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Erythroid Differentiation of Mouse Erythroleukemia Cells Results in Reorganization of Protein-DNA Complexes in the Mouse β^{maj} Globin Promoter but Not Its Distal Enhancer

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Dimethyl sulfoxide (DMSO) induction of mouse erythroleukemia (MEL) cells represents a well-defined in vitro system of terminal erythroid differentiation. We have studied the molecular mechanisms of transcriptional activation of the mouse β^{maj} globin gene during MEL cell differentiation by analyzing nuclear factor-DNA interactions in vivo at the gene's upstream promoter and a distal enhancer, 5'HS-2. Genomic footprinting data indicate that three motifs, CAC, NF-E2/AP1, and GATA-1, of the 5'HS-2 enhancer are bound with nuclear factors in MEL cells both prior to and after DMSO induction. No obvious conformational change of these nuclear factor-DNA complexes could be detected upon terminal differentiation of MEL cells. On the other hand, DMSO induction of MEL cells leads to the formation of specific nuclear factor-DNA complexes at several transcriptional regulatory elements of the mouse β^{maj} globin upstream promoter. Our genomic footprinting data have interesting implications with respect to the molecular mechanisms of transcriptional regulation and chromatin change of the mouse β^{maj} globin gene during erythroid differentiation.

Murine erythroleukemia (MEL) cells provide an interesting in vitro system for the study of terminal differentiation of cells of the erythroid lineage. These cells were established by Friend virus transformation, and they appear to be arrested at a later stage of erythroblast differentiation (17). Upon treatment with dimethyl sulfoxide (DMSO) or other chemical inducers, MEL cells undergo termination differentiation into mature erythroid cells (references 32 and 33 and references therein). This induced terminal differentiation is accompanied by a series of morphological as well as biochemical changes, including terminal cell division, inositol metabolism, increase in the synthesis of heme and enzymes involved in erythropoiesis, chromatin condensation, and DNA methylation.

Among the intracellular changes of MEL cells upon DMSO induction is the turning on and off of transcription of a number of different mouse genes, the analysis of which should provide insight into the mechanisms of induced differentiation of MEL cells and of erythroid differentiation in general. After culturing in the presence of DMSO for 48 h or longer, transcription of the β^{maj} globin gene of MEL cells is enhanced, with the increases ranging from 5- to 100-fold (references 18, 48, and 54 and references therein). β^{maj} is located at the 3' end of mouse β-like globin gene cluster, and it is the major β-like globin gene expressed predominantly in adult mouse erythroid cells (56).

Transfection experiments using mutant or hybrid gene constructs have demonstrated the existence of multiple transcriptional regulatory elements residing in the upstream promoter region of mouse β^{maj} globin gene (references 7, 8, 12, 61, and 66 and references therein). These elements include a TATA box (−31 to −26), CCAAT box (−77 to −72), and CACC box (−94 to −87) (Fig. 1C). All three elements are required for transcriptional initiation of a transfected mouse β^{maj} globin gene in nonerythroid HeLa cells and in MEL cells before and after DMSO induction (6–8, 41). In addition, the DNA region from −53 to −32 consisting of two tandemly arranged copies of a 10-bp sequence appears to be essential for transcriptional activation of the β^{maj} globin gene in induced MEL cells. This region has been termed DRE (61).

As in human cells (reviewed in references 14, 44, and 59), transcription of β^{maj} and other mouse β-like globin genes during erythroid development is probably regulated by distal enhancer sequences located many kilobases upstream of the gene cluster (37) (Fig. 1A). In human cells, a locus control region (LCR) 6 to 17 kb upstream of the human β-like gene cluster has been implicated in the transcriptional regulation in vivo of different human β-like globin genes. One regulatory region within LCR, 5'HS-2, has been shown to be as nearly efficient as LCR in functioning as an erythroid cell-specific enhancer (reviewed in references 14 and 59). The enhancer function of human 5'HS-2 appears to be modulated by several nuclear factor-binding motifs within the enhancer (5, 27, 42, 50, 62). These motifs include (i) NF-E2/AP1, which consists of two tandemly arranged consensus binding sequences of transcription factor AP1 and is recognized by transcription factor NF-E2 in nuclear extracts or living cells of erythroid origin (references 50 and 51 and references therein); (ii) GATA-1, which binds a nuclear factor whose expression is restricted to erythroid cells and monocytes (reviewed in reference 44), and (iii) GT-I, which is the binding site of a ubiquitous nuclear factor(s) (27, 50, 62). Another motif, termed CAC (Fig. 1A), is not required for 5'HS-2 enhancer function in transfection assay, but it binds multiple protein factors in nuclear extracts (62). Recently, DNA sequences analogous to the human 5'HS-2 sequence have been identified upstream of the mouse β-like globin gene cluster. The cloned mouse 5'HS-2 exhibits 70 to 80% sequence homology to human 5'HS-2, and it functions as an erythroid cell-specific enhancer in transfection assays (37). Examination of the DNA sequence of mouse 5'HS-2 (Fig. 1B) indicated that similar to the human sequence, it contains an NF-E2/AP1 motif, a GATA-1 motif, and a CAC motif.

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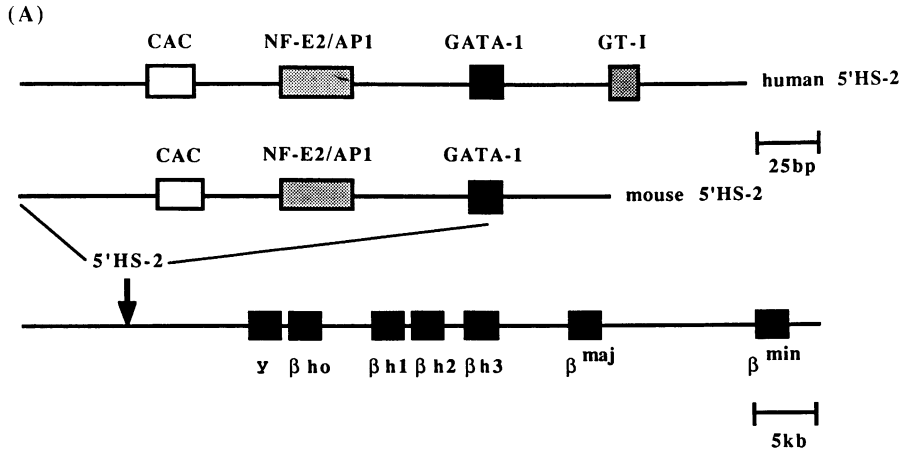

(B)

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ATTATTGCAGTACCACTGTC CAAGGGCAGAGGAGGTTAGC TGGGCCCAGGCGGAGTCAAT  60
TAATAACGTCATGGTGACAG GTTCCCGTCTCCTCCAATCG ACCCGGGTCCGCCTCAGTTA

