The HS2 enhancer in the β-globin locus control region regulates transcription of the globin genes 10–50 kb away. How the HS2 enhancer acts over this distance is not clearly understood. Earlier studies show that in erythroid cells the HS2 enhancer initiates synthesis of intergenic RNAs from sites within and downstream of the enhancer, and the enhancer-initiated RNAs are transcribed through the intervening DNA into the cis-linked promoter and gene. To investigate the functional significance of the enhancer-initiated transcription, here we inserted the lac operator sequence in the intervening DNA between the HS2 enhancer and the e-globin promoter in reporter plasmids and integrated the plasmids into erythroid K562 cells expressing the lac repressor protein. We found that the interposed lac operator/repressor complex blocked the elongation of enhancer-initiated transcription through the intervening DNA and drastically reduced HS2 enhancer function as measured by the level of mRNA synthesized from the e-globin promoter. The results indicate that the tracking and transcription mechanism of the HS2 enhancer-assembled transcriptional machinery from the enhancer through the intervening DNA into the cis-linked promoter can mediate enhancer-promoter interaction over a long distance.

The locus control region (LCR) of the human β-globin gene domain, defined by four erythroid specific DNase I hypersensitive sites (HS1, -2, -3, and -4) and a ubiquitous HS5 site (1–3), regulates transcription of the far downstream embryonic e-globin, fetal Gγ-globin and Aγ-globin, and the adult δ-globin and β-globin genes during erythroid cell differentiation. It is not fully understood whether the LCR acts over the long distance by a looping mechanism in which the LCR complex (the LCR DNA and its associated transcription factors) loops over the intervening DNA to directly interact with the globin promoters or by a tracking mechanism in which the LCR complex or its protein components track along the intervening DNA to reach and activate the downstream promoters (4–7).

In the LCR, the HS2 site located 11 and 55 kb 5′, respectively, of the e- and β-globin genes possesses strong enhancer activity (8) and regulates transcription of the β-like globin genes over a long distance (9, 10). In the endogenous genome of erythroid cells and in recombinant constructs transfected into erythroid cells, the HS2 enhancer recruits erythroid and general transcription factors (11–13) in the assembly of a pol II transcription complex that synthesizes intergenic RNAs from sites within the enhancer in the direction of the downstream gene (14–17). In plasmids integrated into erythroid cells, this genetropic HS2 enhancer transcription, like HS2 enhancer function, is detected regardless of the orientation, position, and distance of the enhancer relative to the cis-linked promoter and gene (15). Moreover, splitting the HS2 enhancer at the enhancer core, thereby preventing assembly of the intact enhancer complex, diminishes both enhancer transcription at the enhancer site and mRNA synthesis at the promoter site (14). These findings suggest that the enhancer-initiated transcription plays a role in mediating enhancer function.

To investigate the contribution of a transcription mechanism to HS2 enhancer function, here we interrupted the enhancer-initiated transcription process with a transcriptional terminator and studied the subsequent effects on enhancer function. We chose the lac operator/repressor (lacO/R) complex of the bacterial lac operon (18) to serve as the transcriptional terminator for this study because of the following considerations: (i) The Lac repressor protein (lacR) binds to the lac operator sequence (lacO) with extremely high specificity and affinity (19). (ii) The lacR bound at the lacO sequence in the lac operon interacts with RNA polymerase and blocks transcriptional elongation without inhibiting transcriptional initiation (20). (iii) The LacR protein can recognize and bind with high affinity to the lacO sequence packaged into nucleosomes (21). (iv) In recombinant plasmids integrated into the eukaryotic genomes, the lacO/R complex blocks mRNA elongation both when it is inserted near the promoter (22, 23) and when it is inserted in the intron of a gene far downstream of the promoter (24). (v) Repression by lacO/R complex in eukaryotic cells can be reversed by dissociating the complex with IPTG (22–24).

We constructed the HS2-lac-1.2-ep-CAT-lac test plasmid, which contained the lacO sequence inserted between the HS2 enhancer and the 1.2-kb DNA located naturally downstream of the HS2 enhancer in the genome (see Fig. 2A), the e-globin promoter, and the bacterial chloramphenical acetyl transferase (CAT) reporter gene. To more closely mimic the endogenous globin gene locus, we also constructed the HS2-lac-1.2–5.5-ep-GFP test plasmid, which contained an additional 5.5 kb of intervening DNA that is contiguous with the e-globin promoter in the genome and the green fluorescent protein (GFP) reporter gene. The test plasmids and the respective reference plasmids that did not contain the interposed lacO sequence were inte-
grated into an erythroid K562 cell line that expressed a steady level of the lacR protein. The effects of the interposed lacO/R complex on synthesis of the enhancer-initiated, intergenic RNAs and of the CAT or GFP mRNA were determined by RT-PCR, RNase protection assay (RPA), and Northern blot. The levels of CAT and GFP proteins were determined by CAT enzymatic assays and FACS analysis of the green fluorescence emitted by GFP in the transfected cells. The results indicate that the interposed lacO/R complex blocked transcriptional elongation of the enhancer-assembled transcription machinery through the intervening DNA into the e-globin promoter and drastically reduced the level of mRNA synthesis at the e-globin promoter. We discuss the tracking and transcription mechanism of the enhancer-assembled transcription machinery in mediating long range enhancer function.

