Interaction of NF-E2 in the Human β-Globin Locus Control Region before Chromatin Remodeling*

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When transcription is initiated under repressive conditions, such as when chromatin is packed together, binding followed by the functioning of key components in the transcriptional apparatus should be appropriately facilitated in the chromatin architecture. We provide evidence that the erythroid-specific enhancer-binding protein NF-E2 interacts with the cognate motif in the human β-globin locus control region in a repressive state. The nucleosome containing the NF-E2-binding site showed characteristic rotational and translational phases in vitro. The binding site had less affinity to the histone octamers than nearby regions while showing greater accessibility to DNase I and micrococcal nuclease. Furthermore, the motif was recognized by the exogenous NF-E2 protein expressed in HeLa cells, which have a repressive state of chromatin at the β-globin locus, as shown by ligation-mediated PCR and chromatin immunoprecipitation assay. These lines of evidence indicate that NF-E2 interacts with the cognate motif on the nucleosome before chromatin is remodeled.

The human β-globin locus (β-LCR) spans 70 kb and contains five developmentally regulated β-like globin genes, 5'-ε, Gγ, Aγ, δ, and β-3', in that order. The expression of these genes is restricted to erythroid tissues and regulated in a developmental stage-specific manner (1). The human β-LCR confers high level and tissue-specific expression to the β-globin genes and is marked by erythroid-specific, DNase I-hypersensitive sites (HS-1 to HS-4), which lie 6–18 kb upstream of the β-globin gene and function together (2). In thalassemia DNA with large deletions encompassing the LCR and the upstream sequence, expression of the downstream globin genes is abolished, and the chromatin structure of the locus becomes resistant to DNase I cleavage (3), which suggests that the LCR mediates activation of transcription of the genes at different stages in development in conjunction with modification of the chromatin structure. Transgenic mouse studies have also indicated that the LCR provides an open, accessible chromatin structure regardless of where on the genome the transgenic gene is integrated and can confer position-independent expression to the integrated globin gene (4). In addition to its enhancer activity, the LCR is important for establishing the timing of DNA replication of the β-globin locus during the S phase of the cell cycle (3, 5, 6). A central question in developmental biology is how enhancers activate gene transcription in a tissue- and developmental stage-specific fashion, a complex process that takes place in the chromatin environment of the nucleus.

Most efforts to elucidate the function of the LCR have focused on identifying the transcription factors, and many of the erythroid-specific transcription factors with conserved binding sites in the LCR have been clarified. These include NF-E2 (7, 8), GATA-1 (9–11), and erythroid Kruppel-like factor (12). Among them, NF-E2 plays a key role in the function of the LCR. NF-E2, a bZIP transcription factor, is a heterodimer of 45- and 18-kDa subunits. Expression of NF-E2 p45 is primarily restricted to erythroid cells, whereas NF-E2 p18 appears to be ubiquitously expressed. There appears to be considerable functional redundancy among polypeptides that recognize NF-E2 sites, and whereas the p18–p45 NF-E2 dimer itself may be required to activate globin gene expression, other species may be able to participate in the formation of the HSs (13). 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 (14–18). From a series of studies using transgenic mice with the β-globin yeast artificial chromosome, it was suggested that the deletion of HS elements markedly reduced the expression of all of the globin genes at all developmental stages accompanying the malformation of DNase I hypersensitivity in the LCR (19). In addition, it was also shown that the protein Bach1, which heterodimerizes with the p18 NF-E2 subunit and interacts with the Maf-responsive element (MARE) or NF-E2-binding site sequences at HS2, HS3, and HS4, is able to cross-link HS sites, thereby dropping out intervening DNA regions (20). These results suggest that a series of protein-protein and protein-DNA interactions establishes the formation of a larger LCR complex (21). Furthermore, NF-E2 is critically involved in remodeling the nucleosome structure over the HS2 region, where it interacts with the tandem MARE sites (22, 23). In contrast with the study supporting a role for NF-E2 in β-globin gene expression, p45 NF-E2 null mice had nearly normal levels of β-globin protein (24), and deletion of HS2 had no significant effect on the timing or extent of expression of the gene (25). Recently, it was reported (26, 27) that the chromatin-opening function of the LCR may not be the primary activity at the endogenous mouse or human globin locus, because the LCR can be deleted without affecting general sensitivity to DNase I. Although the mechanism of long range transactivation by the LCR is still poorly understood, it is true that the LCR is required for conferring high levels of globin gene expression throughout erythroid development.

