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Developmental Changes in Transcription Factors Associated With the Nuclear Matrix of Chicken Erythrocytes

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Abstract The nuclear matrix has roles in organizing nuclear DNA and in controlling transcription. Transcription factors are associated with the nuclear matrix, with the spectra of transcription factors differing from one cell type to another. In this study we identified the transcription factors and enzymes functioning in the regulation of gene expression that were associated with nuclear matrix and nonmatrix nuclear fractions in erythrocytes isolated from chick embryos at different stages of development, anemic and normal adult birds. We found that the primitive erythroid nuclear matrix had the greatest histone deacetylase activity and highest levels of several transcription factors, including GATA-1, CACCC-binding proteins, and NF1. These transcription factors have key roles in erythroid-specific gene expression. The levels of these transcription factors were lower in the nonmatrix and matrix fractions isolated from definitive erythrocytes. For primitive and definitive erythrocytes, the level of CACCC-binding proteins in the nuclear matrix fraction was greater than that of Sp1. The relative levels of these transcription factors were reversed in the nonmatrix fraction. Casein kinase II was not found in erythroid nuclear matrices. The observed erythroid lineage specific alterations in erythroid nuclear matrix transcription factor composition and abundance may be involved in erythroid-specific gene expression.

Key words: nuclear matrix, histone H5, transcription, transcription factors, erythroid development

The nuclear matrix, the nuclear structure remaining after the salt extraction of nuclease treated nuclei, consists of residual nucleoli, nuclear pore–lamina complex, and internal nuclear matrix [He et al., 1990; Belgrader et al., 1991; Berezney, 1991]. Besides its role in organizing nuclear DNA, the nuclear matrix is involved in several nuclear functions, including replication, transcription, and RNA splicing [Bassim Hassan et al., 1994]. These nuclear processes are localized to distinct regions in the nucleus. For example, transcriptionally active genes and RNA transcripts are found in discrete "transcript domains" [Carter et al., 1993; Xing et al., 1993; Bassim Hassan et al., 1994].

Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus [Delcuve and Davie, 1989; Gerdes et al., 1994]. Adult chicken immature erythrocyte nuclear matrix-bound chromatin fragments are enriched in transcriptionally active β-globin and histone H5, but not poised ε-globin, DNA sequences [Delcuve and Davie, 1989; Davie and Hendzel, 1994]. Actively transcribed chromatin regions are thought to be immobilized on the nuclear matrix by multiple dynamic attachment sites. The transcription machinery, specific transcription factors, and nuclear enzymes are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix [van Wijnen et al., 1993; Cook, 1995; Merriman et al., 1995].

Histone deacetylase and histone acetyltransferase are components of the internal nuclear matrix [Hendzel et al., 1991, 1994; Hendzel and Davie, 1992]. Since transcriptionally active DNA is complexed with histones undergoing dynamic acetylation [Boffa et al., 1990; Hendzel et al., 1991], histone deacetylase and acetyltransferase may provide a mechanism by which transcribed genes are associated with the internal nuclear

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matrix [Hendzel et al., 1991; Hendzel and Davie, 1992; Davie and Hendzel, 1994]. Recent reports showing that transcription factors are associated with the nuclear matrix suggest another type of interaction by which transcribed genes are selectively attracted to the nuclear matrix [Merriman et al., 1995]. Sequence-specific DNA-binding proteins that are attached to the nuclear matrix include the estrogen receptor, NMP-2 (member of AML/CBF/PEB2/*Runt Domain* transcription factor family), RAP-1 (repressor-activator binding protein-1), factor F6, Sp1, NF1, and RFP [Gasser et al., 1989; Dworetzky et al., 1992; Isomura et al., 1992; Vassetzky et al., 1993; van Wijnen et al., 1993; Sun et al., 1994; Merriman et al., 1995].

The protein composition of the nuclear matrix varies with cell type and disease state. The distribution of sequence-specific DNA binding proteins associated with the nuclear matrix and nonmatrix nuclear fraction also changes with cell type [van Wijnen et al., 1993]. It has been postulated that the nuclear matrix has a role in the expression of genes by concentrating a subset of transcription factors at specific nuclear sites [Stein et al., 1991; Merriman et al., 1995].

Throughout chicken embryogenesis, there is a temporally regulated expression of sets of tissue-specific genes, for example, the developmental regulated switch in the expression of the β-globin genes. The first hemoglobinized cells appear in the blood islands in chicken embryos at 35 h [Groudine and Weintraub, 1981]; these primitive cells produce embryonic β-globin genes [Bruns and Ingram, 1973]. Between the 5th and 12th days, this primitive group of erythroid cells is replaced by definitive red blood cells which synthesize adult β-globin genes [Brown and Ingram, 1974]. The transcription of the adult globin genes in red blood cells arising from the bone marrow is maximal at about days 9–14 after fertilization. This changing pattern of globin isotypes during development is called hemoglobin switching. In contrast to the adult globin genes, the histone H5 gene is expressed in both primitive and definitive cells [Colman et al., 1983; Trempe et al., 1988].

GATA-1, Sp1, and CACCC-binding proteins are important transcription factors in the expression of erythroid-specific genes [Hartzog and Myers, 1993; Orkin, 1995; Nuez et al., 1995; Perkins et al., 1995]. These factors bind to promoter and enhancer elements of globin, histone H5, and other erythroid-specific genes [Minie et al., 1992; Sun et al., 1992]. The concentrations of Sp1 and GATA-1 are greater in primitive erythroid cells than in definitive cells. These changes in the abundance of Sp1 and GATA-1 correlate with the developmental switch in embryonic gene expression [Minie et al., 1992].

In this study we analyzed the distribution of sequence-specific DNA-binding proteins, particularly those involved in the expression of the H5 and globin genes, in nuclear matrix and nonmatrix nuclear fractions from primitive and definitive erythrocytes. We found that the primitive erythrocyte nuclear matrix binds GATA-1, CACCC-binding proteins, and NF1. The activities of these transcription factors drop sharply in the nuclear matrices of definitive cells.