MATERIALS AND METHODS

Construction of Plasmids—The HS2-lac-1.2-ep-CAT-lac plasmid was created as follows. An oligonucleotide, Stul-lacO3-SmaI-BamHI, containing two tandem natural lacO sequences was inserted into the Stul and SmaI sites located between the 1.2-kb DNA into which the 5.5-kb enhancer/promoter was removed from the pEGFP by AseI and NheI digestion. The brackets are the natural lac operator sequence (18) repeated in the equivalent DNA downstream of the CAT gene. The lacO3 sequence downstream of the CAT gene was similarly inserted as an XhoI-BamHI fragment into the XhoI-BamHI digested pCAT plasmid (Promega, Ref. 39) was inserted into the AseI-NheI-digested pEGFP to make the HS2-1.2-ep-CAT plasmid. To make the final HS2-1.2-ep-CAT plasmid, an XbaI-Stul-lacO3-BamHI oligonucleotide was inserted into the XbaI and BamHI sites downstream of the CAT gene in an enhancer ep-CAT plasmid (15); a PstI-HpaI fragment spanning the lacO sequence was excised from this plasmid and inserted into the HS2-1.2-ep-CAT plasmid similarly cleaved with PstI and HpaI to remove the equivalent DNA downstream of the CAT gene. The lacO3-BamHI oligonucleotide inserted into the polycloning sites of pUC19 was excised by Alwl and SmaI digestions and inserted into the Alwl and SmaI sites located 300 bp upstream of HS2 in HS2-1.2-ep-CAT; the (lacO3)2 sequence downstream of the CAT gene was similarly inserted as an Alwl-BamHI fragment (5 – 3’ sequence of the lacO3) was GACATCTAGAAGGCCCT [GGAATTGTGAGCGGATAACAATT][TGGAATTGTGAGCGGATAACAATT][TGGAATTGTGAGCGGATAACAATT][AATTAAGGGATCGGGATCC][GGATTTGTGAGCGGATAACAATT][TGGAATTGTGAGCGGATAACAATT][GGATACCTGACCTTTACCGCATAAGCTGTGCCTTGC], the DNA of 40 bases within the brackets are the natural lac operator sequence (18) repeated in tandem; underlined bases are respectively XbaI, Stul, SmaI and BamHI sites used in cloning. To conserve the 10-bp helical phasing of HS2 with respect to the downstream 1.2 kb DNA, the inserted (lacO3) between Stul and BamHI sites spanned 55 bases and replaced the 35 bases of DNA between the StuI and BamHI sites used in cloning. To conserve the 10-bp helical phasing of HS2 with respect to the downstream 1.2 kb DNA, the inserted (lacO3) between StuI and BamHI sites spanned 55 bases and replaced the 35 bases of DNA between the StuI and BamHI sites used in cloning. To conserve the 10-bp helical phasing of HS2 with respect to the downstream 1.2 kb DNA, the inserted (lacO3) between StuI and BamHI sites spanned 55 bases and replaced the 35 bases of DNA between the StuI and BamHI sites used in cloning.

The HS2-1.2-5.5-ep-CAT plasmid was created by inserting the pEGFP-C1 vector plasmid (Clontech). First, the cytomegalovirus enhancer/promoter was removed from the pEGFP by Asel and Nhel digestions, and the HS2-1.2-ep DNA with XhoI and Nhel ends made by PCR from the HS2-1.2-ep-CAT plasmid was inserted with an Asel-XhoI adaptor into the Nhel-digested pCDneo plasmid (Stratagene) containing the lacR gene tagged with a nuclear localization signal and the hygromycin selectable marker gene was transfected into K562 cells. Several clonal lines were expanded from single, parental cells, and the line that expressed the highest steady level of the lac repressor was selected. The test or the reference CAT and GFP plasmids were then separately integrated into this (+R) cell line or the wild type (–R) K562 cells with a cotransfected pCD neo plasmid expressing the neomycin resistance gene (15). To obtain multiple copy integrants yet avoiding tandem integration of the plasmids, a modified calcium phosphate precipitation method (15) was used: first, the CAT plasmids were linearized at the unique Alwl site in the vector downstream of the CAT gene, and the GFP-plasmids were linearized at the unique MluI site between the GFP and the downstream kanamycin/neomycin resistance gene. Ten µg of the linearized plasmid and 0.3 µg of the pCneo plasmid were mixed with 20 µg of spacer DNA-K562 genomic DNA fragmented to an average of 5–10 kb by sonication (CAT-plasmid) or by MluI, BglI, and AflII cleavages and sonication (GFP-plasmids). The plasmid DNAs would thus be integrated into the K562 genome not in tandem but interspersed with the spacer DNAs. Cell pools and clonal lines were expanded in medium containing hygromycin and G418. Modes of integration and copy numbers of the integrated plasmids were determined by Southern blots as described (15, supplemental Fig. S1). For IPTG induction, the cells were grown in medium containing 20 mM IPTG for 4–7 days.

Western Blot and Electrophoretic Mobility Shift Assay (EMSA)—For Western blots, 20 µg of protein from (–R) and (+R) K562 cell lysates were resolved in 5–15% gradient SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were soaked in blocking buffer: 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBSW). The membranes were washed three times with TBS and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. The labeled lac repressor band was visualized with an enhanced chemiluminescence detection kit (Pierce). The EMSA reactions (10 µl) contained 2–4 µg of nuclear extract and 40–50 fmol of 32P-labeled probe as described (25). For antibody supershift, 2 µl of the lacR antibody (Upstate Biotechnology) was added prior to the addition of the probe. The reactions were resolved in 5% non-denaturing PAGE. The gels were dried and analyzed in a PhosphorImager.

CAT Assays and FACS Analysis of GFP—Quantitative CAT enzymatic assays were carried out as described (15) with modified conditions. To ensure that the acetylated [3H]chloramphenicol reaction product is linearly proportional to the CAT activity, a 1:1 dilution of the added cell extract, the CAT assays contained an excess of the [3H]chloramphenicol substrate. To achieve this, the percentage conversion of the substrate to the product at the end of the assay reactions was kept within 10–50% of the substrate. Quantitative analyses of GFP fluorescence levels in live cells harboring the integrated test or reference GFP-plasmids were carried out in a FACS Calibur with CellQuest software (BD Biosciences) as described (26). For antibody supershift, 2 µl of the lacR antibody (Upstate Biotechnology) was added prior to the addition of the probe. The reactions were resolved in 5% non-denaturing PAGE. The gels were dried and analyzed in a PhosphorImager.

RNA Isolation, Semiquantitative RT-PCR, Real-time RT-PCR, and RPA—Total cellular RNAs were isolated from K562 cells by the Trizol (Life Technologies) method with RNAse free DNase I digestion. The RNAs were used in RT-PCR (27) and RPA (15) as described. In semiquantitative RT-PCR (see supplemental Fig. S2), random hexamer primers were used for synthesis of master stocks of cDNAs from the respective RNA templates. Aliquots of the cDNA master stocks derived from 0.2 µg of RNA template were then separately amplified with different PCR primer pairs in 50 µl of final volume for 28–30 cycles. The β-actin mRNA amplified by a β-actin primer pair (Stratagene) or the endogenous e-globin mRNA amplified by an appropriate primer pair served as the internal quantitative control as described previously (26). The RT-PCR bands were quantified relative to the β-actin internal control band with an IS1000 Digital Imager (Alpha Innotech). Real-time RT-PCR was carried out in a LightCycler (Roche Applied Science) using the SYBR Green Master kit (Roche Applied Science) according to the vendor’s protocol. The PCR conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s. Quantification of the real-time RT-PCR products was calculated from the PCR cycle number at the initiation of the log-linear phase of amplification by the Fit Points program.

