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Transcription of the hypersensitive site HS2 enhancer in erythroid cells

(enhancer function/long enhancer transcripts/Ap1 sites/RNA protection assays)

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ABSTRACT **In the human genome, the erythroid-specific hypersensitive site HS2 enhancer regulates the transcription of the downstream β-like globin genes 10–50 kilobases away. The mechanism of HS2 enhancer function is not known. The present study employs RNA protection assays to analyze the transcriptional status of the HS2 enhancer in transfected recombinant chloramphenicol acetyltransferase (CAT) plasmids. In erythroid K562 cells in which the HS2 enhancer is active, the HS2 sequence directs the synthesis of long enhancer transcripts that are initiated apparently from within the enhancer and elongated through the intervening DNA into the cis-linked CAT gene. In nonerythroid HL-60 cells in which the HS2 enhancer is inactive, long enhancer transcripts are not detectable. Splitting the HS2 enhancer between two tandem Ap1 sites abolishes the synthesis of a group of long enhancer transcripts and results in loss of enhancer function and transcriptional silencing of the cis-linked CAT gene. In directing the synthesis of RNA through the intervening DNA and the gene by a tracking and transcription mechanism, the HS2 enhancer may (*i*) open up the chromatin structure of a gene domain and (*ii*) deliver enhancer binding proteins to the promoter sequence where they may stimulate the transcription of the gene at the cap site.**

In seeking to identify the cis regulatory elements of the human β-like globin genes (5′-ε-^{G}γ-^{A}γ-δ-β-3′), this laboratory (1) and others (2, 3) have mapped four erythroid-specific and developmentally stable DNase I-hypersensitive sites HS1, -2, -3, and -4 in the locus control region (LCR) between 50 and 70 kilobases (kb) upstream of the β-globin gene. HS2 at −11 kb 5′ of the embryonic ε- and thus at −54 kb 5′ of the β-globin gene has been shown to possess an erythroid-specific and developmentally stable enhancer function (4–6). It is capable of stimulating the transcription of embryonic ε-, fetal γ-, and adult β-globin genes in erythroid cells (7–10). In γδβ-thalassemia, the deletion of HS2 and more upstream DNA is associated with transcriptional silencing of the far downstream β-globin gene (11–13) and possibly of the whole β-like globin gene domain.

How the HS2 enhancer cooperates with distant globin promoter sequences to stimulate the ε-, γ-, and β-globin genes at the respective developmental stage is not clear. Looping (14) and tracking (15) mechanisms have been proposed to explain how a distant enhancer may communicate with the promoter of a cis-linked gene (14, 16, 17). In the present study, the detection of HS2 enhancer transcripts in erythroid cells suggests that the tandem Ap1 sites and other sequence motifs in the enhancer may provide entry sites for transcription factors associated with the transcriptional machinery that track the DNA and synthesize long strands of RNA through the intervening DNA into the cis-linked gene. The possible biological significance of such long transcripts in enhancer function will be discussed.

MATERIALS AND METHODS

Construction of Recombinant Constructs. HS2-εP-CAT and εP-CAT have been described (5). 5′-HS2-εP-CAT was made by triple ligation of the following fragments: the 180-base-pair (bp) *Bam*HI–*Bsp*HI of HS2 with the *Bsp*HI end-blunted, the 202-bp *Bam*HI–*Hin*dIII of ε-globin promoter (5) with the *Bam*HI end-blunted, and a 5.5-kb *Bgl* II–*Hin*dIII vector fragment excised from pA10CAT2 (18). The 3′-HS2-εP-CAT was made by splicing the 520-bp *Bsp*HI (blunted)–*Stu* I of HS2 into HS2-εp-CAT that had been digested with *Sal* I (blunted) and *Stu* I, which removed all HS2 but the 40 bp between the *Stu* I and *Bgl* II sites at the 3′ end.

