Analysis of erythroid nuclear proteins binding to the promoter and enhancer elements of the chicken histone H5 gene

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ABSTRACT

The chicken erythroid proteins binding to the histone H5 5′ promoter and 3′ erythroid-specific enhancer regions were identified. In DNase I footprinting and gel mobility shift experiments with immature adult erythrocyte nuclear extracts, we have demonstrated the binding of proteins to the GC-box, a high affinity Sp1 binding site, and to the upstream promoter element. We have previously demonstrated that a multisubunit complex containing the transcription factor GATA-1 was associated with the enhancer. Here, we show that the enhancer region also has four Sp1 binding sites (one medium and three weak affinity, one of which may also bind the CACCC factor), a potential NF-E4 binding site, and a binding site for a NF1-like factor. The results of gel mobility-shift and competition experiments provide evidence that the Sp1 binding sites are associated with a high molecular mass (greater than 450 kDa), Sp1 containing protein complex. We propose that Sp1 multimers bound at the promoter and enhancer interact to mediate the juxtapositioning of the enhancer and promoter elements, bringing the GATA-1 multisubunit complex next to the initiation site. The GATA-1 complex may contribute to the protein-protein interactions between the enhancer and promoter.

INTRODUCTION

Chicken histone H5 is a H1-like linker histone that is expressed only in nucleated erythrocytes (1). Levels of histone H5 protein increase during the differentiation and maturation of the chicken erythroid cell, and this increase is correlated with chromatin condensation, the shut down of replication and the repression of the transcription of most, but not all, genes (2). Erythroid-specific β-globin and histone H5 genes, for example, are transcriptionally active in adult chicken immature erythrocytes (3).

The chicken erythroid histone H5 gene chromatin has several DNase I hypersensitive sites (DHS) located in the 5′ and 3′ flanking regions of the gene (4). DHS 5 maps at or near a tissue-specific upstream promoter element (5,6), while DHS 7U and 7L map with a 3′ enhancer element which is located in the region +851 to +1064 (6,7). The histone H5 gene promoter has two positive elements: a GC-box (−83 to −75) and an upstream activating sequence (UPE, −54 to −38) which has a sequence similar to the H4 gene subtype-specific element (6). The sequence of the histone H5 gene promoter GC-box is GGGGCGGGG which is a strong Sp1 binding site (8). Sp1 is a ubiquitous transcription factor with a molecular mass of 95 and 105 kDa that binds to the sequence (G/T)(G/A)GGC(G/T)(G/A)(G/A) (G/T) (9). This transcription factor will also bind to sequences where the C is changed to T or A, but these changes reduce the sequences’ affinity for Sp1 binding (10−12). Recently, Mastrangelo et al. (13) presented evidence that Sp1 forms a tetramer which is subsequently assembled into multiple tetramers. The Sp1 multimer complex provides a mechanism for interaction of Sp1 at different sites, resulting in looping out of the intervening DNA sequences (13,14).

At position +1053, the histone H5 gene enhancer region contains the GATA-binding motif AGATAA which binds to the hematopoietic-specific transcription factor GATA-1 (6,15). In gel mobility-shift experiments with a DNA fragment (+893 to +1064) spanning the enhancer region, we reported that GATA-1 binds to its target sequence as a multisubunit complex (15). Our results also indicated that other proteins were associated with the region +893 to +980. To activate transcription, the histone H5 3′ enhancer requires the presence of histone H5 promoter elements (−38 to −90), suggesting that there is an interaction between the proteins associated with the enhancer and promoter elements (6).

In this study, we identified erythroid nuclear proteins that interact with the histone H5 enhancer and promoter regions. Gel mobility-shift and DNase I footprinting experiments revealed that protein factors were associated with the GC box (Sp1 binding site), UPE and TATA box elements of the promoter. The enhancer region had binding sites for ubiquitous (e.g., Sp1) and hematopoietic-specific transcription factors (e.g., GATA-1). Our results provide support for a model in which Sp1 multimers form

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a link between the promoter and enhancer regions, resulting in the positioning of the GATA-1 multisubunit complex next to the promoter.