PCR Primers—For the reference and test plasmids HS2-1.2- and HS2-lac-1.2-ep-CAT plasmids (Fig. 2B and Table I), the respective forward and reverse primers in primer pair 1: GGAACGGCCAGCCTGTTGA (in pA10CAT2), 8747–8771 (U01317) in HS2; in primer pair 2: 10096–10115 (U01317), CAT 2348–2328 (Promega, Ref. 39); in primer pair 3: CAT 2941–2961, CAT 3336–3316 (Promega, Ref. 39). For HS2-1.2- and HS2-lac-1.2-5.5-ep-GFP plasmids (Fig. 6): primer pair 5: 4526–4550 (pEGFP-C1, Clontech), 8747–8771 (U01317); primer pair 6: 10096–10115 (U01317), 14189–14191 (U01317); primer pair 7: 19436–19450 (primer pair 1), 14169–14191 (U01317); primer pair 8: 14169–14191 (U01317).
and detection of the lacR protein by the lacR antibody in Western blot. (Fig. 6) and hybridized to 32P-labeled probe as described (27). radioactive intensities of the RNA bands were quantified with a PhosphorImager. Western blot analysis of proteins isolated from (K562 cell populations harboring the test or the reference plasmids (see lane 5) and (−R) K562 cells. B, EMSA of (+) and (−) K562 nuclear extracts with the lacO sequence as probe. lacR Ab: lacR antibody; 100xself competition with 100-fold excess of the unlabeled lacO sequence.

RESULTS

(+R) K562 Cell Line Expresses the lac Repressor Protein within the Cell Nucleus—The (+R) K562 clonal cell line was created to ensure that the test and the reference plasmids were integrated into (+R) cells expressing an identical level of the lacR protein. To confirm that the integrated lacR expression plasmid indeed expressed the lacR protein, we carried out Western blot analysis of proteins isolated from (+R) and (−R) K562. The result showed that in the (−R) K562 cell line, the lacR antibody detected a protein band of 38 Kd corresponding to the monomeric molecular weight of the lacR protein (18), whereas (−R) K562 cells did not produce this band (Fig. 1A, lanes (+R) and (−R)). To further confirm that the lacR with the nuclear localization signal was transported into the nucleus and could bind to the lacO sequence, we carried out EMSA with (+R) and (−R) K562 nuclear extracts. The result showed that the (+R) K562 nuclear extract produced a strong EMSA band of the lacO/R complex (Fig. 1B, lane 3); this band was abolished by 100× excess of the unlabeled lacO sequence, and it was almost completely abolished by the lacR antibody (Fig. 1B, lanes 5 and 4). The presence of a faint EMSA band that remained after removal of lacR with the antibody (Fig. 1B, lane 4) indicated that in the absence of lacR protein, the lacO sequence could bind weakly to endogenous nuclear protein(s) in K562 cells. Consistent with this interpretation, the (−R) K562 nuclear extract produced a faint EMSA band with the lacO probe (Fig. 1B, lane 2). The results indicate that in the absence of lacR in (−R) K562 nuclear extract or in (+R) nuclear extract treated with lacR antibody, the lacO sequence could bind with weak affinity to an endogenous K562 nuclear protein. However, in the presence of lacR in (+R) K562 cells the lacO sequence bound to lacR with high affinity to form the lacO/R complex in the cell nucleus.
from the enhancer core and mRNA synthesis from the e-globin promoter. To prevent the host genome from interfering with transcription of the CAT gene, an additional lacO sequence was inserted downstream of the CAT gene in the test plasmid (Fig. 2A). In the reference plasmid, lac-HS2-1.2-ep-CAT-lac, the lacO sequence inserted downstream of HS2 in the test plasmid was moved to the vector sequence at a position 0.3 kb upstream of HS2 (Fig. 2A).

The test and the reference plasmids were separately integrated into (+R) or (–R) K562 cells. To prevent tandem integration of the plasmids such that the HS2 enhancer in one plasmid could activate transcription of the CAT gene in the upstream, neighboring plasmid, the plasmids were co-integrated with fragmented K562 spacer DNAs by a modified calcium phosphate precipitation method (Ref. 15, see “Materials and Methods”). Both cell pools and clonal lines were expanded from the transfected cells. Southern blots of the integrated plasmids in clonal lines indicate that the plasmids were integrated not in tandem copies into single host sites but mainly in single copies into multiple host sites (see supplemental Fig. S1, Ref. 15).

To determine the effect of the interposed lac O/R complex on the transcriptional status of the transgene cassette, total cellular RNAs were isolated from (–R) and (+R) cell pools containing the integrated test or the reference plasmids. The RNAs were analyzed by semiquantitative RT-PCR with three primer pairs spanning, respectively, the HS2 enhancer, the 1.2-ep intervening DNA, and the CAT gene in the plasmids (primer pairs 1–3, Fig. 2A). Note that to differentiate the HS2 RNAs transcribed from the plasmids from those transcribed from the endogenous HS2 in the K562 genome, the forward primer for primer pair 1 was located in the vector sequence immediately upstream of HS2 (Fig. 2A). This was possible because RPA studies showed that the HS2 enhancer was able to activate transcription from the immediately upstream vector DNA (Fig. 3, Ref. 15).

In (–R) K562 cells, the interposed lacO sequence in the test plasmid did not inhibit initiation of transcription at the enhancer or synthesis of CAT mRNA at the e-globin promoter because the intensities of the RT-PCR bands amplified by primer pairs 1, 2, and 3 in the test and the reference plasmids were comparable (Fig. 2B, lanes 1–3 in agarose gel and the bar graph below, (–R) panel). In (+R) K562 cells, the levels of HS2 RNAs transcribed from the test and the reference plasmids were again comparable (Fig. 2B, lanes 1, (+R) panel). This indicates that the lacO/R complex located 0.5 kb downstream of the HS2 enhancer in the test plasmid or the lacO/R complex located 0.3 kb upstream of the HS2 enhancer in the reference plasmid did not interfere with initiation of RNA synthesis from the HS2 enhancer.

