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Essential Role of NF-E2 in Remodeling of Chromatin Structure and Transcriptional Activation of the ε-Globin Gene In Vivo by 5′ Hypersensitive Site 2 of the β-Globin Locus Control Region

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Much of our understanding of the process by which enhancers activate transcription has been gained from transient-transfection studies in which the DNA is not assembled with histones and other chromatin proteins as it is in the cell nucleus. To study the activation of a mammalian gene in a natural chromatin context in vivo, we constructed a minichromosome containing the human ε-globin gene and portions of the β-globin locus control region (LCR). The minichromosomes replicate and are maintained at stable copy number in human erythroid cells. Expression of the minichromosomal ε-globin gene requires the presence of β-globin LCR elements in *cis*, as is the case for the chromosomal gene. We determined the chromatin structure of the ε-globin gene in both the active and inactive states. The transcriptionally inactive locus is covered by an array of positioned nucleosomes extending over 1,400 bp. In minichromosomes with a μLCR or DNase I-hypersensitive site 2 (HS2) which actively transcribe the ε-globin gene, the nucleosome at the promoter is altered or disrupted while positioning of nucleosomes in the rest of the locus is retained. All or virtually all minichromosomes are simultaneously hypersensitive to DNase I both at the promoter and at HS2. Transcriptional activation and promoter remodeling, as well as formation of the HS2 structure itself, depended on the presence of the NF-E2 binding motif in HS2. The nucleosome at the promoter which is altered upon activation is positioned over the transcriptional elements of the ε-globin gene, i.e., the TATA, CCAAT, and CACCC elements, and the GATA-1 site at −165. The simple availability of erythroid transcription factors that recognize these motifs is insufficient to allow expression. As in the chromosomal globin locus, regulation also occurs at the level of chromatin structure. These observations are consistent with the idea that one role of the β-globin LCR is to maintain promoters free of nucleosomes. The restricted structural change observed upon transcriptional activation may indicate that the LCR need only make a specific contact with the proximal gene promoter to activate transcription.

Considerable genetic and biochemical evidence demonstrates the active role of chromatin structure in regulating transcription (28, 69, 72). Globin genes have long provided a model system with which to study how alterations in chromatin structure accompany tissue-specific and developmental stage-specific gene expression. The five individual genes (ε, Gγ, Aγ, δ, and β) of the human β-globin locus are expressed sequentially during development (56, 70). In erythroid cells, the entire locus is more open and sensitive to nucleases than it is in nonerythroid tissues (33, 41). In addition, as each gene becomes transcriptionally active, its promoter becomes hypersensitive to DNase I (41). Formation of the DNase I-sensitive chromatin structure of the entire β-globin locus and the activation of individual genes at the appropriate developmental stage are dependent on the locus control region (LCR). This complex regulatory element, located 6 to 22 kb upstream of the ε-globin gene, was first recognized as a series of four sites (DNase I-hypersensitive sites 1 to 4 [HS1 to HS4]) that are constitutively hypersensitive to DNase I in erythroid cells (33, 78). The LCR directs copy number-dependent and integration position-independent expression of a linked human β-globin gene in transgenic mice (40, 67). In naturally occurring deletions of the LCR (19, 21, 75), the entire β-globin cluster remains in a condensed chromatin conformation, the individual gene promoters do not become hypersensitive to DNase I, and the genes are not transcribed (31, 45). Significant enhancer activity in transient assays is displayed only by HS2 (36, 79), but studies using transgenic mice have shown that all four LCR DNase I-hypersensitive sites (HSs) can activate expression of the various β-globin genes, although to different extents (34).

The mechanism by which the LCR activates expression of the globin genes is not known. It has been proposed that the activation of individual globin genes is mediated by direct physical interactions between the LCR and globin promoters, with looping out of the intervening DNA (28). Two types of LCR-promoter structures have been envisioned to account for stage-specific expression of globin genes. There might be preferential interaction between particular HSs and promoters, or alternatively, the LCR may form a complex which interacts with individual promoters in turn (23, 25). While differences have been found among the individual sites in their abilities to activate individual genes, no strict preference has been observed (see, for example, reference 34). Other experiments have shown that deletion of HS2 or HS3 from the normal chromosomal position in mice has little effect on globin gene expression (29, 43). This result suggests that there might be

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functional redundancy among the HSs of the LCR, and if a complex forms, not all of the sites are required for its formation. Complex formation by the HSs is consistent with elegant studies showing that on a single chromosome, the LCR interacts with only one globin gene at a time (80), although serial interactions between the LCR and individual globin genes on a chromosome may occur (9, 80).

The LCR HSs as well as the globin promoters are replete with DNA-binding motifs for the same restricted group of erythroid cell-specific and ubiquitous *trans*-acting factors: NF-E2, GATA-1, and CACCC-binding proteins (26, 59, 60, 71, 74). GATA-1, an erythroid transcription factor, can self-associate as well as interact with EKLF, an erythroid factor that recognizes CACCC motifs, providing a potential basis for physical interactions among these regulatory regions (18, 26, 30, 36, 53). Some evidence suggests that the GATA and CACCC motifs are associated with position-independent globin gene activation (14, 23, 58, 73). NF-E2, a bZIP transcription factor, is a heterodimer of 45- and 18-kDa subunits belonging to the cap 'n' collar family (2, 3, 54). Expression of NF-E2 p45 is primarily restricted to erythroid cells, while NF-E2 p18 appears to be ubiquitously expressed (3). There appears to be considerable functional redundancy among polypeptides which recognize NF-E2 sites, and while the p18-p45 NF-E2 dimer itself may be required to activate globin gene expression, other species may be able to participate in formation of the HSs (46). The overall stimulatory activity of the LCR (at least of HS2) in the chromatin environment of transgenic mice or in stably integrated constructs appears to depend on NF-E2 motifs (13, 14, 23, 47, 73, 74). Furthermore, NF-E2 sites, in conjunction with GATA sites, are also required for formation of the DNase I-sensitive structure of HS4 and of the chick β/ε-globin enhancer (8, 71).

To dissect the interactions between the LCR and a globin promoter in a model chromatin system, we constructed minichromosomes containing HS2 or the μLCR and the human embryonic ε-globin gene with its natural promoter and flanking sequences. These multicopy minichromosomes (typically 15 to 30 copies per cell) are stably maintained in K562 human erythroid cells, in which the endogenous ε-globin gene is actively transcribed (20). HS2 and the μLCR, a fusion of HS1 to HS4, have previously been shown to confer correct tissue- and developmental stage-specific expression on the ε-globin gene in transgenic mice (62, 63, 68). Studies using transient-expression assays indicate that an NF-E2 motif in HS2 and a GATA-1 motif in the ε-globin promoter participate in activated transcription of this gene (36).

We show here that the minichromosome system recapitulates the major aspects of tissue-specific regulation observed in vivo for the ε-globin gene in its chromosomal locus, including dependence on the LCR for expression, and nuclease sensitivity at HS2 and at the promoter when the gene is active (68, 82). Mapping studies reveal that in the inactive state, the promoter and sequences surrounding the ε-globin gene are covered by an array of positioned nucleosomes and that a single nucleosome in this array is disrupted upon activation of transcription. HS2 alone is sufficient to remodel promoter chromatin and activate transcription, and HS2 itself is also present in a nucleosome-free region. Clustered point mutations at the tandem HS2 NF-E2 sites prevent the promoter remodeling and transcriptional activation of the ε-globin gene, and the HS2 site itself does not form. The ε-globin promoter and HS2 on single minichromosomes were similarly and simultaneously sensitive to DNase I, and no other changes in the chromatin structure of the locus were detectable. These results lend support to the idea of a preemptive stable interaction between LCR and promoter which requires occupancy of the LCR NF-E2 motif and results in altered nucleosome structure at the promoter and the activation of transcription.