Creation of K562 and HL-60 Cell Lines and Chloramphenicol Acetyltransferase (CAT) Assays. Each recombinant CAT plasmid at 10 μg and pcDneo (19) at 0.4 μg were introduced into 10^7 K562 or HL-60 cells by calcium phosphate precipitation or electroporation as described (5). G418 at 400 μg/ml was added to the culture medium 24 hr after glycerol shock. Individual pure clones were picked and expanded; the remaining colonies on the dish were expanded for pooled clones. The CAT assays were carried out as described (5).

Purification of RNA. RNA was isolated (20) from 2–4 × 10^7 freshly harvested cells grown for 48 hr in medium with 20 μM hemin. After centrifugation, the RNA pellet was resuspended in an appropriate buffer and digested with RNase-free DNase I (300 units/ml) (from Boehringer) for 15 min at 37°C and then with proteinase K (100 μg/ml) for 15 min at 37°C. The RNA was extracted twice with phenol/chloroform, precipitated in ethanol, and stored at −80°C.

RNA Protection Assays. (*i*) The pGem-HS2-εP-CAT construct for synthesizing P1-P3 probes: The template DNA excised from HS2-εP-CAT by double digestion with *Sal* I, cleaving 5′ of the HS2 sequence, and *Eco*RI, cleaving at base 271 of the CAT gene, was spliced into pGem-3 vector (Promega) between the *Sal* I and *Eco*RI sites in the polylinker. P1 probe was synthesized by T7 polymerase from *Sal* I-linearized template plasmid, P3 probe was synthesized by Sp6 from an *Eco*RI-linearized template plasmid, and P2 probe was synthesized by T7 polymerase from the template construct linearized with *Bam*HI, which cleaves between HS2 and the ε-globin promoter. (*ii*) The protection assays were carried out as described (21). RNA probe (2–4 × 10^6 cpm) and RNA (30–50 μg) were hybridized at 53°C for 16 hr. The reaction mixture was digested for 30 min at 25°C (or 30–37°C) with RNase at 25 (or 35–40) μg/ml and RNase T1 at 2 μg/ml. *Hae* III-cut φX DNA or interleukin 1 sequencing ladders served as size markers in gel electrophoresis. Undigested

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Abbreviations: CAT, chloramphenicol acetyltransferase; HS, hypersensitive site; LCR, locus control region; nt, nucleotide(s).

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probes (2000 cpm) were also loaded into individual lanes. For quantitative analysis, the bands generated by the CAT mRNA [291 nucleotides (nt)] and upstream transcripts of the endogenous ε-globin gene (200 nt) were excised from the gel and radioactivity was measured. The latter served as the internal reference for calculating relative CAT mRNA levels.

RESULTS

HS2 Enhancer Activity in K562 and HL-60 Cells. In erythroid K562 cells, the HS2 enhancer in integrated HS2-εP-CAT plasmids (Fig. 1) activated synthesis of CAT mRNA and CAT enzyme by 600- to 3500-fold over those produced by the enhancerless εP-CAT plasmids (Table 1). In nonerythroid HL-60 cells, the HS2 enhancer did not activate the synthesis of either CAT mRNA or CAT enzyme.

In K562 Cells, the HS2 Enhancer Directs the Synthesis of Long Enhancer Transcripts into the CAT Gene. To analyze the sense transcripts of the integrated HS2-εp-CAT plasmid, two antisense RNA probes were synthesized: a full-length P1 probe of 1300 nt and a shorter P2 probe that spanned essentially the 471 nt at the 5′ end of P1 probe (Fig. 2*a*). Both probes share common 5′ ends corresponding to base 271 of the CAT gene. To analyze the antisense transcripts of the integrated plasmid, a sense P3 probe of 1300 nt was synthesized.