In contrast, the intergenic RNA transcribed from the 1.2-kb DNA and the e-globin promoter was drastically reduced by the interposed lacO/R complex in the test plasmid as compared with the intergenic RNAs transcribed from the reference plasmid (Fig. 2B, lanes 2, (+R) panel). Correspondingly, CAT RNA was also significantly reduced in the test plasmid (Fig. 2B, lanes 3, (+R) panel). Note that because primer pair 3 spanned the 66-bp SV40 intron sequence at the 3’-end of the CAT gene, two RT-PCR bands were produced respectively from the unspliced and the spliced CAT RNAs. Quantification of the RT-PCR bands indicated that CAT mRNA was reduced by ~70% in the test plasmid (Fig. 2B, bar graph, Table 1). Together, these results indicate that the lacO/R complex interposed between the HS2 enhancer and the e-globin promoter in the test plasmid blocked elongation of enhancer transcription and drastically reduced enhancer function as measured by the level of CAT mRNA synthesis at the e-globin promoter.
Integrated plasmids, in the absence of HS2, intergenic RNAs transcribed from the HS2 enhancer-assembled transcriptional complex, because HS2 enhancer and the upstream vector were synthesized by through HS2. The HS2 RNAs initiated from both within the DNA and the upstream vector sequence upstream of HS2 (15), and the P5 probe of 1700 nt of the transgene cassette from the 5'-end of the 1.2-kb DNA through the enhancer core or the lacO/R complex inserted 0.3 kb from the 5'-end of the 1.2-kb DNA and elongated through the enhancer core and the lacO/R complex inserted 0.5 kb downstream of the HS2 enhancer in the reference plasmid did not hinder assembly of the HS2 enhancer complex. Hence, the HS2 enhancer was able to initiate RNA syntheses from similarly located sites in the test and the reference plasmids.

However, the 70% reduction of CAT mRNA in the test plasmid obtained from RT-PCR was an approximate estimation, because RNA analysis by PCR amplification was only semi-quantitative. Pilot studies showed that the amount of PCR product amplified from the template did not increase according to the theoretical yield of 2^n, where n was the PCR cycle number, and for relatively abundant templates the rate of product synthesis decreased with successive PCR cycles and approached zero after 35 cycles under our PCR condition (see supplemental Fig. S2). In addition, RT-PCR analysis could not differentiate between the long enhancer RNAs initiated from the enhancer and elongated through the 1.2-kb intervening DNA into the CAT gene and the shorter CAT mRNA initiated from the \( \epsilon \)-globin promoter. Because RPA did not rely on PCR amplification to detect RNAs and it also could resolve the long enhancer transcripts and the short CAT mRNA into separate bands according to their different sizes, we used RPA to further analyze the effect of the interposed lacO/R complex in (+R) cells.

To analyze the upstream enhancer transcripts and the CAT mRNA, we synthesized two antisense RNA probes, P3 and P5. Because of technical limitations, we were unable to synthesize intact RNA probes longer than 2 kb that spanned the entire 3 kb of the transgene cassette from the 5'-end of HS2 to the 3'-end of the CAT gene. Hence, P3 probe of 825 nucleotides (nt) spanned the 734 bases of the HS2 sequence and 91 bases of the vector sequence upstream of HS2 (15), and the P5 probe of 1700 nt spanned the region immediately downstream of HS2 from the 5'-end of the 1.2-kb DNA through the \( \epsilon \)-globin promoter to the 271th base of the CAT gene (Fig. 3A). The probes were separately hybridized to total cellular RNAs isolated from the (+R) K562 cells harboring the test or the reference plasmids, and the probe-protected RNAs were resolved in polyacrylamide gels.

The RPA results showed that the HS2 enhancer-initiated intergenic RNAs transcribed from both the reference and the test plasmids hybridized to P3 probe and produced similar patterns of protected bands of 825–190 nt (Fig. 3B, lanes 2 and 4, P3 panel). Note that P3-protected bands shorter than 734 nt were generated by RNAs initiated from sites within the HS2 enhancer, but P3 bands longer than 734 nt were synthesized from sites in the vector DNA upstream of HS2 and elongated through HS2. The HS2 RNAs initiated from both within the HS2 enhancer and the upstream vector were synthesized by the HS2 enhancer-assembled transcriptional complex, because in the absence of HS2, intergenic RNAs transcribed from the integrated plasmids, \( \epsilon \)-CAT (15), or the 1.2-\( \epsilon \)-CAT plasmids were not detectable with the P3 probe (supplemental Fig. S3, Ref. 15). The endogenous HS2 RNAs transcribed from the HS2 enhancer in the K562 genome were present at much lower abundance than the HS2 RNAs transcribed from the integrated plasmids; therefore, they did not generate detectable P3-bands in these autoradiograms with short exposure times (supplemental Fig. S3, Ref. 15).

The similar patterns of P3-protected bands generated by the test and the reference plasmids indicate that in the test plasmid the interposed lacO/R complex located 0.5 kb downstream of the enhancer core or the lacO/R complex inserted 0.3 kb upstream of the HS2 enhancer in the reference plasmid did not hinder assembly of the HS2 enhancer complex. Hence, the HS2 enhancer was able to initiate RNA syntheses from similarly located sites in the test and the reference plasmids.

In the reference plasmid, the intergenic RNAs synthesized by the HS2 enhancer were initiated from multiple sites in the 1.2-kb DNA and elongated through the \( \epsilon \)-globin promoter into the CAT gene. They hybridized to P5 probe and produced multiple bands of 1700–300 nt; P5 probe also hybridized to CAT mRNA synthesized from the cap site in the \( \epsilon \)-globin promoter to produce a strong band of 291 nt (Fig. 3B, lane 6). In addition, the 200 nt \( \epsilon \)-globin promoter sequence in P5 probe (Fig. 3A) hybridized to the endogenous RNAs initiated from sites upstream of and elongated through the endogenous \( \epsilon \)-globin promoter (15, 28, 29) to produce a prominent band of 200 nt (Fig. 3B, lane 6), which served as an internal loading control. The detection of the intergenic RNAs by the P5 probe indicates that the HS2-assembled transcriptional complex in the reference plasmid was able to track through the 1.2 kb intervening DNA to reach the \( \epsilon \)-globin promoter and activate synthesis of CAT mRNA.