          CAC
TCTCTACTCCCCACCCTGTG GGTGTGTTCAGCCTTGTGAG CCAGCATCAGGCTTGAGCAC 120
AGAGATGAGGGGTGGGACAC CCACACAAGTCGGAACACTC GGTCGTAGTCCGAACTCGTG

          NF-E2/AP1
AGCAGTGCTGAGTCATGCTG AGTCATGCTGAGGCTTAGGG TGTGTGGCCAGATGTTTTCA 180
TCGTCACGACTCAGTACGAC TCAGTACGACTCCGAATCCC ACACACCGGTCTACAAAAGT

                GATA-1
GCTGTGAGTGATCAGTGCTA TCTGGGTCTCTAGGAGGAAG TCCACAGGGAAGGTGAAAAG 240
CGACACTCACTAGTCACGAT AGACCCAGAGATCCTCCTTC AGGTGTCCCTTCCACTTTTC

AAAATAAGTTGCTCCCTGAA GA-3'
TTTTATTCAACGAGGGACTT CT-5'
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(C)

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CAGAGGGAGTACCCAGAGCT GAGACTCCTAAGCCAGTGAG TGGCACAGCATCCAGGGAGA -132
GTCTCCCTCATGGGTCTCGA CTCTGAGGAGGCGGTCACTC ACCGTGTCGTAGGTCCCTCT

                                    CACC          CCAAT
AATATGCTTGTCATCACCGA AGCCTGATTCCGTAGAGCCA CACCCTGGTAAGGGCCAATC -72
TTATACGAACAGTAGTGGCT TCGGACTAAGGCATCTCGGT GTGGGACCATTCCCGGTTAG

          GATA-1              DRE           TATA
TGCTCACACACGATAGAGAG GGCAGGAGCCAGGGCAGAGC ATATAAGGTGAGGTAGGATC -12
ACGAGTGTGTCCTATCTCTC CCGTCCTCGGTCCCGTCTCG TATATTCCACTCCATCCTAG

AGTTGCTCCTCACATTTGCT TCTGACATAGTTGTGTTGAC TCACAACCCCAGAAACAGAC-3'
TCAACGAGGAGTGTAAACGA AGACTGTATCAACACAACTG AGTGTTGGGGTCTTTGTCTG-5'
          +1
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FIG. 1. (A) The linkage map of the mouse β-like globin gene cluster is shown at the bottom. The map is consistent with that of Shehee et al. (56). The vertical arrow indicates the approximate location of the mouse 5'HS-2 enhancer (18). Above are shown expanded versions of the mouse and human 5'HS-2 enhancers, with the four nuclear factor-binding motifs, CAC, NF-E2/AP1, GATA-1 and GT-I, boxed. The sequence of the mouse enhancer is shown in panel B. (B) DNA sequence of the mouse 5'HS-2 enhancer. A 240-bp sequence of the mouse 5'HS-2 enhancer (18) is shown, with the CAC, NF-E2/AP1, and GATA-1 motifs boxed. (C) DNA sequence of the upstream promoter of the mouse β^{maj} globin gene. Nucleotide sequences from 191 bp upstream (−191) of the β^{maj} globin cap site to 49 bp downstream (+49) are shown. The four promoter elements, CACC, CCAAT, DRE, and TATA, as well as the sequence homologous to the binding consensus sequence of nuclear factor GATA-1 are boxed. Note that the DRE element consists of two tandemly arranged repeating units.

However, no GT-I motif could be found at the orthologous position of mouse 5'HS-2. The striking structural similarity and enhancer function of mouse 5'HS-2 and human 5'HS-2 strongly suggest that the former plays an essential role in the developmental regulation of erythroid lineage-specific transcription of mouse β-like globin genes, including the β^{maj} globin gene.

To further understand the molecular basis of transcriptional regulation of the mouse β^{maj} globin gene during erythroid differentiation, we have analyzed protein-DNA interactions of the mouse 5'HS-2 enhancer as well as the upstream promoter region of the mouse β^{maj} globin gene in living MEL cells before and after DMSO induction and in nonerythroid mouse 3T3 cells in which the β^{maj} globin gene is silent. Our detection of cell-type-specific and differentiation stage-specific binding of nuclear factors at different transcriptional regulatory elements of the β^{maj} globin gene has interesting implications regarding the molecular mechanisms of multistep transcriptional control of this gene in the adult erythroid lineage.

MATERIALS AND METHODS

Cell culture. MEL cells were grown in suspension in RPMI 1640 medium containing 10% fetal bovine serum, 50 U of penicillin per ml, 50 μg of streptomycin (GIBCO) per ml, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES [pH 6.9]; Sigma). To induce differentiation, MEL cells at the log phase of growth, with a density of approximately 5×10^5 cells per ml, were supplemented with 2% DMSO (Sigma), and culture was continued for 4 days in the presence of DMSO. Completion of induction was verified by microscopic analyses of MEL cell morphology and by primer extension assay of the mouse β^{maj} globin transcripts before and after DMSO treatment (data not shown). 3T3 cells (CCL163; American Type Culture Collection) were grown on plates to 80% confluency in Dulbecco's modified Eagle's medium containing 10% calf serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 10 mM HEPES (pH 6.9).

In vivo DMS footprinting. To study protein-DNA interactions in vivo, MEL cells before and after DMSO induction and 3T3 cells were treated with dimethyl sulfate (DMS; Fluka) as described previously (2). Briefly, cells were harvested in the log phase and pelleted. Cells were resuspended at a density of 10^8 cells per ml in culture medium without serum and treated with 0.1% DMS at room temperature for 5 min. Cells were washed twice with 10 to 15 volumes of cold phosphate-buffered saline. Nuclei were then prepared by treating the cells with sucrose buffer containing 0.5% Nonidet P-40. After proteinase K and RNase A digestion, genomic DNAs were extracted by standard methods. As an in vitro control, DNA was prepared from cells not treated with DMS. This protein-free DNA was then empirically treated with DMS for 20 to 30 s at room temperature as described previously (34).

DNA samples treated with DMS in vivo and in vitro were subjected to a β-elimination reaction by 1 M piperidine (Aldrich) in order to cleave them at the modified G residues. Samples were then frozen at −70°C and lyophilized under high vacuum. Trace amounts of piperidine were removed by washing samples two to three times with water and finally by ethanol precipitation.

