by the sense enhancer RNAs synthesized in a direction toward the CAT gene (see the depiction of the P3 probe in Fig. 4b and of the sense strand of the $\overleftarrow{HS2}$-εp-CAT plasmid in Fig. 4a).

Taken together, the above-described results indicate that the HS2 enhancer, irrespective of its orientation relative to the CAT gene, initiated the synthesis of multiple enhancer RNAs in a direction toward the CAT gene. The HS2 enhancer, regardless of its orientation, also activated the synthesis of multiple shorter gene-tropic RNAs, including CAT mRNA, that were initiated from within the ε-globin promoter and elongated into the CAT gene. These findings suggest that either strand of the HS2 enhancer can serve as the template for the synthesis of gene-tropic RNAs.

The HS2 enhancer spliced 3′ of the CAT gene is also transcribed predominantly in a direction toward the gene. To study the effect of enhancer position on enhancer transcription, we analyzed the transcriptional status of the εp-CAT-$\overleftarrow{HS2}$ plasmid, in which the HS2 enhancer was spliced in the reverse genomic orientation in a position 3′ of the CAT gene. Note that because of the downstream position of the HS2 enhancer in the εp-CAT-$\overleftarrow{HS2}$ plasmid, gene-tropic RNAs were now synthesized in the antisense direction (Fig. 5a). Two new RPA probes were synthesized. To detect the antisense, gene-tropic RNAs transcribed in a direction from the HS2 enhancer toward the CAT gene, the sense P5 probe contained the sense strand of the HS2 sequence and also the 548-nt sense sequence between the splice and poly(A) sites 3′ of the CAT gene (Fig. 5a). To detect the sense RNAs of the same region, the antisense P6 probe contained sequences complementary to those of P5 probe (Fig. 5b).

The gene-tropic, antisense RNAs produced multiple P5 bands (Fig. 5c, lane CAT-$\overleftarrow{HS2}$ and depicted in Fig. 5a). The