However, in the test plasmid, P5 probe produced with the intergenic RNAs only two discernible bands of 471–400 nt (Fig. 3B, lane 5); the P5-band of 291 nt produced by CAT mRNA was also much fainter in the test than the reference plasmid (Fig. 3B, compare lanes 5 and 6). Quantification of the intensities of the 291-nt bands showed that the interposed lacO/R complex reduced the CAT mRNA level of the test plasmid by 90% (Table 1). Together, the similar patterns of P3-protected HS2 enhancer RNAs in the test and the reference plasmids and the paucity of P5-protected intergenic RNAs in the test as compared with the reference plasmid indicate that the interposed lacO/R complex in the test plasmid did not hinder transcriptional initiation of enhancer RNAs, but it blocked transcriptional elongation of the HS2-assembled transcriptional machinery through the 1.2-kb intervening DNA into the \( \epsilon \)-globin promoter and drastically reduced synthesis of CAT mRNA from the \( \epsilon \)-globin promoter.

Consistent with the RPA results, CAT assays showed that the CAT enzyme level of the test plasmid in (+R) cells was reduced by nearly 90% (Fig. 4, Table 1). However, ~10% of this reduction could be caused by weak binding to the lacO sequence by an endogenous K562 protein as indicated by the weak

| Cell pools | CAT/\( \mu \)g prot. | Copy/cell | CAT/copy | Rel. CAT | CAT mRNA |
|-----------|------------------|----------|----------|----------|-----------|
| (-R)      |                  |          |          |          |           |
| HS2–1.2   | 0.181 ± 0.05 (n = 4) | 3       | 0.06     | 1        | 0.1       |
| HS2-lac-1.2 | 0.162 ± 0.07 (n = 4) | 3       | 0.054    | 0.9      | 0.92      |
| (+R)      |                  |          |          |          |           |
| HS2–1.2   | 0.33 ± 0.1 (n = 6)  | 6       | 0.055    | 1        | 0.2       |
| HS2-lac-1.2 | 0.028 ± 0.004 (n = 6) | 4       | 0.007    | 0.12     | 0.2 ± 0.1 (0.3, 0.1) |

**A Transcription Mechanism of HS2 Enhancer Function**

**Table I**

Effect of the interposed lacO/R complex on HS2 enhancer activity in the reference HS2–1.2 and the test HS2-lac-1.2 plasmids determined by CAT assays, semiquantitative RT-PCR, and RPA (%). The endogenous HS2 RNAs transcribed from the HS2 enhancer were initiated from multiple sites in the DNA upstream of HS2 and elongated through the enhancer core or the lacO/R complex inserted 0.3 kb upstream of the HS2 enhancer in the reference plasmid did not hinder assembly of the HS2 enhancer complex. Hence, the HS2 enhancer was able to initiate RNA syntheses from similarly located sites in the test and the reference plasmids.

| Cell pools | CAT/\( \mu \)g prot. | Copy/cell | CAT/copy | Rel. CAT | CAT mRNA |
|-----------|------------------|----------|----------|----------|-----------|
| (-R)      |                  |          |          |          |           |
| HS2–1.2   | 0.181 ± 0.05 (n = 4) | 3       | 0.06     | 1        | 0.1       |
| HS2-lac-1.2 | 0.162 ± 0.07 (n = 4) | 3       | 0.054    | 0.9      | 0.92      |
| (+R)      |                  |          |          |          |           |
| HS2–1.2   | 0.33 ± 0.1 (n = 6)  | 6       | 0.055    | 1        | 0.2       |
| HS2-lac-1.2 | 0.028 ± 0.004 (n = 6) | 4       | 0.007    | 0.12     | 0.2 ± 0.1 (0.3, 0.1) |

**Effect of the interposed lacO/R complex on HS2 enhancer activity in the reference HS2–1.2 and the test HS2-lac-1.2 plasmids determined by CAT assays, semiquantitative RT-PCR, and RPA (%).**
Figure 4. CAT enzymatic assays of cellular extracts isolated from (−R) and (+R) K562 cells harboring the reference or the test plasmids. Numbers at the bottom of the autoradiograms, micrograms of the cellular extract used in each CAT enzyme assay. Top spots, acetamidochloramphenicol, the reaction products; bottom spots, chloramphenicol, the substrate.

EMSAs band generated by the nuclear extract of (−R) K562 cells with the lacO probe (Fig. 1B, lane 2) and the ~10% reduction in CAT activity of the test plasmid in (−R) K562 cells (Table I). If this protein could indeed compete with the lacR protein for binding to the lacO sequence in (+R) K562 cells, then the repression in CAT level attributable to the lacO/R complex in the test plasmid would be ~80%. In summary, RNA analyses by semiquantitative RT-PCR and RPA and protein analysis by CAT assays indicate that in (+R) K562 cells the interposed lacO/R complex interrupted the tracking and transcription mechanism of HS2-assembled transcriptional complex through the intervening DNA into the e-globin promoter and drastically reduced HS2 enhancer activity by ~80%.

Reduction of HS2 Enhancer Activity by the Interposed lacO/R Complex Can Be Reversed by IPTG—To determine whether the reduction in HS2 enhancer activity by the lacO/R complex in the test plasmid could be reversed by dissociation of the lacO/R complex with IPTG, nine clonal lines of the test and the reference lines C1–9 did not cause significant differences in the CAT mRNA synthesis, thus in HS2 enhancer activity, was caused mainly by the interposed lacO/R complex.

HS2 Enhancer-initiated Transcription Mediates Long Range Interaction between the HS2 Enhancer and the e-Globin Promoter—in the genome, the HS2 enhancer is separated from the e-globin promoter by 10 kb of intervening DNA (Fig. 2A, top). However, the results presented above were obtained with plasmid constructs containing only the 1.2-kb DNA immediately downstream of HS2 as the intervening DNA. To more closely mimic the endogenous genome, we constructed the test plasmid HS2-lac-1.2-5.5-ep-GFP and the reference plasmid HS2-1.2-5.5-ep-GFP (Fig. 6A). In these plasmids, the 5.5-kb DNA contiguous with the e-globin promoter in the human genome was included. However, the 3.4-kb DNA between the 1.2- and 5.5-kb DNA (Fig. 2A, top) was not included because it spans the HS1 site that possesses weak enhancer activity (30); its inclusion in the plasmids thus would complicate analysis of HS2 enhancer activity. To exclude the possibility that the observed effect of the interposed lacO/R complex in the test CAT-plasmid could be caused by interaction of the lacO/R complex with special DNA sequences in the vector, we used the pEGFP vector with a completely different vector backbone in the construction of the test and reference GFP-plasmids. Because the results of the CAT plasmids showed that the lacO/R complexes flanking the transgenic cassette did not provide complete protection of transgene expression from position variegation (see variations in the levels of intergenic and CAT RNAs among the reference clonal lines, Fig. 5, A and B), the new test and reference GFP-plasmids contained no lacO sequences in the flanking positions of the transgenic cassette (Fig. 6A). The linearized plasmids were introduced again with the spacer DNAs into (+R) K562 cells to prevent tandem integration of the plasmids (see “Materials and Methods”). Cell populations harboring the respective test and reference plasmids were expanded and used for RNA analyses by RT-PCR and Northern blots and for protein analyses by FACS of GFP fluorescence.