MATERIAL AND METHODS

Preparation of nuclear extracts

Adult White Leghorn chickens were made anaemic by injections of phenylhydrazine hydrochloride. Blood was collected and nuclei were isolated from the immature erythrocytes as described previously (16) except for the following modifications. The nuclei were resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 5.2 mM MgCl₂, 0.7 M sucrose, 1 mM PMSF, and the nuclei were collected by centrifugation (2500×g, 10 min). Nuclei were extracted with RSB (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 10 mM sodium butyrate, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.15 μM aprotinin, 5 μM iodoacetamide) containing 0.3 M NaCl (15). The nuclear extract was dialysed against Buffer D (20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and stored at −80°C (15).

DNA fragments and oligonucleotides

The DNA fragments spanning the histone H5 5' promoter and 3' enhancer of H5 gene are shown in Fig.1. US1a (−95 to −1), DS1 (−893 to +1067), and DS1+DS2 (+893 to +1176) were subcloned into pGEM-3. The inserts were excised with appropriate restriction enzymes from the multiple cloning linking sites. DS1a (+893 to +1041) was isolated by digesting the pGEM-3 vector containing DS1 with HindIII and Hpal. DS1a-U (+893 to +980) and DS1a-L (+981 to +1041) were isolated from DS1 which was digested with Mval and Hpal, respectively. The pGEM-3 vector containing DS1+DS2 was digested with Hpal and EcoRI to isolate the DS2 fragment (+1068 to +1177).

Oligonucleotides containing the histone H5 GC-box (−92 to −67), which has the sequence of a high affinity Sp1 binding site, and the UPE (−54 to −26), were synthesized. The oligonucleotides that contained the Sp1 motif 5'-GATGCCTGCGGGGCGATGCAGAGGGG-3' (Sp1-for) and 5'-TCCCTCTGGCCCGGCGGATGCAGACCC-3' (Sp1-rev), 5'-GGGGTGCCGGTGCCGGGG-3' (PII-for), 5'-GGGGTGGAGGTAGAGGAGGGG-3' (PII-rev), 5'-TGCACCAAGGAGGATCCCGTCTCTCTCTCCCCCTCCCCCTC-3' (PIII-rev), 5'-GGGGTGCCGGTGCCGGGG-3' (PIII-for), 5'-GGGGTGCCGGTGCCGGGG-3' (PIII-rev), 5'-GGGGTGCCGGTGCCGGGG-3' (PIII-for), 5'-GGGGTGCCGGTGCCGGGG-3' (PIII-rev), 5'-GGGGTGCCGGTGCCGGGG-3' (PIII-rev). UPE-oligonucleotides were 5'-TA-GGACGTTTCTCCCCGGCGTCTCGTCCGGC-3' (UPE-for) and 5'-GGGCGACGCGACCGCGGAGGAGCTCCGGTCTCCGGC-3' (UPE-rev). Two oligonucleotides with the sequences 5'-GAGGCTGACAGGGGAGGAG-3' (Sp1-for) and 5'-GGGGTGCCGGTGCCGGGG-3' (Sp1-rev) were synthesized. The complementary sequences were annealed to form double-stranded DNA. The double-stranded competition oligonucleotide Sp1 (5'-GATGCATCAGGGGAGGAGGAG-3') was obtained from the Stratagene HotFoot DNase I footprinting kit. The oligonucleotides and DNA fragments were end-labeled as described previously (15).

DNA-binding assays

The gel mobility-shift, competition experiments, diagonal gel mobility-shift (17) and DNase I protection (footprinting) assays were done as previously described (15). The molecular mass determinations were done with 5% polyacrylamide gels as described by Bading (18). The mass determination of DNA-bound protein is based on the assumptions that: (1) the reduced ion mobility of the protein-DNA complex is not influenced by protein structure; (2) the charge of the protein is negligible compared to the charge of the DNA fragment; and (3) the DNA is not bent by the protein.