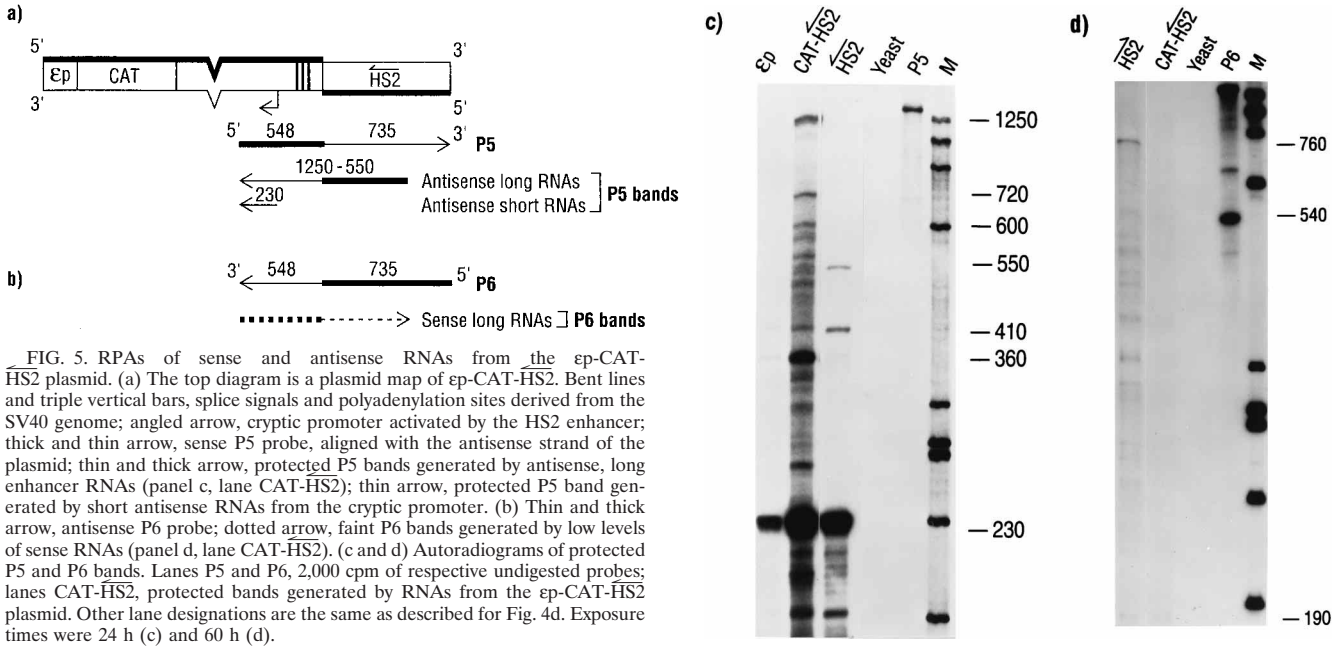

FIG. 5. RPAs of sense and antisense RNAs from the εp-CAT-HS2 plasmid. (a) The top diagram is a plasmid map of εp-CAT-HS2. Bent lines and triple vertical bars, splice signals and polyadenylation sites derived from the SV40 genome; angled arrow, cryptic promoter activated by the HS2 enhancer; thick and thin arrow, sense P5 probe, aligned with the antisense strand of the plasmid; thin and thick arrow, protected P5 bands generated by antisense, long enhancer RNAs (panel c, lane CAT-HS2); thin arrow, protected P5 band generated by short antisense RNAs from the cryptic promoter. (b) Thin and thick arrow, antisense P6 probe; dotted arrow, faint P6 bands generated by low levels of sense RNAs (panel d, lane CAT-HS2). (c and d) Autoradiograms of protected P5 and P6 bands. Lanes P5 and P6, 2,000 cpm of respective undigested probes; lanes CAT-HS2, protected bands generated by RNAs from the εp-CAT-HS2 plasmid. Other lane designations are the same as described for Fig. 4d. Exposure times were 24 h (c) and 60 h (d).

production of protected bands longer than 548 nt suggests that the long enhancer RNAs were initiated from within the HS2 enhancer and elongated in an antisense direction toward the 3′ end of the CAT gene. The strong P5 band of 230 nt was generated apparently by shorter antisense RNAs initiated from a cryptic TATA box located between the splice and poly(A) sites of the CAT gene (37a). The synthesis of the shorter antisense RNAs from the cryptic promoter was also enhanced by the HS2 enhancer, since the enhancerless εp-CAT plasmid present at an average of 300 copies per cell (Table 1) produced a much fainter band of 230 nt (Fig. 5c, lane εp).

In the εp-CAT-HS2 plasmid, sense enhancer RNAs transcribed in a direction away from the CAT gene were present at a very low level and produced only a faint P6 band of 190 nt in the autoradiogram after prolonged exposure (Fig. 5d, lane CAT-HS2). In a neighboring lane, the RNAs from the HS2-εp-CAT plasmid also produced this band and a few faint bands of longer lengths (Fig. 5d, lane HS2). The absence of strong P6 bands in sense RNAs from the HS2-εp-CAT and εp-CAT-HS2 plasmids indicated unexpectedly that even CAT mRNA from these plasmids, which was translatable into active CAT enzyme (Table 1), did not extend into the region between the splice and poly(A) sites. This unexpected premature termination of CAT mRNA was confirmed by RPAs with an RNA probe spanning the entire CAT gene. The result showed that CAT mRNA in those plasmids terminated at two major sites 120 and 215 bases before the 3′ end of CAT gene (data not shown). The truncated CAT mRNA was apparently translatable into active CAT enzyme (Table 1), as the 3′ end of the enzyme molecule does not appear to be essential for CAT enzymatic activity (43).

Regardless of the distance, orientation, and position of the HS2 enhancer relative to the CAT gene, gene-tropic enhancer RNAs are preferentially transcribed. To further study the effects of distance as well as orientation and position of the HS2 enhancer on gene-tropic enhancer transcription, the transcriptional status of the HS2 enhancer in six recombinant CAT plasmids was analyzed with two new RPA probes. In those plasmids, the HS2 enhancer was spliced in the genomic or reverse genomic orientation at a distance between 1 and 6 kb 5′ of the CAT gene or in the genomic orientation at a distance of 1 kb 3′ of the CAT gene (HS2, HS2-1.2, HS2-5.8, HS2, HS2-1.2, and CAT-HS2; see constructs 2 through 6 and 9, respectively, in Fig. 2). In order to compare the transcriptional statuses of only the HS2 enhancer in those constructs, antisense P7 and sense P8 probes were synthesized to span only the HS2 sequence (Fig. 6a).