MATERIALS AND METHODS

Preparation of Nuclear and Nuclear Matrix Extracts

Red blood cells were collected from 5-, 11-, and 15-day chicken embryos as described previously [Minie et al., 1992]. Adult White Leghorn chickens were made anemic by injections of phenylhydrazine hydrochloride. Nuclei were isolated by lysing erythrocytes in CSK buffer (100 mM KCl, 3 mM $MgCl_2$, 10 mM 1,4-piperazine-diethanesulfonic acid, pH 6.8, 1 mM EGTA, 0.3 M sucrose, 0.5% (v/v) thiodiglycol, 1 mM phenylmethylsulfonyl fluoride [PMSF]) with 0.25% (v/v) Triton X-100. The nuclei collected by centrifugation (1,085*g*, 10 min) were resuspended in CSK buffer. The nuclear suspension was passed three times through a 22-gauge needle and then layered on top of CSK buffer with 0.7 M sucrose. Nuclei were collected by centrifugation at 1,900*g* for 10 min. Nuclear extracts (nonmatrix nuclear fraction) were made as described previously, by extracting nuclei (200 A_{260} U/ml) with RSB (10 mM Tris–HCl, pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl, 1 μM leupeptin, 0.15 μM aprotinin, and 1 mM PMSF) containing 0.3 M NaCl [Sun et al., 1992]. Nuclear matrices were prepared using a procedure derived from the protocol of He et al. [1990]. Briefly, purified nuclei were resuspended in digestion buffer (10 mM 1,4-piperazine-diethanesulfonic acid, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% (v/v) Triton X-100, and 1 mM PMSF) at 20 A_{260} U/ml. A sample of the nuclear suspension was taken to determine DNA content using the diphenylamine assay [Delcuve and Davie, 1989]. DNase I (Sigma, D 5025) was added to a final concentration of 100 μg/ml, and

the nuclei were digested for approximately 60 min at 23°C. Ammonium sulfate was added dropwise from a 4 M stock to a final concentration of 0.25 M, and the 0.25 M ammonium sulfate-extracted nuclear matrices (NM1) were collected by centrifugation at 5,100*g* for 10 min. The supernatant S1 contained most of the chromatin. The NM1 nuclear matrices resuspended in digestion buffer were treated with RNase A (10 μg/ml) at room temperature for 10 min and then extracted with 2 M NaCl and 1% (v/v) 2-mercaptoethanol for 30 min on ice. The soluble (S2) and insoluble (NM2) fractions were separated by centrifugation at 5,100*g* for 10 min. *Protein* concentration was determined by the standard BioRad protein assay (BioRad Laboratories, Mississauga, Ontario, Canada).

Histone Deacetylase Assay

Histone deacetylase assay was done in 25 mM sodium phosphate/citric acid buffer, pH 7.0 at 37°C for 30 min with 150,000 dpm (180 μg) total chicken erythrocyte histones radiolabelled with [^{3}H]acetate as described previously [Hendzel and Davie, 1992]. After incubation, the enzyme reaction was stopped by 0.12N/0.72 N (final concentration) of acetic acid/HCl, and two volumes of ethyl acetate were added. After mixing, the samples were centrifuged at 9,000*g* for 1 min, half-vol of the added ethyl acetate was then counted in a scintillation counter to detect the amount of liberated label.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay (EMSA) and competition experiments were done as described previously [Sun et al., 1994]. Oligonucleotides used in this study were Sp1-oligonucleotide (high affinity Sp1 binding site in H5 promoter), NF1-oligonucleotide (NF1 binding site in the H5 enhancer), UPE-oligonucleotide (UPE in H5 promoter), GATA-oligonucleotide (GATA-1 binding site in H5 enhancer), and PV-oligonucleotide (CACCC sequence in H5 enhancer). The sequences of these oligonucleotides can be found in [Sun et al., 1992, 1993, 1994]. The oligonucleotides were end-labelled as described previously [Penner and Davie, 1992]. To determine the levels of complex formed with an oligonucleotide, the autoradiogram was scanned using a PDI 325oe scanning densitometer and PDI ImageMaster evaluation system. The amount of complex formed was proportional to concentration of protein extract. The amount of nuclear- or nuclear matrix-extracted proteins used was kept in the linear range of the assay. For some experiments, nuclear- and nuclear matrix-extracts were incubated with anti-Sp1 rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blot Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transfer of the proteins to nitrocellulose was done as described previously [Delcuve and Davie, 1992]. The nitrocellulose filter was immunochemically stained with anticasein kinase II (α-subunit) antibody [Gietz et al., 1995] and goat antirabbit antibody linked to horseradish peroxidase (HRP) (BioRad Laboratories, Mississauga, Ontario, Canada) using the enhanced chemiluminescence (ECL) detection system (Amersham, Oakville, Ontario, Canada).

RESULTS

Histone Deacetylase Activity in Nuclear Matrix Extracts from Primitive and Definitive Erythrocytes

Chicken erythrocytes were isolated from day 5 (primitive), day 11 (definitive), and day 15 (definitive) embryos and from the peripheral blood of anemic (immature) and untreated (mature) adult birds. For each preparation of nuclei, the concentration of DNA was determined. Nuclei were digested with DNAase I and then extracted with 0.25 M ammonium sulfate. Electron microscopic analysis of nuclear matrices from immature erythrocytes shows a fibrogranular internal nuclear matrix surrounded by nuclear pore–lamina [H.Y. Chen, J-M. Sun, M.J. Hendzel, J.B. Rattner, and J.R. Davie, unpublished observations]. The nuclear matrices were digested with RNase A and then extracted with 2 M NaCl and 1% 2-mercaptoethanol. The content of protein in each nuclear matrix extract was standardized with respect to the amount of nuclear DNA. Figure 1A shows that the nuclear matrix extract from primitive erythrocyte nuclei had a threefold greater amount of protein than that of the definitive erythroid nuclei. The protein contents of the nuclear matrix extracts from 11-day, 15-day, adult immature, and adult mature erythroid cells were similar.

Immature erythrocyte nuclear matrices extracted with 2 M NaCl and 1% 2-mercaptoethanol appeared as empty shells of nuclear pore–

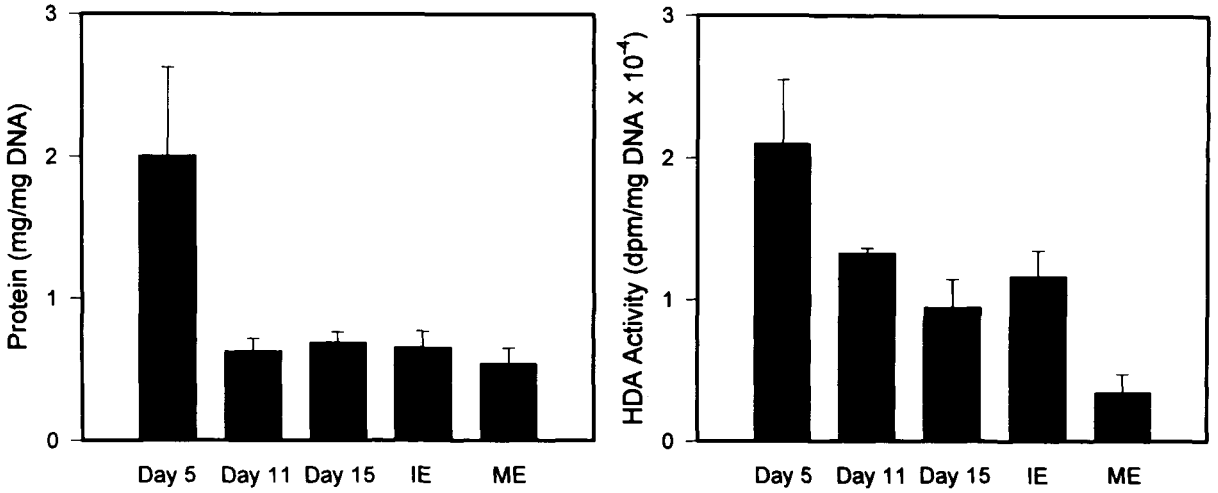

Fig. 1. Levels of protein and histone deacetylase activity in nuclear matrix extracts. **Left:** Extracts were isolated from nuclear matrices of 5-day, 11-day, 15-day, adult immature, and adult mature erythrocytes, as described under Materials and Methods. The protein content in each nuclear matrix extract was standardized relative to the amount of DNA in the nuclear suspension prior to DNase I digestion. Each value represents the mean ±SE (of the mean) of four different preparations. **Right:** Histone deacetylase (HDA) activity in each nuclear matrix extract was determined as described under Materials and Methods. The HDA activity in each nuclear matrix extract was standardized relative to the content of nuclear DNA. Each value represents the mean ±SE (of the mean) of three different preparations.