Five pure clones and one mixed clone of HS2-εp-CAT-transformed K562 cells, containing an average number of 5–10 integrated plasmids per cell (Southern blots not shown), were studied. The P1-protected bands are presented in Fig. 3*a* and analyzed in Fig. 2*b*. In each of the HS2-containing clones, the CAT mRNA initiated at the proper cap site (22) produced a prominent protected band of 291 nt (Figs. 2*b* and 3*a*). In contrast, the enhancerless εP-CAT plasmid integrated at an average copy number of 300 per cell (Table 1) produced only a very faint band of 291 nt (Fig. 3*a*). The intense band of 200 nt, detected in all the clones (Fig. 3*a*), was produced between the 200 nt of ε-globin promoter sequence in the P1 probe and the upstream transcripts of the endogenous ε-globin gene in K562 cells (Fig. 2*b*) (23). In the HS2-εP-CAT-transformed clones, additional bands of longer lengths were detected. The two prominent bands of 435 and 425 nt were generated by upstream transcripts initiated from within the ε-globin promoter. The 471-nt band may be an artifact as it was not consistently detected in all the HS2 clones nor in the same pooled clones of εP-CAT (Fig. 3 *a* and *b*). The bands longer than 471 nt, including the five bands of 1030–750 nt at the top of the gel lanes, were generated by long transcripts that were initiated apparently from within the HS2 enhancer and extended through the ε-globin promoter into the CAT gene (Fig. 2*b*). As most of the long enhancer transcripts extended beyond base 271 of the CAT gene (data using a CAT gene probe not shown), the P1-protected segments of the long transcripts should all share common 3′ ends at base 271 of the CAT gene after RNase digestion. By using this common 3′ end as reference, the 5′ ends of the long transcripts mapped

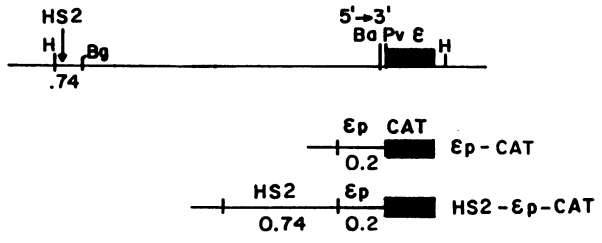

FIG. 1. Recombinant CAT constructs. The globin gene locus upstream of the ε-globin gene (solid box). H, *Hin*dIII; Bg, *Bgl* II; Ba, *Bam*HI; Pv, *Pvu* II. Vertical arrow, DNase I hypersensitive site HS2 (1); εp, the 0.2-kb ε-globin promoter between Ba and Pv; HS2, the 0.74-kb DNA between H and Bg.

Table 1. HS2 enhancer activity measured by CAT enzymatic and RNA protection assays

Plamid	Plasmids, no. per cell	Relative CAT enzymatic activity	Relative CAT mRNA
K562			
εp-CAT	300	1	1
HS2	2	3500 ± 330	1020 ± 360
(HS2)	3	2000	600
5′HS2	20	75	48
3′HS2	6	367	40
HL-60			
(εp-CAT)	20	1	0
(HS2)	4	1.2	0

Recombinant CAT plasmids (Figs. 1 and 4*a*) were stably integrated into K562 or HL-60 cells. Plasmids in parentheses were introduced into cells by electroporation; others were introduced by calcium phosphate precipitation. The average number of copies per cell of integrated plasmids in pooled clones is shown. The relative CAT enzymatic activities and CAT mRNA levels per copy of integrated plasmids are shown; respective values of εp-CAT were used as the standard of comparison.