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‡ The abbreviations used are: β-LCR, β-globin locus control region; HS, DNase I-hypersensitive site; MARE, Maf-responsive element; MNU, micrococcal nuclease; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; LM-PCR, ligation-mediated PCR; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; ERα, estrogen receptor α; DMS, dimethyl sulfate.
Previously, we determined the nucleosome phases around HS2, and we found that there are two major nucleosome positions in the HS2 region in vivo (28). The -60 to +85 phase had a nucleosome dyad axis at its center, where the tandem MARE sites were located, and included the binding sites of the erythropoietin-specific transcription factors HS2NF5 and GATA-1. This phase was shown to be the only phase in HeLa cells, and thus, this is the original state where NF-E2 first interacts with the cognate motif. In contrast, the -100 to +85 phase and several other minor phases were present in erythroid K562 cells. In this state, the region containing HS2NF5 and GATA-1-binding sites was partially or completely open resulting in an increased accessibility to these factors. Furthermore, we also reported that the enhancer activity at HS2 of the human β-LCR can be modulated by the curved DNA located at a distance of two nucleosomes from HS2 and regulates nearby nucleosome phases as a key nucleosome (29). In this paper, we concentrated on characterizing the nucleosome structure over HS2, in particular NF-E2 binding, and we demonstrated that NF-E2 interacts with the cognate motif on the nucleosome before chromatin is remodeled.

### EXPERIMENTAL PROCEDURES

#### Chemicals—Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Cell culture materials were obtained from Invitrogen. Other chemicals used were of the highest quality commercially available and were purchased from Sigma.

#### Reconstitution of the Nucleosomes in Vitro—Nucleosome core particles were prepared from chicken erythrocytes as described previously (28). After the preparation of core particles by gel filtration through Sepharose CL-6B (Amersham Biosciences), the histone octamers were prepared according to the method of Luger et al. (30). Briefly, the core particles were applied to a hydroxyapatite column equilibrated with 0.6 M NaCl, 0.25 mM PMSF, and 0.5 mM dithiothreitol and then with 65 mM NaCl, 50 mM Na/PO4, 0.25 mM PMSF, and 0.5 mM dithiothreitol. The octamer was eluted with 2.5 mM NaCl, 50 mM Na/PO4 (pH 6.0), 0.25 mM PMSF, and 0.5 mM dithiothreitol and dialyzed against 10 mM Tris-HCl (pH 7.5), 2 mM NaCl, and 5 mM 2-mercaptoethanol. Nucleosomes were reconstituted onto DNA fragments either by exchanging them with the DNA from the histone core particles or by dialysis with the histone octamer.

#### Micrococcal Nuclease (MNase) Digestion Assay—The nucleosomes were reconstituted in vitro using the end-labeled DNA fragments corresponding to the -100 to +45 and -60 to +85 nucleosome phases, respectively. MNase digestion was performed at a final concentration of 0.025 units/ml at 25 °C for 2 and 5 min. DNA was purified and electrophoresed on 4% polyacrylamide gels in 40 mM Tris acetate, 1 mM EDTA, and 5% glycerol.

#### Southern Blotting—The nucleosomes were reconstituted in vitro using a DNA fragment containing the entire HS2 region (−10,989 to −10,769 relative to the cap site of the e-globin gene) and treated with MNase as described previously (28). The -146-bp fragments were recovered from 6% polyacrylamide gels and digested with restriction enzymes. DNA was electrophoresed on an 8% polyacrylamide gel and transferred onto the membrane. Hybridization and detection were performed as described previously (29). The probe was the same DNA fragment used in nucleosome reconstitution.

#### Computer Analysis of the Three-dimensional Structure—The three-dimensional structure of the DNA curvature was predicted with TRIF software (sgjsl.weizmann.ac.il/usr/users/Curvature) as described (31).