lamina complexes [H.Y. Chen and colleagues, unpublished observations]. The lack of an internal nuclear matrix in the residual insoluble nuclear material suggested that the extract was enriched in proteins of the internal nuclear matrix. Histone deacetylase is a component of the internal nuclear matrix [Hendzel et al., 1991; Hendzel and Davie, 1992]. Thus, the activity of this enzyme serves as a marker enzyme for the presence of internal nuclear matrix proteins. The nuclear matrix extracts all had histone deacetylase activity (Fig. 1B). However, the activity of histone deacetylase in the nuclear matrix extract from primitive was greater than that from the definitive nuclei. The histone deacetylase activities in extracts from 11-day, 15-day and adult immature erythrocytes were similar, with an average activity approximately 45% less than that present with primitive erythroid nuclear matrices. Mature erythrocyte nuclear matrix extracts had the lowest levels of histone deacetylase activity.

Transcription Factors Associated With Erythrocyte Nuclear Matrices

The histone H5 gene is expressed throughout erythropoiesis [Colman et al., 1983]. Factor binding sites in the H5 gene promoter and enhancer of mature and immature erythrocytes have been identified in vitro and in situ [Sun et al., 1992; Sun, Ferraiuolo, Davie, unpublished observations]. Figure 2 summarizes the results of these studies. The association of these DNA-binding proteins with the nuclear matrices of primitive and definitive erythrocytes was investigated.

The histone H5 enhancer has a NF1 binding site. NF1 was shown to be attached to the nuclear matrices of chicken mature erythrocytes, immature erythrocytes, and liver [Sun et al., 1994]. The NF1 activity in nonmatrix nuclear and nuclear matrix extracts of primitive and definitive cells was determined using the electrophoretic mobility shift assay (EMSA). Several complexes were formed with proteins in the nuclear or nuclear matrix extracts and the NF1-oligonucleotide (Fig. 3A). The slower migrating complexes were presumably formed by proteins associating with NF1 dimer by protein–protein interactions [Sun et al., 1994]. Relative to the content of nuclear DNA, NF1 activity in the primitive erythrocyte nuclear extract was higher than that in extracts from the 11-day, 15-day, immature adult, and mature adult erythrocytes (Fig. 3B). The NF1 activities in the nuclear matrix extracts from 11- and 15-day, immature, and mature erythroid cells were similar, and these activities were considerably less than the NF1 activity of erythrocytes from 5-day embryos. The primitive erythrocyte nuclear matrix NF1 activity was on the average 4.6-fold greater

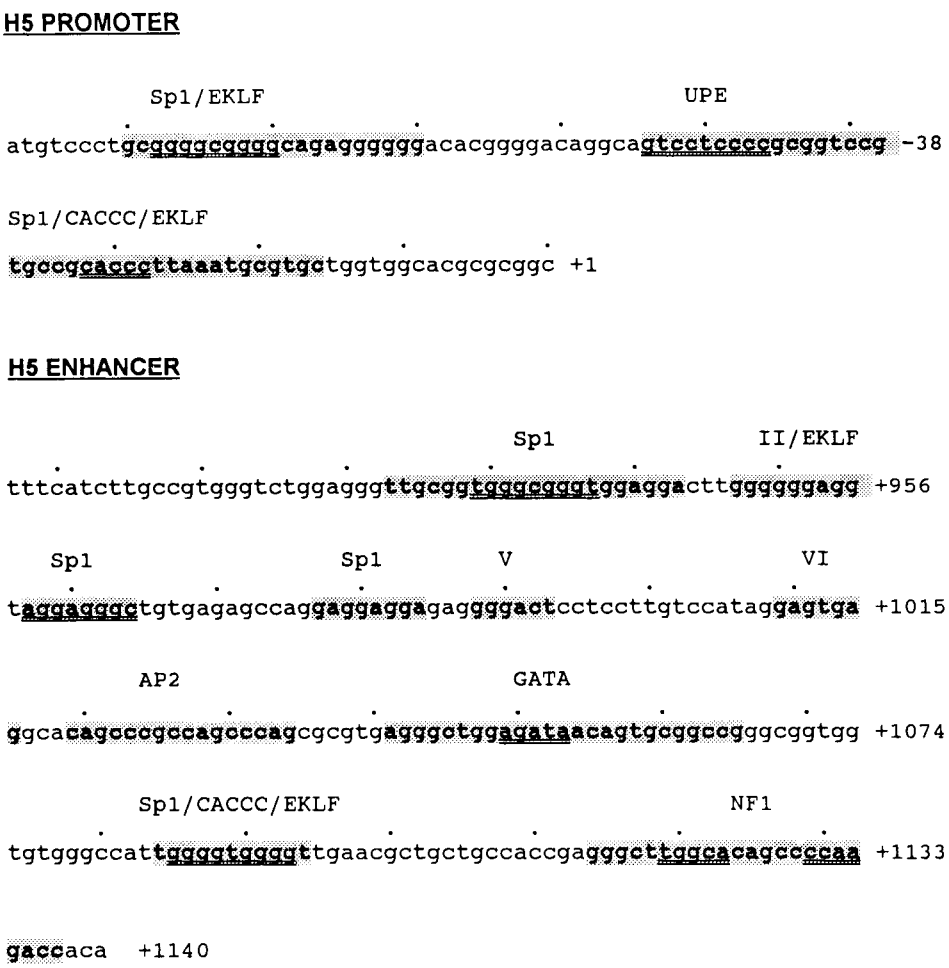

Fig. 2. Map of the factor binding sites in the chicken histone H5 promoter and enhancer [Sun et al., 1992]. EKLF is erythroid-specific Krüppel-like factor, a CACCC-binding protein [Orkin, 1995].

than that of the NF1 activity of definitive erythrocyte nuclear matrices.