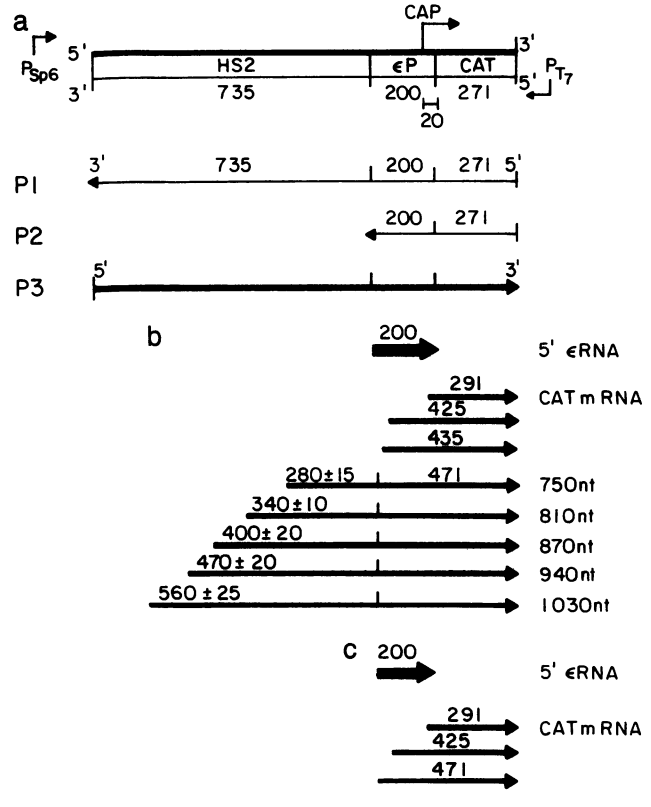

FIG. 2. Analysis of enhancer transcripts in K562 cells. (*a*) Template construct for synthesis of RNA probes. Thick or thin horizontal lines, the sense or antisense strands, respectively, of DNA and RNA; numbers, sizes in bp or nt of the DNA or RNA fragments; CAP, the cap site of CAT mRNA 20 bp into the 3′ end of the ε promoter; small angled arrows above and below the Sp6 and T7 promoters, the direction of synthesis of the RNA probes. (*b*) Analysis of P1-protected sense transcripts. Horizontal arrows, probe-protected sense transcripts generated from 5′ εRNA, the upstream transcripts of the endogenous ε-globin gene, CAT mRNA, and long enhancer transcripts; numbers to the right, average lengths in nt of the protected enhancer transcripts determined from three to six separate experiments; short vertical bars, junction between the enhancer and the promoter that are vertically aligned with those in *a*. (*c*) Analysis of P2-protected sense transcripts.