#### Electrophoretic Mobility Shift Assay (EMSAs)—Nucleosomes were reconstituted with 32P-labeled or fluorescein isothiocyanate-labeled DNA in the presence or absence of competitor DNA by dialysis as described above. Aliquots (10 µl) of the reconstituted nucleosomes were suspended in 20 µl of 16 mM Hepes (pH 7.5), 150 mM KCl, 1% (v/v) glycerol, 1.6 mM MgCl2, 0.8 mM dithiothreitol, 0.4 mM PMSF, 1 mM EDTA, 0.8 mg/ml of bovine serum albumin, 0.06 mg/ml of poly(dI-dC), and 0.04% SDS. These mixtures were electrophoresed on 4% polyacrylamide gels in 40 mM Tris acetate, 1 mM EDTA, and 5% glycerol.

#### Hydroxyl Radical Footprinting—Nucleosomes reconstituted with the end-labeled DNA fragments corresponding to the -60 to +85 nucleosome phase were cleaved with the hydroxyl radical as described (32, 33). After cleavage, the footprinting reaction was initiated by adding an Fe(II) EDTA solution (10 µl), which was prepared immediately before use by mixing equal volumes of freshly prepared 125 mM Fe(II) and 250 mM EDTA, and 28 mM sodium ascorbate (10 µl) and 0.84% hydrogen peroxide (10 µl) on the inner wall of the tube containing the reconstituted nucleosomes. The reaction was allowed to run for 2 min and then quenched by adding 0.1 M thioarsenite (10 µl), 2 mM EDTA (2 µl), TSTA (10 µg), and 0.5% SDS (30 µl). The DNA was extracted with phenol, recovered by ethanol precipitation, and resolved on 8% denaturing polyacrylamide gels.

#### DNase I Footprinting—Nucleosomes reconstituted with end-labeled DNA fragments corresponding to the -60 to +85 nucleosome phase were digested with DNase I as described previously (29). After purification, DNA was resolved on a 6% polyacrylamide, 7 M urea gel under denaturing conditions.

#### Ligation-mediated PCR (LM-PCR)—LM-PCRs were carried out as described (34). Briefly, HeLa cells were transfected with or without the plasmids containing NF-E2 subunits, p18 or p45 (29), and cultured in minimum Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator under a 5% CO2 atmosphere for 24 h. Cells were treated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA, rinsed with PBS twice, and then incubated with 0.2% dimethyl sulfate at 20 °C for 10 min. The reaction was stopped by addition of 1.5 M sodium acetate (pH 7.0) and 1 mM mercaptoethanol followed by washing with PBS twice. The cells were then suspended in 1% SDS and 0.1 M NaHCO3, and heated at 65 °C for 4 h to unite the cross-links. After preparation of the genomic DNA, piperidine was added at 90 °C for 30 min to ensure strand breakage. DNA was then purified and used for first strand synthesis extending up to the cleaved sites using the primer (5'-TTACAGCTCAGTCCTCTCTC'-3'). The synthesized DNA was ligated to the double-stranded linker oligonucleotides (5'-GAATTCAGATC-3' and 5'-GGGTTAGACCGGAGATCTGATCT-3') and amplified by PCR. The PCR primers were 5'-TCCCTCTCCCTCACCTCCTCC-3' and 5'-GGGTTAGACCGGAGATCTGATCT-3'. The conditions for PCR were 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. After amplification, the PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide. DNA from the samples was purified and subjected to PCR. The primers 5'-TCC-3' and 5'-AGG-3' were used for amplification of a 197-bp fragment (positions 779 to 976) on the inner wall of the tube containing the reconstituted nucleosomes.