Gene tropism of enhancer transcription, as suggested by the data presented in Fig. 4 and 5, predicted that the antisense P7 probe should produce strong protected bands with the gene-tropic sense transcripts from the HS2, HS2-1.2, and HS2-5.8 plasmids; as expected, the P7 probe produced prominent bands with these sense RNAs (Fig. 6b, top autoradiogram, left lanes [HS2, HS2-1.2, and HS2-5.8]). Gene tropism also predicted that the P7 probe should not generate prominent protected bands with the very same HS2 transcripts from the HS2 and HS2-1.2 plasmids, since these transcripts now synthesized in an antisense direction away from the CAT gene should be present at much lower levels; as expected, the P7 probe did not produce strong protected bands with such transcripts (Fig. 6b, top autoradiogram, right lanes [HS2 and HS2-1.2]). In contrast, the P8 probe, which contained an HS2 sequence complementary to that in the P7 probe, detected the transcripts synthesized in the sense direction from this latter group of HS2-containing plasmids (Fig. 6b, bottom autoradiogram, lanes HS2 and HS2-1.2); however, it did not detect the very same HS2 transcripts synthesized in the antisense direction from the former group of HS2-containing plasmids (Fig. 6b, bottom autoradiogram, lanes HS2, HS2-1.2, and HS2-5.8).

Consistent with earlier results (Fig. 4 and 5), the enhancer RNAs transcribed in a direction away from the CAT gene, however, were present and produced generally much fainter P7 bands (Fig. 6b, top autoradiogram, right lanes) or P8 bands (Fig. 6b, bottom autoradiogram, left lanes). Hence, regardless of the orientation, position, and distance of the HS2 enhancer with respect to the *cis*-linked CAT gene, the gene-tropic enhancer transcripts (depicted in Fig. 2) appeared to be preferentially transcribed or stabilized.

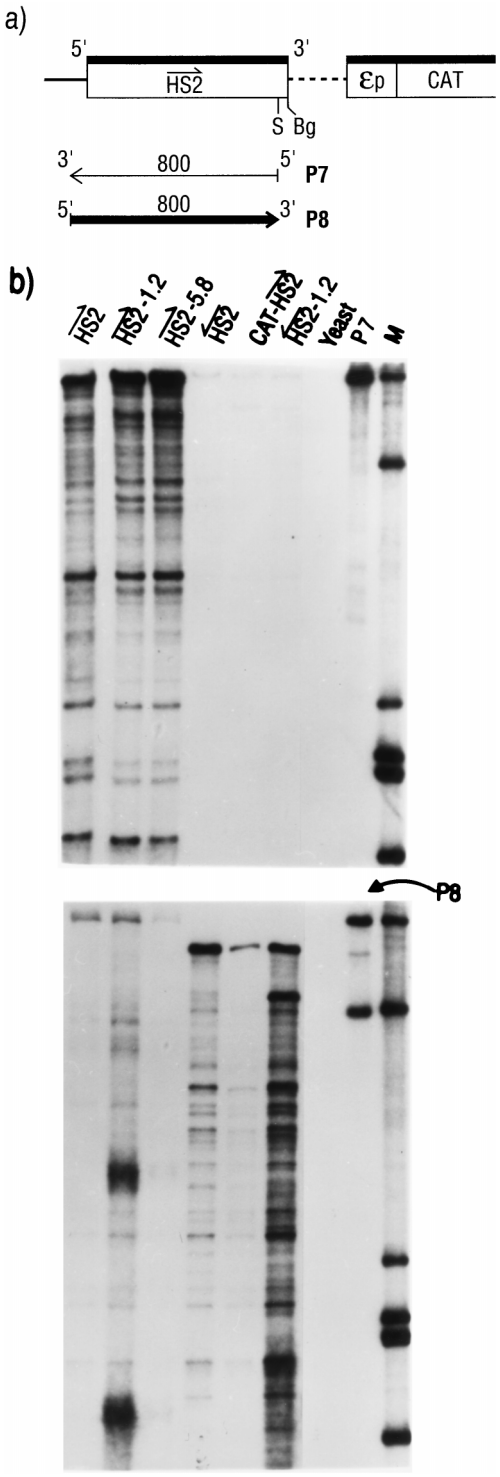

FIG. 6. RPAs of the sense and antisense RNAs of HS2 spliced at various distances from the CAT gene and in either orientation 5′ or 3′ of the gene. (a) Syntheses of the antisense P7 probe and the sense P8 probe. The top diagram shows a representative CAT construct in which HS2 could be ligated to the CAT gene in either orientation 5′ or 3′ of the gene and at various distances from the gene, as denoted by the broken line between them. The horizontal arrows represent the antisense P7 and sense P8 probes of 800 nt. S, *Stu*I; Bg, *Bgl*II. (b) Autoradiograms of protected P7 bands (top autoradiogram) and P8 bands (bottom autoradiogram). The exposure time was 24 h. M, ϕX size marker bands.