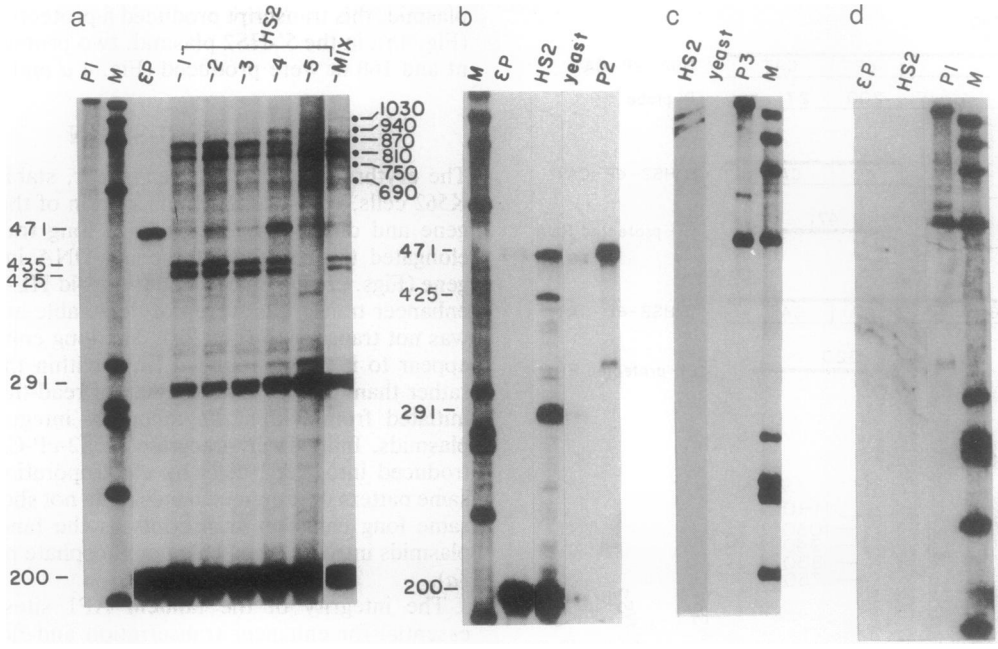

FIG. 3. RNA protection assays of sense and antisense CAT RNAs from K562 and HL-60 cells. (*a*) P1-protected sense transcripts in K562 RNAs. Lanes: εP, RNA isolated from pooled clones transformed by εp-CAT; HS2, 1–5, five pure clones transformed by HS2-εp-CAT; Mix, a mixed population of 10 pure clones transformed by HS2-εP-CAT; P1, undigested P1 probe. Numbers mark sizes in nt of protected bands. Lane M contains *Hae* III-digested φX size markers. Exposure time, 12 hr. (*b*) P2-protected sense transcripts in K562 RNAs. Lanes: εP, a different preparation of RNA from the same pooled clones of εP-CAT used in lane εP of *a*; Yeast, yeast t-RNA. P2, undigested P2 probe; HS2, RNA from pooled clones transformed by HS2-εP-CAT; M, as in *a*. Exposure time, 36 hr. (*c*) P3-protected anti-sense transcripts in K562 cells. The top band in lane P3 is the full-length P3 probe; smaller bands are prematurely terminated P3 probe. Lanes: HS2, yeast, and M, as in *b*. Exposure times: HS2 lane, 52 hr; other lanes, 28 hr. (*d*) P1-protected transcripts in HL-60 cells. Lanes: εP and HS2, RNA from pooled clones of εP-CAT- and HS2-εP-CAT-transformed HL-60 cells, respectively; P1 and M, as in *a*. Exposure time, 60 hr.

within the HS2 enhancer at sites 560, 470, 400, 340, and 280 nt from the 3′ border of the HS2 sequence (Fig. 2*b*).

Since the P1 probe was labeled homogenously throughout its length and not merely at the 5′ end, the P1-protected bands of 1030–750 nt might also be generated from transcripts initiated from sites more upstream of the above mapped sites and terminated prematurely upstream of base 271 of the CAT gene. In this case, the shorter P2 probe spanning only the extreme 5′ 471 nt of P1 probe (Fig. 2*a*) would hybridize to those prematurely terminated transcripts with heterogenous 3′ ends and generate a new ladder of protected bands shorter than 471 nt. On the other hand, if the long transcripts were initiated from the various mapped sites in the enhancer and extended beyond base 271 of the CAT gene, the P2 probe would hybridize to the 3′ part of this array of long transcripts and produce a single prominent band of 471 nt (see Fig. 2 *a–c* for alignment of P2 probe with respect to the enhancer transcripts). The detection of a prominent P2-protected band of 471 nt (Fig. 3*b*) provides support for the latter analysis. The prominent ladder of bands of 425, 291, and 200 nt was detected by both the short P2 probe (Fig. 3*b*) and the long P1 probe (Fig. 3*a*). They were produced not by long enhancer transcripts but, respectively, by shorter transcripts initiated in the ε-globin promoter, CAT mRNA, and upstream transcripts of the endogenous ε-globin gene. Likewise, the many faint bands present in the HS2 lanes of Fig. 3 *a* and *b* were not derived from prematurely terminated upstream transcripts.

The electroporation method was also used to introduce recombinant plasmids into K562 cells. The patterns of P1-protected bands were similar to those shown in Fig. 3*a* (data not shown).

To minimize clonal variations in HS2 enhancer transcription and function caused by a different assortment of host regulatory elements in the vicinity of the clonal integration sites, the current study except for data presented in Fig. 3*a* was carried out using pooled clones containing transfecting plasmids randomly integrated into multiple host chromosomal sites.

The HS2 enhancer in pooled clones of HS2-εP-CAT-transformed K562 cells did not direct the synthesis of detectable levels of antisense transcripts, as the sense P3 probe did not produce clearly discernible bands (Fig. 3*c*). In the nonerythroid HL-60 cells, the HS2-εP-CAT plasmid integrated at an average copy number of 4–20 per cell (Table 1) generated neither the 1030- to 750-nt bands produced by the long enhancer transcripts nor the 291-nt band produced by the CAT mRNA (Fig. 3*d*).