MATERIALS AND METHODS

Episome construction, cell lines, and transfection. Minichromosomes carrying the ε-globin gene were constructed in p220.2, an Epstein-Barr virus vector containing *oriP*, EBNA-1, and a hygromycin resistance gene (37, 81). The ε-globin gene was a 3.7-kb genomic *Eco*RI fragment (GenBank accession numbers 17482 to 21233). The HS2 fragment was a 374-bp *Hin*dIII-to-*Xba*I fragment (GenBank accession numbers 8486 to 8860). Clustered point mutations that eliminate the binding of NF-E2 to the HS2 sequence were as described previously (36, 55). The μLCR was a 2.5-kb fusion of HS1 to HS4 (32) that appropriately regulates expression of the ε-globin gene in transgenic mice (62). K562 cells were maintained in RPMI 1640 medium with 10% fetal calf serum. Electroporation of minichromosomes was performed as described previously (39). Individual clones were selected by limiting dilution in the presence of 200 μg of hygromycin B per ml. The minichromosome copy number for each clone and the lack of rearrangement or chromosomal integration of the minichromosomal DNA were verified (37, 38).

RNase protection assay. RNA was prepared from 5×10^7 cells of K562 clones carrying minichromosomes (15), and RNase digestion and gel analysis of the products were performed as recommended by the manufacturer of the reagents (Ambion). The RNA probe used was generated with T7 polymerase from plasmid pBS458 after linearization with *Nco*I. The plasmid contains an *Eco*RV-to-*Ssp*I fragment from the ε-globin gene promoter and 5′ structural region (GenBank accession numbers 19215 to 19670) inserted into pBS at the *Eco*RV site.

Preparation of nuclei and nuclease treatment. Nuclei of K562 cell clones were prepared as described previously (17), with minor modifications. Typically, 1×10^8 to 2×10^8 cells were washed with ice-cold phosphate-buffered saline and resuspended in 6 ml of homogenization buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 0.1 mM EGTA, 15 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% Nonidet P-40, 5% [wt/vol] sucrose). Lysis of greater than 90% of the cells, observed microscopically, was obtained after a 2- to 3-min incubation on ice. The suspension was centrifuged (20 min, 2,300 rpm, 4°C) through a 3.5-ml cushion of homogenization buffer containing 10% sucrose. Nuclei were resuspended gently in 4 to 6 ml of wash buffer (10 mM Tris [pH 7.4], 15 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 8.5% sucrose), and aliquots of 1 ml were digested with 0, 3, 6, 12, or 25 μg of DNase per ml for 10 min at room temperature in the presence of 3 mM $CaCl_2$ or with 0, 30, 60, or 90 U of micrococcal nuclease (MNase) per ml for 6 min at room temperature in the presence of 1 mM $CaCl_2$. Alternatively, nuclei were resuspended to a volume of 360 μl in wash buffer, and six 60-μl aliquots were digested with 100 U of various restriction enzymes in a final volume of 400 μl of the recommended enzyme buffer (New England Biolabs) as described previously (16). The reactions were stopped by bringing the samples to 0.5 M NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate, and 0.1 mg of proteinase K per ml and then incubating the mixtures overnight at 37°C. The DNA was then purified by multiple phenol-chloroform extractions and analyzed by gel electrophoresis and Southern blotting as described in the figure legends or was digested to completion with a restriction enzyme before analysis. Southern blots were hybridized with probe labeled by random priming to a specific activity of 1×10^9 to 2×10^9 cpm/μg of DNA, and the intensity of bands was quantified with a PhosphorImager (Molecular Dynamics).

RESULTS

Minichromosomes containing the human ε-globin gene were constructed by using a vector based on the Epstein-Barr virus origin of replication, *oriP* (81). The globin gene sequences inserted into the minichromosomes are shown in Fig. 1. The minichromosome pεA contains the 3.7-kb genomic *Eco*RI fragment of the ε-globin gene marked by a 2-bp mutation in the 5′ untranslated region of the gene. The minichromosome pεHS2A carries, in addition, the β-globin LCR HS2 enhancer fragment, while pεμLCRA carries the μLCR, a fusion of the four HS sites of the β-globin LCR which has been shown to allow stage-specific regulation of an ε-globin gene in transgenic mice (62). Each of the constructs was separately inserted into a parental episome in two directions (designated A [transcription of the ε-globin gene in the same direction as transcription of EBNA-1] and B [opposite direction]).

Transcription of ε-globin minichromosomes. Minichromosomes were introduced into human erythroid K562 cells that actively transcribe the endogenous ε-globin gene. We used an

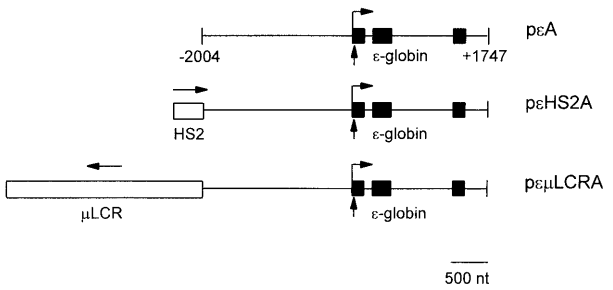

FIG. 1. Structures of ε-globin insertions into minichromosomes. Three constructs inserted into the episomal vector p220.2 (42) by blunt-end ligation into the unique, filled *Sal*I site are illustrated. Each contained the 3.7-kb *Eco*RI human genomic ε-globin fragment with a 2-bp mutation at position +11 in the 5′ untranslated region (vertical arrow). Coding regions of the gene are indicated by filled boxes. pεHS2A also contained HS2 of the β-globin LCR, while pεμLCRA contained the μLCR (denoted by open boxes). Horizontal arrows indicate the direction of transcription for the ε-globin gene or the 5′-to-3′ orientation of LCR sequences. The constructs were inserted into p220.2 such that transcription of the ε-globin gene was either in the same direction as transcription of EBNA-1 (pεA, pεHS2A, and pεμLCRA are illustrated) or in the opposite direction (pεB, pεHS2B, and pεμLCRB).

RNase protection assay to distinguish minichromosomal ε-globin transcripts from those of the endogenous gene as illustrated in Fig. 2. RNA from K562 cell clones carrying minichromosomes without enhancer elements (lanes 2 and 3) produced one protected band at the position observed for the endogenous genomic transcript in K562 cells (lane 1). However, with RNA from clones with either HS2 (lanes 4 and 5) or the μLCR (lanes 6 and 7), we observed an additional protected band of the expected size for transcripts of the minichromosomal ε-globin gene. Thus, either HS2 or the μLCR was sufficient to activate transcription of the ε-globin gene on the minichromosome. However, the ε-globin gene alone was not transcribed, even in the erythroid environment of K562 cells, where the endogenous ε-globin gene is active.