Sites of piperidine cleavage were mapped by the ligation-mediated polymerase chain reaction (PCR) method of genomic DNA sequencing (19, 38, 47). Piperidine-cleaved DNA fragments (2 to 3 μg) were first hybridized with one set of target-specific oligonucleotide primers corresponding to sequences flanking one side of the mouse 5'HS-2 enhancer (A1 or B1; see below) or the β^{maj} globin promoter (C1 or D1; see below), respectively, and extended with Vent DNA polymerase (New England Biolabs). The Vent DNA polymerase-extended samples were then ligated to a common linker as described previously (19). Ligated samples were amplified by heating at 95°C for 2 min, hybridized at 60°C with a second set of primers (A2 or B2 for the 5'HS-2 enhancer; C2 or D2 for the β^{maj} promoter) for 2 min and then extended at 76°C for 3 min. After 20 to 22 cycles of PCR, samples were hybridized with end-labeled primers (A3 or B3 for the 5'HS-2 enhancer and C3 or D3 for the β^{maj} promoter) and amplified by two more rounds of PCR (19).

The final products were purified by extraction with phenol-chloroform and by ethanol precipitation. DNA fragments were analyzed on denaturing 6% urea–polyacrylamide gels. The bands on the autoradiogram were visualized after 24 to 48 h of exposure. The reproducibility of the in vivo footprinting data was checked by analyzing genomic DNA samples prepared from three or more separate batches of DMS-treated cells.

Primers. To analyze protein-DNA interactions of the coding strand of the mouse 5'HS-2 enhancer, overlapping oligonucleotide set A (A1, 5'-GTTTCTTCAGGGAGCAAA C-3'; A2, 5'-CAGGGAGCAAACTTATTTTCTTTTCACC-3'; A3, 5'-TTCTTTTCACCTTCCCTGTGGAC-3') was used. For the noncoding strand, primer set B (B1, 5'-TT GCAGTACCACTGTCC-3'; B2, 5'-GCAGTACCACTGTC CAAGGGCAGA-3'; B3, 5'-TCCAAGGGCAGAGGAGGT TAGC-3') was used.

For mapping of the mouse β^{maj} promoter region, primer set C (C1, 5'-GGGGTTGTGAGTCAACAC-3'; C2, 5'-GTG GATCAACTCAACTATGTCAGAAGC-3'; C3, 5'-GTCAG AAGCAAATGTGAGGAGC-3') and primer set D (D1, 5'-GAGTACCCAGAGCTGAGAC-3'; D2, 5'-GAGCTGAGA CTCCTAAGCCGATGAG-3'; D3, 5'-GCCAGTGAGTGGC ACAGCATCC-3') were used for the coding and noncoding strands, respectively.

RESULTS

To map sites of nuclear factor binding of the 5'HS-2 enhancer and β^{maj} globin promoter of the mouse β globin gene locus, uninduced MEL cells, MEL cells after DMSO induction, and nonerythroid 3T3 cells were treated with DMS. After treatment, genomic DNAs were purified, and the extents of DMS reaction in vivo of different G residues were determined by ligation-mediation PCR as described in Materials and Methods. The final extension products, which were labeled with ^{32}P at their 5' ends (19), were analyzed by denaturing 6 M urea–polyacrylamide gel electrophoresis and autoradiography on Kodak X-ray films. Multiple sets of experiments were carried out, and G residues reproducibly exhibiting protection or hyperreactivity to DMS in vivo, compared with naked DNA controls, are indicated in the autoradiographs in Fig. 2 to 5. In most cases, the presence of protection or hyperreactivity of a particular G residue was determined by eye. When the extents of changes of DMS reactivities appeared to be subtle, approximate quantitation of band intensities on the autoradiographs was done by scanning the autoradiographs with a video image system from Millipore. The scanning data are presented in Table 1.

Genomic DMS footprints of the mouse 5'HS-2 enhancer. The genomic footprinting data for the CAC motif at the

TABLE 1. Quantitation of DMS reactivities in vivo[a]

G residue	Reactivity	
	M	IM
78	50	40
80	40	40
82	40	30
84	40	30
86	50	40
208	110	110
202	70	60
193	80	60
−76	100	90
−87	130	80
−88	80	70
−89	80	50
−91	80	50
−93	120	100
−94	70	70
−32	70	50
−37	110	120
−42	160	140
−43	100	90
−49	100	100

[a] Small changes of DMS reactivities of certain G residues in uninduced (M) or DMSO-induced (IM) MEL cells were confirmed by densitometric tracing of the autoradiographs shown in Fig. 2 to 5. The reactivities were estimated by calculating the relative intensities of individual bands on the autoradiographs, with the intensities of corresponding bands in the naked DNA control lanes being 100. The data for G residues 78 through 86, 193 through 208, −76 through −94, and −32 through −49 were derived from densitometric tracing of autoradiographs of Fig. 2A, 4, and 5A, respectively. Differences of 10% or less are considered as indications of no change in the DMS reactivities, and they are usually not detectable by eye.

mouse 5′HS-2 enhancer are shown in Fig. 2. While no apparent alterations of reactivity of G residues to DMS in vivo could be detected within and flanking this motif in nonerythroid 3T3 cells (Fig. 2, lanes 4 and 8), specific G's are

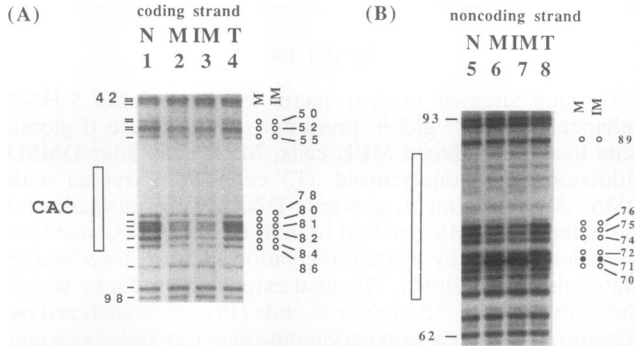

FIG. 2. Genomic DMS footprints within and flanking the CAC motif of the mouse 5′HS-2 enhancer. Shown are representative autoradiographs of the in vivo DMS protection analyses of the coding (A) and noncoding (B) strands. The horizontal lines to the left of each autoradiograph indicate the positions of G residues, with the top and bottom G's numbered according to Fig. 1B. The location of the CAC motif is indicated by the vertical open bar. Protected and hyperreactive G residues are denoted by open and closed circles, respectively, on the right side of the autoradiographs. The protection of residues G78 through G86 was confirmed by densitometric tracing of the autoradiographs (Table 1). Lanes: 1 and 5, naked DNA controls (N); 2 and 6, uninduced MEL cells (M); 3 and 7, DMSO-induced MEL cells (IM); 4 and 8, mouse 3T3 cells (T). Note that G71 is also hyperreactive in 3T3 cells, even though it is not indicated in panel B.