DISCUSSION

In this study we have shown that in erythroid K562 cells, which express the globin gene program, both the endogenous HS2 enhancer and the transfected HS2 enhancer in integrated plasmids were transcribed predominantly in a direction toward the *cis*-linked gene. In the K562 genome, the HS2 enhancer is located approximately 10 kb upstream of the ε-globin gene. Using RT-PCR to detect HS2 transcripts, we found that the endogenous HS2 sequence as well as 1 kb of the immediate downstream DNA was transcribed in a direction toward the ε-globin gene in erythroid K562 cells but not in nonerythroid RB cells; antisense transcripts synthesized from these regions in a direction away from the gene were not detected (Fig. 1). In K562 cells, the endogenous ε-globin promoter located 10 kb downstream of HS2 was similarly transcribed in a sense direction as shown by RPAs (Fig. 4). The intervening DNA between the HS2 enhancer and the ε-globin promoter has been reported to be also transcribed in K562 cells in a sense direction from several initiation sites located as far as 4.5 kb upstream of the ε-globin gene; these sense RNAs were elongated through the ε-globin gene at least up to the polyadenylation sites 3′ of the gene (1). The levels of these upstream RNAs varied according to the location of the initiation sites, i.e., the farther the initiation site from the gene, the lower the level of the transcripts (1). In keeping with this finding, the endogenous HS2 enhancer transcripts were present at a relatively low level and were therefore readily detectable not by RPAs but by the more sensitive RT-PCR, whereas the sense transcripts of the endogenous ε-globin promoter were present in more abundance and were readily detectable by RPAs. Similarly, because of the relative proximity of the HS2 enhancer to the *cis*-linked promoter and the CAT gene in the transfected plasmids, the enhancer RNAs of the transfected HS2 sequence were also present at higher levels than the endogenous HS2 transcripts and were therefore detectable by RPAs. Taken together, these observations suggest that the entire 10 kb of the intervening DNA between the HS2 enhancer and the ε-globin gene may be transcribed in erythroid cells in a sense direction through the promoter and the gene.

In transfected plasmids, the HS2 sequence exhibited prominent enhancer activity that was independent of its orientation, position, and distance with respect to the *cis*-linked gene, a unique functional feature shared by many eukaryotic enhancers (19). In addition, the HS2 enhancer activity was independent of whether the plasmids were integrated in tandem or single copies into the K562 genome. Our observation that single copies of the integrated HS2 sequence possess enhancer function is in agreement with similar findings in K562 cells reported by other investigators (31, 54). However, it has been reported recently that single copies of the HS2 sequence integrated into the fetal liver erythroid cells of transgenic mice did not exhibit enhancer activity (13). This apparently discordant observation may be due to the relative inactivity of the HS2 enhancer in fetal liver erythroid cells, since the HS2 site, as compared with the HS3 site, exists in a relatively inaccessible chromatin structure in fetal liver erythroid cells (17), whereas it exists in a more accessible chromatin structure in K562 cells (50, 56).

In correlation with the orientation, position, and distance independence in enhancer function, the HS2 enhancer was transcribed in a direction toward the *cis*-linked CAT gene also independent of the orientation, position, and distance of the enhancer with respect to the gene (Fig. 4 and 5). This gene-tropic enhancer transcription does not appear to be restricted only to plasmids containing the ε-globin promoter, since it has

also been observed in plasmids containing the SV40 promoter (52a), nor does it appear to be dependent on common vector sequences present in these transfected plasmids since HS2 enhancer transcription was detected also in plasmids containing a different vector backbone (6a). Gene-tropic HS2 enhancer transcription also does not appear to be dependent on common host sequences flanking the integrated plasmids, since the plasmids transfected by electroporation and the modified calcium phosphate precipitation methods apparently were integrated into different host sites as shown by the Southern blots (Fig. 3).

