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Transcription of the Histone *H5* Gene Is Regulated by Three Differentiation-Specific Enhancers

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Histone H5, an early marker of the avian erythroid lineage, is expressed at low levels in early erythroid precursors and at higher levels in more mature cells. We show that the increase in H5 expression is due to transcriptional activation of the *H5* gene following differentiation of precursor CFU(E). We have found and characterized two upstream enhancers, E1 (between −2233 and −1878 from the site of transcription initiation, +1) and E3 (between −1321 and −1163), and confirmed the presence of a downstream enhancer (C. D. Trainor, S. J. Stamler, and J. D. Engel, Nature [London] 328:827–830, 1987) E7 (between +846 and +1181) which are responsible for the increase in *H5* gene transcription. The enhancers had a weak effect in nondifferentiated CFU(E) but a strong effect when the cells were induced to differentiate. Cooperation among the three enhancers, however, was not required for *H5* gene activity in the differentiated cells. The enhancers contain binding sites for several ubiquitous and erythroid cell-specific nuclear proteins, including GATA-1, as demonstrated with GATA-1-specific antibodies. Although the GATA sites were required for enhancer function, the concentration of GATA-1, GATA-2, and GATA-3 decreased during cell differentiation, and overexpression of these factors had little effect on *H5* transcription. Hence, the differentiation-specific effect of the enhancers is not mediated by changes in relative levels of the GATA factors. Functional analysis of the *H5* promoter indicated that the requirement of several elements, including a GC box necessary for transcription enhancement, did not change during the early stages of CFU(E) differentiation. However, the UPE, a positive element in proliferating CFU(E) recognized by the transcription factor H4TF2, was dispensable in the differentiated cells. These results suggest that as the cells enter the final stages of differentiation, there is a reprogramming of the regulatory factors that control *H5* transcription and that the enhancers rescue and increase the activity of the promoter.

Vertebrate erythropoiesis is characterized by the expression of sets of tissue-specific genes which are temporally regulated during maturation of the committed precursors. The chicken histone *H5* gene is an interesting marker by which to study how these processes are regulated, because it responds both to early and late differentiation cues during erythropoiesis. Contrary to late erythroid markers, including the adult globin chains and cell surface proteins (2), H5 is already present in the early precursor CFU(E) (3, 45), and its expression increases further with the onset of the expression of these late genes (1). Transcription run-on analysis of erythroid cells at different stages of maturation have also indicated that the activity of the *H5* gene is lower in proliferating erythroid precursors than in more mature cells (1). Thus, H5 expression during maturation is predominantly regulated at the transcription level.

In an attempt to understand the molecular mechanisms accounting for the tissue-specific and stage-specific regulation of the *H5* gene, we have studied the activity of *H5* reporter genes during the in vitro differentiation of chicken HD3 cells. These cells are differentiation-blocked CFU(E) transformed by the v-*erbA* and ts-v-*erbB* oncogenes of *ts*34 avian erythroblastosis virus (2). HD3 can be grown under conditions that do not allow differentiation to occur, but they can also be induced to differentiate at the nonpermissive ts-v-*erbB* temperature in the presence of the protein kinase inhibitor H-7 (58). We have previously shown that nondifferentiated HD3 cells express low levels of endogenous H5 compared with that expressed by more mature cells (1) and that the relative concentration of H5 increases during in vitro differentiation (unpublished observations; this paper). Similarly, expression of adult globin chains and that of other differentiation-specific erythroid products are induced under the same conditions (2). Hence, the HD3 cell line provides a good model system in which to examine potential regulatory elements controlling activation of the *H5* gene during erythrocyte maturation.

Previous functional analyses (14, 41) have indicated that the activity of the −120 *H5* promoter is regulated by several *cis*-acting sequences including a negative element (UNE), a GC box, UPE (a sequence homologous to the subtype-specific element of histone H4), a TATA box, and sequences spanning the transcription initiation site. Although the activity of the *H5* promoter was modulated by upstream and downstream flanking regions in an erythroid cell-specific fashion, the effect of those flanking regions in a noninducible HD3 cell line [blocked at the CFU(E) stage] was smaller than expected for typical tissue-specific enhancers (41).

Using differentiation-competent cells, we describe here that the region upstream of the *H5* gene harbors, in fact, two additional differentiation-specific enhancers (E1 and E3). Each of these enhancers is capable of increasing the rate of *H5* transcription during differentiation of HD3 cells to levels comparable to those obtained with the downstream enhancer (E7) previously characterized (50). We also report an analysis of the enhancer and promoter sequences recognized by nuclear DNA-binding proteins and changes in DNA-binding patterns during differentiation. Interestingly, although

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GATA-1 binds the GATA sites necessary for enhancer function, the cellular concentration of all GATA factors decreased during differentiation. This suggests that GATA-1 must act in concert with other differentiation-specific factors to establish the pattern of H5 expression or that factors preventing enhancer function are down regulated during differentiation.

MATERIALS AND METHODS

Cells and transfections. Chicken erythroid HD3 cells [*ts*34 avian erythroblastosis virus-transformed CFU(E)], transformed quail fibroblasts (H32), and T-lymphoblastoma MSB-1 cells were grown as previously described (14, 44). Mature and immature erythrocytes were purified from the peripheral blood of adult hens (34).

Nondifferentiated HD3 cells (2×10^6 to 1×10^7) were transfected by using DEAE-dextran (14) or Lipofectin (GIBCO-BRL) with 1.5 to 2.0 μg of reporter H5CAT plasmid DNA or with a mixture of reporter plasmid and GATA expression vectors in the sense (RSVGATA-S) or antisense (RSVGATA-AS) orientations (57), as indicated. RSV*lacZ* was also cotransfected for normalization purposes (41). Cells were incubated after transfection for 16 h at 36°C in 6% CO_2. Half of the culture was then induced to differentiate for 48 h at 40.8°C in the presence of 15 μM H7 (Seikagaku) and 24 to 30 h at 36°C in 4% CO_2, while the remainder was kept under conditions that do not allow differentiation (36°C and 6% CO_2). Cell differentiation, monitored by benzidine staining, varied between 50 and 80%. Chloramphenicol acetyltransferase (CAT) activity was determined as previously described (41, 46), and the values given in the text are the averages of three to five independent transfections. RNA analysis indicated that the H5CAT mRNA started at the cap site of H5 mRNA (41).

Nuclear extracts and protein fractionation. Proteins were extracted from isolated nuclei with 0.42 M KCl in buffer A [25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.9), 20% glycerol, 3 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,-*N'*,*N'*-tetraacetic acid (EGTA), 6 mM 2-mercaptoethanol, 10 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 50 μg (each) of leupeptin and pepstatin per 100 ml]. Nuclear proteins were concentrated by precipitation with 70% ammonium sulfate, solubilized in buffer A containing 0.1 M KCl, and stored in liquid nitrogen after 12 h of dialysis against the same buffer. Extracts from mature erythrocyte were further fractionated by precipitation with 60% ammonium sulfate. This step removed most of the contaminating hemoglobin without noticeable loss of the DNA-binding proteins tested. Wheat germ agglutinin-Sepharose 4B chromatography (Pharmacia) was as previously described (14).

Plasmids and probes. The series of 5′ deletion mutations p3612, p2014, p1658, p1321, p1163, p120, p95, p86, p53, and p43 contain the indicated sequences upstream of the transcription initiation site of the *H5* gene (+1) abutting at +72, fused to the *CAT* reporter gene and inserted at the *Eco*RV site of pAT153 as previously described (41). Deletions were made with the appropriate restriction nucleases or *Bal* 31.

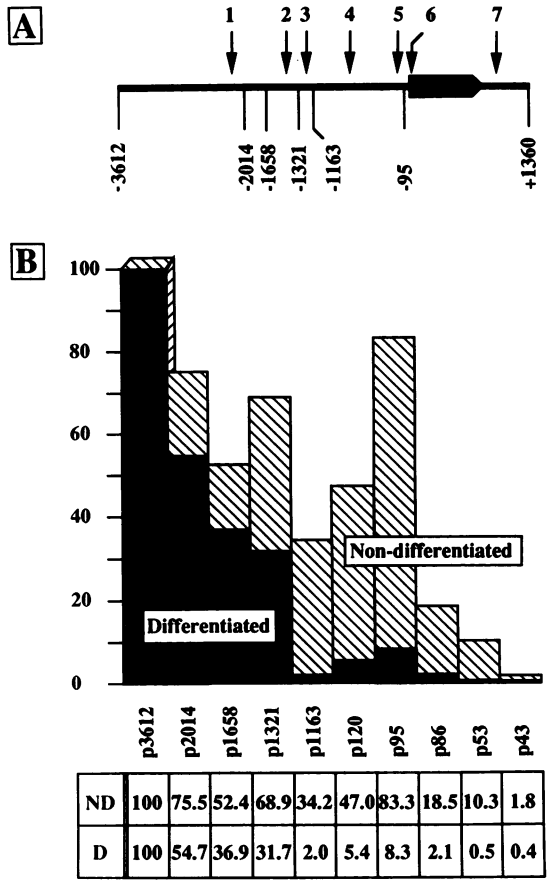
The series of constructs p1163DEFGH, p1163EFG, p1163H, p95ABC, p95AB, p95A, p95B, p95EFG, p95F, p95EF, p95FG, and p95IJ contain the A to J fragments indicated in the text inserted at a *Nhe*I site of the vector, upstream of the −1163 to +72 or the −95 to +72 *H5* promoters in the natural or reverse (−) orientation, respectively. Plasmids p95(3′)ABC, p95(3′)IJ, and p43(3′)IJ contain the −95 or the −43 promoters and fragments ABC or IJ inserted downstream of the *CAT* gene in the natural or reverse (−) orientation at a *Cla*I site of the vector. Plasmids p3612IJ and p3612I contain the −3612 promoter and the downstream sequences IJ or I of the *H5* gene substituting the simian virus 40 T-antigen fragment of the *CAT* reporter gene.