Tandem Ap1 Sites in HS2 Enhancer Transcription and Function. Within the HS2 enhancer, the 26-bp sequence TGCTGAGTCATGATGAGTCATGCTGA encompassing two tandem Ap1 sites (underlined) (24) is located at the 5′ end of the HS2 enhancer and has been shown to be essential for enhancer function (R. Cavallesco and D.T., unpublished observation) (10, 25, 26). Cleaving the 735-bp HS2 enhancer between the two tandem Ap1 sites (Fig. 4*a*) caused a precipitous drop in enhancer transcription and function. The 5′-HS2- and the 3′-HS2-εP-CAT plasmids, integrated at average copy numbers 3–10 times that of the parental HS2 plasmids (Table 1), produced very faint bands of 291 nt (Fig. 4*b*) and accordingly an amount of CAT mRNA ≈20-fold lower than the parental plasmid (Table 1). In agreement, the CAT enzymatic activities were 10- to 50-fold lower (Fig. 4*c* and Table 1).

Associated with the drastic drop in enhancer activity, the 3′-HS2 plasmid produced none of the major P1-protected bands of 1030–750 nt that were observed with the parental HS2 plasmid (Fig. 4*b*), even though the 560-bp 3′ subfragment contains all the potential transcriptional initiation sites for these transcripts (Fig. 4*a*). The prominent new band of 520 nt (Fig. 4*b*) was apparently generated by an enhancer transcript initiated at a new site 50 nt upstream of the *Bgl* II site