The transcription factor GATA-1 binds to the enhancer and promoter regions of erythroid-specific genes (e.g., histone H5, globin), and it plays a pivotal role in primitive and definitive erythropoiesis [Simon et al., 1992; Penner and Davie, 1994; Pevny et al., 1995]. GATA-1 exists in a multiprotein complexes in primitive and definitive erythrocytes [Penner and Davie, 1994]. These complexes are formed by proteins binding to GATA-1. The predominating complex in nuclear extracts from the erythrocytes of day 11 and day 15 embryos, immature and mature erythrocytes was usually C3 (Fig. 4A) [Penner and Davie, 1994]. In primitive erythroid cells the faster migrating C1 was the major complex (Fig. 4A). The C1 complex contained GATA-1 and the GATA-oligonucleotide, but none of the proteins binding to the transcription factor GATA-1 [Penner and Davie, 1992]. Consistent with the reports of others, GATA-1 activity in nuclear extracts from primitive erythroid nuclei was greater than the GATA-1 activity from definitive erythroid cells (Fig. 4B) [Minie et al., 1992]. GATA-1 activity in the primitive erythrocyte nuclear extracts was approximately 8-fold greater than the GATA-1 activity of immature erythrocyte nuclear extracts.

All erythrocyte nuclear matrix extracts with the exception of those from mature erythrocytes had GATA-1 activity. Primitive erythrocyte nuclear matrix extracts had the highest amount of GATA-1 activity, while extracts from immature erythrocyte nuclear matrices had the lowest levels of GATA-1 activity (Fig. 4B). For 11-day, 15-day, and immature adult erythrocytes GATA-1 activity in the nuclear matrix extract was primarily in the C2 or C3 complexes (Fig. 4A). In primitive erythrocyte nuclear matrix

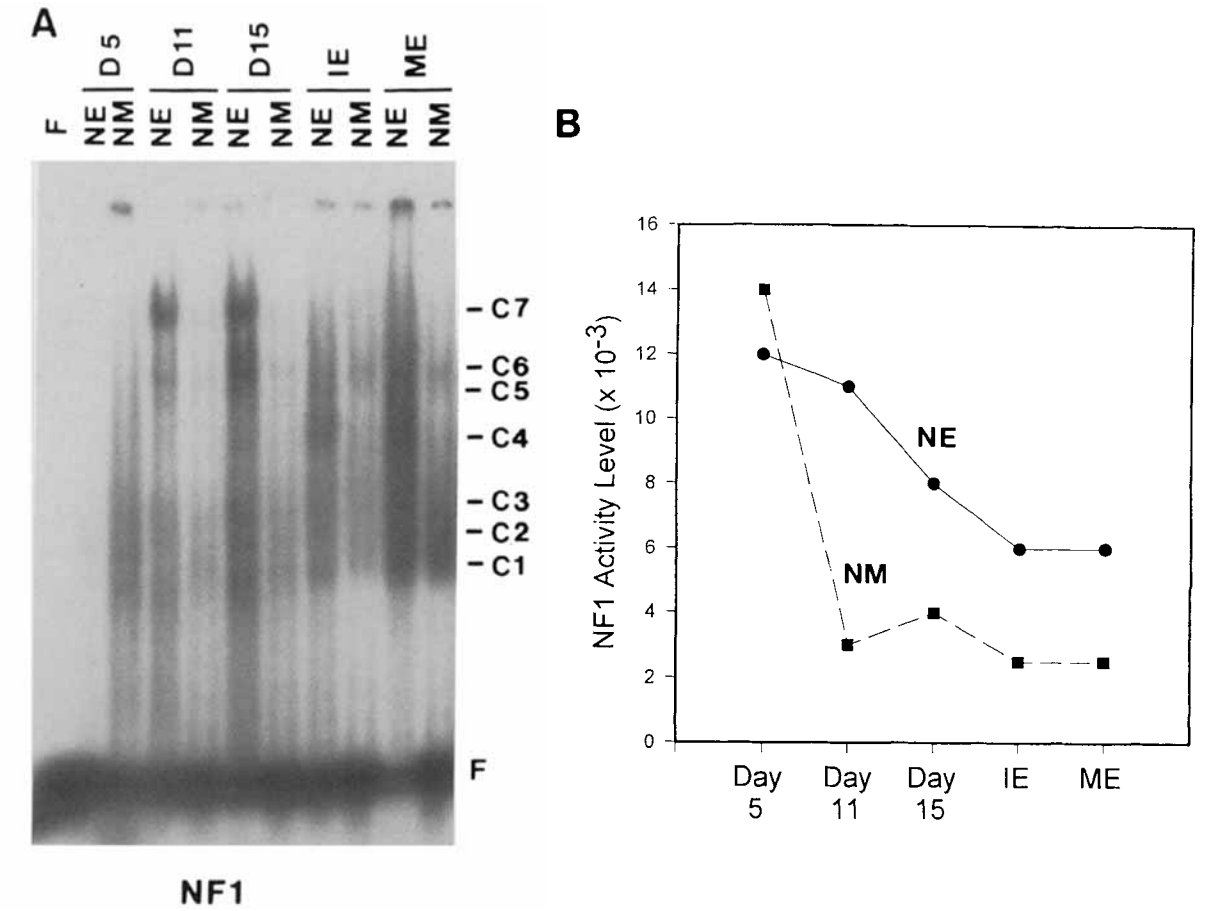

Fig. 3. NF1 activity is associated with nuclear matrices of primitive and definitive erythrocytes. **A:** Proteins (10 μg) in extracts from nuclei (NE, nonmatrix nuclear fraction) and nuclear matrices (NM, nuclear matrix fraction) from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes were incubated with end-labelled NF1-oligonucleotide (1 ng), and then electrophoretically resolved on a 4% polyacrylamide gel. The autoradiogram is shown. F is the free labelled oligonucleotide, and C1-7 are the protein–DNA complexes. **B:** Autoradiogram scanned to measure the level of complexes in each extract. This value was standardized relative to the content of nuclear DNA (factor activity/mg DNA). This result is representative of the findings with three different preparations. ●, nonmatrix nuclear fraction (NE, nuclear extract); ■, nuclear matrix fraction (NM).

extracts GATA-1 activity was found equally distributed among the C1 and C2 complexes (Fig. 4A). Thus, the GATA-1 multiprotein complex was attached to the erythroid nuclear matrix.