Constructs were verified by restriction analysis and DNA sequencing of the junctions. The sequence of the two DNA strands from −3612 to −1201 was determined by the dideoxy termination method with a kit (Pharmacia) and specific oligonucleotides as primers. DNA concentration was determined by fluorometry and protein concentration by the method of Bradford (4a) (Bio-Rad).

Oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer. The following oligonucleotides were used (only the sequence of the top strand is given). H5TF2 wild type (−64 to −34), AAGGGACAGG CAGTCCTCCCCGCGGTCCGTGCCG; mutated H5TF2, GGACAGGCATTCCGACCCGCACTACGTGCC; chicken H4TF2 (53), CCGCCCCTGGTTTCAATCAGGTCCGACC AT; human H4TF2 (19), CTTCTTTCAGGTTCTCAGTTCG GTCCGCCAACTGTCGTAT; *H5* E7-VI (+1107 to +1133), GATCCACCGAGGGCTTGGCACAGCCCCAAG; *H5* E3-Id (−1263 to −1241), CCCTTTCTGTTTTCCCTCTTTGG; *H5* E1II-GATA (−2132 to −2114), CTGTTCCCGATAAAGG CTT; *H5* E1V-GATA (−2024 to −2006), CCGATAGCGAT ATCGCCTG; *H5* E3-GATA (−1186 to −1168), CAGGCA GATAAGATTCCAT; *H5* E7-GATA (+1044 to +1061), GCTGGAGATAACAGTGCG; *H5* Sp1 (−91 to −76), GTGCGGGGCGGGGCAGA; *H5* CACCC (−46 to −18), CGCGGTCCGTGCCGCACCCTTAAATGCGT; rat α_1-fetoprotein NFI (−128 to −103 [18]), AATTCAATTATTGGCA AATTGCCTAACTTCG.

DNA binding assays. DNase I footprints were as previously described (14, 41). Poly(dI-dC) was used as nonspecific competitor unless otherwise indicated. Briefly, footprint reactions (20 μl) were carried out in buffer A containing 0.1 M KCl, 5 to 10 ng of ^{32}P-end-labeled DNA (1×10^4 to 3×10^4 cpm), 0 to 1 μg of poly(dI-dC), 0 to 160 μg of nuclear protein, a 50- to 400-fold molar excess of competing oligonucleotide, 50 ng of $pd(N)_5$ (Pharmacia), and 0.1% Nonidet P-40 for 30 min at 25°C. DNase I (10 ng to 1 μg) was added at the end of the incubation for 2 min. Digestions were stopped after 2 min by addition of sodium dodecyl sulfate to 1%, EDTA to 25 mM, and proteinase K (Boehringer) to 100 μg/ml. DNA was purified and analyzed in 4 to 6% denaturing polyacrylamide gels. Gel retardation and methylation interference assays were carried out by incubation of the probes with 5 to 10 μg of protein for 30 min at 4°C as described previously (14, 41). In the case of purified human H4TF2 (6), the binding reaction contained 50 mM HEPES (pH 7.9), 40 mM KCl, 4% Ficoll, 1 ng of probe, 50 ng of sheared salmon sperm DNA, and the indicated amounts of competitor oligonucleotides. Samples were analyzed in native 4% polyacrylamide gels. Supershifts with anti-GATA-1 antibodies were carried out as described above, except that the nuclear extracts (5 to 10 μg of protein) were incubated with purified immunoglobulin G fractions for 3 h at 25°C prior to the binding reaction.

Antibodies. The synthetic peptide GPDAGSPTPFPD(C), corresponding to residues 7 to 18 of chicken GATA-1 (9, 57), was used to elicit polyclonal antibodies in rabbits. The immunoglobulin G fraction of preimmune and immune sera was purified as previously described (29). Immunoblots with



	p3612	p2014	p1658	p1321	p1163	p120	p95	p86	p53	p43
ND	100	75.5	52.4	68.9	34.2	47.0	83.3	18.5	10.3	1.8
D	100	54.7	36.9	31.7	2.0	5.4	8.3	2.1	0.5	0.4

FIG. 1. The 5′ flanking region of the *H5* gene contains differentiation-specific enhancers. (A) Location of the nuclease hypersensitive sites of the *H5* locus in chromatin from erythroid cells. (B) Relative CAT activity of 5′ deletion mutations determined by transfection. ND, hatched bars, ND-HD3; D, solid bars, D-HD3. CAT activity is given relative to that of p3612.

the anti-GATA-1 antibodies were carried out by the method of Rózalski et al. (42).

RNA analysis. RNA from whole cells was purified and analyzed by blot hybridization as previously described (48). The probes were the *Xba*I-*Cla*I fragments of RSVGATA-1 (1.1 kb), RSVGATA-2 (2.4 kb), and RSVGATA-3 (1.8 kb) (57) and the *Not*I-*Xmn*I fragment of pchV2.5B/H (43). Probes were labeled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; NEN) by random priming with a DNA labeling kit (GIBCO-BRL).

RESULTS

The 5′ flanking region of the *H5* promoter harbors differentiation-specific enhancers. To identify regulatory elements responsible for the transcriptional activation of the *H5* gene, we have examined the effect of 5′ deletion mutations of the p3612 *CAT* reporter gene, encompassing the nuclease hypersensitive sites DHS 1 to 6 (Fig. 1A) of the *H5* locus (39). The constructs were transfected in nondifferentiated HD3 cells (ND-HD3), and the cells were either allowed to differentiate (D-HD3) or kept under nondifferentiation conditions.

CAT activity of transfected ND-HD3 indicated that removal of sequences from −3612 to −1163 resulted in progressively reduced levels of expression (Fig. 1B), although the effect was relatively modest (2.8-fold). Deletions down to −120 did not have any significant additional effect, whereas deletion to −95 resulted in a significant increase of promoter activity. This effect was due to the removal of a negative element (UNE), which is also able to repress transcription from the heterologous herpes simplex virus thymidine kinase promoter (41). Deletions to −86, −53, and −43 further inactivated the promoter by eliminating a GC box (p86), the upstream part of UPE (p53), and two-thirds of UPE (p43), a sequence homologous to the histone *H4* subtype consensus sequence (41; see below). These results are in full agreement with those previously obtained with a noninducible HD3 cell line and a different transfection protocol (41).

The effect of the upstream sequences was more dramatic in D-HD3, the deletion removing DHS 1 (−3612 to −1658) having a somewhat more marked effect than in ND-HD3 (Fig. 1B). More strikingly, however, removal of the region harboring DHS 3 (−1321 to −1163) resulted in a 15-fold reduction in the level of transcription compared with a twofold decrease in ND-HD3. Differentiation also altered the requirement for some of the promoter-proximal elements (Fig. 1B). Deletion of UPE (−53 to −43) inactivated the promoter in ND-HD3, but it had no effect in D-HD3. On the other hand, the effect of UNE and the GC box did not change during differentiation.

These results suggested that the sequences harboring DHS 3 contain an enhancer that activates *H5* transcription during the transition of CFU(E) to erythroblasts. This effect was increased even further by the sequences encompassing DHS 1, resulting in a 50-fold increase in stimulation of transcription. These activation values are minimal estimates, since not all cells underwent differentiation, as determined by benzidine staining.

Functional mapping of the upstream enhancer E3. Typical enhancer elements have the ability to increase transcription independently of orientation and position with respect to the promoter. To ascertain whether the DHS 3 region contained an enhancer, we constructed a series of *CAT* reporter genes containing the −95 promoter and various portions of the −1321 to −1070 region (Fig. 2A). Fragment ABC(p95ABC) increased promoter activity by a 3-fold factor in ND-HD3 and by about a 12-fold factor in D-HD3 (Fig. 2B), independently of position [cf. p95ABC with p95(3′)ABC] and orientation [cf. the activity of the above constructs with that of p95ABC$^-$ and p95(3′)ABC$^-$]. In addition, the magnitude of the effect of the upstream sequences was comparable to that produced in the wild-type sequence context (Fig. 1B, cf. p1321 and p1163). Therefore, we concluded that the −1321 to −1070 region harbors a differentiation-specific enhancer, termed E3.