#### Chromatin Immunoprecipitation Assay (ChIP Assay)—The assay followed a procedure published previously (35, 36). K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidiﬁed incubator under a 5% CO2 atmosphere. After the introduction of plasmids, HeLa cells were cultured as mentioned above. Cells were then treated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA before being rinsed with PBS twice. The cells were lysed by adding 150 µl of lysis buffer (25 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA, and 1 mM PMSF) and were allowed to incubate on ice for 10 min. Sonication was followed by centrifugation, and the supernatants containing soluble chromatin were collected after digestion with or without MNase. The chromatin sample was cleared with salmon sperm DNA and protein A-Sepharose for 2 h before overnight incubation with 10 µg of anti-NF-E2 (p45) polyclonal antibody (Santa Cruz Biotechnology). Fifty microliters of 50% protein A-Sepharose was added to the samples and incubation carried out at 4 °C for 2 h followed by centrifugation. The pellets were washed three times, once in 150 µl of wash buffer 1 (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% SDS, and 2 mM EDTA), once with 150 µl of wash buffer 2 (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), and once in 150 µl of wash buffer 3 (10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA). Each wash was performed at 4 °C for 10 min. Next, the samples were washed 3 times with 150 µl of TE buffer. The immunocomplexes were then eluted off with 1% SDS and 0.1 M NaHCO3 and heated at 65 °C for 4 h to reverse the cross-links. The cross-links of DNA input samples were reversed in a similar manner. DNA from the samples was purified and subjected to PCR. The primers 5'-GGTCTAGTCGACCCCCCGCTC-3' and 5'-AGGTTAGATCTGATCTCTC-3' were used for amplification of a 197-bp fragment (positions 969 to 1165) on the inner wall of the tube containing the reconstituted nucleosomes.
RESULTS

Translational Phase at HS2—The HS2 region is very important for β-globin gene expression and contains many transcription factor binding sites, NF-E2 and GATA-1, for example (Fig. 1A). The binding of these factors to their cognate motifs is critical in regulating the gene expression, suggesting that it is crucial to investigate the status of these cis-elements around the HS2 region. We have previously reported (28) that there were two major nucleosome phases at HS2 in vitro. One is the −60 to +85 phase which has a nucleosome dyad axis at its center, where the tandem MAREs are located, and the other is the −100 to +45 phase. We have also reported that the translational phase of the nucleosome containing the tandem MAREs and the enhancer activity was modulated by the curved DNA located at a distance of two nucleosomes from HS2 (29).

First, we analyzed the translational phase of the nucleosomes at HS2 when the effect from the key nucleosome was absent. As shown in Fig. 1A, we used two end-labeled DNA fragments corresponding to the −100 to +45 and −60 to +85 nucleosome phases, respectively, for reconstitution of the nucleosomes in vitro. After their reconstitution, the nucleosomes were treated with MNase and then resolved on 4% native polyacrylamide gel. Interestingly, the band of the nucleosome containing the 5′-end-labeled DNA fragment corresponding to the −100 to +45 nucleosome phase disappeared immediately after MNase digestion, whereas the bands containing the other DNA fragments remained undigested, indicating that the 5′-region of the DNA fragment corresponding to the −100 to +45 nucleosome phase did not bind to histone octamers but was free because this region was not protected from MNase digestion (Fig. 1B). These results suggest that the nucleosome over the HS2 region bore a propensity to show a specific position. To determine the exact translational phase, the −146-bp core DNA fragments were recovered after MNase digestion from the nucleosomes reconstituted in vitro using a 200-bp DNA fragment containing whole regions of the −100 to +45 and −60 to +85 phases and were analyzed by restriction digestion. As shown in Fig. 1C, Sau96I did not create any digested bands, whereas BspHI and BfaI produced mainly two bands of −70 and 120 bp, respectively, suggesting that the major translational phases were −60 to +85. In BspHI or BfaI digests, some minor bands were observed, indicating that several minor phases were also present in this region as we reported previously (28). The position shown here in the nucleosome reconstituted in vitro was almost identical to the −60 to +85 phase observed as in a repressive state in HeLa cells. The results are summarized in Fig. 1D.

Characterization of the Nucleosome Structure at HS2—Previously, we reported that DNA bend sites around eB-17 and eB-16 showed nucleosome positioning activity with almost unique phases, respectively (28). The positions of the neighboring nucleosomes were abolished after removal of these sites (29). Here the HS2 region also showed several preferential nucleosome positions (Fig. 1), although typical curved DNA was not found by circular permutation assay (28). In contrast, TRIP 1.00 computer software (31) predicted significant DNA curvatures not only in the regions containing eB-17 and eB-16 but also at HS2, although the HS2 region showed right-handed superhelix, whereas both eB-17 and eB-16 showed left-handed superhelix (middle in Fig. 2). The B, NF30, and E subregions in eB-17, HS2, and eB-16, respectively, contained curved DNA. When we performed a competition assay (bottom in Fig. 2), the oligonucleotides containing these subregions did not compete strongly in nucleosome formation, whereas those from the immediate vicinity (C in eB-17, B and C in HS2, and D in eB-16) showed the strongest competition in each region.