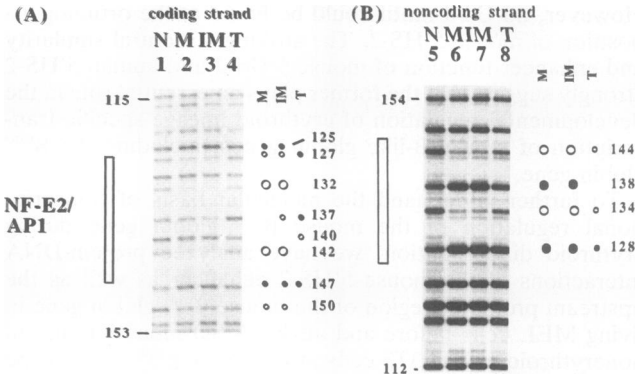

FIG. 3. Genomic DMS footprints within and flanking the NF-E2/AP1 motif of the mouse 5′HS-2 enhancer. Sizes of the circles indicate the relative extents of DMS protection or hyperreactivities in vivo. Lanes: 1 and 5, naked DNA controls (N); 2 and 6, uninduced MEL cells (M); 3 and 7, DMSO-induced MEL cells (IM); 4 and 8, 3T3 cells (T). Note that the protection and hyperreactivity at G127 and G137 in MEL and 3T3 cells, respectively, may result from differential binding of NF-E2 and AP1 in these two types of cells (see text for further details).

protected or become hyperreactive in MEL cells with or without DMSO induction. Thus, relative to naked DNA controls (lanes 1 and 5), G50, G52, G55, G78, G80, G81, G82, G84, and G86 on the coding strand are partially protected in uninduced as well as induced MEL cells. On the noncoding strand, G71 showed hyperreactivity, while G70, G72, G74, G75, and G76 showed partial protection in uninduced and DMSO-induced MEL cells. These results suggest that nuclear factors are bound at the CAC motif in erythroid MEL cells, and this protein-DNA interaction is not altered upon DMSO induction.

A second genomic footprint of the mouse 5′HS-2 enhancer consists of both enhancement and protection of DMS reactivities in vivo of specific G residues at the NF-E2/AP1 motif (Fig. 3). Protections of the coding (or upper) strand were detected at G127, G132, G142, and G150 in uninduced as well as in induced MEL cells. G137 appeared to be weakly protected in induced MEL cells but not in uninduced MEL (Fig. 3A, lanes 2 and 3). In nonerythroid 3T3 cells, none of these G residues shows protection, but G140 is lightly protected, and both G127 and G137 are partially hyperreactive (lane 4). Finally, G125 is somewhat hyperreactive in both uninduced and induced MEL cells, while G147 is hyperreactive in all three types of cells (Fig. 3A).

On the noncoding strand of the NF-E2/AP1 motif, G134 and G144 are protected, and G128 and G138 are hyperreactive in uninduced and induced MEL cells (Fig. 3B, lanes 6 and 7). This pattern of DMS footprinting in vivo appears to be similar to that in nonerythroid 3T3 cells, although the extent of protection of G124 and G134 is much less than in MEL cells (compare lane 8 with lanes 6 and 7). These data suggest that the NF-E2/AP1 motif of mouse 5′HS-2 binds nuclear factors in vivo in both erythroid MEL and nonerythroid 3T3 cells. However, the unique hyperreactivity at G127 and G137 in 3T3 cells is indicative that protein-DNA complexes formed at this motif may be conformationally different between the two types of cells (see further discussion later).

Another DMS footprint is present around nucleotides 198 to 203, which constitute the core binding sequence of transcription factor GATA-1. On the noncoding strand, G202 is

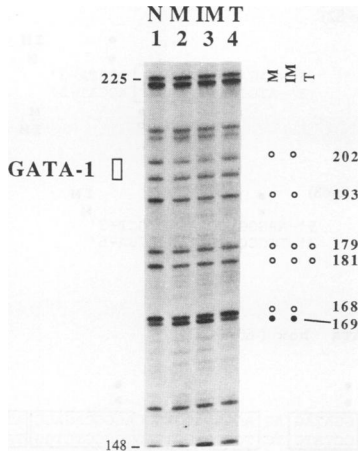

FIG. 4. Genomic DMS footprints within and flanking GATA-1 motif of the mouse 5′HS-2 enhancer. Shown in the autoradiograph is the in vivo DMS protection pattern of the noncoding strand. Lanes: 1, naked DNA control (N); 2, uninduced MEL cells (M); 3, DMSO-induced MEL cells (IM); 4, 3T3 cells (T). The weak protection of G202 was confirmed by densitometric tracing of the autoradiograph (Table 1).

protected in uninduced and induced MEL cells but not in 3T3 cells. G193 also exhibits light protection patterns in both types of MEL cells (Fig. 4 and Table 1). No significant alteration of G reactivities could be seen on the coding strand around the GATA-1 motif in all three cell types (results not shown). The data shown in Fig. 4 and Table 1 suggest that the GATA-1 factor binds to the 5′HS-2 enhancer in vivo in MEL cells but not in 3T3 cells.

Genomic DMS footprints of the mouse β^{maj} globin promoter. Protein-DNA interactions in the upstream promoter region of the mouse β^{maj} globin gene were also analyzed by genomic footprinting (Fig. 5 and 6; Table 1). Between nucleotides −99 and −82, a region containing the CACC promoter box, G−94, G−91, G−89, and G−88 showed partial protection, while G−93, G−87, and G−85 were somewhat hyperreactive in uninduced MEL cells (Fig. 5, lanes 2 and 8). Upon DMSO induction, the hyperreactivities at G−93 and G−87 disappeared, while G−95 and G−87 became protected. Also, the extents of the protection at G−91 and G−89 increased (lanes 3 and 9). These results suggest that prior to DMSO induction, there is nuclear factor binding at the CACC promoter box in MEL cells, and this protein-DNA interaction, as analyzed by the DMS protection assay, is altered and becomes more extensive upon erythroid differentiation of MEL cells. There is no detectable footprint at the CACC promoter box in nonerythroid 3T3 cells (data not shown).

As shown in Fig. 5, no obvious protein-DNA interaction could be detected in the vicinities of the CCAAT promoter box and the GATA-1 motif in MEL cells before and after DMSO induction (lanes 2, 3, 8, and 9). A weak genomic footprint, on the other hand, is mapped at the 3′ half of the DRE promoter element immediately upstream of the TATA