The gene-tropic HS2 transcripts appeared to be initiated from defined sites within the HS2 enhancer and did not appear to be the random degradation products of longer RNAs initiated from more-upstream sites in the host genome or the tandemly integrated, neighboring plasmids, since the enhancer RNAs from different plasmids generated similar patterns of P7 and P8 bands (Fig. 6b). The similar patterns of protected P7 and P8 bands also suggest that the enhancer RNAs of different plasmids were initiated from a common set of initiation sites in HS2. The possibility of the existence of a common set of initiation sites suggests that specific DNA motifs in HS2 may be capable of initiating the transcription of enhancer RNAs. Indeed, the HS2 sequence has been shown to contain DNA motifs that bind to many transcription factors such as NF-E2 and Ap-1 (33, 35, 46, 48, 49), GATA (22, 39, 49), YY1 (5, 12), and USF (4, 5, 12, 40, 49). NF-E2, AP-1, and GATA have been suggested to be capable of recruiting and assembling the transcriptional machinery (16, 53), and USF and YY1 have been reported to be capable of stimulating transcriptional initiation from their respective binding sites (25, 26, 41, 44). The presence in the enhancer complex of transcription factors intimately associated with the transcriptional machinery suggests that the HS2 enhancer complex may also constitute a transcriptional complex (6) and thus may be capable of initiating enhancer transcription from defined sites in the HS2 enhancer.

The Ap-1 and GATA motifs should potentially be capable of initiating enhancer transcription in both the sense and antisense directions, since Ap-1 binding sites show dyad symmetry (32) and the GATA motif has been found in either orientation in the promoter of many erythroid genes (16, 30, 36, 38, 57). Indeed, both strands of the HS2 enhancer were transcribed; gene-tropic enhancer RNAs, however, appeared to be preferentially synthesized or stabilized (Fig. 4 to 6). The basis of this apparent gene tropism is not known.

The correlation of orientation, position, and distance independence in enhancer function and enhancer transcription suggests that gene-tropic enhancer transcription may play a role in enhancer function. The coordinated syntheses of gene-tropic enhancer RNAs and globin mRNA in K562 cells (Fig. 1 and 4) suggest that the long enhancer RNAs may be processed to produce CAT mRNA. However, this appears to be unlikely since in the εp-CAT-$\widetilde{HS2}$ plasmid in which HS2 was placed downstream of the CAT gene, the gene-tropic, long enhancer RNAs were antisense to and not colinear with CAT mRNA (Fig. 5a). It also appears to be unlikely that the enhancer RNAs may encode other translatable proteins, as the HS2 sequence contained no long open reading frames (27) and were found mainly in the nucleus (25a). The above observations suggest that the actual enhancer RNAs may not possess relevant functional roles. However, the process of gene-tropic transcription itself appears to be important and is preserved irrespective of the orientation, position, and distance of the HS2 enhancer.

It is possible that HS2 enhancer transcription has no biological significance and is not related to HS2 enhancer function. On the other hand, previous studies of targeted replacement of HS2 and HS3 in the murine LCR (15, 21) suggest that the transcription of a foreign gene at the location of the HS2 or the HS3 site disrupts proper LCR function. In this study we have shown that the HS2 enhancer is transcribed predominantly in a direction toward the *cis*-linked gene apparently from defined DNA motifs within the HS2 sequence. Hence, a regulated transcription process at the HS2 or the HS3 site may be important to LCR and enhancer function. The process of gene-tropic enhancer transcription through the intervening DNA into the gene, either continuously to produce a long strand of RNA or in a relay fashion to produce many overlapping shorter RNA fragments, may unravel and modify nucleosomes through acetylation of histones (55) and open up the chromatin structure of a *cis*-linked gene domain. This transcription process may also deliver the transcription factors associated with the enhancer complex to the *cis*-linked promoter, where they may activate mRNA synthesis. Thus, opening up the chromatin structure of the gene domain and delivering transcription factors from the enhancer to the promoter may be two functional facets of the same underlying process of enhancer transcription. Whether gene-tropic enhancer transcription possesses these functional roles remains to be determined.