The functional limits of E3 were refined by introducing subfragments A, B, and AB in either orientation upstream of the −95 promoter. Figure 2B indicates that fragment AB(p95AB) increased the level of expression in a differentiation-specific manner but that neither subfragment A (p95A) nor B (p95B) had any effect. Therefore, the E3 core enhancer overlaps DHS 3 and was included within fragment AB. Although fragment C contributed to E3 activity, we have not been able to detect any specific protein interaction on this fragment (see below). Furthermore, a computer search for known factor DNA-binding sites revealed only the sequence TTTCCCGC(GC) at −1126, homologous to the E2F binding site of c-*myc*. Methylation of the CpG with *Hha*I methylase, to prevent E2F binding in vitro (26), had no effect on the activity of p95ABC (data not shown). It is possible that the function of the C fragment is to separate the

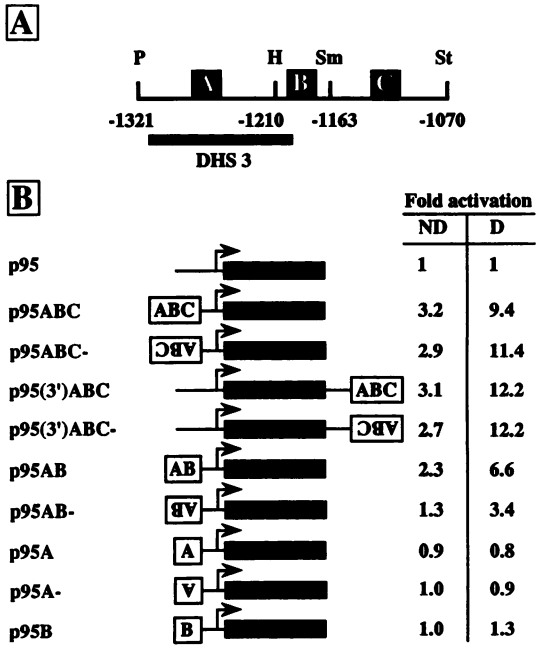


FIG. 2. Functional mapping of enhancer E3. (A) Schematic diagram of the region containing E3. P, *Pst*I; H, *Hin*dIII; Sm, *Sma*I; St, *Sty*I. (B) A fragment from −1321 to −1070, or subfragments from it, was inserted upstream or downstream of the −95 *H5* promoter (p95) in the natural or reverse (−) orientation. ND, ND-HD3; D, D-HD3. CAT activity is given relative to that of p95.

AB from the promoter elements, thus allowing a proper steric interaction between the respective binding factors.

Functional mapping of the upstream enhancer E1. The positive effect of the sequences upstream of E3 (Fig. 1B) suggested the presence of an additional enhancer in that region. To investigate this possibility, we constructed a p3204 reporter (p1163DEFGH [Fig. 3A]) carrying a deletion of fragment B that inactivates E3 (Fig. 2A). The transfection results showed that the DEFGH fragment contributed, respectively, to 3- and 25-fold increases in transcription in ND-HD3 and D-HD3 relative to that in the parent construct p1163 (Fig. 3B), confirming the presence of an enhancer in the 5′ flanking region of *H5*. The data obtained with p1163H and p1163EFG further suggested that the enhancer did not include the DHS 2 region, but only the DHS 1 region (Fig. 3A and B). To locate more precisely the active sequences, we inserted the subfragments EFG, EF, F, and FG upstream of the −95 promoter. Figure 3C shows that fragment EFG enhanced transcription in ND-HD3 and D-HD3, regardless of its orientation, and that the effect was between four and six times higher in D-HD3. The activities of p95EF$^-$, p95FG, p95F, and p95F$^-$ further indicated that the core enhancer is located in fragment F that harbors DHS 1 (Fig. 3C). Therefore, we concluded that the sequences from −2233 to −1878 contained a second differentiation-specific enhancer, termed E1. The results shown in Fig. 1B also indicate that there was a degree of synergy in the transcriptional effect of E1 and E3, since their combined effect was higher than that of the individual enhancers.

The downstream enhancer, E7, does not cooperate with the upstream enhancers to activate *H5* transcription in differentiated HD3 cells. Sequences from +797 to +1360, overlapping DHS 7, were previously shown to contain an erythroid cell-specific element that induced a modest increase in the

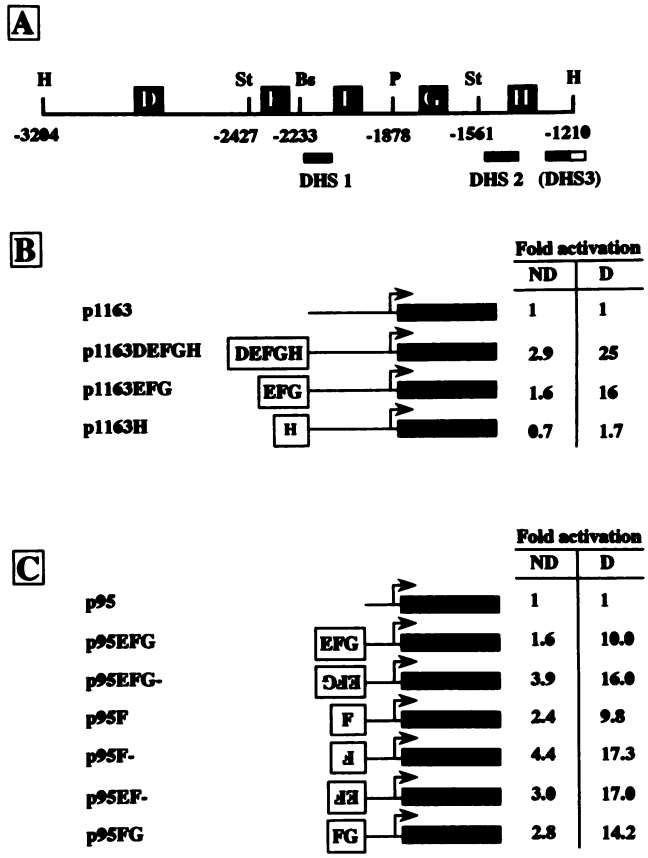


FIG. 3. Functional mapping of enhancer E1. (A) Schematic diagram of the region containing E1. H, *Hin*dIII; St, *Sty*I; Bs, *Bsr*I; P, *Pst*I. A fragment from −3204 to −1210, or subfragments from it, was inserted upstream of the −1163 *H5* promoter (p1163) (B) or upstream of the −95 promoter (p95) (C) in the natural or reverse (−) orientation. ND, ND-HD3; D, D-HD3. CAT activity is given relative to that of the parent plasmids p1163 and p95.

level of transcription in a noninducible HD3 cell line (41) and a stronger increase in differentiated cells (50). To investigate this element in a cellular and gene context comparable to those used for the enhancers E1 and E3, fragment IJ was inserted upstream and downstream of the −95 promoter in either orientation (Fig. 4A). These constructs, p95IJ$^-$, p95(3′)IJ, and p95(3′)IJ$^-$, were severalfold more active than the −95 promoter in D-HD3 than in ND-HD3 (Fig. 4B). These results confirmed the presence of a third differentiation-specific enhancer, hereafter referred to as E7. Additional mapping further indicated that E7 is contained within the +845 and +1181 boundaries (data not shown).

Given the number of enhancers, the question arose as to whether E7 represented a redundant element or whether it contributed with the upstream enhancers to increase the level of transcription. Therefore, we constructed p3612IJ, containing the three enhancers, and p3612I, in which E7 had been inactivated by deletion of fragment J (Fig. 4B) (41). The transfection results indicated that p3612IJ was twofold more active than p3612I in ND-HD3 but equally active in D-HD3 (Fig. 4C). The lack of effect of E7 upon cell differentiation suggests that a cooperation among the three enhancers is not needed for *H5* gene transcription in the more mature cells (see Discussion).

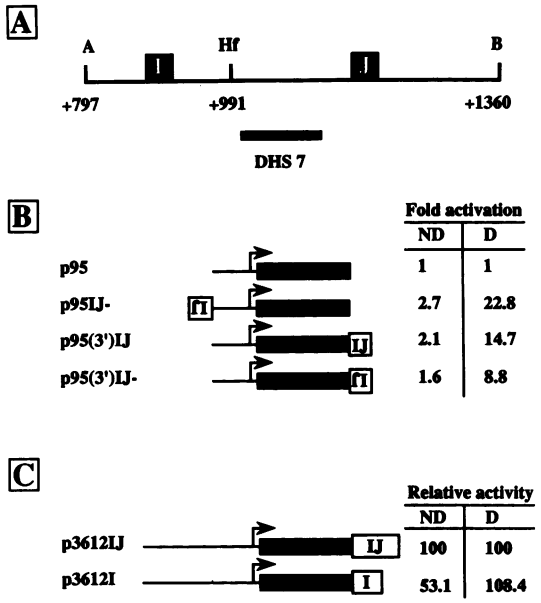


FIG. 4. Enhancer E7 does not cooperate with E1/E3 in D-HD3. (A) Schematic diagram of the region containing E7. A, *Alu*I; Hf, *Hin*fI; B, *Bam*HI. (B) A fragment from +797 to +1360 was inserted upstream or downstream of the −95 *H5* promoter (p95) in the natural or reverse (−) orientation. (C) A fragment from +797 to +1360 or from +797 to +991 was inserted downstream of the *CAT* gene in place of the simian virus 40 T-antigen fragment. ND, ND-HD3; D, D-HD3. CAT activity is given relative to that of the parent plasmids p95 and p3612IJ.

Nuclear protein binding to the *H5* promoter. DNase I protection experiments were carried out with nuclear proteins from erythroid cells (ND-HD3, D-HD3, and mature erythrocytes) and the T-lymphoblastoma cell line MSB-1. The nuclear extracts from erythroid and nonerythroid cells protected the same seven regions of the *H5* promoter, between −170 and +14 (Fig. 5A and C), although the extent and details of the protection differed with the source of the extract.

Footprint VII, spanning the transcription initiation site (Fig. 5A, lanes 4 to 6), is produced by cIBR (mature erythrocytes) and cIBF (HD3 and MSB-1). Erythroid cIBR and cIBF have been recently purified, and cIBR has been shown to specifically repress transcription from the *H5* promoter (14). Footprint VI, better seen when poly(dI-dC) rather than poly(dA-dT) is used as nonspecific competitor (Fig. 5A, cf. lanes 3 and 4), corresponds to a consensus CACCC sequence for TEF-2 (7, 56) or related factors like CON (8). Although Sp1 can recognize the CACCC motif (56) (Fig. 5B, lanes 3 and 4), gel shift competition assays indicated that the CACCC-binding protein(s) has a higher affinity than Sp1 for the sequence present in the *H5* promoter (not shown). The transcriptional effect of the CACCC sequence has not yet been directly tested by mutagenesis. However, the very low activities of p43 (Fig. 1B) and p43(3')IJ (not shown; see reference 41) suggest that this putative element may not be functional.