To investigate the transcriptional status of the integrated plasmids, total cellular RNAs were isolated from the respective cell populations and analyzed first by semiquantitative RT-PCR with primer pairs 5–8 (Fig. 6A). The primer pairs were designed again to amplify only the RNAs transcribed from the plasmids but not the RNAs transcribed from the equivalent regions in the endogenous globin gene locus of the (+R) K562 cells. In primer pair 5 (pp5), which amplified the HS2 region, the forward primer was located in the vector immediately upstream of HS2 as in primer pair 1 (Fig. 2A); pp6 spanned the junction region between the 1.2-kb and 5.5-kb DNA, which in the genome is separated by 3.4 kb of DNA spanning HS1, a distance not efficiently amplified in the presence of the shorter, competing RNAs transcribed from the shorter DNA template in the integrated plasmid. pp7 with the reverse primer located in the GFP reporter gene amplified the region from the 5.5 kb DNA through the e-globin promoter to the 5′-end of the GFP gene, and pp8 amplified only the GFP gene.

The RT-PCR results showed that the level of HS2 RNAs amplified by pp5 did not vary significantly between the test and the reference plasmids (Fig. 6B, lane 5). This indicates that in the test plasmid the lacO/R complex did not interfere with assembly of the HS2 enhancer complex and transcriptional initiation of the HS2 enhancer. However, the lacO/R complex reduced by 50–60% the intensities of the RT-PCR bands amplified from RNAs transcribed from the 1.2- and 5.5-kb intervening regions by pp6 and pp7 (Fig. 6B, lanes 6 and 7). Accordingly, GFP RNA transcribed from the test plasmid was reduced by ~50% (Fig. 6B, lane 8).

Again, RNA analysis by RT-PCR could not differentiate between the long upstream transcripts, initiated from the 5.5-kb DNA or further upstream DNA, and elongated through the
GFP gene and the shorter GFP mRNA synthesized from the cap site in the \(/H9280/-globin promoter. Because we were unable to synthesize intact RPA probes of longer than 2 kb, we used Northern blot to resolve the long, upstream RNAs and the short GFP mRNA. The Northern blot generated by the GFP probe showed that the RNAs transcribed from the reference plasmid produced two prominent bands (Fig. 6C, HS2 lane, left panel): a 1.5-kb band produced by GFP mRNA, containing 1.25 kb of GFP RNA from the cap site in the \(/H9280/-globin promoter to the SV40 polyadenylation signal of the GFP gene and a poly(A) tail of 0.25 kb, and a longer 3.3 kb band produced by the upstream RNA transcribed from a site 2 kb upstream of the \(/H9280/-globin promoter (Fig. 6A). Because RNA bands of larger sizes were not detected (top of the Northern blot not shown), longer RNAs transcribed from further upstream sites in the HS2 enhancer, 1.2 and 5.5 kb DNAs and elongated into the GFP gene were apparently not present, even though RT-PCR did detect RNAs transcribed from these upstream regions (Fig. 6B). This observation indicates that upstream RNAs were shorter than 3.3 kb in length and is consistent with our earlier finding that LCR transcripts of longer than 3 kb were not detectable. The test plasmid produced two corresponding bands of much fainter intensities (Fig. 6C, HS2-lac lane). Quantification of the GFP mRNA bands indicated that the interposed lacO/R complex in the test plasmid reduced the level of GFP mRNA by \(\sim 60\%\) (Fig. 6C, right panel).

To quantify the GFP level, the green fluorescence of cell populations harboring either the test or the reference plasmid was analyzed by FACS dot plots (Fig. 6D, top, see sample dot plots). The GFP level of each cell population was calculated from the parameters of the dot plots (Fig. 6D, bottom). Comparison of the GFP levels of the test and the reference cell populations showed that in agreement with the level of GFP mRNA, the average level of GFP synthesized from the test plasmid was reduced by 55–60% (Fig. 6D, bottom).

\[ J. Ling, B. Baibakov, W. Pi, B. Emerson, and D. Tuan, submitted for publication. \]
FIG. 6. RT-PCR and Northern blot of RNAs and FACS analyses of GFP from (+R) K562 cells harboring the integrated reference and test plasmids HS2-1.2-5.5-ep-GFP and HS2-lac-1.2-5.5-ep-GFP. 

A, plasmid maps of the reference and test plasmids, HS2 and HS2-lac. Mlu, the unique MluI site in the vector used to linearize the plasmids before transfection; other designations, same as Figs. 2A and 3A. Horizontal lines with triangular ends marked with 5, 6, 7, and 8, RT-PCR fragments amplified by primer pairs 5, 6, 7 and 8. Hatched bar, probe for Northern blot. 

B, left panel, semiquantitative RT-PCR of RNAs transcribed from the reference and the test plasmids. Lanes 5–8, RNAs amplified by primer pairs 5–8 respectively. In lanes 8, the RT-PCR product of GFP mRNA was amplified by two fewer PCR cycles than the intergenic RNAs. Right panel, bar graphs of the RT-PCR band intensities normalized with respect to the band intensity of the β-actin RNA internal control and the relative copy number of the integrated plasmids; the data were averages from two independent RNA preparations. 

C, left panel, Northern blot with the GFP probe. Lanes HS2 and HS2-lac, RNAs isolated from (+R) cells harboring either the reference or the test plasmids; lane 0, RNA isolated from control, non-transfected (+R) K562 cells. Numbers in the left margin, sizes in kb of the RNA bands; Ups. RNA, mRNA, RNA transcribed from the 5.5 kb upstream DNA; mRNA, GFP mRNA transcribed from the cap site in the e-globin promoter. To serve as a loading control, 18 S ribosomal RNA band in the same gel was viewed under UV light. Right panel, bar graph of the relative intensity of the GFP mRNA band in the respective samples; the intensity of the GFP mRNA band of the reference plasmid was set at 100 to serve as the standard of comparison. The results were averages from two Northern gels. 