These results suggest that the NF30 fragment in the HS2 region that had curved DNA with right-handed superhelicity bound to the histone octamer less tightly than the other subregions.

We next examined the accessibility to MNase of these fragments in the nucleosome (Fig. 3). We subcloned HS2 fragments, A–D and NF30, which had been used as competitors (Fig. 2), into pBluescript SK+ and reconstituted nucleosomes in vitro using the 305-bp fragments from these subclones as probes. As shown in Fig. 3A, all constructs, including the DNA fragment without an insert, showed similar band intensities at the mononucleosome position, indicating that there was little, if any, difference in nucleosome formation among these constructs. However, when the nucleosomes reconstituted in vitro were digested with MNase and the −146-bp core DNA fragments recovered were analyzed on a 6% polyacrylamide gel, only the nucleosome containing the NF30 fragment showed little recovery of the core DNA (shown by an arrowhead in Fig. 3B), which was even less than that from the nucleosome with a pBluescript fragment. Because the DNA fragments used in nucleosome reconstitution in this experiment had the same DNA sequence except for the 30-bp insert regions and showed
FIG. 2. DNA structure and affinity for the histone octamer of the fragments in eB-17, HS2, and eB-16. Upper panel, the nucleosome phases around HS2 of the human β-LCR. The phases are indicated by the circles as reported before (28). The open and solid boxes show the sites of curved DNA determined by circular permutation assay and the bend centers, respectively (28). The probes and the oligonucleotides are the DNA fragments and the oligonucleotides used for the nucleosome reconstitution and for the competition assay as competitors, respectively. The probes used are as follows: eB-17, /H11002 11,498 to /H11002 11,250; HS2, /H11002 10,989 to /H11002 10,769; eB-16, /H11002 10,609 to /H11002 10,408. The positions of the oligonucleotides are as follows: for eB-17: A, /H11002 11,369 to /H11002 11,340; B, /H11002 11,339 to /H11002 11,310; C, /H11002 11,309 to /H11002 11,280; D, /H11002 11,279 to /H11002 11,250. For HS2: A, /H11002 10,918 to /H11002 10,889; B, /H11002 10,888 to /H11002 10,859; NF30, /H11002 10,858 to /H11002 10,829; C, /H11002 10,828 to /H11002 10,799; D, /H11002 10,798 to /H11002 10,769. For eB-16: A, /H11002 10,597 to /H11002 10,568; B, /H11002 10,567 to /H11002 10,538; C, /H11002 10,537 to /H11002 10,508; D, /H11002 10,507 to /H11002 10,478; E, /H11002 10,477 to /H11002 10,448; and F, /H11002 10,447 to /H11002 10,418. AT, the control oligonucleotides, (dA)30 and (dT)30.

Middle panel, the results of curvature analysis for eB-17 and eB-16 and HS2 using TRIF software. Bottom panel, the results of EMSA using the nucleosomes reconstituted in vitro in the presence of competitors. The positions of the mononucleosome and the free DNA probe are indicated as Nuc and Probe, respectively.

FIG. 3. Sensitivity of cloned DNA fragments from the HS2 region to MNase after nucleosome formation. A, EMSA of the nucleosomes. B, sensitivity to MNase. The ~146-bp core fragments generated after MNase digestion are indicated by arrows. pBSK, DNA fragments amplified from pBluescript SK. The positions of the subcloned inserts are: A, −10,918 to −10,889; B, −10,888 to −10,859; NF30, −10,858 to −10,829; C, −10,828 to −10,799; D, −10,798 to −10,769. Mononuc, mononucleosome.
nucleosome formation at the same level, the difference in MNase sensitivity should be attributable to the insert. These results suggest that the NF-30 fragment containing the tandem MAREs was more accessible to MNase on the nucleosome and did not bind to the histone octamer tightly.