At least 10 clones of each of the six types of minichromosome from three different electroporation experiments have been studied. In all cases, the presence of either HS2 or the μLCR resulted in minichromosomal ε-globin expression. The dominant effect of the LCR elements on transcription in the minichromosomes appears to recapitulate the effect of the β-globin LCR in the genome and in transgenic mice.

Chromatin structure of ε-globin minichromosomes. We next examined whether changes in chromatin structure of the ε-globin gene on the minichromosomes could be correlated with transcriptional activity. We digested intact nuclei from K562 clones carrying inactive (pεB) or actively transcribed (pεμLCRB) minichromosomes with MNase, which cleaves linker DNA between nucleosome cores. Partial digestion of chromatin produces an array of fragments that are multiples of the length of nucleosome monomer DNA. Figure 3B shows the extent of MNase digestion of bulk nuclear DNA, and Fig. 3C shows results of probing a blot of this gel with a segment of DNA upstream of the ε-globin gene. The nucleosome ladder produced indicates that minichromosomes are assembled into nucleosomes in this region in both the active and inactive states. Nuclei from K562 cells and HeLa cells, in which the endogenous gene is active and inactive, respectively, gave the same result. The data in Fig. 3C were used to calculate the nucleosome repeat lengths, which were found to be 189 bp for K562 cells and 189 and 186 bp for pεB and pεμLCRB, respectively.

We next investigated the chromatin structure of the proximal ε-globin gene promoter by hybridizing the same blot with a DNA probe spanning these sequences (Fig. 3D). The promoter region is nucleosomal when the gene is inactive but not when it is actively transcribed, as indicated by a less distinct ladder and poor hybridization to mono- and dinucleosome bands for the latter. We also investigated the organization of the μLCR region, as shown in Fig. 3E. A smear rather than a nucleosome ladder was detected in active pεμLCRB. This probe does not hybridize to pεB, which does not contain LCR sequences. The relative faintness of the nucleosome ladder in Fig. 3E compared with Fig. 3D may result from the greater extent of the sensitive area surrounding the HS2 probe sequences in chromatin. Finally, the blot was rehybridized to a DNA segment outside the globin sequences (Fig. 3F). In the region of *oriP*, a nucleosome ladder was observed for both minichromosomes, but the pattern was different from that seen upstream of the globin promoter (Fig. 3C) and suggests irregular spacing of nucleosomes in this region. Together, these experiments show disruption of nucleosome structure at the ε-globin promoter and at HS2 on the minichromosomes when transcription of the ε-globin gene is ongoing.

Accessibility of the ε-globin proximal promoter in chromatin. To examine ε-globin promoter structure at higher resolution, we studied the accessibility of various promoter restriction sites to their cognate endonucleases in isolated nuclei. Figure 4B illustrates the results for nontranscribed minichromosome pεA and actively transcribed minichromosomes pεHS2A and pεμLCRA. Substantial cleavage of all minichromosomes

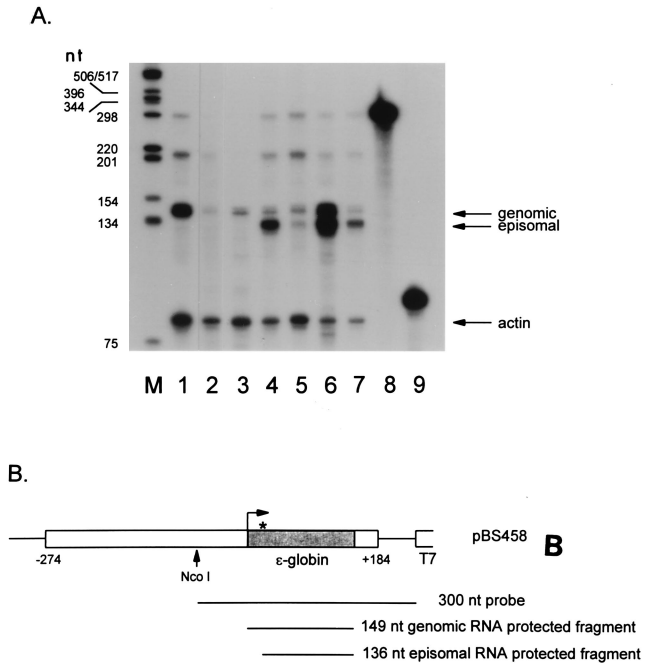

FIG. 2. Activation of ε-globin transcription from minichromosomal templates is dependent on β-globin LCR sequences. RNase protection analysis was performed on RNA isolated from K562 clones carrying different ε-globin minichromosomes. The labeled antisense probe was synthesized from pBS458 (bottom). The 149-nt band protected by endogenous ε-globin transcripts is indicated by the upper arrow. The lower arrow indicates the 136-nt band protected by minichromosomal ε-globin transcripts which contain a mismatch cleavable by RNase as a result of a 2-bp mutation. An actin probe was included as a load control. For lane 2, a longer exposure of the blot was used. Lanes: M, ^{32}P-labeled 1-kb marker DNA (Gibco/BRL); 1, K562; 2, pεA; 3, pεB; 4, pεHS2A; 5, pεHS2B; 6, pεμLCRA; 7, pεμLCRB; 8, ε-globin probe; 9, actin probe. The K562 clones carried between 12 and 26 copies of the indicated minichromosomes.