RESULTS

Identification of proteins that bind to DNA fragments spanning the histone H5 3' enhancer

The histone H5 3' enhancer is localized in the region (+851 to +1064). In a previous study, we demonstrated that GATA-1 and proteins associated with it formed several protein-DNA complexes with the DNA fragment DS1 (see Fig.1; 15). Our results also indicated that protein(s) other than GATA-1 were forming protein-DNA complexes with DS1 and that these proteins were binding in the region +893 to +980 (15). The binding sites of the chicken immature adult erythrocyte nuclear proteins were identified in DNase I footprinting experiments. Fig.2A and 2B show the DNase I protection sites generated by proteins binding to DNA sequences present in DNA fragments DS1a and DS1+DS2, respectively. Three footprint activity were observed with DNA fragment DS1a with the level of protection being I > II > III. Footprints I and II contained potential Sp1 binding sites (TGCCCG+59GGGGG and TAGGGA+96GGGC), and these footprints covered approximately 22 and 24 bp, respectively, which is similar to protection generated by human Sp1 (19,20). Both of these Sp1 binding sites are present in the region spanned by DNA fragment DS1a-U. Interestingly, DNase I hypersensitive sites generated in vitro are localized at sites approximately equidistant between the two Sp1 binding sites (boxes I and II), suggesting that the binding of the Sp1-like proteins distorts the intervening DNA structure. The third protected region (footprint III), which is in DNA fragment DS1a-L, also harbored a potential Sp1 binding site (CTCCT+100GGGC). The protected region III also had the sequence GAGAGGGG which is the sequence of the β-globin developmental stage selector element that binds the 65-kDa protein NF-E4 (21). Three protected regions (footprints IV, V and VI) were observed between +1041 and +1177. Protected region IV had the AGATAA motif at +1053, in agreement with our previous study (15). A potential Sp1 binding site (GGGGTG+106GGGG) was found in the 24 bp protected region V. This sequence is also recognized by the CACCC factor (22). Protected region VI harbored a TTGGCCACGCCGCA sequence (+1122 to +1135) which is similar to the NF1 binding sequence (YTGGCANNNTGCCCC; 23,24).

The gel mobility-shift assay with the DNA fragment DS1a (+893 to +1041; see Fig.1) was also used to detect chicken erythroid nuclear proteins that were binding to this region of the histone H5 enhancer. Fig.3A shows that the DNA fragment DS1a formed four complexes. The approximate molecular masses of the DNA-bound proteins were determined (see Materials and Methods). The linear range of this assay described by Bading (18) is to 450 kDa, with an error of 10%. This method is subject
proteins of complexes C2, C3 and C4 were greater than 450 kDa. The protein of complex C1 had a molecular mass of approximately 200 kDa, while the molecular masses of the proteins of complexes C2, C3 and C4 were greater than 450 kDa.

Three of the four complexes showed specific binding to the DS1a fragment. A 100-fold molar excess of the unlabelled DNA fragment DS1a competed effectively for all four complexes (Fig.3A). The non-specific competitor salmon sperm DNA did not effectively compete for C2, C3 and C4, but salmon sperm DNA did compete for complex C1 (not shown), suggesting that complex C1 is non-specific. Competition studies were also done with a DNA fragment spanning the promoter region of the histone H5 gene (fragment US1a, see Fig.1). The US1a DNA fragment competed for the four complexes forming with DS1a with the following efficiencies: C3, C4 > C2 > C1 (Fig.3A).

The results of the DNase I footprinting indicated that several potential Sp1 binding sites were localized in the region +893 to +1041. Since the histone H5 promoter region has a strong Sp1 binding site (GC-box), it was possible that the US1a DNA fragment was competing with the DS1a DNA fragment for the binding of a Sp1-like protein. This possibility was tested by using an oligonucleotide containing a high affinity Sp1 DNA fragment (see 'Materials and Methods'). The Sp1-oligonucleotide competed effectively for complexes C3, C4 and, to a lesser extent, C2 that formed with the DNA fragment DS1a (Fig.3A). The Sp1-oligonucleotide did not compete with complex C1. These observations suggested that a Sp1-like protein was involved in the generation of complexes C3 and C4.