**Rotational Phase at HS2**—To confirm whether the NF30 region has a similar structure on the nucleosome at HS2, we first determined the rotational phase of the nucleosome reconstituted with the DNA fragment corresponding to the $\sim-60$ to $+85$ phase. Fig. 4 shows the results of hydroxyl radical footprinting, where a 10-bp ladder pattern characteristic of the rotational phases of nucleosomes was observed. In the NF30 region, some of the nucleotides at each NF-E2-binding site showed accessibility to hydroxyl radicals, indicating that a part of the NF-E2-binding site faces outside of the histone octamer. A simple calculation based on hydroxyl radical footprinting results (Fig. 4) predicted that DNA wrapped around the histone core had an average number of 10.2 bp per helical turn, which agreed with the corresponding number from the nucleosomes in general (38). Next, we performed DNase I footprinting to analyze the accessibility to nuclease of the tandem MAREs on the nucleosome. The periodic 10-bp cleavage sites shown as open triangles indicate the rotational nucleosome phases. Gel patterns of the antisense strand of HS2 after LM-PCR with DNA from the NF-E2-expressing or non-expressing cells are shown in Fig. 5. Note that the antisense strand exhibits more prominent footprinting patterns than the sense strand (39). The positions of guanine nucleotides, the targets of DMS modification, matched very well those of the cleaved bands except for the region at the tandem MAREs in cells expressing NF-E2. The region showed significant protection against methylation. Ikuta and Kan (40) and Reddy and Shen (41) reported that the

**NF-E2 Binding to Its Cognate Motifs on the Nucleosome in Vivo**—To confirm that NF-E2 can bind to its cognate motifs while it is located on the nucleosome, we performed a DMS LM-PCR using non-erythroid HeLa cells. We have already determined nucleosome positions around the HS2 region in active and inactive states using K562 and HeLa cells, respectively (28). HeLa cells do not express the $\beta$-like globin genes and exhibit a packed chromatin structure at this locus as revealed by the absence of the erythroid-specific DNase I-hypersensitive sites. The translational phase at HS2 in HeLa cells mainly showed the $-60$ to $+85$ phase which is the same as observed in the nucleosome reconstituted in vitro (Fig. 1). After introducing NF-E2 expression plasmids in HeLa cells, the binding of NF-E2 to its cognate motif on the nucleosome in vivo was analyzed by genomic DMS footprinting followed by LM-PCR. Gel patterns of the antisense strand of HS2 after LM-PCR with DNA from the NF-E2-expressing or non-expressing cells are shown in Fig. 6. Note that the antisense strand exhibits more prominent footprinting patterns than the sense strand (39). The positions of guanine nucleotides, the targets of DMS modification, matched very well those of the cleaved bands except for the region at the tandem MAREs in cells expressing NF-E2. The region showed significant protection against methylation. Ikuta and Kan (40) and Reddy and Shen (41) reported that the
binding of NF-E2 to its motif generated hyper-reactive patterns of guanine nucleotides within and flanking the tandem MAREs in K562 cells. Previously, we also reported that inducing NF-E2 expression in HeLa cells generated an increased DNase I sensitivity at the MAREs perhaps corresponding to the changes in the chromatin structure (29). As shown in Fig. 6, we also observed hyper-reactive patterns of guanine nucleotides within and flanking the tandem MAREs in HeLa cells when NF-E2 expression was induced (indicated as closed circles). These results suggest that NF-E2 can interact with its binding motifs on the nucleosome structure in HeLa cells.