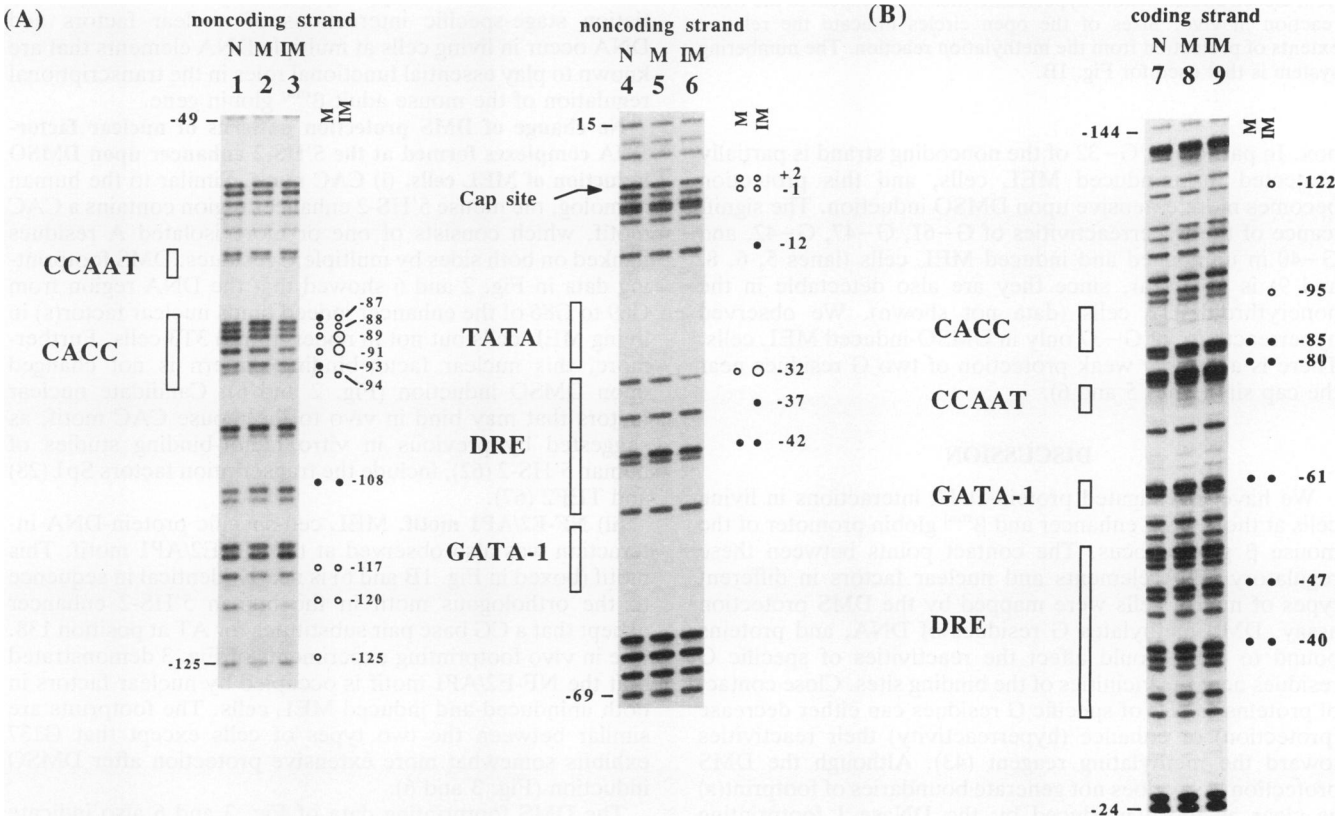

FIG. 5. Genomic DMS footprints within and flanking CACC, CCAAT, and DRE promoter boxes of the mouse β^{maj} globin gene. Lanes: 1 and 4, naked DNA controls (N); 2 and 5, uninduced MEL cells (M); 3 and 6, DMSO-induced MEL cells (IM); 7, naked DNA control; 8, uninduced MEL cells; 9, DMSO-induced MEL cells. Note that the changes in the DMS reactivity patterns of G−37 and G−32 are relatively subtle, and they were confirmed by densitometric tracing (Table 1).

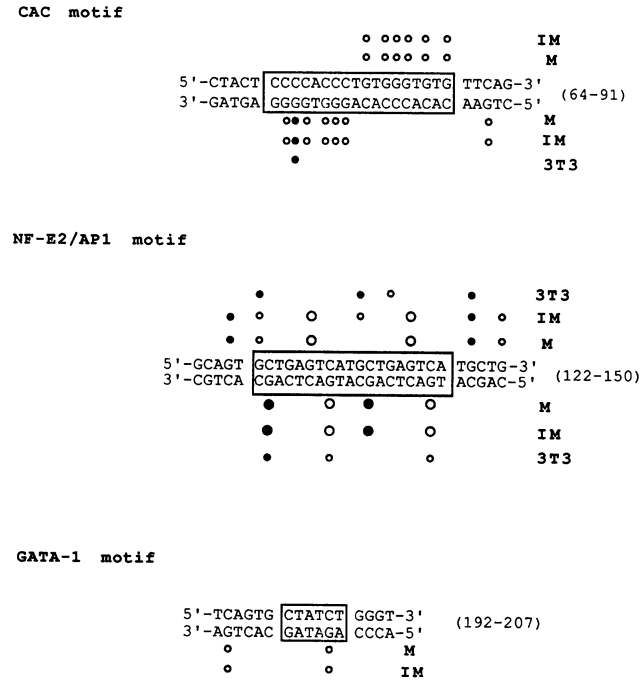

FIG. 6. Summary of genomic DMS footprinting data for the mouse 5'HS-2 enhancer. The methylation patterns of G residues of both DNA strands within and flanking the three nuclear factor-binding motifs of the mouse 5'HS-2 enhancer, as deduced from data shown in Fig. 2 to 4, are summarized. Open circles denote protection; closed circles denote hyperreactivities with respect to the DMS reaction in vivo. Sizes of the open circles indicate the relative extents of protection from the methylation reaction. The numbering system is that used for Fig. 1B.

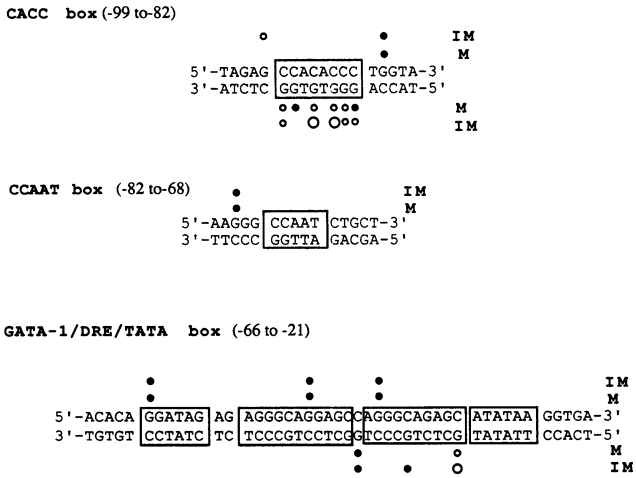

FIG. 7. Summary of genomic DMS footprinting data for the mouse β^{maj} globin promoter. Data from the methylation protection assay in living cells at the upstream promoter of the mouse β^{maj} globin gene, as deduced from data shown in Fig. 5, are summarized individually for the three promoter elements. The numbering is that used for Fig. 1C.

box. In particular, G−32 of the noncoding strand is partially protected in uninduced MEL cells, and this protection becomes more extensive upon DMSO induction. The significance of the hyperreactivities of G−61, G−47, G−42, and G−40 in uninduced and induced MEL cells (lanes 5, 6, 8, and 9) is not clear, since they are also detectable in the nonerythroid 3T3 cells (data not shown). We observed hyperreactivity of G−37 only in DMSO-induced MEL cells. There is also very weak protection of two G residues near the cap site (lanes 5 and 6).