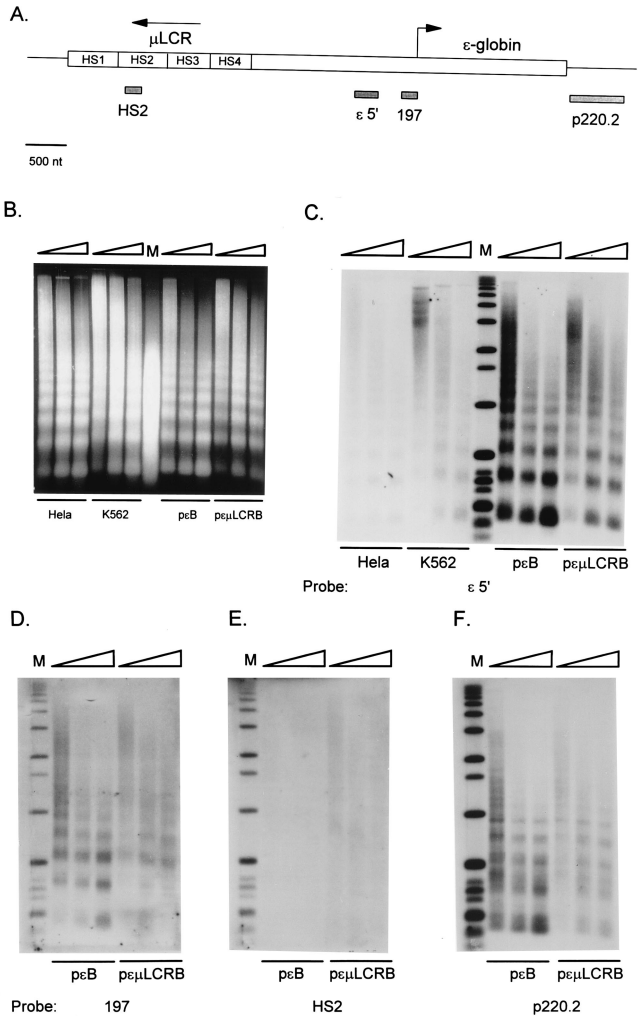

FIG. 3. In vivo chromatin structure of actively transcribed and nontranscribed minichromosomes. Nuclei from HeLa cells, K562 cells, and two K562 clones carrying different minichromosome constructs were cleaved with increasing amounts of MNase (30, 60, and 90 U/ml; indicated by the wedges). pεB contains an ε-globin gene that is not transcribed. pεμLCRB contains the β-globin μLCR fusion and an actively transcribed ε-globin gene. The purified DNA (20 to 40 μg per lane) was separated on a 0.8% agarose gel, blotted to a nylon membrane, and hybridized successively with probes to different regions of the minichromosomes. Lanes M, ^{32}P-labeled marker DNA. (A) Linear map of minichromosome pεμLCRB showing the location of probe sequences as shaded rectangles below the map. The thin line represents vector sequences. (B) Ethidium bromide-stained gel used for Southern blotting. (C) Hybridization with a 300-bp ε-globin 5′ probe extending from −536 (*Xba*I) to −836 (*Cla*I). (D) Hybridization with probe 197 extending from −179 (*Bam*HI) to +18 (*Pvu*II) in the ε-globin promoter. (E) Hybridization with a 374-bp HS2 probe (*Hin*dIII-*Xba*I fragment). (F) Hybridization with a 623-bp p220.2 probe (*Sal*I to *Eco*RV in the Epstein-Barr virus *oriP* of the minichromosome).

was observed at the *Bam*HI and *Pvu*II sites (Fig. 4B, lanes 3, 6, 9, 12, 15, and 18). However, only actively transcribed minichromosomes were cleaved at the *Ava*II and *Nco*I sites (Fig. 4B; compare lanes 4 and 5 with lanes 10, 11, 16, and 17). The same result was obtained with chromatin from K562 clones carrying minichromosomes with insertions in the opposite direction relative to p220.2 sequences: pεB, pεHS2B, and pεμLCRB (not shown). The efficiency of restriction enzyme cleavage was quantitated and confirms the greater accessibility of the *Ava*II and *Nco*I sites in actively transcribed minichromosomes (Table 1). The *Bam*HI and *Pvu*II sites are separated by 204 bp of DNA, suggesting that they lie in linker regions that flank a nucleosome on the ε-globin gene promoter in inactive chromatin. The *Ava*II and *Nco*I sites were protected from cleavage in the inactive minichromosomes, consistent with the presence of a nucleosome over these sites which is altered or removed upon activation of the gene.

The pattern of digestion with restriction enzymes for the minichromosomal ε-globin gene was compared with that obtained with K562 cells, which transcribe the ε-globin gene, and HeLa cells, which do not (Fig. 4C and Table 1). *Bam*HI was the only enzyme to cleave HeLa cell chromatin in the ε-globin promoter region even after a 60-min incubation period. In K562 chromatin, the *Bam*HI and *Pvu*II sites were cleaved as were the *Ava*II and *Nco*I sites that are protected in the chromatin of nonexpressing minichromosomes and in HeLa chromatin. That the *Bam*HI site appears to be in an accessible

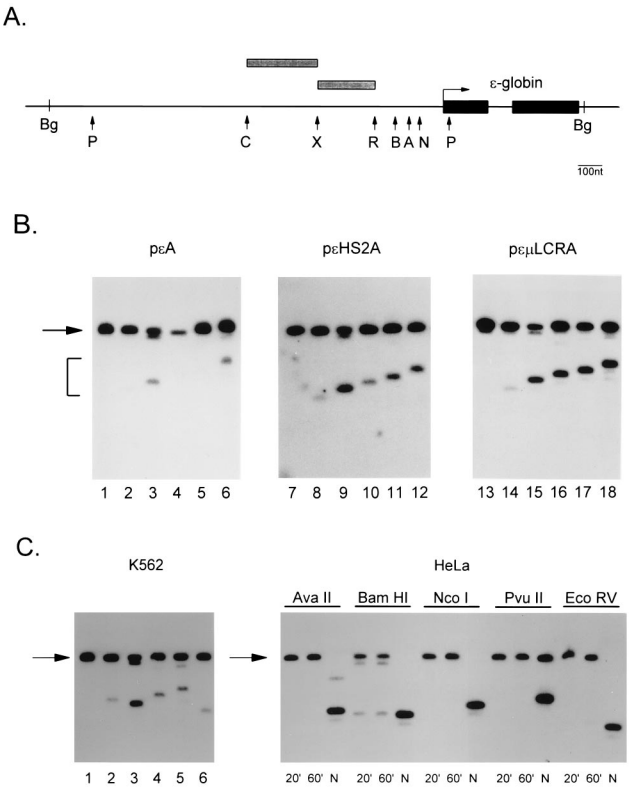

FIG. 4. Restriction enzyme access to sites in ε-globin promoter chromatin. (A) Locations of restriction enzyme sites in the *Bgl*II fragment containing the ε-globin promoter. The arrow indicates the transcription start site for the ε-globin gene, and the filled rectangles represent the coding exons of the gene present in the *Bgl*II parent fragment. Bg, *Bgl*II; C, *Cla*I; X, *Xba*I; R, *Eco*RV; B, *Bam*HI; A, *Ava*II; N, *Nco*I; P, *Pvu*II. Blots were hybridized with the probe fragments indicated by the shaded rectangles above the map. (B) Nuclei of K562 clones carrying the indicated minichromosomes were either mock digested (lanes 1, 7, and 13) or digested for 20 min at 37°C with *Eco*RV (lanes 2, 8, and 14), *Bam*HI (lanes 3, 9, and 15), *Ava*II (lanes 4, 10, and 16), *Nco*I (lanes 5, 11, and 17), or *Pvu*II (lanes 6, 12, and 18). After purification of the products, the DNA was cut to yield the *Bgl*II parent fragment (arrow). DNA (5 μg) was separated on a 0.8% agarose gel and blotted to a nylon membrane. The bracket indicates the bands resulting from cleavage with the various restriction enzymes in nuclei. (C) Nuclei from K562 cells were either mock digested (lane 1) or digested for 20 min at 37°C with *Ava*II (lane 2), *Bam*HI (lane 3), *Nco*I (lane 4), *Pvu*II (lane 5), or *Eco*RV (lane 6). HeLa cell nuclei were digested with the indicated restriction enzymes for either 20 or 60 min at 37°C. The products were purified and digested to completion with *Bgl*II and analyzed as described for panel A (20 μg of DNA). Lanes N contained naked DNA cut with *Bgl*II and then digested with the indicated restriction enzyme.

TABLE 1. Accessibility of the ε-globin promoter to restriction enzymes

Cleavage with:	% Cleavage of ε-globin promoter as chromatin[a]				
	pεA	pεHS2	pεμLCRA	K562	HeLa
*Eco*RV	6 ± 1	4 ± 1	7	8 ± 3	<1
*Bam*HI	28 ± 2	40 ± 7	35	32 ± 3	25 ± 10
*Ava*II	<1	8 ± 1	16	1 ± 1	<1
*Nco*I	2 ± 1	18 ± 3	21	9 ± 4	<1
*Pvu*II	8 ± 2	26 ± 3	24	2.5	<1

[a] Determined by dividing the intensity of the cleaved band by the sum of the intensity of the cleaved plus uncleaved bands. When results are given as means ± standard errors of the means, three to seven nuclear isolations and digestions were performed. When the standard error is not given, the results are averages of two separate such experiments. The theoretical contribution of cleavage of genomic K562 chromatin to the cleaved bands measured in the minichromosome containing K562 clones is about 0.25 to 2%. However, we believe this to be an upper limit because in experiments analyzing 5 μg of K562 DNA (the amount used for the K562 minichromosome clones), cleaved bands were not detectable by a PhosphorImager for any enzyme.

linker region in HeLa cell DNA is consistent with positioning of a nucleosome between the *Bam*HI and *Pvu*II sites in inactive chromatin in both an erythroid and a nonerythroid nuclear environment.