D, FACS analyses of (+R) K562 cells harboring the reference and the test plasmids. Top panels, representative FACS dot plots of the reference and test cell populations. x axis, the fluorescent intensities of the gated cells, each dot represented one cell among the 20,000 cells analyzed for each cell population; y axis, side scatter; R2 and R3, regions of gated non-fluorescent and fluorescent cells, respectively. Bottom panels, % Gated, the percentages of the gated non-fluorescent and fluorescent cells in the R2 and R3 regions, respectively; Xmean, the mean fluorescent intensities of the cells in the R2 or the R3 regions; Copy#, the relative per cell copy number of the test plasmid with respect to that of the reference plasmid, which was set at 1. GFP level, the percentage of gated fluorescent cells in R3 times the Xmean of the fluorescent cells divided by the relative per cell copy numbers of the reference or the test plasmid (26); numbers in parentheses, GFP level of the test plasmid relative to that of the reference plasmid set at 100; numbers were mean ± standard deviation from three independent test cell populations.
the levels of GFP mRNA and GFP synthesized from the far downstream GFP gene.

**DISCUSSION**

In this study, we showed that in the test plasmids HS2-lac-1.2-ep-CAT-lac and HS2-lac-1.2-5.5-ep-GFP integrated into (+R) K562 cells, the lacO/R complex present in the intervening DNA at the 3’-end of the HS2 enhancer blocked transcriptional elongation of the HS2-assembled transcriptional machinery through the 1.2- and 5.5-kb intervening DNA into the ε-globin promoter and drastically reduced HS2 enhancer activity by 50–80%. Earlier, we showed that the 1.2-kb intervening DNA by itself in the 1.2-ep-CAT plasmid was unable to initiate transcription and did not exhibit enhancer activity (15). However, in the presence of the HS2 enhancer in HS2-1.2-ep-CAT plasmid, the enhancer-assembled transcriptional machinery apparently could track and transcribe through the 1.2-kb DNA to produce many intergenic RNAs that were initiated from multiple sites within the 1.2-kb DNA and elongated into the ε-globin promoter and the CAT gene (Fig. 3 and supplemental Fig. S3). Through this tracking and transcription mechanism, the HS2 enhancer complex could reach the ε-globin promoter to activate synthesis of the CAT/GFP mRNA. In the test plasmids, the interposed lacO/R complex interacted with and apparently arrested the RNA polymerase in the enhancer complex at the lacO site (20). In the presence of the lacO/R complex serving as a roadblock, the HS2 transcriptional complex was thus unable to track through the intervening DNA to reach the ε-globin promoter and activate synthesis of CAT/GFP mRNA.

This tracking and transcription mechanism of enhancer function indicates that the intervening DNA between the enhancer and the promoter participates in transmitting enhancer activity to the promoter. In the contrary, the looping mechanism leads one to postulate that the enhancer and the promoter complexes directly interact to loop out the intervening DNA, which thus does not participate in enhancer function. Viewing our data within the framework of the looping mechanism, one could, however, argue that the interposed lacO/R complex in the intervening DNA at 0.5 kb downstream of the enhancer core sterically hindered the assembly of a fully functional HS2 enhancer complex. The functionally impaired enhancer complex could not efficiently loop with the ε-globin promoter complex, thus causing the observed reduction in HS2 enhancer activity in the test plasmids.

However, the HS2 enhancer complex in the test plasmids appeared to be fully functional because both the initiation sites and the levels of HS2 RNAs transcribed from the test plasmids did not differ significantly from those of the reference plasmids (Figs. 2, 3, and 6). In particular, in the reference plasmid lac-HS2-1.2-ep-CAT-lac, the lacO/R complex located 0.3 kb upstream of the HS2 enhancer (thus at 0.5 kb upstream of the enhancer core) did not appear to sterically hinder the assembly and thus the function of the HS2 enhancer complex. Similarly, in the test plasmid the ε-globin promoter located 1.2–6.7 kb downstream of the lacO/R complex should also be fully functional because the 5’ and 3’ lacO/R complexes present in the reference plasmid lac-HS2-1.2-ep-CAT-lac, respectively, at 0.3 kb upstream of the HS2 enhancer and thus 4 kb upstream of the promoter and at 1.8 kb downstream of the promoter did not appear to interfere with the activity of the promoter complex.

Hence, the fully functional HS2 enhancer complex in the test as in the reference plasmids should be able to loop over the interposed lac O/R complex to interact with the functional ε-globin promoter complex and efficiently activate synthesis of the CAT/GFP mRNA, if the HS2 enhancer activated primarily through a looping mechanism. The drastic reduction in HS2 enhancer activity of 50–80% by the interposed lacO/R complex in the test plasmids indicates that the tracking and transcription mechanism of the HS2 enhancer complex from the enhancer through the intervening DNA into the ε-globin promoter constitutes a primary functional mechanism in transmitting HS2 enhancer activity over a long distance.

In the endogenous genome of K562 cells, the transcriptional machinery assembled by the HS2 enhancer may activate the far downstream ε-globin promoter also by a tracking and transcription mechanism because the entire 10 kb of intergenic DNA between HS2 and the ε-globin gene in the K562 genome is transcribed by pol II in a sense direction co-linear with transcription of the globin genes (15, 16, 29). These intergenic RNAs were synthesized from multiple sites within and downstream of HS2 and were polyadenylated at multiple further downstream sites less than 3 kb from their initiation sites. Thus, the HS2-assembled pol II transcriptional machinery, in a relay fashion to produce not one long contiguous RNA but many short, overlapping, polyadenylated RNAs, could track and transcribe the 10 kb of intervening DNA to reach the ε-globin promoter and activate synthesis of the ε-globin mRNA.

The tracking and transcription mechanism of the HS2-assembled pol II transcription machinery is consistent with findings in yeast that the predominant function of enhancers is to recruit and deliver pol II to the cis-linked promoters (31, 32) because the promoters, packaged in nucleosomes, are unable to stably bind TBP and recruit the pol II machinery (33). In the 340-kb Drosophila bithorax complex where enhancers act over long intergenic distances to regulate transcription of distant cis-linked genes, the intergenic DNA was also transcribed, and interfering with synthesis of the intergenic RNAs disrupts transcription of the far downstream genes (34, 35).