DISCUSSION

We have investigated protein-DNA interactions in living cells at the 5'HS-2 enhancer and β^{maj} globin promoter of the mouse β globin locus. The contact points between these regulatory DNA elements and nuclear factors in different types of mouse cells were mapped by the DMS protection assay. DMS methylates G residues of DNA, and proteins bound to DNA could affect the reactivities of specific G residues near the vicinities of the binding sites. Close contact of proteins to N-7 of specific G residues can either decrease (protection) or enhance (hyperreactivity) their reactivities toward the methylating reagent (43). Although the DMS protection assay does not generate boundaries of footprint(s) as clear as those produced by the DNase I footprinting technique, it can reveal subtle differences in the modes of nuclear factor binding to the DNA helix on a nucleotide sequence level. It should be pointed out that with appropriate modification of the DMS reaction in vivo, it is possible to detect DMS reactivities of A as well as G residues (60). This method may help to confirm the nuclear factor binding at sequences lacking G residues, such as the GATA-1 motifs. The data presented in Fig. 2 to 5 and summarized in Fig. 6 and 7 indicate that cell-type-specific as well as cell differentiation stage-specific interactions of nuclear factors and DNA occur in living cells at multiple DNA elements that are known to play essential functional roles in the transcriptional regulation of the mouse adult β^{maj} globin gene.

No change of DMS protection patterns of nuclear factor-DNA complexes formed at the 5'HS-2 enhancer upon DMSO induction of MEL cells. **(i) CAC motif.** Similar to the human homolog, the mouse 5'HS-2 enhancer region contains a CAC motif, which consists of one or more isolated A residues flanked on both sides by multiple C residues. DMS footprinting data in Fig. 2 and 6 showed that the DNA region from G69 to G86 of the enhancer indeed binds nuclear factor(s) in living MEL cells but not in nonerythroid 3T3 cells. Furthermore, this nuclear factor-binding pattern is not changed upon DMSO induction (Fig. 2 and 6). Candidate nuclear factors that may bind in vivo to the mouse CAC motif, as suggested by previous in vitro factor-binding studies of human 5'HS-2 (62), include the transcription factors Sp1 (28) and TEF2 (67).

(ii) NF-E2/AP1 motif. MEL cell-specific protein-DNA interaction was also observed at the NF-E2/AP1 motif. This motif (boxed in Fig. 1B and 6) is nearly identical in sequence to the orthologous motif in the human 5'HS-2 enhancer except that a CG base pair substitutes for AT at position 138. The in vivo footprinting experiments of Fig. 3 demonstrated that the NF-E2/AP1 motif is occupied by nuclear factors in both uninduced and induced MEL cells. The footprints are similar between the two types of cells except that G137 exhibits somewhat more extensive protection after DMSO induction (Fig. 3 and 6).

The DMS footprinting data of Fig. 3 and 6 also indicate less extensive binding of nuclear factors at the NF-E2/AP1 motif of 5'HS-2 in nonerythroid 3T3 cells. However, G127 and G137 of the coding strand are hyperreactive in 3T3 cells, instead of being protected as in MEL cells. This difference

suggests that different protein-DNA complexes form at the NF-E2/AP1 motifs in these two types of cells.

The mouse NF-E2/AP1 motif consists of a dimer of the sequence 5′-GCTGAGTCA-3′, which is the binding consensus sequence for transcription factor AP1 or NF-E2. Previous nuclear factor-binding studies of the NF-E2/AP1 motif in the human 5′HS-2 enhancer have suggested that AP1 factor binding to the motif would render the first G residue of the above consensus sequence hyperreactive toward DMS, while an erythroid-specific AP1 homolog(s), possibly NF-E2, protects it from the methylating reagent (50). Furthermore, mutational analyses of the human 5′HS-2 showed that a change of the first G residue of the binding consensus sequence, 5′-GCTGAGTCA-3′, of NF-E2/AP1 to T not only drastically reduced the enhancer function of human 5′HS-2 in transfected human erythroid K562 cells but also greatly reduced the binding affinity of 5′HS-2 with NF-E2, but not with AP1, in K562 nuclear extracts (35, 36, 42, 58). These studies together with the footprint data of Fig. 3 suggest that NF-E2, but not AP1, also binds in vivo to the mouse 5′HS-2 enhancer in MEL cells, and NF-E2 protein-DNA interaction may very well be essential for the enhancer function of mouse 5′HS-2 in erythroid-specific transcription of the mouse β globin locus.

(iii) GATA-1 motif. The binding site of GATA-1, a nuclear factor present in erythroid cells and monocytes, is present in the promoters and/or enhancers of a number of erythroid cell-specific genes, and it has been implicated in the transcriptional regulation of these genes. Our observation of a DMS footprint at the GATA-1-binding site (Fig. 4) suggests that it is also involved in the erythroid cell-specific enhancer function of mouse 5′HS-2.

In summary, we have identified specific nuclear factor binding at the CAC motif, NF-E2/AP1 motif, and GATA-1 motif of the mouse 5′HS-2 enhancer in living MEL cells. Of these three motifs, CAC and GATA-1 do not appear to bind nuclear factors, while NF-E2/AP1 appears to bind weakly AP1, instead of NF-E2 as in MEL cells, in nonerythroid 3T3 cells. The close proximity of the three motifs in 5′HS-2 suggests that nuclear factor-DNA complexes formed at the three motifs may cooperatively interact in vivo with each other and also with protein-DNA complexes formed at the promoters of different mouse β-like globin genes as suggested for the human β globin locus (reviewed in references 14, 44, and 59; see further discussion below). Interestingly, the in vivo factor-binding pattern of 5′HS-2 in MEL cells did not change significantly upon DMSO induction (Fig. 6) when both β^{maj} globin transcription (18, 48, 54) and the amount of DNA-binding NF-E2 molecules (mobility shift assay by Bastiani and Shen [1a]) were greatly enhanced. Thus, it appears that at the resolution of DMS footprinting, the induction of the erythroid maturation process of MEL cells does not significantly alter the conformation of multiple protein-DNA complexes formed at the 5′HS-2 enhancer. This finding is similar to results of one genomic footprinting study (50), but not another (27), of the human 5′HS-2 enhancer in K562 cells during hemin induction.

Nuclear factor binding at mouse β^{maj} globin upstream promoter is both cell type specific and differentiation stage specific. Contrary to the 5′HS-2 enhancer, DMS footprints in vivo of the upstream promoter of the mouse β^{maj} globin gene showed cell type specificity as well as differentiation stage specificity.