Like GATA-1, Sp1 plays a major role in the expression of erythroid-specific genes. The H5 promoter has a high-affinity Sp1 binding site, while the enhancer has several weak and low-affinity binding sites for Sp1 (Fig. 2) [Sun et al., 1992]. One of the low-affinity Sp1 binding sites has the CACCC sequence and will bind to CACCC-binding proteins. EMSA with an oligonucleotide with a high-affinity Sp1 binding site (promoter) was done with nuclear and nuclear matrix extracts from the erythrocytes. Several complexes were formed with the Sp1-oligonucleotide and the nuclear- or nuclear matrix-extracted proteins (Fig. 5). To determine which of the complexes contained Sp1, nuclear extracts from 15-day definitive erythrocytes were incubated with anti-Sp1 antibodies. We and others have shown that complexes formed with Sp1 are absent when anti-Sp1 antibodies are added to the nuclear extract [DesJardins and Hay, 1993; Miller et al., 1996]. Figure 6B shows that the presence of anti-Sp1 antibodies reduced the abundance of complex 7 formed with the Sp1-oligonucleotide and nuclear-extracted proteins. However, the levels of all complexes formed with the Sp1-oligonucleotide were decreased when unlabelled Sp1-oligonucleotide was added. Thus, the slowest migrating complex (C7) that predominates the complexes generated with erythrocyte nuclear extracts was formed with Sp1.

EMSA was also done with an oligonucleotide (PV), which has the CACCC sequence and the consensus sequence (CCNCNCCCN) for ery-

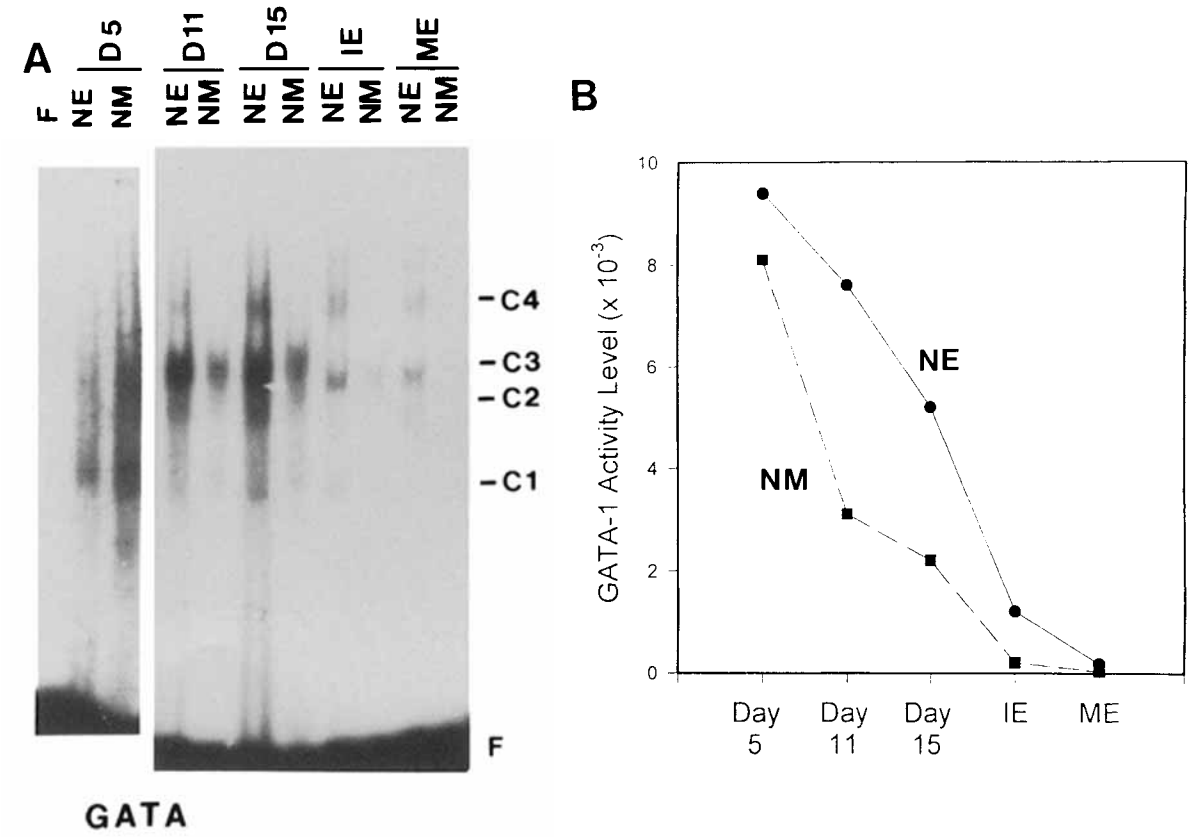

Fig. 4. GATA-1 protein complexes are associated with the nuclear matrices of primitive and definitive erythrocytes. **A:** Proteins (10 μg) in extracts from nuclei (NE) and nuclear matrices (NM) from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes were incubated with end-labelled GATA-oligonucleotide (0.9 ng). EMSA was done as described under Materials and Methods. F is the free labelled oligonucleotide, and C1-4 are the protein–DNA complexes. **B:** The level of the complexes formed in each extract was standardized relative to the content of nuclear DNA (factor activity/mg DNA). The results shown are representative of the findings with three different preparations. ●, nonmatrix nuclear fraction (NE, nuclear extract); ■, nuclear matrix extract (NM).

throid Krüppel-like factor (EKLF) [Orkin, 1995]. The high affinity Sp1 oligonucleotide also contained the EKLF consensus sequence (Fig. 2). Complexes similar to those shown in Figure 5A were generated with the PV-oligonucleotide, with the exception that the intensities of complexes 2-5 were enhanced, and the intensity of complex 7 reduced (Fig. 6A, cf. Fig. 5A). The inclusion of anti-Sp1 antibodies in EMSA did not prevent the formation of complexes 2-4 (Fig. 6B). These observations suggest that complexes 2-5 were generated by CACCC-binding proteins (perhaps the chicken equivalent of EKLF).

Sp1 and CACCC-binding protein activities were high in primitive erythroid nuclear extracts and declined progressively in the nuclear extracts from erythrocytes of 11-day, 15-day, immature adult, and mature adult erythrocytes (Figs. 5A, 6A). There was a 13.5-fold and 21.5-fold difference in Sp1 and Sp1-like (including CACCC-binding protein) activities, respectively, between 5-day and immature adult erythrocyte nuclear extracts (Fig. 5B,C).

Nuclear matrix extracts from primitive and definitive erythrocytes formed complexes with the Sp1-oligonucleotide, but there was a distinct difference in the distribution of complexes formed with nuclear- and nuclear matrix-extracted protein. The majority of the complexes formed with nuclear-extracted proteins migrated slower than those formed with nuclear matrix-extracted proteins. The nuclear matrix extracts had some Sp1 activity (complex C7), but most complexes were formed with Sp1-like proteins and/or CACCC-binding proteins. There was a marked decline in nuclear matrix activity of Sp1-like and CACCC-binding proteins from primitive to definitive erythrocytes (Fig. 5C). Comparing the activity of these proteins in nuclear matrix extracts of 5-day and immature