To delineate further the location of the complexes, gel mobility-shift assays were done with DNA fragments DS1a-U (+893 to +980) and DS1a-L (+981 to +1041, see Fig.1). Two strong and several weaker complexes were formed with fragment DS1a-U (Fig.3B). Other gel patterns showed that the major complex formed with DS1a-U was two closely migrating complexes with mobilities slightly greater than complexes C3 and C4 that formed with DS1a. One major complex with electrophoretic mobility similar to the major complex generated with DS1a-U formed with the fragment DS1a-L (Fig.3B). The intensity of this complex was similar to the intensity of the major complexes formed with DS1a or DS1a-L. However, in other gel mobility-shift assays we found the intensity of the major complex formed with DS1a-L to be usually less than that of the major complexes generated with DS1a-U. To determine whether DS1a-U and DS1a-L contained Sp1 binding sites, competition experiments with an oligonucleotide containing the high affinity Sp1 binding site of the histone H5 promoter (−92 to −67) were done. This Sp1-oligonucleotide effectively competed for the major complexes forming with DS1a-U and DS1a-L (not shown).

Oligonucleotides containing the potential Sp1 binding sites present in DS1a were synthesized (see Materials and Methods). These oligonucleotides, which are referred to as PI, PII and PIII, correspond to footprints I, II and III (see Fig.2A). Two complexes, one strong and the other weak, formed with the oligonucleotides P1, PII and PIII (Fig.4B). The observation that several complexes form with the Sp1-oligonucleotide is consistent with the observations of others, indicating factors other than Sp1 bind to these sequences (22,25–27). The mobilities of the major complexes formed with these oligonucleotides were identical. However, the relative intensities of the major complex were different with PI > PIII > PII, reflecting their relative binding affinities for Sp1. The mobilities of the two complexes formed with oligonucleotide P1 were identical to those generated with DS1a-L and slightly greater than the migration of the major complexes formed with DS1a and DS1a-U (Fig.3B). Thus, although the lengths of the DNA fragments DS1a (156 bp), DS1a-U (91 bp), DS1a-L (63 bp) and the PI oligonucleotide (26 bp) were quite different, the migration of the major complexes formed with these fragments were similar. These observations demonstrated that a Sp1-like protein was involved in the generation of the major complexes formed with DNA fragments DS1a, DS1a-U and DS1a-L.
shows that an oligonucleotide containing the high affinity Sp1 binding site of the histone H5 promoter (−92 to −67) effectively competed for complexes C5 and C6 (DS2 DNA fragment). An oligonucleotide (PV) containing the potential Sp1 binding site (footprint V in Fig.2B) in DS2 was synthesized. One major complex, which had a mobility similar to C5 and C6, and several less intense complexes were generated with the oligonucleotide PV. This demonstrated that a Sp1-like protein(s) was involved in the generation of the major complexes formed with DNA fragment DS2.

Comparison of the intensities of the major complexes formed with DNA fragments DS2, DS1a and US1a indicated that the Sp1 binding site of DS2 had the lowest relative affinity for Sp1. This was confirmed by comparing the relative intensities of the major complexes formed with oligonucleotides PI, PII, PIII, PV and Sp1 which has the high affinity Sp1 binding site of the histone H5 promoter. The relative intensities of the major complex were Sp1 > PI > PII > PIII > PV (Fig.4B).

In agreement with our previous studies (15), a 40 bp DNA fragment spanning the region +1042 to +1067 that contained the GATA sequence and an oligonucleotide containing the GATA sequence formed several protein-DNA complexes (Fig.5). We have demonstrated previously that complex C1 contained GATA-1, while complexes C2, C3 and C4 were generated by the association of non-DNA-binding proteins with GATA-1, forming multisubunit complexes (15). We have recently observed that the GATA-1 multisubunit complexes readily dissociated under certain dialysis conditions. Fig.5 shows that complexes C3 and C4 predominated when the GATA-oligonucleotide was incubated with the nuclear extract or the extract dialysed against RSB-0.3 M NaCl. However, complex C1 predominated when the GATA-oligonucleotide was incubated with the nuclear extract that had been dialyzed against Buffer D. The relative abundance of C1 versus C2 and C3 in nuclear extracts dialyzed against buffer D was variable, but for all buffer D-dialysed extracts the level of complex C1 was elevated. The gel mobility shift patterns generated with DNA fragments DS1a, DS2, US1a and the Sp1 oligonucleotides were the same with nuclear extracts that were or were not dialysed against RSB-0.3 M NaCl or buffer D.