To confirm the ability of NF-E2 to bind the nucleosome, we next performed a ChIP assay. After the expression of NF-E2 in HeLa cells, protein-DNA cross-linking was performed by incubating the sample with formaldehyde, and the complexes that formed were fragmented by sonication. The NF-E2-containing complexes were recovered by immunoprecipitation using anti-p45 antibody, and the DNA fragments that bound with NF-E2 were prepared after release from the fixation. The HS2 region was then amplified by PCR using the recovered DNA fragments as templates to identify whether the HS2 region was included in these fragments or not. A region from the ERα gene promoter, which lacks MAREs, was used as a negative control. Based on our previous study on the nucleosome structure in this region (42), PCR was designed to amplify the region within a nucleosome phase. As shown in Fig. 7A, the HS2 region was amplified from K562 cells, which express endogenous NF-E2, and also recovered from the HeLa cells expressing NF-E2. Apparently, no bands were amplified from the original HeLa cells, which do not express NF-E2. We then confirmed that the recovered HS2 region from HeLa cells was derived from the nucleosome. When the cell lysate was digested with MNase after cross-linking, the protein-DNA complexes became as small as mononucleosomes suggesting that most linker regions were digested, whereas nucleosome regions were retained under these conditions (data not shown). When these samples were used for ChIP assay (Fig. 7B), the HS2 region was amplified by PCR even after digestion with MNase, and the quantity of the amplified band differed little between the samples with or without digestion. The control fragment from the ERα gene, on the other hand, was not amplified from the samples except for the input because of the absence of MAREs. This indicates that the recovered HS2 fragments were derived from the nucleosome. These results revealed that NF-E2 can bind to its cognate motifs even if the binding site is located on the nucleosome.

**DISCUSSION**

**What Happens When NF-E2 Binds to the MAREs?**—The central role of the human β-LCR is to regulate and enhance β-globin gene expression in a tissue- and stage-specific manner and is mediated by specific transcription factors (43). Two mechanisms were proposed for the activation of globin gene expression. In the dominant chromatin opening model, regulation of the expression of individual genes is autonomous and is dependent on the state of transcription factors during development (44). In this model, the LCR is simply responsible for creating a decondensed and favorable chromatin structure in which the globin gene promoters can interact with stage-specific transcription factors. In the mutual interaction model, on
the other hand, the LCR is physically in contact with the individual promoters and activates them sequentially, switching expression of the genes in response to stage-specific factors (45, 46). The transcription factors that bind to LCR HSs and globin promoters can homo- and heterodimerize (47–49) and can interact with TAFII130 or cAMP-response element-binding protein/p300, components of the transcriptional machinery (50–53). Some transcription factors function by altering the chromatin structure. Erythroid Krüppel-like factor binds to SWI/SNF remodeling complex after acetylation and induces adult-type \( \beta \)-globin gene expression (54). NF-E2 is also involved in the ATP-dependent nucleosome remodeling process (22, 23, 28). NF-E2 is one of the most important factors for regulating \( \beta \)-globin gene expression and consists of p45 and p18 subunits (7, 8). The p18 subunit, MafK, also heterodimerizes with Bach1 (55), Nrf1 (56), and Nrf2 (57). Interestingly, the Bach1 heterodimer binds to MAREs located in different regulatory elements within the human \( \beta \)-LCR and creates a large looped DNA structure (20), suggesting that this structure is involved in opening chromatin. Recently, it was reported that the chromatin-opening function of the LCR may not be the primary activity in the endogenous mouse or human globin locus (26, 27). The relationship among transcriptional activation, an open chromatin structure, and DNase I-hypersensitive sites has not been completely elucidated, and further study is needed. Nevertheless, it is clear that the binding of NF-E2 to the MAREs at HS of the \( \beta \)-LCR is critical for high level \( \beta \)-globin gene expression.

Levings and Bungert (58) reported that the transcriptional activation process of the \( \beta \)-globin locus is conceptually divided into four steps as follows: (a) generation of a highly accessible LCR holocomplex; (b) recruitment of transcription and chromatin-modifying complexes to the LCR; (c) establishment of chromatin domains permissive for transcription; and (d) transfer of transcription complexes to the globin gene promoters. Forsberg et al. (36) demonstrated that NF-E2 binds directly in vivo to the tandem MAREs at HS2 of the human \( \beta \)-LCR in K562 cells. Previously, we also reported that NF-E2 binds to the tandem MAREs at HS2 of the human \( \beta \)-LCR in vitro and remodels the reconstituted nucleosome containing the tandem MAREs at HS2 in the presence of remodeling factors and ATP (28). From these results, we speculate that NF-E2 first binds directly to the tandem MAREs at HS2 on the chromatin structure, then remodels the chromatin structure in the presence of remodeling factors and ATP, and enhances the globin gene expression. The first step in the binding of NF-E2 to the nucleosome may be rate-limiting and critical for the activation of globin gene expression. Therefore, in this report we focused on this first step and examined whether NF-E2 can bind to the tandem MAREs at HS2 on the chromatin.