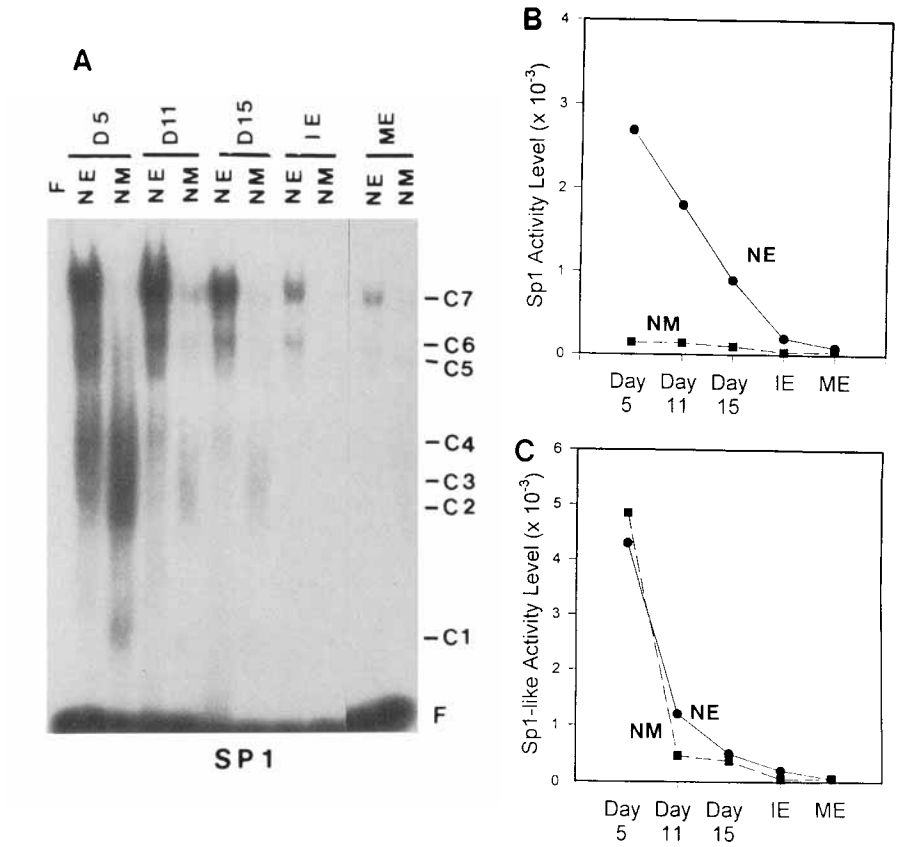

Fig. 5. Sp1-like and CACCC-binding proteins are associated with the nuclear matrix of primitive erythroid cells. **A:** Protein extracts from nuclei (NE) and nuclear matrices (NM) derived from 0.5 A_{260} units of nuclei from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes were incubated with end-labelled Sp1-oligonucleotide (0.4 ng). EMSA was done as described under Materials and Methods. F is the free labelled oligonucleotide, and C1-7 are the protein-DNA complexes. **B,C:** Amount of complex 7 (Sp1, shown in B) and complexes 1–6 (Sp1-like, includes CACCC-binding proteins, C) formed in each extract was standardized relative to the content of nuclear DNA (factor activity/mg DNA). This result is representative of the findings with three different preparations. ●, nonmatrix nuclear fraction (NE, nuclear extract); ■, nuclear matrix extract (NM).

adult erythrocytes, the decline in Sp1-like (including CACCC-binding) protein activity was approximately 70-fold.

The H5 promoter has an upstream promoter element (UPE) that is recognized by a DNA-binding protein present in mature and immature erythrocyte nuclei [Sun et al., 1992]. The UPE-oligonucleotide was used in EMSAs with extracts from the erythrocyte nuclei or nuclear matrices (Fig. 7A). All erythrocyte nuclear extracts formed complexes with the UPE-oligonucleotide, with the activity of the UPE-binding protein lower in the immature and mature adult extracts than nuclear extracts from 5-, 11-, and 15-day cells (Fig. 7B). The nuclear matrix extract from the primitive erythroid cells had DNA-binding activity with UPE-oligonucleotide, but the activity of UPE-binding protein was very low in nuclear matrix extracts from 11-day (9-fold less than 5-day cells), 15-day, immature adult (45-fold less than 5-day cells), and mature adult cells (Fig. 7B).

Casein Kinase II Is Not a Component of the Erythrocyte Nuclear Matrix

Casein kinase II was recently shown to be a component of the rat liver and prostate nuclear matrix [Tawfic and Ahmed, 1994]. Casein kinase II, a serine/threonine protein kinase, is a tetrameric enzyme consisting of two α (and/or α′) and two β-subunits. This enzyme phosphorylates several transcription factors, including c-*Myc*, c-*Myb*, and c-*Jun* [Litchfield and Lüscher, 1993]. In Western blot experiments with an antibody to casein kinase II subunit α, casein kinase II was found to be present in nuclear, but not nuclear matrix, extracts (Fig. 8). Thus, ca-