Identification of proteins that bind to DNA fragments spanning the histone H5' promoter

Fig.6 shows the DNase I protected regions generated by proteins binding to DNA sequences present in the US1a DNA fragment, which spans the promoter region (−95 to −1, see Fig.1). Three protected regions were observed. Footprint I protected a 21 bp region that harbored the strong Sp1 binding site (GGGCGTGGG), footprint II contained the UPE, and footprint III spanned the TATA box at −22 (TTAAAT).

Gel mobility-shift assays were done with DNA fragment US1a, and a nuclear extract from chicken immature adult erythrocytes. Fig.7 shows that several protein-DNA complexes were formed with the US1a DNA fragment. Of the eight complexes detected, complexes C7 and C8 were the most intense followed by complexes C3, C4 and C5. Complexes C1, C2 and C6 were weak. The approximate molecular masses of the proteins present in the various complexes were as follows: C1, 90 kDa; C2, 117 kDa; C3, 334 kDa; C4, 380 kDa. The proteins of complexes C5 to C8 were greater than 450 kDa. All the protein-DNA complexes showed specific binding to the US1a fragment. A 100-fold molar excess of the unlabelled DNA fragment US1a
Figure 3. Interactions between the histone H5 enhancer region and proteins from adult chicken immature erythrocyte nuclear extracts. The DNA fragment used in the gel mobility-shift assay is indicated at the bottom of each panel. Panel A: End-labelled DNA fragment DS1a (207 bp, 0.3 ng) was incubated with 0.36 μg poly (dI-dC)/μl in the presence (+, 5 μg) or absence (−) of nuclear proteins extracted (NE) from adult chicken immature erythrocytes. The amount in molar excess of competitor DNA fragment (DS1a, US1a or Spl-oligonucleotide from the Stratagene Hotfoot kit), as indicated at the top of the panel, was 12.5, 25, 50, 100, and 200. Panel B: End-labelled DNA fragments DS1a, DS1a-U (91 bp), DS1a-L (63 bp), or oligonucleotide PI were incubated in the presence (+) of immature erythroid nuclear proteins. The oligonucleotide was incubated with 0.05 μg poly (dI-dC)/μl. The arrow heads show the complexes formed with the oligonucleotide PI. Panel C: End-labelled DNA fragment DS2 (124 bp, 1 ng) was incubated with 0.36 μg poly (dI-dC)/μl in the presence (+, 12 μg) or absence (−) of erythroid nuclear proteins. The amount in molar excess of competitor Spl-oligonucleotide (histone H5 promoter) added was 12.5, 50 and 200. C1 to C6 are the protein-DNA complexes. Panel D: End-labelled DNA fragment DS2 and oligonucleotide PV were incubated with 0.36 or 0.05 μg poly (dT-dC)/μl, respectively, in the presence (+, 12 μg) of erythroid nuclear proteins. The arrow heads point to the complexes formed with the oligonucleotide PV.

Figure 4. Analysis of protein binding to oligonucleotides containing Spl sites. Panel A: End-labelled DNA fragments US1a (182 bp, 0.5 ng), DS1a (207 bp, 0.5 ng) and DS2 (124 bp, 0.5 ng) were incubated with 0.36 μg poly (dI-dC)/μl in the presence (+, 5 μg) of nuclear proteins extracted (NE) from adult chicken immature erythrocytes. The arrow heads point to the major complexes formed with these DNA fragments. Panel B: End-labelled oligonucleotides PI, PII, PIII, PV, and Spl were incubated with 0.36 μg poly (dI-dC)/μl in the presence (+, 5 μg) of nuclear proteins extracted (NE) from adult chicken immature erythrocytes. The arrow heads point to the minor complexes formed with these oligonucleotides.

Figure 5. Multisubunit complexes forming with an oligonucleotide containing the GATA binding site. An end-labelled oligonucleotide (0.025 ng) containing the GATA motif was incubated in the presence (+, 2 μg) of nuclear proteins extracted (NE) from adult chicken immature erythrocytes. Nuclear extract was either not dialyzed (none) or dialyzed against RSB-0.3 M NaCl, 20% glycerol and 1 mM dithiothreitol (RSB) or buffer D (Hepes). C1 to C4 are the protein-DNA complexes.