On the other hand, in the test plasmids, the interposed lacO/R complex blocked HS2 enhancer activity by 50–80%, not 100%. This partial blockage could be caused by the lacO/R complex not completely inhibiting transcriptional elongation of the enhancer-assembled transcription machinery as indicated by the presence of low levels of RNAs transcribed from the 1.2-kb and 5.5-kb intervening DNA in the test plasmids. Alternatively, this partial blockage of enhancer activity by the interposed lacO/R complex could indicate that direct interaction of the enhancer and the promoter complexes by a looping mechanism also participated in HS2 enhancer function. Indeed, in murine fetal liver erythroid cells, the HS2 enhancer has been shown to be in physical proximity to the actively transcribed β-globin gene located far downstream of the HS2 enhancer (36, 37). This finding indicates that in erythroid cells expressing the adult β-globin gene program, the HS2 enhancer could interact directly with the distant β-globin promoter by a looping mechanism. However, it is not clear how the HS2 enhancer translocates through the nucleoplasm space to precisely loop with the far downstream β-globin promoter without interacting with and activating nearby heterologous promoters in trans.

It is possible that HS2 enhancer function may involve both the tracking and the looping mechanisms in a dynamic equilibrium during erythroid cell development. As suggested by the facilitated tracking model of long range enhancer function (7, 14, 38), a tracking mechanism can establish precise loop formation between the enhancer and the promoter over long intervening distances. In the endogenous genome of human erythroid cells, the functional significance of the HS2-assembled pol II machinery in transmitting long-range enhancer/LCR activity to the embryonic ε-globin gene and the further downstream adult β-globin gene still remains to be investigated. A critical experiment will be to introduce an appropriate transcriptional terminator downstream of the endogenous HS2 to block the tracking and transcription process of the HS2-
assembled pol II complex and study the subsequent effects on transcription of the far downstream ε- and β-globin genes during erythroid cell development.

Acknowledgments—We thank Drs. M. Sodofsky and D. Levin for critical review of the manuscript.

REFERENCES
1. Tuan, D., Solomon, W., Li, Q., and London, I. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6384–6388
2. Forrester, W., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G., and Groudine, M. (1987) Nucleic Acids Res. 15, 10159–10177
3. Grosveld, F., Van Assendelft, G. B., and G. Kollias. (1987) Cell 51, 975–985
4. Bulger, M., and Groudine, M. (1999) Genes Dev. 13, 2465–2476
5. Engel, J. D., and Tanimoto, K. (2000) Cell 100, 499–502
6. Higgs, D. R. (1998) Cell 95, 299–302
7. Li, Q., and Peterson, K. (1999) Trends Genet. 10, 403–408
8. Lee, J., and Goldfarb, A. (1991) Cell 66, 793–798
9. Chao, M., Gralla, J., and Martinson, H. (1980) Biochemistry 19, 3254–3260
10. Bungert, J., Tanimoto, K., Patel, S., Liu, Q., Fear, M., and Engel, J. D., (1999) Mol. Cell. Biol. 19, 3062–3072
11. Armstrong, J., and Emerson, B. (1996) Mol. Cell. Biol. 16, 5634–5644
12. Sawado, T., Igarashi, K., and Groudine, M. (2001) Proc. Natl. Acad. Sci. U.S.A. 18, 10226–10231
13. Johnson, K., Gruss, J., Boyer, M., Kriekhaefer, C., Blobel, G., Weise, M., and Bresnick, E. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 11760–11765
14. Tuan, D., Kong, S., and Hu, K. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 51713
15. Kong, S., Bohl, D., Li, C., and Tuan, D. (1997) Mol. Cell. Biol. 17, 3955–3965
16. Ashe, H., Wijerde, M., Fraser, P., and Proudfoot, N. (1997) Genes Dev. 11, 2494–2509
17. Leach, K., Nightingale, K., Igarashi, K., Levinge, P., Engel, D., Becker, P., and Bungert, J. (2001) Mol. Cell. Biol. 21, 2829–2840
18. Gilbert, W. (1972) CIBA Found. Symp. 7, 245–259
19. Riggs, A., and Bourgeois, S. (1970) J. Mol. Biol. 48, 67–75
20. Lee, J., and Goldfarb, A. (1991) Cell 66, 793–798
21. Chen, M., Grolla, J., and Martinson, H. (1980) Biochemistry 19, 3254–3260
22. Hu, M., and Davidson, N. (1997) Cell 84, 355–366
23. Li, Q., Figge, J., Hansen, U., Wright, C., Heang, K.-T., Khoury, G., Livingston, D., and Roberts, T. (1987). Cell 49, 603–612
24. Deuschle, U., Hipskind, R., and Bjuard, H. (1990) Science 248, 480–483
25. Ramchandran, R., Bengra, C., Whitney, B., Lanclots, K., and Tuan, D. (2000) Am. J. Hematol. 65, 14–24
26. Ling, J., Pi, W., Bellag, R., Zeng, S., Keskinetpe, M., Saliman, H., Krantz, S., Whitney, B., Tuan, D. (2002) J. Virol. 76, 2410–2434
27. Ling, J., Pi, W., Bengra, C., Long, Q., Jin, H., Seyfang, A., and Tuan, D. (2003) Nucleic Acids Res. 31, 4582–4596
28. Plant, K., Routledge, S., and Proudfoot, N. (2001) Mol. Cell. Biol. 21, 6507–6514
29. Allan, M., Lanyon, W. G., and Paul, J. (1983) Cell 35, 187–197
30. Tuan, D., Oldham, A., Lee-Oldham, M., and Lee, D. (1987) Developmental Control of Globin Gene Expression, pp. 211–220, Alan R. Liss, Inc., New York
31. Keaveney, M., and Struhl, K. (1998) Mol. Cell. 1, 917–924
32. Ptashne, M., and Gann, A. (1997) Nature 386, 569–576
33. Inbalamano, A. N., Kwon, H., Green, M. R., and Kingston, R. E. (1994) Nature 370, 481–485
34. Drewell, R., Bae, E., Burr, J., and Lewis, E. B. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 16853–16858
35. Keaveney, M., and Struhl, K. (2000) Science 281, 4915–4922
36. Carter, D., Chakalova, L., Osborne, C., Dai, Y., and Fraser, P. (2002) Nat. Genet. 32, 1–4
37. Tolhuis, B., Palstra, R., Splinter, E., Grosveld, F., and Laat, W. (2002) Mol. Cell. Biol. 10, 1453–1465
38. Blackwood, E., and Kadonaga, T. (1998) Science 281, 60–63
39. Promega (1989) Bulletin 80, Madison, WI