Footprint V mapped within the UPE, an element homologous to the histone H4 subtype-specific element (41). Since the factor binding to the *H4* sequences, H4TF2, is present in very low concentrations (6), we further characterized the UPE interaction by gel shift assays. Analysis with ND-HD3 nuclear extracts and a probe of the UPE region (−64 to −34)

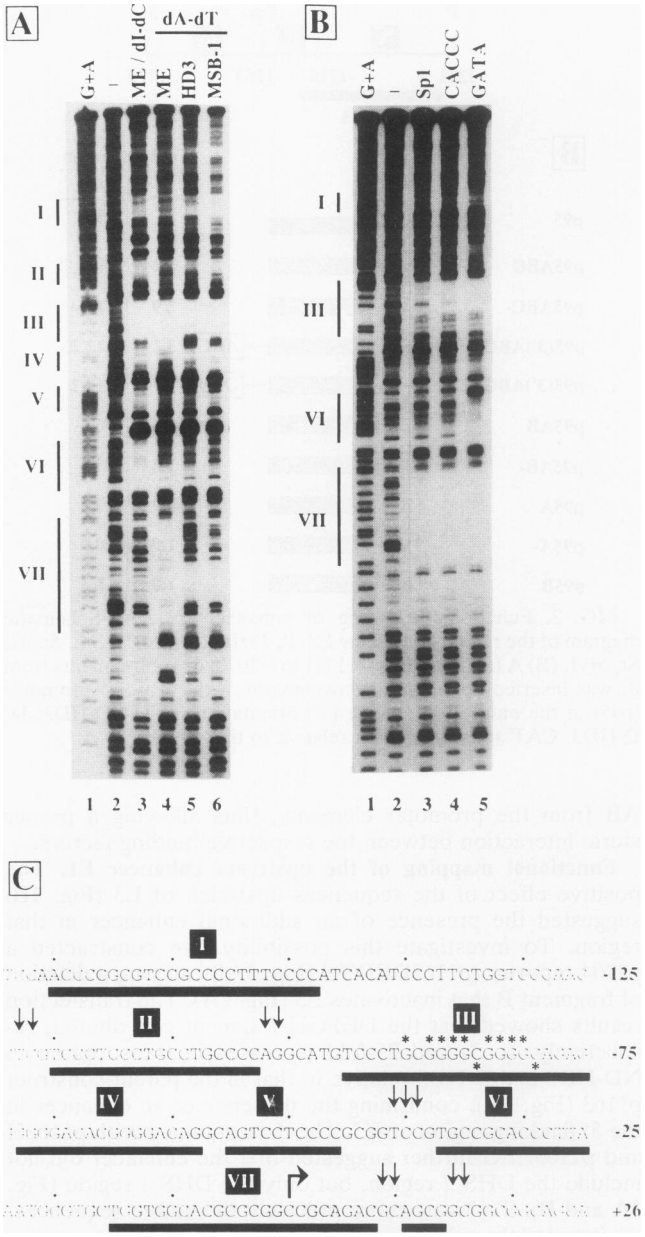


FIG. 5. DNase I footprinting of the *H5* promoter. (A) The probe was from −308 to +72 labeled at the 5′ end of the noncoding strand. Poly(dI-dC) was used as nonspecific competitor in lane 3 and poly(dA-dT) was used in all the others. Lanes: 1, G+A sequence ladder; 2, no protein; 3 and 4, mature erythrocytes (100 μg); 5, ND-HD3 (50 μg); 6, MSB-1 (50 μg). (B) Footprint competition experiments. The same probe was incubated with 250 ng of wheat germ agglutinin-binding proteins from a mature erythrocyte nuclear extract in the presence of a 300-fold molar excess of the indicated oligonucleotide before digestion with DNase I. Lanes: 1, G+A sequence ladder; 2, no protein; 3, Sp1; 4, CACCC; 5, E7-GATA. (C) Summary of the sequences protected by the DNA-binding proteins from erythroid cells. Roman numerals correspond to the footprints in panel A. Hypersensitive sequences are indicated by arrows. Nucleotides showing methylation interference are indicated with stars above the sequence and below the sequence for the complementary strand.

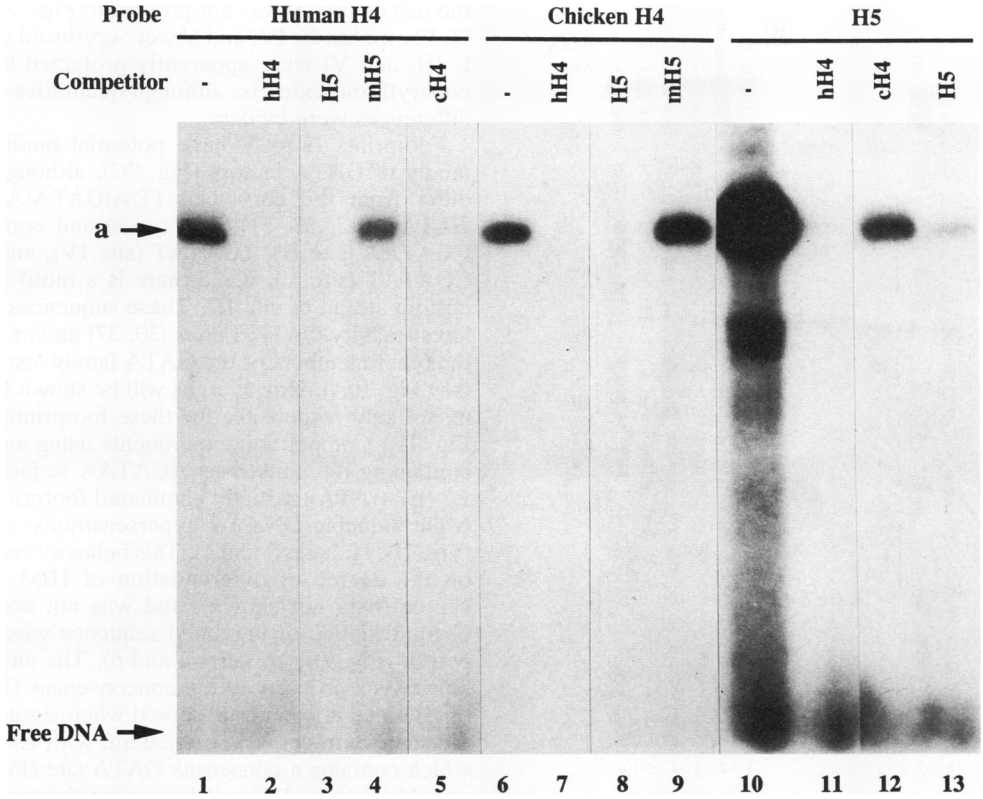


FIG. 6. The UPE is recognized by H4TF2. Purified human H4TF2 (6) was incubated with 5'-labeled oligonucleotide probes of the human *H4* gene pHuH4A (18) (lanes 1 to 5), chicken *H4* gene H4L (48) (lanes 6 to 9), and *H5* gene (lanes 10 to 13), all containing the *H4* subtype-specific region found upstream of the respective TATA boxes. The competitor oligonucleotides, where indicated, were present at a 50-fold molar excess during the binding reaction. Lanes: 1, no competitor; 2, human *H4*; 3, *H5*; 4, mutated *H5*; 5, chicken *H4*; 6, no competitor; 7, human *H4*; 8, *H5*; 9, mutant *H5*; 10, no competitor; 11, human *H4*; 12, chicken *H4*; 13, *H5*.

revealed the presence of two specific complexes. Competition with the appropriate wild-type and mutated oligonucleotides indicated that one of the complexes was produced by Sp1, presumably by interaction with the CCTCCC portion of the UPE, while the second complex was inhibited by a chicken H4 oligonucleotide containing the H4TF2 cognate sequence (not shown). To determine whether this factor was the chicken homolog of human H4TF2, a series of gel retardation assays were carried out with purified human H4TF2 (6), in collaboration with S. B. Roberts and N. Heintz. Figure 6 (lane 1) shows that purified H4TF2 produced a single complex (*a*) with an oligonucleotide containing the cognate sequence of the human histone *H4* gene pHu4A (19). This complex was specifically inhibited by oligonucleotides containing the homologous sequence or the corresponding regions of the chicken *H4L* (53) and *H5* genes but not by an *H5* oligonucleotide carrying a mutation of the relevant region (Fig. 6, lanes 2 to 5). A complex of similar mobility was also observed when the chicken *H4* motif was used as probe (Fig. 6, lane 6). This complex was specifically inhibited by the wild-type human *H4* and *H5* oligonucleotides but not by the mutated *H5* oligonucleotide (Fig. 6, lanes 7 to 9). Finally, when the wild-type *H5* oligonucleotide was used as probe, a complex of mobility similar to those obtained with the *H4* probes was observed (Fig. 6, lane 10). This complex could be equally inhibited with oligonucleotides containing the wild-type chicken *H4* and *H5* and human *H4* elements (Fig. 6, lanes 11 to 13). Therefore, the human H4TF2 factor recognizes the *H5* and *H4* elements with comparable affinity, indicating that the UPE can be recognized by its chicken homolog.