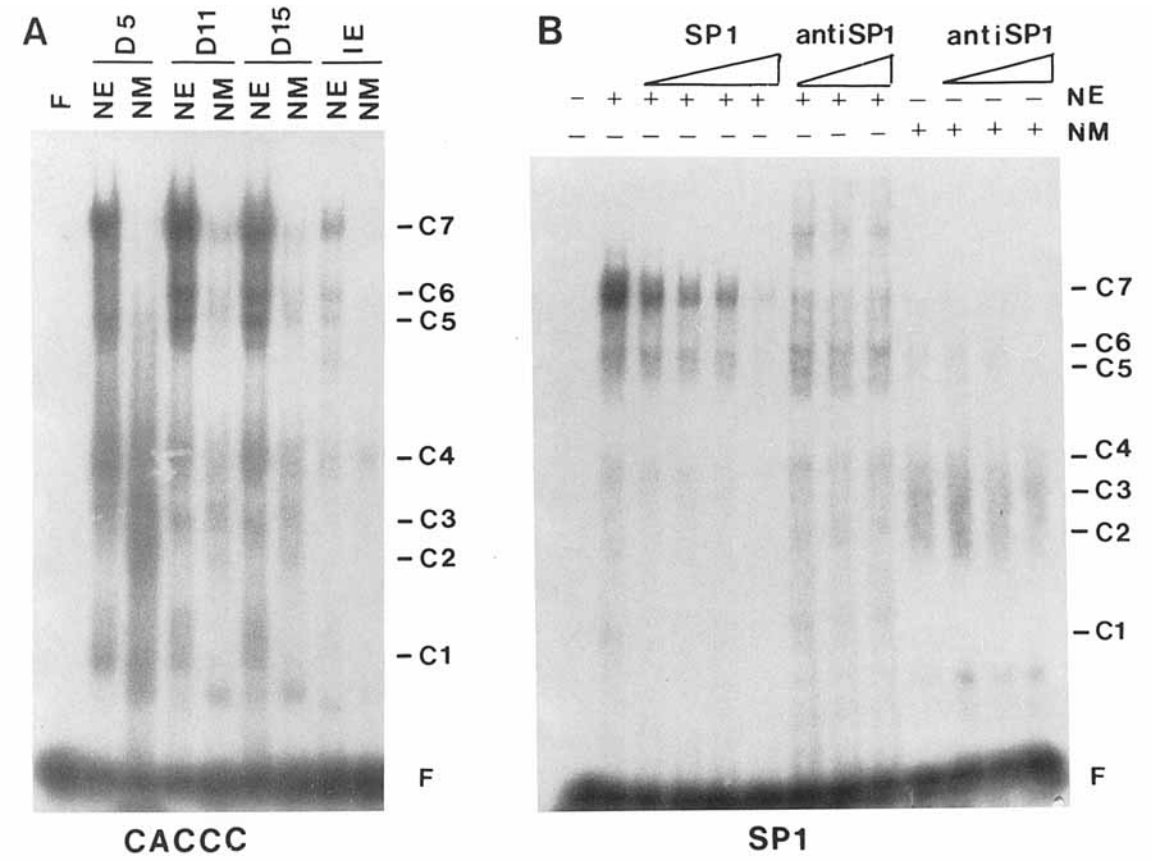

Fig. 6. CACCC-binding proteins are associated with the primitive and definitive erythrocyte nuclear matrix. **A:** Protein extracts from nuclei (NE) and nuclear matrices (NM) derived from 0.5 A_{260} units of nuclei from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes were incubated with end-labelled PV (CACCC)-oligonucleotide (0.4 ng). EMSA was done as described under Materials and Methods. F is the free labelled oligonucleotide, and C1–7 are the protein–DNA complexes. **B:** *Lanes 2–9,* nuclear extracted protein (10 μg) from 15-day erythrocytes was incubated with end-labelled Sp1-oligonucleotide (0.15 ng) (*lane 2*) and with either 25, 50, 100, and 200 molar excess of unlabelled Sp1-oligonucleotide (*lanes 3–6,* respectively) or 0.5, 1, and 2 μg of anti-Sp1 antibody (*lanes 7–9,* respectively). *Lanes 10–13,* nuclear matrix-extracted protein (10 μg) from 15-day definitive erythrocytes was incubated with end-labelled Sp1-oligonucleotide (0.15 ng) (*lane 10*) and with 0.5, 1, and 2 μg of anti-Sp1 antibody (*lanes 11–13,* respectively). F is the free labelled oligonucleotide, and C1–7 are the protein–DNA complexes.

sein kinase II was not a component of chicken erythrocyte nuclear matrices.

DISCUSSION

Our results show that the activity of several transcription factors is elevated in five day embryonic erythrocyte nuclei. In agreement with the observations of Minie et al. [1992], GATA-1 and Sp1 activities were greater in the nuclear extracts of the primitive erythrocytes than in extracts from definitive erythrocyte nuclei. The activities of CACCC-binding proteins and NF1 were also greatest in the primitive erythrocytes.

Transcription factors GATA-1, CACCC-binding proteins, and NF1 were associated with the primitive and definitive erythrocyte nuclear matrix, with the primitive erythroid nuclear matrix having the highest concentration of these factors. None of these proteins was exclusively bound to the nuclear matrix. In marked contrast to the Sp1-like and CACCC-binding proteins, Sp1 was found mostly in the nonmatrix nuclear fraction. In five day embryonic erythrocytes, the embryonic ϵ- and ρ-globin genes are expressed. The preferential binding of CACCC-binding proteins with the primitive erythrocyte nuclear matrix may allow the selective binding of CACCC-binding proteins to Sp1 binding sites in the embryonic globin promoter/enhancer. CACCC-binding proteins (e.g., EKLF and CON) are also important in the expression of the β-globin genes [Emerson et al., 1989; Perkins et al., 1995]. Interestingly, in situ occupancy at the CACCC site in the β-globin promoter progres-