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REFERENCES

1. **Allan, M., W. G. Lanyon, and J. Paul.** 1983. Multiple origins of transcription in the 4.5 kb upstream of the ε-globin gene. Cell **35:**187–197.
2. **Baralle, F., C. Shoulder, and N. Proudfoot.** 1980. The primary structure of the human ε-globin gene. Cell **21:**621–630.
3. **Benz, E., M. J. Murnane, B. L. Tonkonow, B. W. Berman, E. M. Mazur, R. Cavallesco, T. Jenko, E. L. Snyder, B. G. Forget, and R. Hoffman.** 1980. Embryonic-fetal erythroid characteristics of a human leukemic cell line. Proc. Natl. Acad. Sci. USA **77:**3509–3513.
4. **Bresnick, E., and G. Fesenfeld.** 1993. Evidence that the transcription factor USF is a component of the human β-globin locus control region heteromeric protein complex. J. Biol. Chem. **268:**18824–18833.
5. **Caterina, J., D. Ciavatta, D. Donze, R. Behringer, and T. Townes.** 1994. Multiple elements in human β-globin locus control region 5′ HS2 are involved in enhancer activity and position-independent, transgene expression. Nucleic Acids Res. **22:**1006–1011.
6. **Cavallesco, R., and D. Tuan.** 1997. Modulatory subdomains of the HS2 enhancer differentially regulate enhancer activity in erythroid cells at different developmental stages. Blood Cells Mol. Dis. **23:**8–26.

6a. **Cavallesco, R., and D. Tuan.** Unpublished observation.

7. **Charnay, P., and T. Maniatis.** 1983. Transcriptional regulation of globin gene expression in human erythroid cell line K562. Science **220:**1281–1283.
8. **Curtin, P., M. Pirastu, Y. W. Kan, J. A. Gobert-Jones, A. D. Stephens, and H. Lehmann.** 1985. A distant gene deletion affects β-globin gene function in an atypical γδβ-thalassemia. J. Clin. Invest. **76:**1554–1558.
9. **Curtin, P., D. Liu, W. Liu, J. C. Chang, and Y. W. Kan.** 1989. Human β-globin gene expression in transgenic mice is enhanced by a distant DNase I hypersensitive site. Proc. Natl. Acad. Sci. USA **86:**7082–7086.
10. **Dean, A., T. Ley, K. Humphries, M. Fordis, and A. Schechter.** 1983. Inducible transcription of five globin genes in K562 human leukemia cells. Proc. Natl. Acad. Sci. USA **80:**5515–5519.
11. **Driscoll, K., C. S. Dobkin, and B. P. Alter.** 1989. δγβ-Thalassemia due to a *de novo* mutation deleting the 5′ β-globin gene activation region hypersensitive sites. Proc. Natl. Acad. Sci. USA **86:**7470–7474.
12. **Ellis, J., D. Talbot, N. Dillion, and F. Grosveld.** 1993. A dominant chromatin-opening activity in 5′ hypersensitive site 3 of the human β-globin locus control region. EMBO J. **12:**127–134.
13. **Ellis, J., K. Tan-Un, A. Harper, D. Michalovich, N. Yannoutsos, S. Philipsen, and F. Grosveld.** 1996. A dominant chromatin-opening activity in 5′ hypersensitive site 3 of the human β-globin locus control region. EMBO J. **15:**562–568.
14. **Enver, T., N. Raich, A. J. Ebens, T. Papayannopoulou, F. Costantini, and G.**