Footprint IV corresponds to the direct repeat GGGGACA CGGGGACA, a sequence motif also found in several histone gene promoters of *Caenorhabditis elegans* (40). This sequence may not be functional, since its deletion had no apparent effect on *H5* promoter activity (41). Footprints III and I correspond to GC boxes which were also protected by wheat germ agglutinin-binding proteins from the extract (Fig. 5B, lane 5) (14). Since Sp1 is known to be glycosylated (22) and the footprints were efficiently inhibited by the Sp1 oligonucleotide (Fig. 5B, lanes 3 and 4), we concluded that these sites bind Sp1, a conclusion further supported by the pattern of methylation interference (shown in Fig. 5C), and by competition with an oligonucleotide containing the SP1-binding sites of the simian virus 40 promoter (data not shown). While the upstream GC box is probably not functional (41), promoter activity is severely impaired by removal of the proximal GC box (Fig. 1B, cf. p95 and p86) (41). Our data also indicate that the proximal GC box is needed for activated transcription, since E7 cannot rescue the activity of the −43 promoter (41) in which the sequence had been deleted.

Footprint II mapped within UNE, a polypyrimidine sequence that has a negative effect on transcription in erythroid (Fig. 1B) and nonerythroid cells (41). Consistent with this, a similar footprint was observed with nuclear proteins from liver (data not shown).

Nuclear protein binding to enhancer E1. DNase I protec-

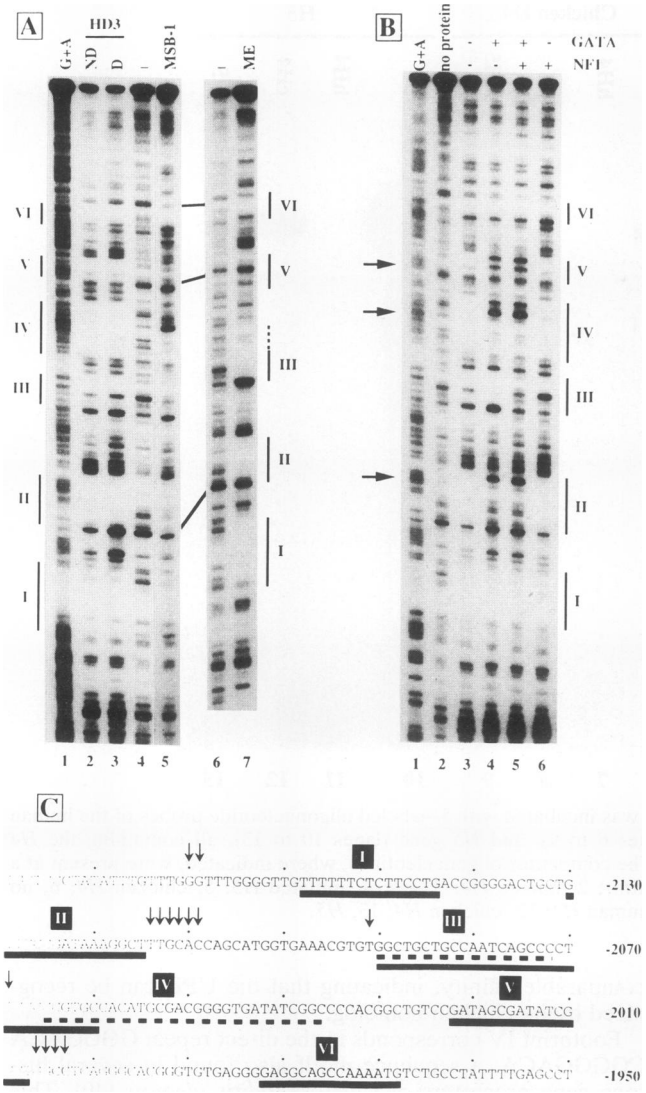


FIG. 7. DNase I footprinting of the enhancer E1 region. (A) The probe was from −2296 to −1817 labeled at the 5′ end of the top strand. Lanes: 1, G+A sequence ladder; 2, ND-HD3 (35 μg); 3, D-HD3 (20 μg); 4, no protein; 5, MSB-1 (50 μg); 6, no protein; 7, mature erythrocytes (160 μg). (B) Footprint competition experiments. The same probe was incubated with nuclear proteins from D-HD3 cells (40 μg) in the presence of a 300-fold molar excess of the indicated oligonucleotide before digestion with DNase I. The arrows indicate hypersensitive regions induced by competition with the GATA oligonucleotide. Lanes: 1, G+A sequence ladder; 2, no protein; 3, no competitor; 4, GATA; 5, GATA plus α_1-fetoprotein NFI; 6, α_1-fetoprotein NFI. (C) Summary of the sequences protected by the DNA-binding proteins from erythroid cells. Roman numerals correspond to the footprints in panels A and B. Arrows indicate regions of hypersensitivity. Sites III and IV were differentially protected by HD3 (broken line) and erythrocyte (solid line) proteins.

tion experiments, with the same extracts described above, indicated that proteins from ND-HD3 and D-HD3 protected the same six regions (footprints I to VI, from −2160 to −1960) (Fig. 7A, lanes 2 and 3). However, proteins from transcriptionally inactive mature erythrocytes produced an extended footprint III, partially overlapping site IV, while the rest of site IV was not protected (Fig. 7A, lanes 2, 3, and 7). Footprints II, IV, and V were erythroid specific, whereas I, III, and VI were apparently protected by erythroid and nonerythroid extracts, although qualitative and quantitative differences were evident.

Footprints II to V have potential binding sites for the family of GATA factors (Fig. 7C), although the sequences differ from the consensus (T/A)GATA(A/G) derived for GATA-1 (12, 33, 51). The top strand contains the motifs CGATAA (site II), TGATAT (site IV), and CGATAG and CGATAT (site V), while there is a motif TGATTG in the bottom strand of site III. These sequences could represent lower-affinity GATA-1 sites (30, 37) and/or binding sites for the other members of the GATA family also present in HD3 (see Fig. 10A). However, as will be shown later, GATA-1 is most likely responsible for these footprints (see below and Fig. 11). Competition experiments using an oligonucleotide containing the consensus AGATAA sequence of enhancer E7 (E7-GATA) partially eliminated footprints II, IV, and V while inducing DNase I hypersensitivity at the same sites (Fig. 7B, cf. lanes 2 and 4). This behavior was not dependent on the degree of differentiation of HD3 used as protein source (data not shown), and was not observed when an oligonucleotide of unrelated sequence was used as a competitor (Fig. 7B, cf. lanes 4 and 6). The nuclease hypersensitivity is a property of the nonconsensus GATA sequences of E1 since it was not observed when similar analysis, with the same extracts, was carried out with E3 and E7, each of which contains a consensus GATA site (Fig. 8 and 9). It is possible that the DNase I hypersensitivity in the presence of the competitor E7-GATA reflects the interaction of non-GATA factors with these GATA sites.

In the case of footprint III, competition with the E7-GATA oligonucleotide did not eliminate the footprint but, instead, resulted in a more complete protection of the site (Fig. 7B, cf. lanes 3 and 4). Since footprint III contains the sequence TGGCN$_4$GCCAA that could bind a member of the NF1 family (15, 16), we carried out single and double competitions with E7-GATA and an oligonucleotide containing a NF1 sequence of the rat α_1-fetoprotein gene (18). Double competition completely abolished footprint III, whereas competition with the NF1 oligonucleotide alone affected only the 5′ side of the footprint (Fig. 7B, cf. lanes 3, 5, and 6). Since the NF1 and GATA sites partially overlap (i.e., the dinucleotide CA of the 3′ half of the NF1 palindrome forms part of the GATA site TGATTG), the results suggest that both factors can compete for site III binding. Factor occupancy of site III appears to change during erythrocyte maturation, since proteins from the mature cells produced a longer footprint III, extending into the upstream portion of site IV (Fig. 7A, lane 7, and Fig. 7C), while leaving unprotected the TGATAT sequence of site IV. This behavior may reflect changes in relative activity of NF1 and GATA factors during terminal maturation.

The proteins binding to sites I and VI are unknown, although the sequence of site VI is homologous to a region of the chicken β^A-globin promoter reported to bind nuclear factors from erythrocytes and brain tissue and activate transcription in vitro (8). We also note that the polypyrimidine sequence of site I has homology to the binding site of the erythroid factor NF-E4 on the β^A-globin promoter (13).