(i) CACC and CCAAT boxes. Transcription of both endogenous and exogenously transfected mouse β^{maj} globin genes could be induced by more than 20-fold after DMSO treatment. The relatively large difference of transcriptional activity of the endogenous mouse β^{maj} globin gene of MEL cells before and after induction is reflected in our in vivo DMS footprinting at the CACC promoter box. Although nuclear factor binding was detectable at this motif in uninduced MEL cells, specific G residues became protected or more extensively protected after DMSO treatment (Fig. 5 and 7). Thus, the functioning of CACC box in transcriptional initiation of the mouse β^{maj} globin gene in matured erythroid cells is most likely modulated by binding of nuclear factors such as Sp1 (28; see reference 68 for references) and TEF2 (67).

In contrast to the human β globin gene, whose CACC as well as CCAAT promoter boxes bind nuclear factors in vivo in transcriptionally active, nucleated human erythroblasts (50a), the CCAAT box of the mouse β^{maj} globin gene did not appear to bind factors in vivo (Fig. 7). Although the molecular basis of this observation in induced MEL cells is not clear at the moment, the absence of factor binding of this motif in uninduced MEL cells argues against the suggestion that a DNA-binding repressor at the CCAAT box may be responsible for transcriptional inactivity of the β^{maj} globin promoter in this cell line (30).

(ii) The DRE motif. Like CCAAT and CACC, the DRE element located between the CCAAT and TATA boxes functions as an upstream promoter element of the mouse β^{maj} globin gene in transfected cell cultures (61). Removal of both, but not one, of the repeating units of the DRE element reduced the transcriptional level of the transfected β^{maj} globin gene in erythroid MEL cells as well as in nonerythroid HeLa or 3T3 cells (7, 40, 61). However, the reduction of β^{maj} gene transcription upon deletion or mutation of DRE was most drastic when the transfection assay was carried out in DMSO-induced MEL cells (61). Thus, DRE appears to play an essential role in transcriptional activation of the β^{maj} globin gene in differentiated MEL cells. Since the DRE sequence is highly conserved among all mammalian adult β globin genes, it may function as both a developmental and a differentiation stage-specific promoter element of all mammalian β globin genes in mature adult erythroid cells. In correlation with these observations, we have detected induced protein-DNA contacts around the 3′ copy of the DRE element in DMSO-induced MEL cells (Fig. 7).

Comparison with other promoters. The DMSO-induced change of protein-DNA contacts at the CACC and DRE promoter elements of the mouse β^{maj} promoter is similar to what has been found for several induced promoter systems but not the others. Changes in the DNase I footprint pattern have been detected in the upstream regulatory region of the human beta interferon gene after its induction with poly(dI)-poly(dC) (69). Upon cyclic AMP induction, the tyrosine aminotransferase gene promoter also showed prominent in vivo footprints at its CRE promoter element (64). Hemin induction of human erythroleukemia K562 cells also induced extensive in vivo footprints at the CACC and CCAAT promoter boxes of the human γ globin gene (27). On the other hand, the patterns of protein-DNA interaction at the alpha-1 interferon promoter are identical before and after viral induction (45). Epidermal growth factor induction did not alter the in vivo footprinting pattern of the serum response element of the mouse c-*fos* promoter regardless of the changes of its transcription state (23). The proximal promoter of the human H4 histone gene also showed similar DMS reactivities in vivo before and after transcriptional activation of the gene (46). The different relationships between nuclear factor-DNA contacts and promoter activities

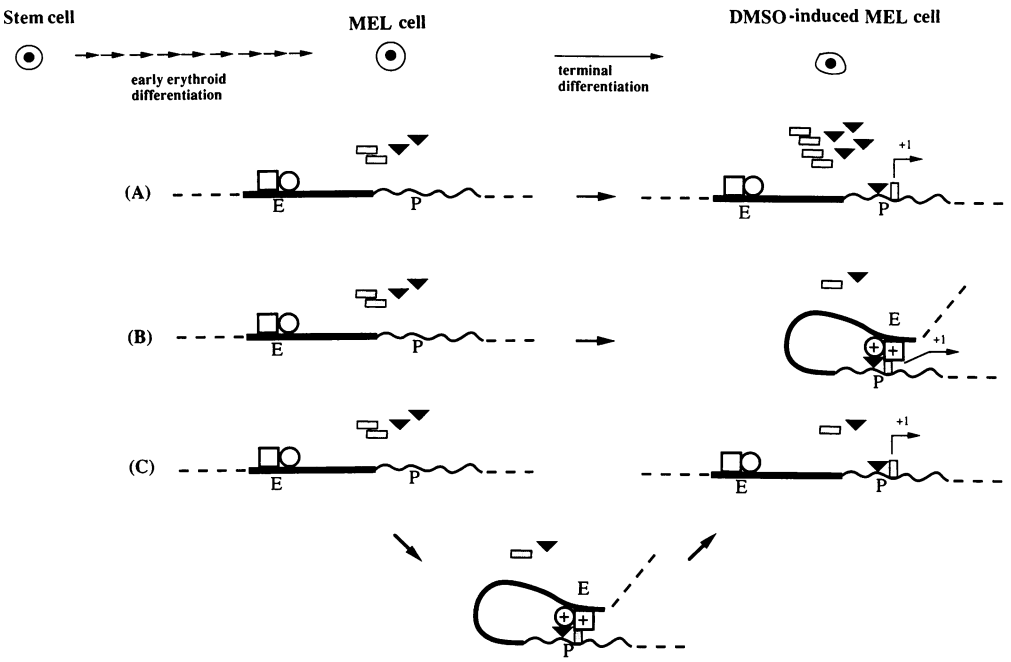

FIG. 8. Models of transcriptional activation the mouse β^{maj} globin promoter during terminal erythroid differentiation. Three models of transcriptional activation of the mouse β^{maj} globin promoter during terminational differentiation of MEL cells induced by DMSO are proposed in relation to nuclear factor-DNA interactions at the distal 5'HS-2 enhancer (E) and at the β^{maj} upstream promoter (P). In all three models, the upstream promoter and a portion of the coding regions of the β^{maj} globin gene adopt a preactivated chromatin structure before DMSO induction (see text). This preactivation of chromatin may be modulated by nuclear factor-DNA complexes of the distal LCR enhancer, including 5'HS-2, which are assembled at an earlier stage of erythroid differentiation. In model A, transcriptional activation of the β^{maj} globin gene upon DMSO induction results directly from the increases of the cellular concentrations of active transcription factors, which assemble at the upstream promoter to form active or open initiation complexes. In model B, the cellular concentrations of the active transcription factors do not increase, but a stable DNA looping between the distal enhancer and upstream promoter facilitates the entry and/or assembly of the transcription factors at the upstream promoter to form open or active initiation complexes. In this case, the conformation of multiple nuclear protein-DNA complexes of the enhancer, upon contact with the upstream promoter, is expected to be different from that in uninduced MEL cells. In model C, the DNA looping between enhancer and promoter in terminally differentiated MEL cells is only transient. Once the open or active initiation complex is formed at the upstream promoter, the enhancer separates from the promoter and the multiple nuclear factor-DNA complex of the distal enhancer adopts its original conformation (i.e., that prior to DMSO induction). The nature of the molecular mechanisms of induction of DNA looping between enhancer and upstream promoter during MEL differentiation is not specified in models B and C. See text for more discussion of the models.

of the above two categories of gene systems may result partly from certain basic difference in the mechanisms of their transcriptional induction.