DNase hypersensitivity of the ε-globin promoter and LCR. The development of an HS in the promoter is a hallmark of an active gene (22), and globin gene promoters show such sites during the developmental stage at which they are active (41). We therefore performed DNase I digestions of nuclei from K562 clones carrying active or inactive ε-globin minichromosomes. Figure 5A shows that actively transcribing minichromosomes pεHS2B and pεμLCRB have a site sensitive to cleavage by DNase I close to the *Nco*I restriction site in the ε-globin promoter which is absent in chromatin of nontranscribing minichromosome pεB. The same samples were used to look at the DNase I sensitivity at HS2 in minichromosomes (Fig. 5B). No DNase I-sensitive site is seen with pεB, which lacks HS2. In contrast, highly sensitive DNase I cleavage sites in pεHS2B and pεμLCRB which map to the position of HS2 were detected. Identical results were obtained with K562 clones carrying minichromosomes pεA, pεHS2A, and pεμLCRA (data not shown). To demonstrate that the chromatin of pεB was digested with DNase to an extent similar to that for pεHS2B and pεμLCRB, the samples were probed with a p220.2 vector fragment, and strong previously mapped DNase I cut sites were detected (not shown) (42). The pattern of HSs detected in minichromosomes is a faithful reproduction of those sites at HS2 and at the ε-globin promoter in the K562 genome and correlates with active transcription of the gene (11, 77).

We next examined whether HS2 and the ε-globin promoter on the same minichromosome are hypersensitive to DNase I simultaneously, as would be expected if both participate in a chromatin structure necessary for active transcription of the gene. In the minichromosomes, HS2 has been brought closer to the ε-globin gene than it is in the genome, making it possible to assess hypersensitivity at the promoter and HS2 on individual molecules. Figure 6 illustrates the *Spe*I restriction fragment of the minichromosomes which contains both sites. Nuclei from actively transcribed pεHS2A were incubated with DNase I for increasing periods of time. Molecules which were cut singly at either the promoter or enhancer appeared by 1 min, and most of these were chased to doubly cut molecules by 10 min. At 10 min, only about 2% of the parent fragment remained. These results indicate that when the minichromosomes are transcribed, virtually all ε-globin genes are hypersensitive to DNase simultaneously at the promoter and at HS2. Further, it appears that the structures comprising the two HSs are sensitive to cleavage at the same time point in digestion.

Chromatin structure of the ε-globin gene. To extend nucleosome mapping to the region surrounding the proximal promoter, we used MNase-digested nuclei in indirect end labeling experiments. In Fig. 7, the positions of cleavages by MNase are indicated by arrows along the left relative to the ε-globin promoter and coding sequences being probed. A regular series of cut sites at average intervals of 184 bp, separated by protected regions, can be seen for the chromatin of both inactive pεB and actively transcribed pεHS2B. This pattern, which was not seen with naked DNA, is consistent with nonrandom positioning of nucleosomes (indicated as ovals along the ε-globin promoter and proximal coding regions) in both the active and inactive states. The position of the nucleosome at the promoter revealed by indirect end labeling coincides with that predicted from restriction enzyme accessibility studies (Fig. 4).

A comparison of the cleavage sites in chromatin for the active (Fig. 7, lane 5) and inactive (lane 9) minichromosomes revealed the appearance of three strong cut sites at the prox-

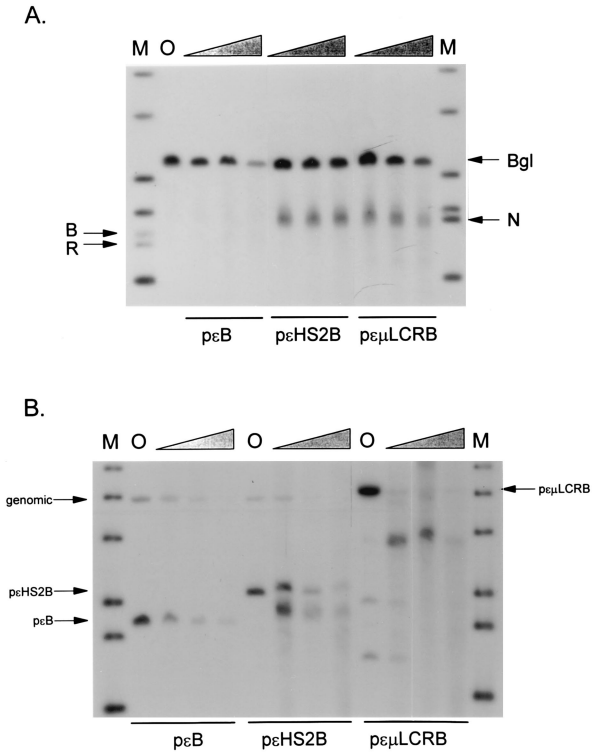

FIG. 5. HSs in the ε-globin promoter and LCR sequences in inactive and actively transcribed minichromosomes. (A) Nuclei from K562 clones carrying the indicated minichromosomes were incubated without DNase (lane O, pεB) or with increasing amounts of DNase I (6, 12, and 25 μg/ml; indicated by the wedges) for 10 min at 25°C. The DNA was purified and cut to completion with *Bgl*II to analyze promoter hypersensitivity, and 20-μg aliquots were analyzed by gel electrophoresis and Southern blotting using the *Xba*I-to-*Eco*RV probe (Fig. 4A). The *Bgl*II parent fragment is indicated by an arrow. Lanes M contained ^{32}P-labeled 1-kb marker DNA fragments plus DNA fragments from pεB which had been cleaved with *Bgl*II followed by *Bam*HI (B; 1,421 nt), *Eco*RV (R; 1,329 nt), or *Nco*I (N; 1,534 nt). (B) The same DNase-digested samples as in panel A were cleaved with *Hin*dIII and *Eco*RV to look at HS2 hypersensitivity. DNase I concentrations were as in panel A. The parent fragments resulting from *Hin*dIII and *Eco*RV digestion of pεB (1.7 kb), pεHS2 (2.4 kb), and pεμLCR (4.3 kb) are indicated and are unique to the minichromosome. An arrow indicates the K562 genomic fragment 5′ to the ε-globin gene that hybridizes with the probe. M, marker DNA as described above.