Nuclear protein binding to enhancer E3. DNase I protection analysis with the same extracts described above revealed that the erythroid proteins protected only two regions from −1290 to −1170 of the core E3 enhancer (Fig. 8A and

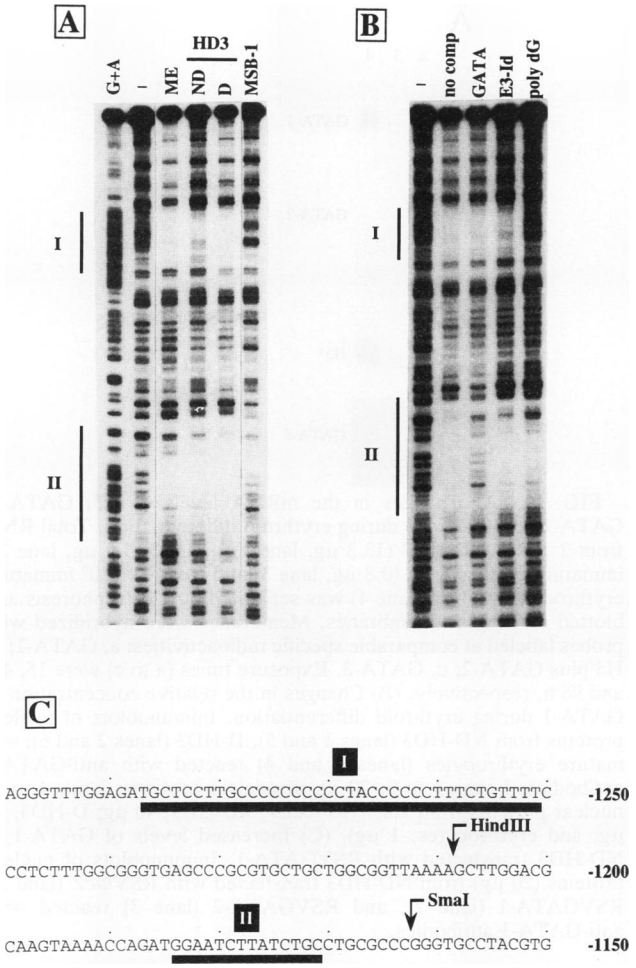


FIG. 8. DNase I footprinting of the enhancer E3 region. (A) The probe was from −1321 to −1070 labeled at the 5′ end of the bottom strand. Lanes: 1, G+A sequence ladder; 2, no protein; 3, mature erythrocytes (40 μg); 4, ND-HD3 (35 μg); 5, D-HD3 (20 μg); 6, MSB-1 (50 μg). (B) Footprint competition experiments. The same probe was incubated with 40 μg of nuclear proteins from D-HD3 in the presence of a 300-fold molar excess of the indicated oligonucleotide before digestion with DNase I. Lanes: 1, no protein; 2, no competitor; 3, GATA; 4, E3-Id; 5, 200 ng of poly(dG) · poly(dC). (C) Summary of the sequences protected by the DNA-binding proteins from erythroid cells. Roman numerals correspond to the footprints in panels A and B.

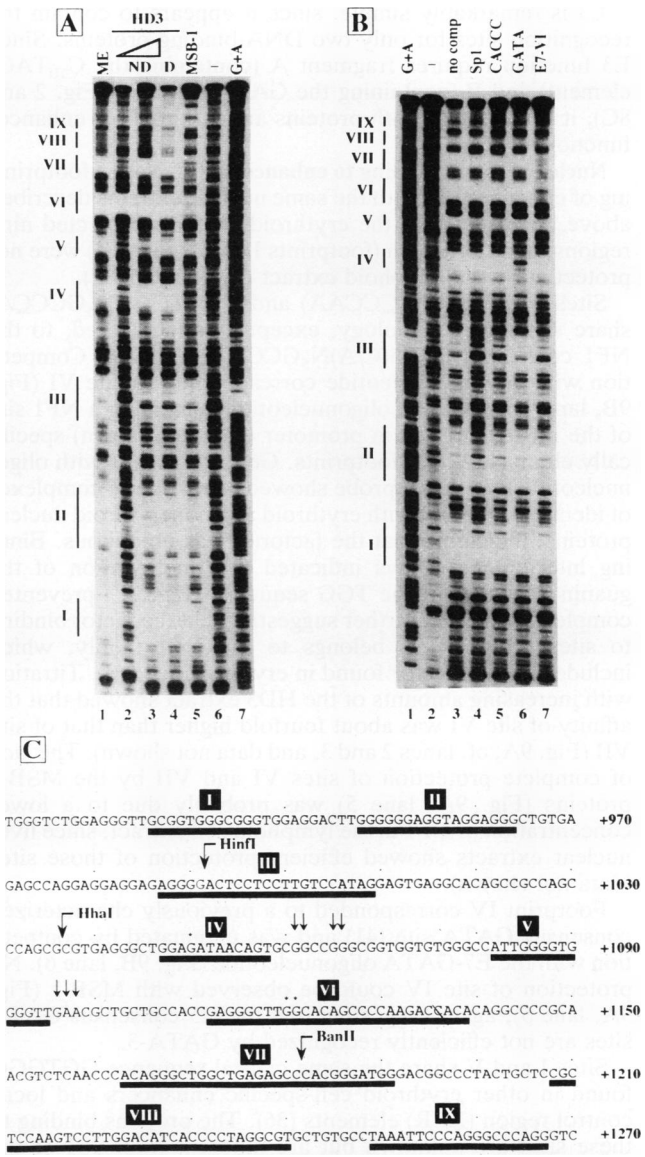


FIG. 9. DNase I footprinting of the enhancer E7 region. (A) The probe was from +846 to +1360 labeled at the 5′ end of the top strand. Lanes: 1, mature erythrocytes (40 μg); 2, ND-HD3 (5 μg); 3, ND-HD3 (35 μg); 4, D-HD3 (20 μg); 5, MSB-1 (50 μg); 6, no protein; 7, G+A sequence ladder. (B) Footprint competition experiments. The same probe was incubated with nuclear proteins from D-HD3 cells (40 μg) in the presence of a 300-fold molar excess of the indicated oligonucleotide before digestion with DNase I. Lanes: 1, G+A sequence ladder; 2, no protein; 3, no competitor; 4, Sp1; 5, CACCC; 6, GATA; 7, H5 E7-VI. (C) Summary of the sequences protected by the DNA-binding proteins from erythroid cells. Roman numerals correspond to the footprints in panels A and B. Hypersensitive sequences are indicated by arrows. Nucleotides showing dimethyl sulfate methylation interference are indicated with stars. The restriction nuclease sites used in the construction of the reporter genes shown in Fig. 4 and others discussed in the text are given as references.

C), and no additional footprints were observed in sequences down to −1070 (data not shown).

Footprint II includes a consensus GATA site (AGATAA) in the bottom strand. This footprint was specifically inhibited by the E7-GATA oligonucleotide (Fig. 8B, lanes 3 to 5), and unlike E1, no DNase I hypersensitivity was induced. Interestingly, the MSB-1 proteins, containing GATA-3 (20, 23, 57), did not protect site II (Fig. 8A, cf. lanes 3 to 6).

Footprint I, which covers a $C_{10}TAC_6$ sequence and its flanks, was specifically inhibited with poly(dG) · poly(dC) (Fig. 8B, cf. lanes 3 and 5) or with cloned $(dG)_{29} \cdot (dC)_{29}$ tracts (data not shown), but not with oligonucleotide E3-Id, containing the −1263 to −1241 sequences downstream of the C_6 tract (Fig. 8B, lane 4). Gel shift assays with cloned dG · dC tracts also indicated the formation of specific complexes (5a). Therefore, the factor binding to site I belongs to the family of G-string binding proteins described for several systems (24, 25, 55), including erythrocytes (28). Changes in the degree of protection of footprints I and II (Fig. 8A) suggest that the activity of the $C_{10}TAC_6$-binding factor increases relative to that of GATA-1 during maturation.

E3 is remarkably simple, since it appears to contain the recognition sites for only two DNA-binding proteins. Since E3 function requires fragment A (containing the $C_{10}TAC_6$ element) and B (containing the GATA element) (Fig. 2 and 8C), it follows that both proteins are essential for enhancer function.

Nuclear protein binding to enhancer E7. DNase I footprinting of enhancer E7, with the same nuclear extracts described above, indicated that the erythroid proteins protected nine regions, three of which (footprints IV, VI, and VII) were not protected by the lymphoid extract (Fig. 9A and C).

Sites VI ($TGGCN_5\underline{C}CCAA$) and VII ($TGGCN_5GCC\underline{C}A$) share significant homology, except as underscored, to the NF1 consensus [$TGG(C/A)N_5GCCAA$] (15, 16). Competition with an oligonucleotide corresponding to site VI (Fig. 9B, lane 7) or with an oligonucleotide containing a NF1 site of the rat α_1-fetoprotein promoter (data not shown) specifically eliminated both footprints. Gel shift assays with oligonucleotide E7-VI as a probe showed formation of complexes of identical mobility with erythroid and nonerythroid nuclear proteins, indicating that the factor(s) was ubiquitous. Binding interference assays indicated that methylation of the guanine residues of the TGG sequence (Fig. 9C) prevented complex formation, further suggesting that the factor binding to sites VI and VII belongs to the NF1 family, which includes the Pal factor found in erythroid cells (8). Titration with increasing amounts of the HD3 extract showed that the affinity of site VI was about fourfold higher than that of site VII (Fig. 9A, cf. lanes 2 and 3, and data not shown). The lack of complete protection of sites VI and VII by the MSB-1 proteins (Fig. 9A, lane 5) was probably due to a lower concentration of NF1 in the lymphoid cell extract, since liver nuclear extracts showed efficient protection of those sites (data not shown).

Footprint IV corresponded to a previously characterized consensus GATA site (41) and was eliminated by competition with the E7-GATA oligonucleotide (Fig. 9B, lane 6). No protection of site IV could be observed with MSB-1 (Fig. 9A, lane 5), again suggesting that the *H5* consensus GATA sites are not efficiently recognized by GATA-3.

Sites I and V share the same central sequence GGTGGG found in other erythroid cell-specific enhancers and locus control region (LCR) elements (36). The proteins binding to these sites are unknown but are different from Sp1 or the CACCC-binding factor(s) (Fig. 9B, lanes 4 and 5). Footprints II (polypurine) and III (polypyrimidine) share homology to the NF-E4 site of the β^A-globin promoter (13). However, the Sp1 and CACCC oligonucleotides eliminated these footprints (Fig. 9B, lanes 4 and 5), indicating that these factors can also bind to sites II and III.

Although site IX matches the consensus for AP-2 (31), the nature and possible function of the proteins binding to sites VIII and IX are unknown, since deletion of these sites (by cleavage at *Ban*II [Fig. 9C]) did not impair enhancer function (data not shown).