Multistep activation of the mouse β^{maj} globin locus during erythroid differentiation: nuclear factor-DNA complexes, chromatin structure, and enhancer-upstream promoter interaction. As for the enhancer sequences of other eukaryotic gene systems, the molecular mechanisms by which 5'HS-2 and other distal enhancer sequences in the mammalian LCR regulate the transcription of different mammalian β-like globin genes during erythroid development are unknown. In the following text, we discuss our genomic footprinting data in terms of three models (Fig. 8) in relation to the multistep transcriptional activation of the mouse β^{maj} globin gene during terminal differentiation of MEL cells (references 4, 8, and 11 and references therein).

From many previous studies, it is clear that the chromatin structure of the β^{maj} globin gene and its flanking DNA adopts an altered or preactivated conformation in uninduced MEL cells prior to terminal differentiation, and the existence of this preactivated chromatin state is not sufficient for active transcription of the gene (1, 4, 11, 26, 55, 57). In nonerythroid cells, nucleosomes are well phased along the β^{maj} globin gene and its flanking DNA region. However, this phasing of nucleosomes is disrupted around the structural gene in MEL cells, both before and after DMSO induction (4, 11). It was further suggested that the disruption of nucleosomes in the upstream promoter region is probably due to the replacement of nucleosomes by regulatory factors that are DNA sequence specific. Furthermore, the 5' end of the β^{maj} globin gene is sensitive to DNase I digestion in uninduced MEL cells. After DMSO or HMBA (*N,N'*-hexamethylene bisacetamide) induction, the DNase I hypersensitivity of the promoter region increases by severalfold (1, 26, 55, 57). This alteration in the chromatin structure, manifested by increased hypersensitivity to DNase I, usually precedes the onset of inducible transcription in MEL cells (53).

As depicted in model A of Fig. 8, it is possible that the mouse LCR, including 5'HS-2, functions by modifying the chromatin structure of the mouse β^{maj} globin gene and its flanking DNA into the preactivated state (15, 16, 22, 65) at an earlier stage(s) of differentiation of the adult erythroid lineage. Furthermore, this active chromatin structure is established and maintained throughout the subsequent and terminal stages of erythroid differentiation by nuclear factor-DNA complexes formed at the mouse LCR, such as those identified in 5'HS-2 by our genomic footprinting assay (Fig. 6).

The nature of the altered chromatin structure is not known, but it allows only modest binding of nuclear factors to the upstream promoter region (Fig. 5 and 7). Whether this inactive β^{maj} globin promoter of uninduced MEL cells is also bound with the general transcription factor TFIID and transcriptionally engaged but blocked RNA polymerase II molecules, as in the case of the *Drosophila hsp70* promoter prior to heat shock (21, 52), remains an interesting question. DMSO induction would then increase the cellular concentrations of active molecules of one or more transcription factors, and it is these increases that directly enhance the transcriptional initiation of the β^{maj} globin gene (positive regulation). Consistent with this notion is the enhanced binding, as manifested by the DMS footprinting assay, of nuclear factors at the CACC and DRE promoter boxes (Fig. 7). The increases of active nuclear factors could be due to either increased de novo synthesis of the factor molecules or posttranscriptional modification of preexisting factors (references 20 and 24 and references therein). Although the observed nuclear factor assembly process at the mouse β^{maj} globin promoter remains to be analyzed fully, at least the enhancement of factor binding at the DRE promoter element could be partly due to a higher concentration of an active DRE-element-binding protein(s) in induced MEL cells (cited in reference 40).

Alternatively, the induced nuclear factor binding in the mouse β^{maj} globin promoter region in DMSO-treated MEL cells could result from the increase of the local concentration of the factor(s) at the vicinity of the upstream promoter. This increase could be modulated by the physical interaction between the mouse LCR, including 5'HS-2, and the β^{maj} promoter, as depicted in model B of Fig. 8. The mode of this interaction could be analogous to those suggested for other eukaryotic gene systems; i.e., it may be modulated by DNA looping via physical contacts of multiple nuclear factor-DNA complexes formed at the enhancer and the upstream promoter (references 29, 31, 39, 49, and 63 and references therein). This enhancer-promoter interaction would facilitate the formation of stable or active initiation complexes at the upstream promoter. There is much evidence supporting this functional, and probably physical as well, enhancer-promoter interaction in globin gene regulation. In chickens, the developmental stage-specific transcription of *cis*-linked adult and embryonic β-like globin genes appears to be regulated by the competitive interaction of the upstream promoters of the two globin genes with an enhancer located between them, as shown by transient expression studies of transfected DNA clones in definitive and primitive erythroid cell lineages (9, 10, 25). Similar analysis of human β and γ globin genes in transgenic mice also suggests that the human LCR or 5'HS-2 enhancer interacts with different globin promoters in erythroid cells of different developmental stages and thus activates the appropriate human β-like globin genes during development (3, 13). However, it is not obvious from our genomic footprinting data that nuclear factor-DNA complexes formed at the mouse 5'HS-2 enhancer become physically associated with the upstream promoter during DMSO induction. This is because 5'HS-2 is bound in MEL cells with nuclear factors at the NF-E2/AP1, CAC, and GATA-1 motifs prior to DMSO induction, and within the limits of resolution of the DMS protection assay, the conformations of these complexes are not apparently modified after DMSO induction (Fig. 2 to 4 and 6), despite the significantly induced binding of nuclear factors at the upstream promoter (Fig. 5 and 7).

We speculate that, as depicted in model C of Fig. 8, the 5'HS-2 enhancer physically interacts with the upstream promoter in induced MEL cells, but only transiently. However, this transient interaction would facilitate the assembly of an active transcription complex at the promoter. Once the active complex is formed and/or after transcription starts, the enhancer would separate from the upstream promoter (Fig. 8, model C). The conformational changes of the nuclear factor-DNA complexes of 5'HS-2 during their transient interaction with the promoter could have escaped detection by the genomic DMS footprinting assay. Determination of whether one or a combination of these three models operates in the multistep activation of β^{maj} globin gene transcription during terminal differentiation of MEL cells awaits further investigation.

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