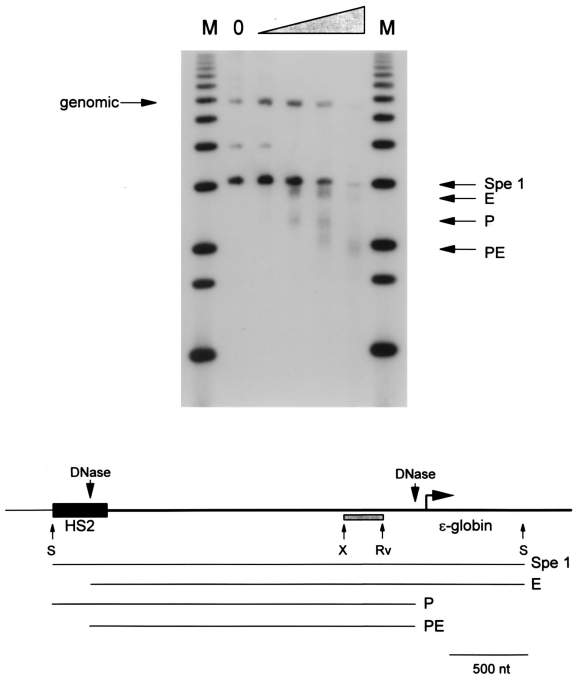

FIG. 6. The promoter of the ε-globin gene and HS2 are cut simultaneously on individual molecules of pεHS2A. Nuclei from K562 clones carrying pεHS2A were incubated without DNase I (lane 0) or with 12 μg of DNase I per ml for increasing time periods (15 s, 1 min, 3 min, and 10 min; indicated by the wedge). The DNA was purified, cut to completion with *Spe*I to give the parent fragment indicated, and then subjected to gel electrophoresis and Southern blotting. The blot was hybridized with the *Xba*I-to-*Eco*RV probe shown in the diagram by a shaded box. The positions of migration of restriction fragments that had been cut singly by DNase at the promoter (P) or at HS2 (E), or cut at both positions (PE), are indicated. The position of the *Spe*I genomic fragment is shown by an arrow. S, *Spe*I; X, *Xba*I; Rv, *Eco*RV. Lane M contained ^{32}P-labeled 1-kb marker DNA.

imal promoter for actively transcribed pεHS2B (cuts indicated by starred arrows at −38, −110, and −118 and dots beside lane 9). The same result was obtained with pεμLCRB, pεHS2A, and pεμLCRA (data not shown). Thus, activation of the ε-globin gene for transcription by β-globin LCR elements involves the alteration or removal of a particular nucleosome occluding the proximal promoter of the gene. The altered nucleosome covers sequences including the TATA motif and the −165 GATA-1 site as indicated in Fig. 7, as well as the *Ava*I and *Nco*I restriction enzyme sites which became accessible to cleavage after transcription activation (Fig. 4).

In additional indirect end labeling experiments, we observed positioned nucleosomes extending from −628 to +830 with respect to the start site for transcription of the ε-globin gene in the chromatin of active and inactive minichromosomes (not shown). The average length of protected DNA was 184 bp, which agrees well with the data in Fig. 3. The protected region of about 160 nucleotides (nt) between −200 and −360 was consistently shorter than would be expected for a nucleosome. Further experiments will be needed to determine whether this region contains a close packed nucleosome or perhaps some other structure. Figures 4, 5, and 7 indicate that when transcription is activated, the only change which occurs in the array of positioned nucleosomes over the ε-globin gene 5′ flank and proximal coding regions is the disruption of a single nucleosome at the promoter.

Role of NF-E2 in HS formation and transcriptional activation. The NF-E2 sites in HS2 have been shown to play a crucial role in its ability to enhance activity of a linked β-globin gene in the chromatin environment of transgenic animals or stably integrated constructs (13, 14, 23, 47, 73, 74). We therefore wished to ascertain the role of the NF-E2 motif of HS2 in promoter remodeling and the transcriptional activation of the ε-globin gene. We introduced clustered point mutations which prevent NF-E2 binding into pεHS2A to create pεHS2A(NF-E2mut). RNA was isolated from K562 clones carrying these minichromosomes, and ε-globin transcripts were assayed by RNase protection as in Fig. 2. No ε-globin RNA was detected for minichromosomes containing the mutated NF-E2 motif (Fig. 8; compare lane 3 with lanes 5 and 6). We also analyzed the DNase I sensitivity of the ε-globin promoter and HS2 in pεHS2A and pεHS2A(NF-E2mut). Increasing amounts of DNase were used to digest nuclei, and the DNA was subsequently cleaved with either *Bgl*II or *Eco*RV as for Fig. 5. Figure 8B shows that neither the promoter of the ε-globin gene nor HS2 itself became hypersensitive to DNase I when the HS2 motif for NF-E2 was mutated. We conclude that formation of active structure(s) at the LCR and ε-globin promoter requires the NF-E2 binding sites.

DISCUSSION

Our goal is to understand how promoter-enhancer interactions give rise to transcription activation in chromatin. We used a minichromosome system to look at the architecture of a model gene in chromatin in either a transcriptionally active or inactive state. Without an activation element (HS2 or μLCR) in the minichromosome, ε-globin is transcriptionally inactive and nucleosomes are positioned over the ε-globin promoter.

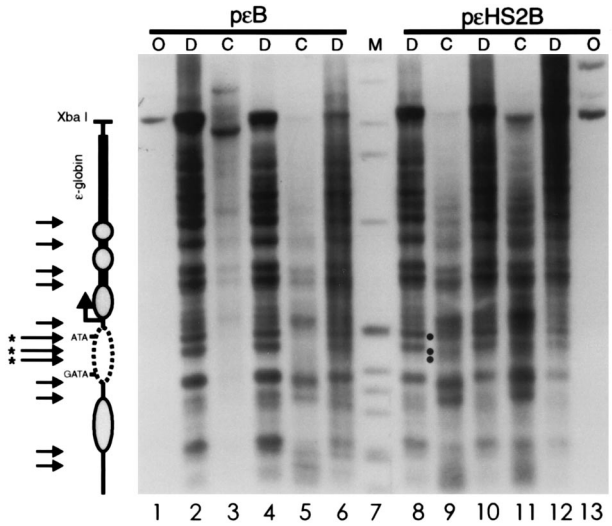

FIG. 7. Nucleosome structure in chromatin surrounding the ε-globin promoter in actively transcribing and nontranscribing minichromosomes. Nuclei from K562 cell clones carrying actively transcribed pεHS2B or inactive pεB (C) or naked DNA (D) were mock digested (O) or digested with MNase. After purification of the DNA and digestion with *Xba*I, the products (20 to 40 μg of DNA) were separated by gel electrophoresis and analyzed by Southern blotting using an *Xba*I-to-*Eco*RV probe (Fig. 4A). Coding sequences of the ε-globin gene in the *Xba*I fragment are indicated by the heavy line in the map at the left. The vertical arrow indicates the ε-globin gene transcription start site, and the positions of the TATA and −165 GATA sites are shown. Horizontal arrows indicate cut sites observed in the chromatin of the minichromosomes. Starred arrows indicate cut sites unique to the actively transcribed minichromosome pεHS2B. Shaded ellipses represent the positions of nucleosomes present for both types of minichromosomes. The dotted ellipse represents a nucleosome detected in chromatin from pεB but not in pεHS2B. MNase concentrations were 0 (lanes 1 and 13), 8 (lanes 2 and 12), 20 (lane 3), 50 (lane 11), 12 (lanes 4 and 10), 80 (lanes 5 and 9), and (lanes 6 and 8) 16 μg/ml. Lane 7, 1-kb ladder marker DNA (M).