Expression of GATA factor and H5 mRNAs during differentiation. Since the GATA sites were required for enhancer function, it was relevant to determine the steady-state mRNA levels of the GATA factors during HD3 differentiation. Blots of total RNA from the same number of cells were hybridized with cDNA probes for H5, GATA-1, GATA-2, and GATA-3, labeled at comparable specific radioactivities. Figure 10A (lane 1) shows that GATA-1 mRNA was, by and large, the most abundant mRNA in ND-HD3, followed by those of GATA-2 and GATA-3, the latter representing a relatively minor species. H5 mRNA, barely detectable in the autoradiography (lane 1), could be better seen when the blot was hybridized with the H5 probe alone. Differentiation resulted in a reduction in the levels of all GATA mRNAs (lanes 2), the relative decrease being smaller for GATA-1 mRNA. This trend in the expression of GATA mRNAs is presumably a continuous process during maturation, since GATA-1 mRNA was the only detectable species in immature erythrocytes (Fig. 10A, lanes 3 and 4). On the other hand, H5 mRNA levels increased with the onset of differentiation and were still high in immature erythrocytes (Fig. 10A) (1). Blots of proteins from whole cells probed with GATA-1 antibodies also indicated that the concentration of GATA-1 decreased during differentiation by a factor of 2 in D-HD3 and by more than 20 in immature erythrocytes (Fig. 10B).

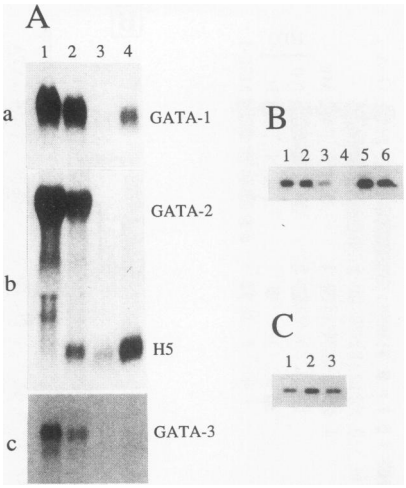


FIG. 10. (A) Changes in the mRNA levels of H5, GATA-1, GATA-2, and GATA-3 during erythroid differentiation. Total RNA from 2×10^6 ND-HD3 (13.3 μg, lane 1), D-HD3 (5.5 μg, lane 2), immature erythrocytes (0.8 μg, lane 3) and from 6×10^6 immature erythrocytes (2.4 μg, lane 4) was separated by electrophoresis and blotted onto nylon membranes. Membranes were hybridized with probes labeled at comparable specific radioactivities: a, GATA-1; b, H5 plus GATA-2; c, GATA-3. Exposure times (a to c) were 15, 48, and 96 h, respectively. (B) Changes in the relative concentration of GATA-1 during erythroid differentiation. Immunoblots of nuclear proteins from ND-HD3 (lanes 1 and 5), D-HD3 (lanes 2 and 6), and mature erythrocytes (lanes 3 and 4) reacted with anti-GATA-1 antibodies. Lanes 1 to 3, 20 μg of nuclear proteins; lanes 4 to 6, nuclear proteins from 1.6×10^6 cells (ND-HD3, 40 μg; D-HD3, 20 μg; and erythrocytes, 1 μg). (C) Increased levels of GATA-1 in ND-HD3 transfected with RSVGATA-1. Immunoblots of nuclear proteins (20 μg) from ND-HD3 transfected with RSV*lacZ* (lane 1), RSVGATA-1 (lane 2), and RSVGATA-2 (lane 3) reacted with anti-GATA-1 antibodies.

GATA-1 binds to the GATA sites of the enhancers. The abundance of GATA mRNAs in HD3 makes it possible that the GATA sites of the enhancers can be recognized by any of the three factors. Gel shift assays with probes containing the nonconsensus sites of E1 (E1-II-GATA and E1-V-GATA) and the consensus sites of E3 (E3-GATA) and E7 (E7-GATA) produced a major (*a*) and a minor (*b*) complex, the mobilities of which were independent on the degree of cell maturity (Fig. 11A). Competition with the homologous sequences (e.g., Fig. 11B), as well as with heterologous GATA sites (not shown), but not with oligonucleotides of unrelated sequence (Fig. 11B) indicated that the complexes were specific. Incubation of the nuclear extracts with GATA-1 antibody, prior to the binding reactions, resulted in disap-

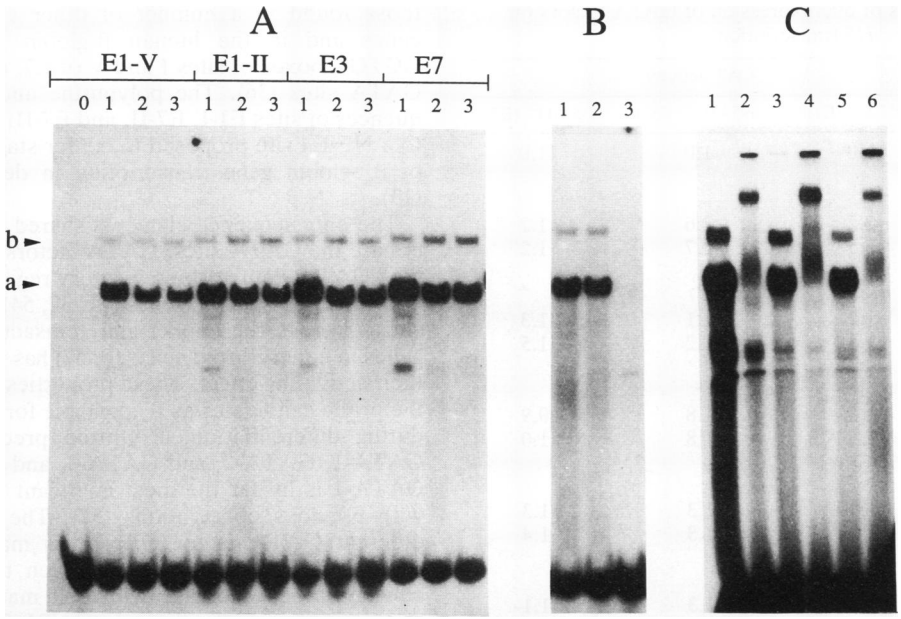


FIG. 11. Interaction of GATA-1 with enhancers E1, E3, and E7. (A) Gel shifts of nuclear proteins from erythrocytes (lanes 1), D-HD3 (lanes 2), and ND-HD3 (lanes 3) with 1 ng of oligonucleotide probes corresponding to GATA footprints of the enhancers labeled at a comparable specific radioactivity. Lane 0, no extract. (B) Probe E7-GATA was incubated with nuclear proteins from D-HD3 in the absence of competitor (lane 1), and in the presence of a 100-fold molar excess of heterologous (E7-VI, lane 2) or homologous (E7-GATA, lane 3) competitor oligonucleotides. (C) Gel shifts of nuclear proteins from mature erythrocytes (lanes 1 and 2), ND-HD3 (lanes 3 and 4), and D-HD3 (lanes 5 and 6) with probe E7-GATA. In lanes 2, 4, and 6, the nuclear extracts were preincubated with anti-GATA-1 antibodies.

pearance of complexes *a* and *b* with the concomitant appearance of supershifts (e.g., Fig. 11C, cf. lanes 1, 3, and 5 with 2, 4, and 6). On the other hand, incubation with preimmune sera or with antibodies raised against cIBR (14) had no effect on the formation and mobility of complexes *a* and *b* (not shown). Since the same results were obtained with the different probes, we concluded that GATA-1 binds to the enhancer sequences in vitro. Methylation and carbetoxylation interference assays further indicated that the contacts made by the proteins in complexes *a* and *b* were the same (not shown) (41). Complex *a* probably represents binding of GATA-1 monomer, whereas complex *b* probably contains a GATA-1 homodimer or heterodimer, since only the E1-V-GATA probe has two close GATA sites, and only one of them was occupied. Fractionation of the erythrocyte extract by sedimentation in glycerol gradients further supported this interpretation.

Activation of *H5* transcription is independent of changes in the concentration of the GATA factors. Although GATA-1 binds to the enhancer GATA sites in vitro, the possibility that the situation in vivo was different could not be ruled out. If GATA-2 and GATA-3 were to compete in vivo with GATA-1, occupancy of the enhancer GATA sites by any of these factors might change during differentiation and be responsible for the activation of the *H5* gene. This possibility was explored by transfecting HD3 with reporter constructs containing enhancers E1, E3, or E7 together with expression vectors of the GATA factors or with the appropriate antisense controls (57). Previous experiments indicated that despite the high levels of endogenous GATA-1, transfection with RSVGATA-1 increased the cellular concentration of GATA-1 by a factor of two (Fig. 10C), a minimal estimate, considering that not all cells were transfected. Transfection with the GATA-2 and GATA-3 expression vectors probably resulted in higher relative increases of these factors because of their lower endogenous concentrations (Fig. 10A).

Table 1 shows that the activity of the E1 and E3 reporters, but not that of the E7 reporter, was slightly increased by overexpression of GATA-1 and GATA-2, while overexpression of GATA-3 had no effect. Since these effects were much smaller than the activation observed during HD3 differentiation, these results indicated that enhancer activation is not related to the changes in relative concentration of the GATA factors. Although small, the different response of the enhancers to supernumerary GATA-1 and GATA-2 factors was reproducible and might reflect the affinity of these factors for the GATA sites. The lack of effect of GATA-3 and the inability of the MSB-1 extract (containing GATA-3) to footprint the GATA sites of the enhancers (Fig. 7 to 9) further suggests that this factor plays no role in *H5* gene activity.