Competed effectively for all the complexes (Fig.7A), whereas the non-specific competitor salmon sperm DNA did not effectively compete (not shown). DNA fragment DS1 (enhancer region) also competed effectively for all the complexes, with the exception of complex C6 (not shown).

The two positive elements of the histone H5 gene promoter are a GC-box (the strong Spl binding site) and an upstream promoter element (UPE) (6). Oligonucleotides containing these elements were used in competition and gel mobility shift experiments. A 25-fold molar excess of the Spl-oligonucleotide competed effectively for complexes C5, C7 and C8, but not
Figure 6. DNase I footprint analysis of proteins binding to the histone H5 promoter region. DNA fragment US1a was end-labelled on the template (T) strand and incubated in the absence (−) or presence (+) of the nuclear extract (20 μg protein per ng DNA) isolated from chicken immature erythroid nuclei. Identical footprints were obtained with the end-labelled non-template strand (not shown). C+T indicates the chemical sequencing reaction. The amount of DNase I added to 50 μl was as follows (left to right): 0.1, 0.2, 0.1, 0.01, and 0.1 units. The arrowheads are the DNase I hypersensitive sites of the complex. The Roman numerals indicate the protected regions.

complex C6 (Fig.7C). Similar results were obtained when this study was done with another oligonucleotide containing a strong Sp1 binding site from the Stratagene HotFoot DNase I footprinting kit (see Materials and Methods). A 50-fold excess of the UPE-oligonucleotide effectively competed for complex C6 (Fig.7B). However, this oligonucleotide was not effective at competing for the formation of complexes C7 and C8. An oligonucleotide containing the GATA sequence did not compete (not shown). The Sp1 oligonucleotide generated a major complex and several minor complexes that had mobilities similar to complexes of similar intensities formed with the much longer US1a fragment (Fig.7D). This result demonstrated that the Sp1 was involved in the formation of the major complexes C7 and C8. One of the minor complexes formed with the PV oligonucleotide would be generated by the binding of the CACCC factor (22). The DNase I footprint II over the histone H5 upstream promoter region was interesting in that it had protected regions interspersed with DNase I hypersensitive sites (see Fig.6). To provide further evidence that a protein was associated with the histone H5 upstream promoter element (UPE), an oligonucleotide containing this element was used in gel mobility-shift assays. Fig.7D shows that the UPE-oligonucleotide formed a complex with electrophoretic mobility slightly slower than the free US1a fragment.

DISCUSSION

We have identified in vitro the DNA binding sites of chicken immature erythrocyte nuclear proteins that are associated with the histone H5 gene promoter and 3' erythroid-specific enhancer regions. Our results are summarized in Fig.8.

The chicken histone H5 promoter region has two positive elements, a GC-box at −83 to −75 and a proximal element (UPE) at −54 to −38 (6). Deletion of either the GC-box or UPE reduced the strength of the histone H5 promoter, while deletion of sequences between the GC-box and UPE had no effect (6). In DNase I footprinting experiments, we observed three protected regions, corresponding to a high affinity Sp1 binding site (footprint I), UPE (footprint II) and TATA box (footprint III). These results suggest that these proteins are involved in regulating the activity of the histone H5 gene promoter.

The US1a DNA fragment (promoter region) formed several protein-DNA complexes. We have presented evidence that shows that several of the major and some of the minor complexes can be accounted for by the binding of protein(s) to footprint I, a strong Sp1 binding site. We propose that several of the less abundant complexes are formed by the binding of the UPE-
binding protein and TATA-box-binding proteins to the US1a DNA fragment. Further, we suggest that the slowest migrating complex C8 is generated by the combined association of Sp1-like proteins and other proteins (e.g., proteins binding to the TATA box). The approximate mass of the protein(s) of the major complexes C7 and C8 was greater than 450 kDa which is much greater than the molecular mass of the Sp1-like protein (typically 95/105 kDa, 9). In preliminary studies, we have observed the molecular mass of the chicken erythrocyte nuclear proteins that bind to the Sp1 oligonucleotide to be 75/97/105 kDa. It has been demonstrated by several groups that Sp1 forms multimers (13,14). Mastangelo et al. (13) have shown that Sp1 is first organized as a tetramer which is subsequently assembled into multiple tetramers. It is possible that complexes C7 and C8 consist of several stacked Sp1 tetramers.