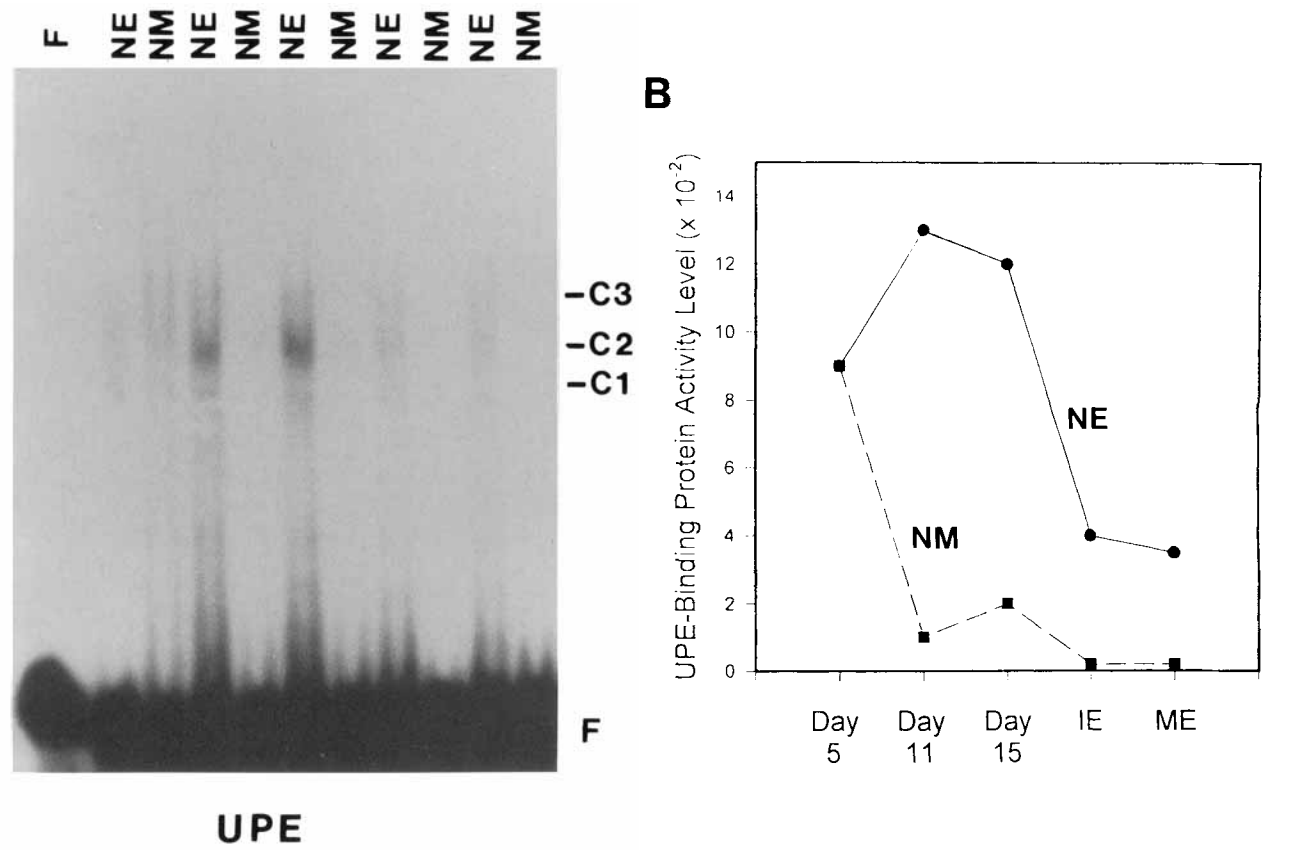

Fig. 7. UPE-binding protein is bound to the primitive erythrocyte nuclear matrix. **A:** Protein extracts from nuclei (NE) and nuclear matrices (NM) derived from 0.5 A_{260} units of nuclei from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes were incubated with end-labelled UPE-oligonucleotide (2 ng). EMSA was done as described under Materials and Methods. F is the free labelled oligonucleotide, and C1–3 are the protein–DNA complexes. **B:** The amount of complexes formed in each extract with the UPE-oligonucleotide was standardized relative to the content of nuclear DNA (factor activity/mg DNA). This result is representative of the findings with three different preparations. ●, nonmatrix nuclear fraction (NE, nuclear extract); ■, *nuclear matrix* extract (NM).

sively declines in 11-day, 15-day and adult mature erythrocytes [Jackson et al., 1991]. This parallels the decline in CACCC-binding protein activity in the nuclei of these cells. There was also a progressive decline in nonmatrix and matrix nuclear GATA-1 activity, a factor important in activity of erythroid enhancers and promoters. GATA-1 interactions with Sp1 and CACCC-binding proteins (e.g., EKLF) appear to important in promoter-enhancer complex formation (embryonic and adult β-globin genes, H5 gene) [Gallarda et al., 1989; Sun et al., 1992; Merika and Orkin, 1995]. The recruitment of GATA-1 and CACCC-binding proteins to the nuclear matrix may enhance the formation of these promoter-enhancer complexes in primitive and definitive erythrocytes.

Vassetzky et al. [1993] identified a GATA-like factor (F6) that was associated with the nuclear matrix of avian erythroblastosis virus-transformed chicken erythroblasts. The nuclear matrix-bound GATA-like factor appeared to be involved in an interaction between matrix associated regions (MARs) in the α-globin gene domain and the nuclear matrix. The molecular mass of the F6 protein and its association with the nuclear matrix would suggest that the F6 factor is GATA-1, a result consistent with our findings.

GATA-1 exists as a multiprotein complex. Consistent with previous observations [Sun et al., 1993], we found that the majority of GATA-1 activity in primitive erythrocytes tends to be free of GATA-1 binding proteins. In primitive erythrocyte nuclear extracts, the C1 complex (GATA-1) is more prevalent than the C2-4 complexes that have proteins binding to GATA-1. In definitive erythrocyte nuclear extracts most GATA-1 activity is in complexes 2 and 3. The prevalence of the C1 complex in 5-day embry-

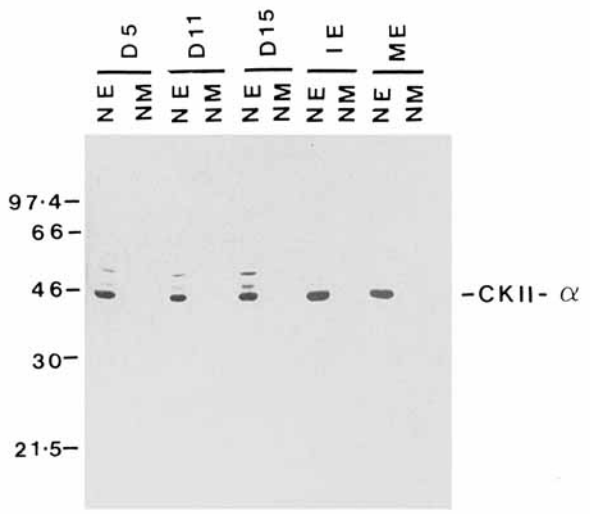

Fig. 8. Casein kinase II is not bound to the nuclear matrices of chicken erythroid cells. Ten μg of nuclear- (NE) and nuclear matrix-extracted (NM) protein from 5-day (D5), 11-day (D11), 15-day (D15), adult immature (IE), and adult mature (ME) erythrocytes was electrophoretically separated on a SDS-10% polyacrylamide gel. The proteins were transferred to nitrocellulose. The blot was immunochemically stained with the anticasein kinase II subunit α antibody as described under Materials and Methods. The molecular masses of the marker proteins run in the same gel are shown in kDa on the left.

onic red blood cell nuclei may be a consequence of the higher amounts of GATA-1 relative to the GATA-1 binding proteins. GATA-1 and GATA-1 with its associated proteins (complexes C2-4) were components of the nuclear matrices of day 5, 11 and 15 embryonic erythrocyte nuclear matrices. These results suggest that the GATA-1 domain that interacts with protein(s) in the C2-4 complexes is different from the domain binding to the nuclear matrix acceptor for GATA-1.

The histone H5 gene is expressed in primitive erythrocytes and adult immature erythrocytes [Colman et al., 1983; Trempe et al., 1988]. NF1, which binds to the 3′ enhancer of histone H5 gene, may mediate an interaction between the H5 enhancer and the nuclear matrix in primitive and definitive erythroid cells. In contrast to other transcription factor activities (e.g., GATA-1, CACCC-binding proteins), NF1 activity remained constant in nuclear matrices of 11-day, 15-day, adult immature, and mature erythrocytes. In primitive cells the H5 enhancer and promoter would potentially make multiple attachments to the nuclear matrix. Interactions between nuclear matrix bound factors (NF1, GATA-1, CACCC-binding proteins, and UPE-binding protein) and the H5 promoter/enhancer would be possible.

The internal nuclear matrix of definitive erythrocytes is less extensive than the nuclear matrix of primitive erythroid cells [Lafond and Woodcock, 1983]. In parallel, there is a progressive loss of constrained supercoils in 5-, 12-, and 18-day embryonic erythroid nucleoids which are nuclei treated with 1 M NaCl [Cook and Brazell, 1976]. The loss of constrained supercoils and reduction in internal nuclear matrix correlates with a decrease in histone deacetylase activity and transcription factors associated with the internal nuclear matrix of 11- and 15-day embryonic erythrocytes. The reduction in these nuclear matrix proteins and reduction in nuclear RNA may contribute to weakening the structural integrity of the internal nuclear matrix [Gasaryan, 1982; Belgrader et al., 1991; Hozák et al., 1995]. However, it should be noted that although there is a general reduction in constrained supercoils in definitive erythroid cells, transcriptionally active chromatin domains (e.g., the β-globin domain in 12-day red blood cells) are torsionally stressed [Villeponteau and Martinson, 1987]. Dynamic interactions between nuclear matrix bound histone acetyltransferase, histone deacetylase and transcription factors may contribute to constraining the supercoils of transcriptionally active chromatin domains in both primitive and definitive erythrocytes.

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