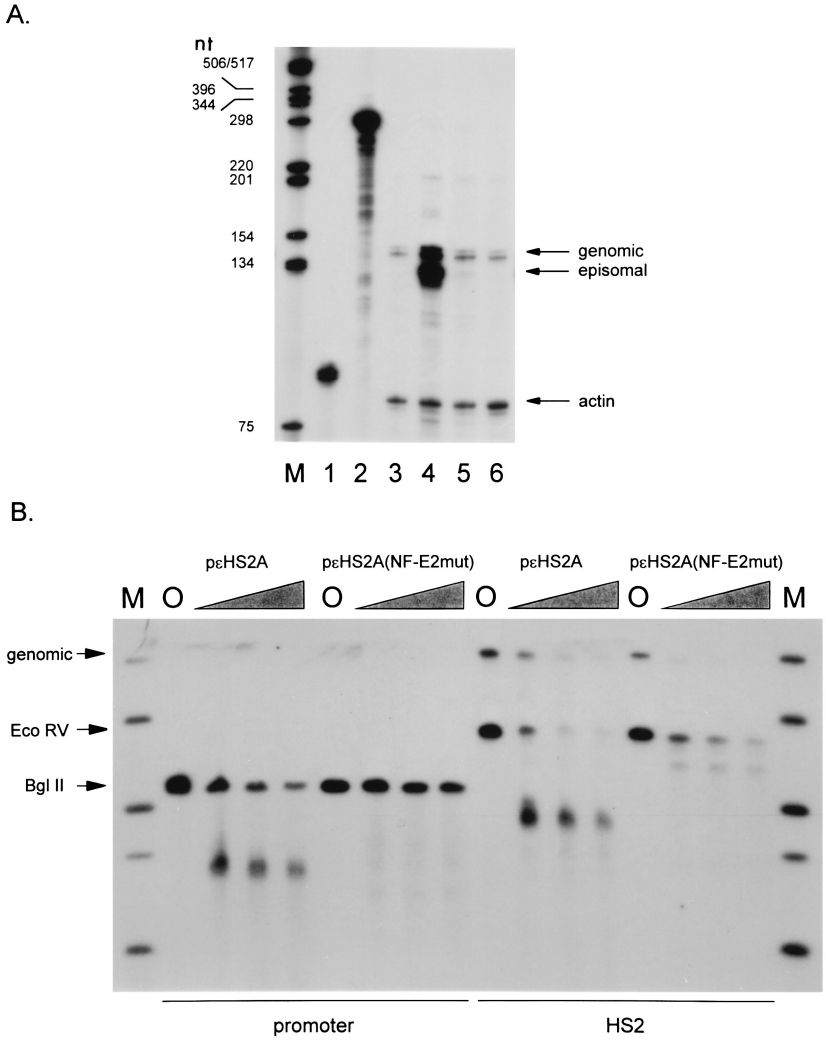

FIG. 8. The HS2 NF-E2 site is critical for remodeling the promoter of the ε-globin gene and for transcriptional activation of the gene. (A) RNase protection analysis was performed on RNA isolated from K562 clones carrying pεHS2A or pεHS2A(NF-E2mut) as described in the legend to Fig. 2. The 149-nt band protected by transcripts from the endogenous ε-globin gene is indicated by the upper arrow. The lower arrow indicates the 136-nt band protected by minichromosomal ε-globin transcripts. An actin probe was used as a load control. Lanes: M, ^{32}P-labeled 1-kb marker fragments; 1, actin probe; 2, ε-globin probe; 3, pεA; 4, pεHS2A; 5 and 6, individual clones of pεHS2A(NF-E2mut). (B) Nuclei from K562 clones carrying pεHS2A or pεHS2A(NF-E2mut) were digested for 10 min at 25°C with no DNase (lanes O) or increasing concentrations of DNase I (12, 19, and 25 μg/ml; indicated by the wedges). The DNA was purified, cut as for Fig. 5 with either *Bgl*II to analyze the promoter region or *Eco*RV to analyze HS2 (parent fragment indicated), and then subjected to gel electrophoresis and Southern blotting. The blot was hybridized with the *Xba*I-to-*Eco*RV probe (Fig. 4A). Lane M contained ^{32}P-labeled 1-kb marker DNA.

However, when the β-globin μLCR or HS2 is included, activation of ε-globin transcription occurs and the nucleosome positioned over the proximal promoter is lost. These results are consistent with the idea that transcription factors can exclude a canonical nucleosome from DNA and are summarized in Fig. 9.

Long-range and promoter chromatin structure of the ε-globin gene. The ε-globin gene in minichromosomes is covered with an array of positioned nucleosomes from −800 to +600 bp relative to the promoter. This strong positioning pattern weakens significantly upstream of −800, and in other experiments we observed occasional very weak cleavage sites for DNase I between this position and the end of the ε-globin fragment in the minichromosomes which extends to −2004 (38). Limited organized chromatin domains similar to this exist surrounding some regulated genes in yeast cells (35). Although positive and negative *cis*-acting sequences in the region −250 to −800 that affect transcription of the ε-globin gene have been described (12, 61, 76), we detected no structural alterations in chromatin, either with MNase or with DNase I, that might shed light on their mechanism of action.

Arrays of positioned nucleosomes have also been observed over the chick and murine β-globin genes (7, 51). As in our study, a gap in the regular disposition of nucleosomes has been observed at the transcriptionally active chick β-globin promoter, while in murine MEL cells, interruption in the array over 500 nt is constitutively present (7, 51). This gap is larger than that observed in the present study or at the chick β-globin promoter, perhaps because a small chemical probe, methidiumpropyl-EDTA-Fe(II), was used rather than a relatively bulky nuclease. We do not believe that the smaller organized domain and limited nucleosome alteration that we observe reflect the environment of the minichromosome, since in vivo studies of DNase I sensitivity of the chromosomal ε-globin gene in K562 cells reveal a highly nuclease sensitive region encompassing only the 200 bp of the proximal promoter (11).

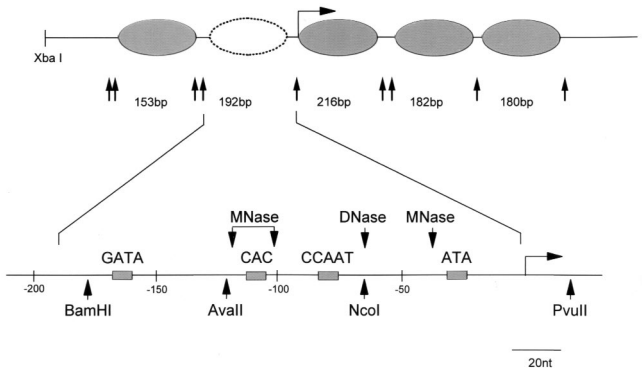

FIG. 9. Disruption of a nucleosome at the proximal promoter of the ε-globin gene when the gene is activated for transcription. The illustration summarizes the results shown in Fig. 4, 5, and 7. It depicts the positioned nucleosomes along the body of the ε-globin gene. The expanded portion illustrates sequences occluded by a nucleosome in the inactive state but which become accessible when the gene is activated for transcription. Sites of cleavage by nucleases in the active state and recognition motifs for transcription factors in this region of the promoter are shown.

Taken together, these and other results indicate that positioned nucleosomes can affect chromatin function (4, 27, 44, 52, 66).