**How Does NF-E2 Bind to the MAREs?**—Previously, we found that the nucleosome over HS2 was aligned by a key nucleosome located at a distance of two nucleosomes from HS2, and the enhancer activity can be modulated by changing the distance between the nucleosome over HS2 and the key nucleosome (29). The key nucleosome was marked by curved DNA. These findings suggest that the nucleosome over HS2 is crucial for the enhancer activity.

The nucleosome reconstituted in vitro using only the HS2 fragment showed preferred nucleosome positions, which corresponded mostly to the −60 to +85 phase (Fig. 1). The −60 to +85 phase had a nucleosome dyad axis and tandem MAREs at its center, which was an appropriate position for NF-E2 to bind (29). The advantage of aligning nucleosomes by the key nucleosome may be to reduce deviation in the nucleosome phase at HS2 and to facilitate the interaction of NF-E2 with the MAREs by adjusting the site of interaction at the dyad axis. If a motif is well exposed to an incoming factor because it faces a specific direction as a result of specific nucleosome positioning, protein accessibility should be greatly improved (59). This idea has already been demonstrated in part in a nucleosome containing a thyroid hormone-responsive element (60). The DNA fragment at HS2 showed an affinity to the histone octamer as high as the DNA bend sites, \( \varepsilon \)-B-16 and \( \varepsilon \)-B-17, although the HS2 fragment did not show nucleosome positioning activity like the curved DNAs (28). All the DNA fragments, HS2, \( \varepsilon \)-B-16, and \( \varepsilon \)-B-17, showed almost the same structural characteristics where the sequences adjacent to the 30-mer curved DNA but not the curved DNA sequences themselves had the highest affinity to the histone octamer. The major difference was that HS2 showed a right-handed superhelicity and could not determine nucleosome positions alone, whereas the DNA bend sites, \( \varepsilon \)-B-16 and \( \varepsilon \)-B-17, showed left-handed superhelicity and could determine unique nucleosome positions (Fig. 2). It was reported that right-handed DNA was difficult to incorporate into nucleosomes (61), and that is why the HS2 region did not show a fixed nucleosome position even though it showed a higher affinity to the histone octamer. Moreover, the tandem MAREs showed accessibility to MNase and DNase I on the nucleosome, suggesting that the proteins can interact with the tandem MAREs on the nucleosome (Figs. 3–5). A simple calculation based on hydroxyl radical footprinting (Fig. 4) predicted that the DNA wrapped around the histone core of the in

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**Fig. 7. Binding of exogenous NF-E2 to the tandem MAREs on the chromatin revealed by ChIP assay.** A, samples were prepared from HeLa cells expressing (+) or not expressing (−) NF-E2 and from K562 cells. Templates for PCR are the input chromatin (Input) and the DNA precipitated with (+Ab) or without (−Ab) anti-NF-E2 antibody. B, ChIP assay with MNase digestion. Arrowheads indicate the positions of the PCR products of HS2. The human ERα gene was used as a control.
In vitro reconstituted nucleosome with HS2 had an average number of base pairs per helical turn of 10.2, which agreed with the corresponding number for the nucleosomes in general (38). This suggests that the nucleosome at HS2 is structurally the same as general nucleosomes. Although the central three turns at the dyad axis in the nucleosome have a greater helical density (10.7 bp per turn) than the rest of the nucleosomes, we could not identify a difference in structure at HS2 in Fig. 4. We are currently investigating the structure of the nucleosome over HS2 in more detail.

Increased sensitivity to DNase I at the tandem MAREs on the HS2 nucleosome was observed (Fig. 5), which seemed to be caused by the modification of the nucleosome structure. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. However, it was reported that the chromatin-opening function of the LCR is not related to the formation of DNase I HSs. 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Interaction of NF-E2 in the Human $\beta$-Globin Locus Control Region before Chromatin Remodeling
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