Stamatoyannopoulos. 1990. Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. Nature **344:**309–313.
15. **Fiering, S., E. Epner, K. Robinson, Y. Zhuang, A. Telling, M. Hu, D. Martin, T. Enver, T. Ley, and M. Groudine.** 1995. Targeted deletion of 5 HS2 of the murine β-globin LCR reveals that it is not essential for proper regulation of the β-globin locus. Genes Dev. **9:**2203–2213.
16. **Fong, T. C., and B. Emerson.** 1993. The erythroid protein cGATA-1 mediates distal enhancer activity through a specialized β-globin TATA box. Genes Dev. **6:**521–532.
17. **Forrester, W., S. Takegawa, T. Papayannopoulou, G. Stamatoyannopoulos, and M. Groudine.** 1987. Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. Nucleic Acids Res. **15:**10159–10177.
18. **Forrester, W. C., U. Novak, R. Gelinas, and M. Groudine.** 1989. Molecular analysis of the human β-globin locus activation region. Proc. Natl. Acad. Sci. USA **86:**5439–5443.
19. **Gluzman, Y., and T. Shenk.** 1983. Enhancers and eukaryotic gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Grosveld, F., G. B. Van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
21. **Hug, B., R. Wesselschmidt, S. Fiering, R. Wesselschmidt, M. Bender, E. Epner, M. Groudine, and T. Ley.** 1996. Analysis of mice containing a targeted deletion of β-globin locus control region 5′ hypersensitive site 3. Mol. Cell. Biol. **16:**2906–2912.
22. **Ikuta, T., and Y. W. Kan.** 1991. In vivo protein-DNA interactions at the β-globin gene locus. Proc. Natl. Acad. Sci. USA **88:**10188–10192.
23. **Kim, C., E. Epner, W. Forrester, and M. Groudine.** 1992. Inactivation of the human β-globin gene by targeted insertion into the β-globin locus control region. Genes Dev. **6:**928–938.
24. **Kioussis, D., E. Vanin, T. DeLange, R. Flavell, and F. G. Grosveld.** 1983. β-Globin gene inactivation by DNA translocation in γβ-thalassaemia. Nature **306:**662–666.
25. **Kirschbaum, B. J., P. Pognonec, and R. G. Roeder.** 1992. Definition of the transcriptional activation domain of recombinant 43-kilodalton USF. Mol. Cell. Biol. **12:**5094–5101.
25a. **Kong, S., et al.** Unpublished data.
26. **Lee, J. S., K. M. Galvin, and Y. Shi.** 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc. Natl. Acad. Sci. USA **90:**6145–6149.
27. **Li, Q. L., P. Powers, and O. Smithies.** 1985. Nucleotide sequence of 16-kilobase pairs of DNA 5′ to the human ε-globin gene. J. Biol. Chem. **260:** 14901–14910.
28. **Lloyd, J. A., J. M. Krakowsky, S. C. Crable, and J. B. Lingrel.** 1992. Human γ- to β-globin gene switching using a mini construct in transgenic mice. Mol. Cell. Biol. **12:**1561–1567.
29. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
30. **Mignotte, V., L. Wall, E. deBoer, F. Grosveld, and P. H. Romeo.** 1989. Two tissue-specific factors bind the erythroid promoter of the human PBGD gene. Nucleic Acids Res. **17:**37–54.
31. **Miller, J., C. Walsh, P. Ney, J. Samulski, and A. Nienhuis.** 1993. Single-copy transduction and expression of human γ-globin in K562 cells using recombinant adeno-associated virus vectors: the effect of mutations in NF-E2 and GATA-1 binding motifs within the HS2 enhancer. Blood **82:**1900–1906.
32. **Mitchell, P., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
33. **Moi, P., and Y. W. Kan.** 1990. Synergistic enhancement of globin gene expression by activator protein-1-like proteins. Proc. Natl. Acad. Sci. USA **87:**9000–9004.
34. **Morley, B. J., C. A. Abbott, J. A. Sharpe, J. Lida, P. S. Chan-Thomas, and W. G. Wood.** 1992. A single β-globin locus control region element (5′ hypersensitive site 2) is sufficient for developmental regulation of human globin genes in transgenic mice. Mol. Cell. Biol. **12:**2057–2066.
35. **Ney, P., B. Sorrentino, K. McDonagh, and A. Nienhuis.** 1990. Tandem Ap-1 binding sites within the human β-globin dominant control region function as an inducible enhancer in erythroid cells. Genes Dev. **4:**993–1006.
36. **Noguchi, T., K. Yamada, H. Inoue, T. Matsuda, and T. Tanaka.** 1987. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. J. Biol. Chem. **262:**14316–14321.
37. **Orkin, S.** 1990. Globin gene regulation and switching: circa 1990. Cell **63:** 665–672.
37a. **Promega.** 1989. Technical bulletin 80. CAT reporter system. Promega, Madison, Wis.