Dependence of promoter chromatin structure on LCR sequences. Even in the erythroid environment of K562 cells, the ε-globin promoter depended on LCR sequences to remain nucleosome free. The LCR dependence is consistent with the behavior of the chromosomal globin genes (19, 21, 75) and of the chick β-globin promoter in transgenic mice and in synthetic nuclei (6, 16, 65). Results of the experiments using synthetic nuclei support a model for transcriptional activation in which passage of a replication fork is followed by a period during which transcription factors can bind to newly replicated DNA and interfere with nucleosome deposition (6). While transcription factors may bind transiently to the ε-globin promoter following replication of the minichromosomes, the formation of a nucleoprotein complex which can prevent the deposition of a nucleosome at the promoter requires the participation of distant LCR sequences, suggesting that there is a physical interaction between LCR and promoter nucleoprotein complexes.

The native LCR HSs are nonnucleosomal. This property has been reproduced in transgenic model systems using the chicken β/ε-globin enhancer and HS2 and HS4 of the human β-globin LCR (10, 57, 71). The feature is also reproduced by the minichromosome LCR sequences. Removal of a nucleosome from the chick β/ε-globin enhancer required the presence of a *cis*-linked promoter, supporting the idea of mutual interaction of the promoter and enhancer (65). In contrast, human HS4 and HS2 may be able to form independently in an erythroid environment (49, 57). Other data indicate that HS2 can form in minichromosomes in the absence of a globin gene (50). These observations are consistent with the LCR being the dominant regulatory element in both minichromosomes and chromosomes.

When ε-globin transcription was active, virtually all minichromosome molecules were cleaved by DNase I at both the promoter and HS2. These results are consistent with a looping model for enhancer-promoter interaction in which these regulatory elements physically interact with one another (28). Simultaneous sensitivity to DNase I of two widely separated regulatory sequences of the mouse serum albumin gene has been interpreted as evidence that they may participate in a specific structure required for high-level transcription (48). The promoter and HS2 on the minichromosomes are sensitive to similar levels of DNase I, in contrast to erythroid cells, in which HS1 to HS4 of the LCR are more sensitive to DNase than are the globin promoters (33, 78). In studies with K562 cells, we have observed that the endogenous HS2 is as sensitive to DNase I as the HS2 in the minichromosomes (38). This finding suggests that the ε-globin promoter is more sensitive on minichromosomes than in the genome. Our interpretation is that most or all of the minichromosome promoters are in a sensitive configuration and are actively transcribing, while in a population of K562 cells, fewer ε-globin promoters than LCRs are sensitive. This explanation is consistent with the lower level of access to restriction enzymes for K562 cells than for actively transcribing minichromosomes (Table 1) and may explain clonal variation in levels of the endogenous ε-globin gene transcript in K562 cells.

Role of NF-E2 in HS formation and promoter activation. We showed that HS2 is sufficient for activation of the ε-globin promoter and that its NF-E2 sites are required for this function as well as for formation of HS2 itself. Two recent studies have addressed the formation of HSs in globin chromatin (8, 71). Both used constructs stably integrated into erythroid cell lines and found that formation of the hypersensitive chromatin domain required both an AP-1/NF-E2 motif and GATA-binding sites. In the first study, HS4 formation was investigated in a construct with HS3 present as a control, but no globin promoter (71). Formation of HS4 was severely impaired but not abolished when either the AP-1/NF-E2 site or individual GATA sites were mutated. In our study, it is possible that a much less sensitive site forms at HS2, although this seems unlikely since we also did not detect transcription of the ε-globin gene with the mutated HS2. Formation of the chick β/ε-globin enhancer HS was studied in a construct containing the chick β-globin gene (8). Both the NF-E2 and GATA motifs contributed to the accessibility of the enhancer, and the data were interpreted to mean that binding to these sites increased the probability that the enhancer would be nucleosome free. Our observations and those for HS4 formation are not inconsistent with this interpretation, and studies with restriction enzyme access could be used to verify it.

AP-1/NF-E2 mutation did not completely eliminate HS formation for HS4 or the chick β/ε-globin enhancer, and it was concluded that the GATA sites participated in HS formation either cooperatively (71) or additively (8). In contrast, we demonstrated an essential role for NF-E2 sites in HS2 formation. Several factors might explain this difference. First, the role of NF-E2 motifs may differ among the LCR HSs, particularly since only HS2 has duplicated sites. Recent in vitro studies using an HS2–β-globin template show that NF-E2 alone is sufficient to disrupt a nucleosome assembled over HS2 and to form HS2 and, furthermore, that GATA-1 cannot bind to its HS2 in a nucleosomal template until NF-E2 has bound and presumably disrupted the nucleosome (5). Second, the influence of HS3 or of a globin promoter on the formation of a neighboring HS is not clear. Third, and possibly of more importance, is a fundamental difference between the model systems. The minichromosomal HS2–ε-globin gene is essentially a single-copy transgene integrated each time in the same chromosomal position. The chromosomal environment is probably analogous to the decondensed state of the globin locus in erythroid cells, and the transgene is probably not subject to position variegation effects. What we assay is the promoter-activating function of an LCR HS in chromatin. It is not clear if we are asking the HS to perform all of the functions of an

LCR. The data on whether a single copy of HS2 can function as an LCR in transgenic mice are conflicting (13, 24).

In our studies, we looked at promoter remodeling and transcriptional activation of a linked gene as well as formation of HS2 itself. Since we observed that HS2 (or μLCR) is required for transcription activation of the ε-globin gene, the lack of formation of HS2 when the duplicated NF-E2 sites are mutated is presumably responsible for the absence of promoter remodeling and transcription. The NF-E2 sites of HS2 are required for enhancer activity on linked globin genes in transiently transfected K562 cells and in stably transfected MEL cells (23, 36, 55, 64, 73, 74). Several studies have shown that in transgenic mice, deletion or mutation of the NF-E2 sites of HS2 severely reduced expression of the linked β-globin gene (13, 14, 23, 47, 73, 74). Mutation of GATA or CACCC sites in HS2 had less deleterious effects, suggesting that multiple factor-binding sites cooperate for promoter-enhancer communication. Our results that NF-E2 is required do not rule out a role for other sites. Interestingly, it was shown recently that NF-E2 may interact directly with elements of the transcriptional machinery (1). Further experiments will be required to determine the role of other factors and of transcription per se in promoter remodeling in minichromosomes.

A mechanism for LCR activation. We favor the view that the LCR HSs form a complex (23). This complex might be able to form in more than one way as a result of the arrangement of binding sites in each LCR HS, with potentially differing consequences for function. This might explain the finding that deletion of either HS2 or HS3 from the mouse genome has little effect on globin gene expression (29, 43). How does an LCR complex interact with a promoter? A likely mechanism is progressive interaction with looping out of the intervening DNA (80). For accessibility reasons, and consistent with our findings, we believe that a single HS and a single promoter would interact and result in promoter remodeling and transcription activation. However, other HSs might have the potential to interact with each globin promoter, helping to explain the results for the HS2 and HS3 genomic deletions. The interactions between particular LCR HSs and globin promoters might differ because of the different arrangements of protein factor-binding sites (or other stage-specific factors). We have shown that HS2 keeps the ε-globin promoter free of nucleosomes and activates transcription in a chromatin environment. We have begun to study the role of factors that bind HS2 and the promoter in the structural transition and in gene activation. We anticipate that this approach will be useful in understanding further how an LCR activates a gene for transcription.

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