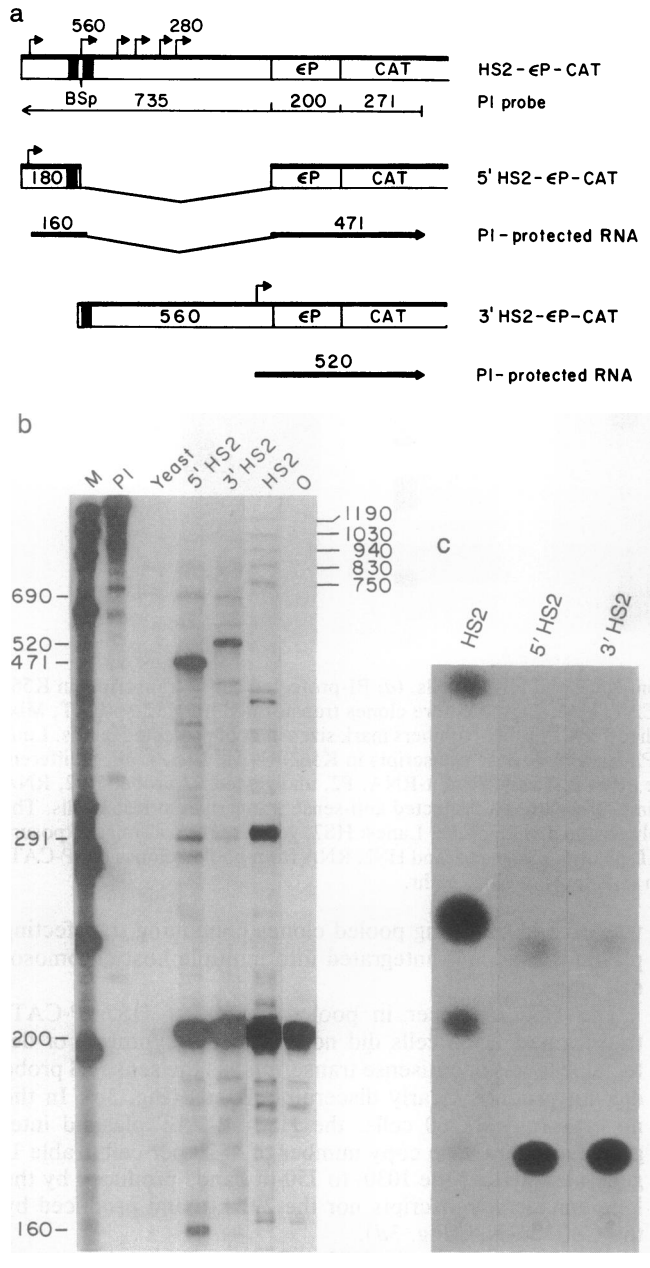

FIG. 4. Contiguity of tandem Ap1 sites in enhancer transcription and function. (*a*) Transcription of recombinant plasmids containing the 5′ or 3′ split enhancer. The parental HS2-εP-CAT plasmid is shown at the top. Double solid boxes, the two tandem Ap1 sites; Bsp, *Bsp*HI site; 5′-HS2-εp-CAT or 3′-HS2-εP-CAT, the 5′ or 3′ split enhancer containing the single Ap1 site (solid box) linked to εP and the CAT gene; bent line in 5′-HS2-εP-CAT, absence of the 560-nt 3′ sequence; heavy horizontal arrows or lines, P1-protected transcripts from the 5′ or 3′ split enhancer; numbers, sizes in bp or nt; angled arrows, apparent initiation sites of enhancer transcripts; numbers above the arrows, locations of the initiation sites relative to the Bg site at the 3′ border of HS2. (*b*) P1-protected transcripts. Lanes: 5′-HS2, 3′-HS2, and HS2, RNA isolated from 5′-HS2-εP-CAT-, 3′-HS2-εP-CAT-, and the parental HS2-εP-CAT-transformed K562 cells; 0, RNA from nontransfected K562 cells; M, as in Fig. 3*b*; P1 and yeast, as in Fig. 3 *a* and *b*. Exposure times: HS2 and 0, 11 hr; other lanes, 20 hr. (*c*) CAT assays for HS2, 5′-HS2, and 3′-HS2.

at the 3′ border of HS2 (Fig. 4*a*). The 690-nt band, produced by both the 5′- and 3′-HS2 plasmids and very faintly by yeast RNA but not reproducibly by the parental HS2 plasmid (Figs. 3*a* and 4*b*) might be generated in part by undigested probe. In both the parental and the 5′-HS2 plasmids, an apparent transcriptional initiation site is located 20 nt downstream of the *Hin*dIII site at the 5′ border of HS2. In the parental HS2 plasmid, this transcript produced a protected band of 1190 nt (Fig. 4*b*); in the 5′-HS2 plasmid, two protected bands of 471 nt and 160 nt were produced (Fig. 4 *a* and *b*).

DISCUSSION

The erythroid-specific HS2 enhancer, stably integrated into K562 cells, activates the transcription of the cis-linked CAT gene and directs the synthesis of long transcripts that are elongated through the intervening DNA into the cis-linked gene (Figs. 2*b* and 3*a*). In nonerythroid HL-60 cells, the long enhancer transcripts were not detectable and the CAT gene was not transcribed (Fig. 3*d*). The long enhancer transcripts appear to be RNA initiated from within the HS2 enhancer rather than degradation products of read-through transcripts initiated from within the tandemly integrated neighboring plasmids. Individually integrated HS2-εP-CAT plasmids, introduced into K562 cells by electroporation, produced the same pattern of protected bands (data not shown) and thus the same long enhancer transcripts as the tandemly integrated plasmids introduced by calcium phosphate precipitation (Fig. 3*a*).

The integrity of the tandem AP1 sites appears to be essential for enhancer transcription and function. Splitting the enhancer between the AP1 sites results in disappearance of the multiple enhancer-initiated long transcripts and loss of enhancer function (Fig. 4 and Table 1). The tandem AP1 sites may drive enhancer function by communicating with the far downstream promoter through synthesis of long enhancer transcripts by a tracking and transcription mechanism. The tandem Ap1 sites bind to both ubiquitous Ap1 factors (24) and erythroid-specific transcription factor NFE-2 (27). The Ap1 site also shares homology with the binding motifs of the yeast transcription factor GCN4 (28). The GCN4 binding motifs possess TATA box function and are capable of initiating the transcription of mRNA (29). The Ap1 sites in the HS2 enhancer may thus also bind to mammalian transcription factors similar to GCN4 and initiate the transcription of the long enhancer transcripts.