DISCUSSION

The observations reported here and in our previous work (14, 41) show that transcription of the *H5* gene in CFU(E) is predominantly controlled by positive (GC box and UPE) and negative (UNE) upstream promoter elements. The flanking sequences up to −3.6 kb and +1.4 kb contribute little to transcription, although their effect compensates for that of UNE. However, when CFU(E) are induced to differentiate, these flanking regions reveal the presence of enhancers E1, E3, and E7 responsible for the increased levels of transcription.

We have shown that E1 and E3 cooperate in HD3 regardless of their state of maturity, while a cooperation among the three enhancers was only observed in the nondifferentiated cells. A possible role of the enhancers is to provide redun-

TABLE 1. Effects of overexpression of GATA factors on *H5* transcription[a]

GATA vector (μg)	CAT activity[b]		
	p95EF⁻ (E1)	p95ABC⁻ (E3)	p95IJ⁻ (E7)
None	1.0	1.0	1.0
RSVGATA-1-S			
0.25	2.2	2.6	1.2
0.50	2.5	2.7	1.2
RSVGATA-1-AS			
0.25	1.2	1.1	1.3
0.50	1.3	1.2	1.5
RSVGATA-2-S			
0.25	1.6	1.8	0.9
0.50	1.5	1.8	1.0
RSVGATA-2-AS			
0.25	1.4	1.3	1.3
0.50	1.3	1.3	1.4
RSVGATA-3-S			
0.25	1.3	1.3	1.1
0.50	1.1	1.4	1.3
RSVGATA-3-AS			
0.25	1.4	1.3	1.3
0.50	1.5	1.4	1.3

[a] *H5* reporter genes (1 μg) were cotransfected in ND-HD3 with RSV*lacZ* (0.25 μg) and the indicated amounts of the expression GATA vectors.
[b] CAT activity of each *H5* construct is expressed relative to that in the absence of the GATA vectors. Values in rows are not to be compared.

dant elements to ensure activation of the gene. A second, more appealing, alternative is that they serve different purposes during development of the erythroid lineage to secure timely expression of the gene. The number of enhancers and hypersensitive sites of the *H5* locus suggest that some of them may have a role equivalent to that of the LCR of the human β-globin gene cluster (17). The LCR is composed of several *cis*-acting elements, marked by four DNase I hypersensitive sites, located some 50 kb upstream of the β-globin gene that confers high-level, copy-number-dependent and position-independent expression to associated genes (4). Two of the LCR domains (sites 2 and 3) that provide about half of the LCR activity and contain binding sites for GATA factors (36, 49) act as erythroid cell-specific enhancers when assayed by transfection. The other two sites can cooperate with sites 2 and 3 but have little activity on their own (5). The parallelism between the upstream region of the *H5* gene, also containing four DNase I hypersensitive domains, two of which have been identified as enhancers, and the β-globin LCR suggest the testable hypothesis that the *H5* elements constitute the LCR of the H5 locus.

It is reasonable to predict that the differentiation-specific enhancers respond to qualitative and/or quantitative alterations in the population of transcription factors brought about by the release of the cells from the differentiation block. Analysis of the DNA-binding proteins from cells with different degrees of maturity has revealed that enhancers E1 and E7, which activate transcription to comparable levels, are complex and contain a large array of binding sites for transcription factors, while E3 appears to be very simple, since it contains sites for only two DNA-binding proteins (Fig. 12). Elements of these enhancers share homology with those found in a number of other erythroid cell-specific genes and in the human β-globin LCR, including the GGTGG boxes of sites I and V of E7, often associated with GATA sites (36). The polypurine and polypyrimidine sequences of sites E1-I, E7-II, and E7-III are also homologous to a NF-E4 site proposed to confer stage-specific activation of β^A-globin gene transcription in definitive erythrocytes (13).

The only sequence elements shared by the three enhancers are the GATA sites. GATA factors have been shown to bind GATA elements in regulatory regions of the majority of erythroid cell-specific genes (11, 32, 54). Mutation of GATA sites (38, 51; this work) and transactivation of erythroid genes in nonerythroid cells (10, 57) has provided evidence of their activating effect. These properties made GATA factors the prime candidates as responsible for the activation of *H5* during differentiation. Erythroid precursor cells express GATA-1, GATA-2, and GATA-3, and we have shown that GATA-1 is by far the most abundant factor, in agreement with previous observations (57). The balance among the different GATA factor mRNAs is maintained during the early stages of differentiation, even though their cellular concentrations decrease with cell maturity. However, as maturation proceeds, the only GATA factor mRNA detectable in cells which still transcribe the *H5* gene was that of GATA-1. These observations implicate GATA-1 in *H5* enhancer function and are consistent with the fact that GATA-1 recognizes the GATA sites of the three enhancers, whether or not they contain the consensus WGATAR sequence (12, 33, 52).

Despite the central role of GATA-1 in *H5* gene activity, it appears that this factor is not sufficient for activation of *H5* transcription during differentiation. Increasing the cellular concentration of GATA-1 had minimal effects on transcription, as expected from the high levels of the endogenous factor, and overexpression of GATA-2 and GATA-3 did not produce significant effects. This makes it unlikely that activation of *H5* reflects changes in the type of GATA factor occupying the GATA sites. It is equally unlikely that *H5* activation is due to possible stage-specific modifications of GATA-1 resulting in an increase of its activator character, since GATA-1 is required much earlier in development (35). Therefore, it is reasonable to consider that *H5* activation is mediated by changes in the activity of other factors. Although we have observed differences in the population of E1-binding factors from cells expressing different levels of H5 mRNA, this was not observed for E3 or E7, yet the latter can activate *H5* gene transcription to levels comparable to those for E1. In the case of E3, it is particularly revealing that the two binding factors, GATA-1 and a G-string binding protein, presumably BGP-1 (28), are already present in the nondifferentiated cells that express little H5 mRNA. Although it is possible that the same sites can be recognized by diverse factors having different properties, it is equally possible that activation of the *H5* gene is brought about by changes in promoter factors.

Functional analysis of the *H5* promoter has shown that the UPE, which is required for activity in the nondifferentiated cells, was dispensable following differentiation. The UPE was proposed to be analogous to the histone *H4* subtype-specific element on the basis of sequence homology (41), and we have shown that it binds human H4TF2, a factor that activates *H4* transcription (6, 27). Factors interacting with the *H4* element are cell cycle regulated (21), and occupancy of the *H4* element in vivo by HiNF-D decreases following the onset of HL-60 differentiation (47). The timing at which

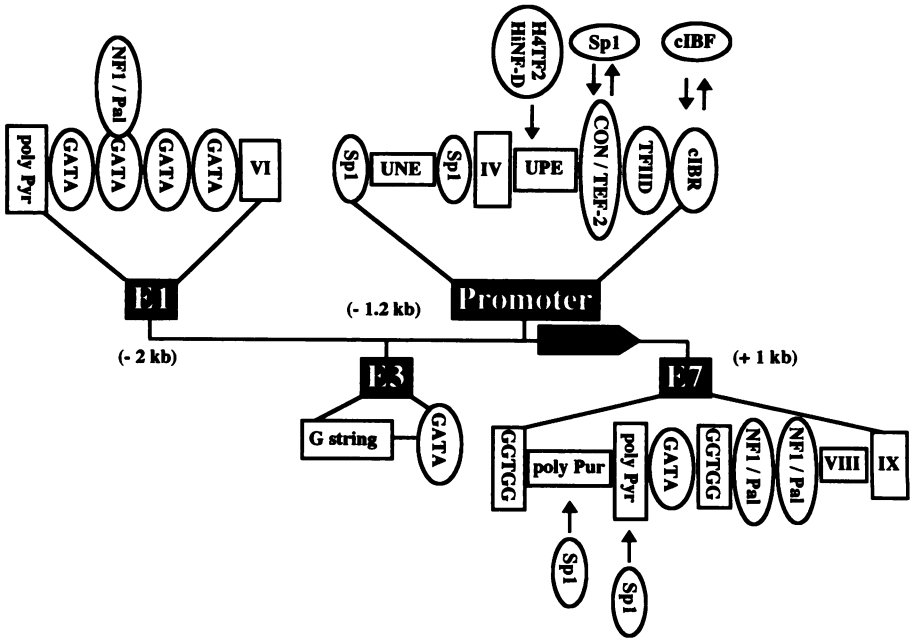


FIG. 12. Schematic summary of the control regions and binding factors of the chicken *H5* gene. The H5 mRNA coding region is indicated by the thick arrow pointing to the orientation of transcription. Sequence elements are indicated by square boxes and binding factors by round boxes. For details, see the text.

the UPE becomes dispensable overlaps with the increase in enhancer function, as if the *H5* enhancers prevented the promoter from being repressed by the down regulation of the UPE-binding factor(s). This mechanism would be in keeping with the biology of the system, since although transcription of *H4* is replication dependent, transcription of *H5* is not restricted to the S phase (1, and references therein). Therefore, when the cells are not in S phase and when their proliferation potential decreases because of differentiation, alternative mechanisms must exist not only to prevent inactivation of the *H5* gene but also to increase it to the final levels. It will be interesting to determine whether the UPE-binding factor shields the promoter from enhancer action in the nondifferentiated HD3 cells. Since the *H4* subtype-specific sequence is structurally and functionally related to the UPE (and probably to a highly homologous element of the $H1^0$ gene [41]), we propose that this element be referred to as the proliferation responding element, a term that better describes its effects.

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