38. **Rahuel, C., A. Vignal, J. London, S. Hamel, P. H. Romeo, Y. Colin, and J. P. Cartron.** 1989. Structure of the 5′ flanking region of the gene encoding human glycophorin A and analysis of its multiple transcripts. Gene **85:**471–477.
39. **Reddy, P. M. S., and C. K. J. Shen.** 1991. Protein-DNA interactions in vivo of an erythroid-specific, human β-globin locus enhancer. Proc. Natl. Acad. Sci. USA **88:**8676–8680.
40. **Reddy, P. M. S., G. Stamatoyannopoulos, T. Papayannopoulou, and C. K. Shen.** 1994. Genomic footprinting and sequencing of human β-globin locus. J. Biol. Chem. **269:**8287–8295.
41. **Roy, A., M. Meisterernst, P. Pogonec, and R. Roeder.** 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature **354:**245–248.
42. **Ryan, T. M., R. R. Behringer, N. C. Martin, T. M. Townes, R. D. Palmiter, and R. L. Brinster.** 1989. A single erythroid-specific DNase I super-hypersensitive site activates high levels of human β-globin gene expression in transgenic mice. Genes Dev. **3:**314–323.
43. **Shaw, W. V., L. C. Packman, B. D. Burleigh, A. Dell, H. R. Morris, and B. S. Hartley.** 1979. Primary structure of a chloramphenicol acetyltransferase. Nature **282:**870–872.
44. **Shi, Y., E. Seto, L. S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-kruppel-related protein and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
45. **Shih, D., R. Wall, and S. Shapiro.** 1993. A 5′ control region of the human ε-globin gene is sufficient for embryonic specificity in transgenic mice. J. Biol. Chem. **268:**3066–3071.
46. **Solomon, W., C. H. Lin, J. Palma, X. Y. Gao, and S. Wu.** 1993. Suppression of a cellular differentiation program by phorbol esters coincides with inhibition of binding of a cell-specific transcription factor (NF-E2) to an enhancer required for expression of an erythroid-specific gene. J. Biol. Chem. **268:**5089–5096.
47. **Sorrentino, B., P. Ney, D. Bodine, and A. Nienhuis.** 1990. A 46 base pair enhancer sequence within the locus activating region is required for induced expression of the γ-globin gene during erythroid differentiation. Nucleic Acids Res. **18:**2721–2731.
48. **Talbot, D., S. Philipsen, P. Fraser, and F. Grosveld.** 1990. Detailed analysis of the site 3 region of the human β-globin dominant control region. EMBO J. **9:**2169–2178.
49. **Talbot, D., and F. Grosveld.** 1991. The 5′ HS2 of the globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. EMBO J. **10:**1391–1398.
50. **Tuan, D., W. Solomon, Q. Li, and L. M. London.** 1985. The β-like globin gene domain in human erythroid cells. Proc. Natl. Acad. Sci. USA **82:**6384–6388.
51. **Tuan, D., A. Abeliovich, M. Lee-Oldham, and D. Lee.** 1987. Identification of regulatory elements of human β-like globin genes, p. 211–220. *In* G. Stamatoyannopoulos and A. Nienhuis (ed.), Developmental control of globin gene expression. Alan R. Liss, Inc., New York, N.Y.
52. **Tuan, D. Y. H., W. B. Solomon, I. M. London, and D. P. Lee.** 1989. An erythroid-specific, developmental-stage-independent enhancer far upstream of the human "β-like globin" genes. Proc. Natl. Acad. Sci. USA **86:**2554–2558.
52a. **Tuan, D., W. Solomon, R. Cavallesco, G. Huang, and I. London.** 1989. Characterization of a human globin enhancer element, p. 63–72. *In* G. Stamatoyannopoulos and A. Nienhuis (ed.), Hemoglobin switching. Part A. Transcriptional regulation. Alan R. Liss, Inc., New York, N.Y.
53. **Tuan, D., S. Kong, and K. Hu.** 1992. Transcription of the hypersensitive HS2 enhancer in erythroid cells. Proc. Natl. Acad. Sci. USA **89:**11219–11223.
54. **Walters, M., W. Magis, S. Fiering, J. Eidemiller, D. Scalzo, M. Groudine, and D. Martin.** 1995. Transcriptional enhancers act in cis to suppress position-effect variegation. Genes Dev. **10:**185–195.
55. **Wang, L., C. Mizzen, C. Ying, R. Candau, N. Barlev, J. Brownell, D. C. Allis, and S. L. Berger.** 1997. Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. Mol. Cell. Biol. **17:**519–527.
56. **Yoo, J., L. Herman, C. Li, S. Krantz, and D. Tuan.** 1996. Dynamic changes in the locus control region of erythroid progenitor cells demonstrated by polymerase chain reaction. Blood **87:**2558–2567.
57. **Youssoufian, H., L. I. Zon, S. Orkin, A. D. D'Andrea, and H. F. Lodish.** 1990. Structure and transcription of the mouse erythropoietin receptor gene. Mol. Cell. Biol. **10:**3675–3682.