The results of gel mobility-shift, DNase I footprinting and competition experiments provide evidence that the Sp1-like high molecular mass complexes also form with the DNA fragments DS1a, DS1a-U, DS1a-L, and DS2 which span the histone H5 3' enhancer region. Several of the DNase I protected regions (I, II, III and V) present in the region spanned by DNA fragments DS1 and DS2 contained Sp1 binding sites (see Fig.8). The sequences of these Sp1 binding sites propose that their relative affinities for the Sp1 protein would be medium (footprint I) and weak (footprints II, III and V) (8, 10–12). The level of footprint activity (e.g., footprint I was stronger than footprint II with DS1a) and the relative intensities of the complexes formed with oligonucleotides PI, PII, PIII and PV agrees with the proposed affinities.

The binding of several factors other than Sp1 were detected in the region (+893 to +1177) spanned by DNA fragments DS1 and DS2. In addition to a weak Sp1 binding site, footprint III has the sequence GAGAGGGA which is similar to the sequence recognized by NF-E4 (G/AAGAGGA/GGG) (21). The DNA fragment (DS1a-L) containing this sequence forms protein-DNA complexes poorly, suggesting that the abundance of NF-E4 is low in the nuclear extracts or NF-E4 has a weak affinity for this sequence in the histone H5 enhancer. Footprint IV harbors the GATA binding motif that binds GATA-I which is a component of a multissubunit complex (15). The CACCC sequence of footprint V may bind Sp1 or the CACCC factor (22). Protected region VI has an inverted repeat TTGGCACAGCCCAA which is similar to the inverted repeat found in the chicken β-globin gene promoter region and the consensus recognition sequence for the chicken TGGCA protein/NF1 (23,24,28). The TGGCA sequence is also found in the chicken β-globin enhancer (footprint activity 1, 29). Deletion of this region did not affect β-globin enhancer activity (30). Further, the 3' flanking region of the histone H5 gene containing the TGGCA protein/NF1 (23,24,28). The TGGCA sequence is also found in the chicken β-globin enhancer (footprint activity 1, 29). Deletion of this region did not affect β-globin enhancer activity (30). Further, the 3' flanking region of the histone H5 gene containing the TGGCA sequence is not required for enhancer activity (7). These observations suggest that the NF1-like protein that generates footprint VI is not involved in histone H5 enhancer function.

In chicken immature erythrocyte chromatin, DNase I hypersensitive site (DHS) 5 maps at -117 ± 6 and DHS 5L maps at -25 ± 6 in the promoter region (4). Thus, the DHS 5 is located immediately 5' to the Sp1 binding site, while DHS 5L co-maps with the protected regions II (the UPE) and III (TATA box) (see Fig.8). In the histone H5 enhancer, DHS 7L (+1003 ± 12) and 7U (+1095 ± 12) co-map with protected regions III and V, respectively (see Fig.8).Lowery et al. (31) demonstrated that interactions between ubiquitous factors (e.g., Sp1) and erythroid-specific nuclear proteins (e.g., GATA-I) were
required to form the DNase I hypersensitive site within the human globin locus control region. The binding sites for GATA-1 and Sp1 (e.g., at CACCC; 12) are commonly found in the promoter and enhancers of globin and other erythroid-specific genes (e.g., porphobilinogen deaminase gene) (12,32,33). Thus, Sp1 and GATA-1 may be involved in the formation of hypersensitive sites in chromatin within the histone H5 enhancer as well as the globin enhancer and promoter regions. However, the CACCC sequence does not have enhancer activity (7). Our results show that the region +851 to +1185 by Rousseau et al. (7) and between +851 to +1064 by Trainor et al. (7) did not have enhancer activity (7). This model the role of GATA-1 may be to contribute to the protein-protein interactions between the enhancer and promoter (35). Alternatively, the GATA-1 multissubunit complex may have a role in recruiting or stabilizing the initiation complex.

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