One possible functional role of the gene-tropic long enhancer transcripts may be to open up the chromatin structure of a cis-linked gene domain. The β-like globin gene domain of erythroid cells has been shown to possess overall DNase I hypersensitivity (30, 31) and exists in an open and accessible chromatin structure. The detection of long enhancer transcripts in the present study suggests that the HS2 enhancer in the LCR may unravel and open up the chromatin structure of the β-like globin gene domain by directing synthesis of long strands of RNA through the DNA duplex of the enhancer, intervening DNA, and β-like globin genes. In support of this functional role of the HS2 enhancer, the endogenous HS2 sequence of K562 cells has been found to be transcribed in low abundance and also in a direction toward the β-like globin genes (RNA-PCR data not shown). Moreover, deletion of HS2 and more upstream DNA has been reported to cause the chromatin structure of the whole β-like globin gene domain to revert to a closed form and the far downstream β-globin gene to be transcriptionally inaccessible and inactive (11–13, 32).

A second possible function of the enhancer transcripts may be to deliver enhancer binding proteins to the promoter where these proteins may interact with promoter binding proteins to stimulate transcription of mRNA at the cap site. Thus, when synthesis of the long enhancer transcripts is blocked, cap transcription of mRNA is suppressed (Fig. 4). As synthesis of enhancer transcripts initiated from the split enhancers outside of the region between bases 560 and 280 in HS2 does not appreciably activate the CAT gene (Fig. 4), it is possible that only the initiation complexes containing transcription factors that bind to sequence motifs within this

region of HS2, when delivered to the promoter, can interact fruitfully with the promoter binding proteins.

The tandem Ap1 sites may drive enhancer function also by a looping mechanism. In transgenic animals, an apparent competition between the γ- and the β-globin transgene for binding to regulatory elements in the LCR (33) has been interpreted to result from alternative loop formation (34) between the γ- or β-globin promoter and the LCR. As Ap1 factors can bend the target site (35), it is conceivable that the HS2 enhancer may bend at the tandem Ap1 sites to enable more upstream DNA to loop back and interact with the far downstream globin promoters. The long enhancer transcripts in this case may then be merely incorrectly initiated upstream transcripts without any biological function. So far, direct evidence for loop formation between the enhancer and the promoter sequences is provided by the prokaryotic and viral systems (36, 37). The looping model of enhancer function predicts that transcriptional terminators, which are spliced into the loop region to block tracking or transcription through the loop, should not interfere with enhancer–promoter interaction and, therefore, enhancer-directed activation of mRNA transcription. Transcriptional terminators or psoralen photoadducts inserted between an upstream yeast or viral enhancer and the cap site have, however, been reported to block mRNA synthesis (38, 39). Furthermore, Herendeen *et al.* (40) reported that a viral T4 enhancer element functions primarily by a tracking mechanism.

We speculate that the tracking and transcription mechanism of HS2 enhancer function and the looping mechanism may not be mutually exclusive. The transcriptional machinery assembled at the tandem Ap1 sites in the HS2 enhancer, presumably contains the Ap1 factors and may bind tightly to the enhancer, since Ap1 factors have been reported to grip the target site by a scissors-grip mechanism (41). As this transcriptional machinery tracks and transcribes the DNA, it may bend the enhancer onto the DNA being transcribed and bring the tightly bound enhancer with it. This may then form, as the transcription process proceeds, an increasingly enlarging loop between the enhancer and the DNA bases being transcribed and finally between the enhancer and the promoter sequences. In DNA replication, such dynamic loop formation by a tracking mechanism has been suggested to exist between the ends of the lagging strand and the DNA at the advancing replication fork (42). Thus, a tracking and transcription mechanism may establish the initial contacts between the HS2 enhancer and the promoter sequences and between their respective binding proteins for possible subsequent loop formation between these elements.

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