not examined in detail.

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Association of Globin Ribonucleic Acid and Its Precursors with the Chicken Erythroblast Nuclear Matrix†

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ABSTRACT: Nuclear matrix was prepared from both erythroblasts and erythrocytes of chicken red blood cells. Greater than 90% of the globin nuclear RNA remains bound to the erythroblast nuclear matrix. There are approximately 1000 copies of globin RNA in the nucleus per cell, and most of these contain a poly(A) tail. Precursor β globin RNA exists in four high molecular weight forms, some of which are larger than the natural β globin gene. Most of the ribosomal RNA is lost during the preparation of an erythroblast nuclear matrix. In contrast, some of the snRNAs are specifically enriched in the erythroblast nuclear matrix. There is little or no globin nuclear RNA in the erythrocyte nuclear matrix. There appears to be no selective attachment of the globin genes to the erythroblast nuclear matrix. The nuclear matrix is postulated to be a platform for the differential processing of nuclear RNA.

An underlying structure of all eukaryotic nuclei is the nuclear matrix, nuclear lamina, or nuclear skeleton which morphologically resembles the nucleus but which is mainly composed of polymeric protein fibrils and RNA (Grebanier & Pogo, 1979). The nuclear matrix, obtained by digestion with DNase I or endogenous nucleases and repeated extraction with 2 M NaCl, contains a small amount of tightly bound and highly protected DNA and much of the nuclear RNA (Miller et al., 1978a,b; Berezney & Coffey, 1975). The nuclear lamina, the peripheral layer of the nuclear matrix, or nuclear skeleton can be prepared by slight variation of the digestion and washing steps used in preparing the nuclear matrix (Kaufman et al., 1981). Although it is not yet clear if the matrix has any nuclear function other than providing the structural framework of the cell nucleus, several pieces of evidence suggest that the nuclear matrix may serve as a platform for various important nuclear events. For example,

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pulse-labeling experiments show that DNA replication (Pardoll et al., 1980) and processing of ribosomal RNA (Herlan et al., 1979) occur in association with the matrix. Chromosomal DNA is anchored with the nuclear matrix via repeated sequences (Razin et al., 1979) just as in metaphase chromosomes (Laemmli et al., 1978). hnRNA (Miller et al., 1978a) and snRNA (Miller et al., 1978b), which has been implicated in the removal of intron sequences in hnRNA (Lerner et al., 1980), were found to be tightly associated with the nuclear matrix.

The nuclear matrix may provide sites for gene transcription, RNA processing, and RNA transport (Wunderlich et al., 1976; Herlan et al., 1979). As a first step to address this question, we studied the possible binding of the globin genes and globin RNA to the nuclear matrix of erythroblasts and erythrocytes of chicken red blood cells. We find that practically all of the globin nuclear RNA is tightly associated with the erythroblast nuclear matrix and some of the matrix-bound globin RNA is precursor RNA. Only a fraction (30%) of the nuclear RNA, but nearly all of the globin nuclear RNA, is associated with the nuclear matrix, suggesting that not all nuclear RNA is tightly associated with the nuclear matrix.

Some gene systems, like the SV40 virus DNA, are specifically enriched in the nuclear matrix (Nelkin et al., 1980). The globin genes were investigated to see if they are also enriched on the nuclear matrix in erythroblasts. In contrast to globin nuclear RNA, there does not appear to be any direct and selective linkage of the globin gene DNA to the nuclear matrix.

Experimental Procedures

Preparation of Erythrocyte and Erythroblast Nuclei. Erythrocytes were taken directly from White Leghorn chicken. Erythroblasts were prepared by phenylhydrazine injection of chickens as described (Gadski & Chae, 1978). The cells were washed and nuclei prepared as described (Gadski & Chae, 1978) except that all solutions were treated with 0.1% diethyl pyrocarbonate. Nuclei were washed 3–4 times with 0.25 M sucrose, 10 mM KH_2PO_4, pH 6.8, and 3 mM $MgCl_2$.

Preparation of the Nuclear Matrix. The nuclear matrix was prepared by the method of Berezney & Coffey (1975) with some modifications: for preparations of the nuclear matrix, nuclei were washed with 0.35 M NaCl, 10 mM Tris, pH 7.5, and 0.2 mM $MgCl_2$ (3 times) and 0.2 mM $MgCl_2$ and 10 mM Tris, pH 7.5 (TM0.2) (once). The washed nuclei were suspended in 0.2 mM $MgCl_2$ and 10 mM Tris, pH 7.5, at 1 mg of DNA/mL and digested with 10 μg/mL DNase I for 1–2 h at 25 °C. The nuclei were then washed with 2 M NaCl, 0.2 mM $MgCl_2$, and 10 mM Tris, pH 7.5 (3 times), and 1% Triton X-100, 5 mM $MgCl_2$, and 10 mM Tris, pH 7.5 (once), and finally with 10 mM Tris, pH 7.5, and 5 mM $MgCl_2$ (TM5) (3 times). All solutions contained 0.01 mM aurintricarboxylic acid and 0.1 mM PMSF. All washing steps were done at 4 °C with centrifugation at 3000*g* for 15 min. The final material showed intact nuclear morphology under a light microscope after staining with 0.1% Coomassie Blue in 10% acetic acid.

DNase I (Worthington; RNase-free grade) was treated to remove RNase and proteases. Essentially DNase I was applied to a lima bean trypsin inhibitor–Sepharose column, treated with bentonite, and then purified over a 5′-[(4-aminophenyl)phosphoryl]uridine 2′(3′)-phosphate–Sepharose column (Miles) (Wang & Moore, 1978).

RNA and DNA Extraction. The nuclear matrix or total washed nuclei were digested with proteinase K (50 μg/mL) in 0.5% sodium dodecyl sulfate ($NaDodSO_4$), 1 mM EDTA, 10 mM Tris, pH 7.5, and 150 mM NaCl and extracted with phenol–$CHCl_3$ (1:1) and then $CHCl_3$. Nucleic acids were precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate at −20 °C. For isolation of RNA, DNA was removed by further incubation with DNase I and subsequent extraction with phenol–$CHCl_3$. For isolation of DNA, RNA was removed by treatment with RNase A and 0.3 M NaOH at 68 °C for 30 min. Aurintricarboxylic acid was present in all steps for RNA isolation; the inhibitor can be removed with Sephadex. Vanadyl sulfate adenylate was also used as an RNase inhibitor in some experiments. Poly(A) RNA was prepared by using poly(U)–Sepharose exactly as described by Payvar & Schimke (1979).

cDNA Preparation. [^{3}H]- and [^{32}P]cDNA were prepared from chicken globin mRNA (mixture of α and β globin) by using reverse transcriptase as described (Friedman & Rosbash, 1977; Gadski & Chae, 1978). Hybridization was carried out in 0.4 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, and 10 mM Tris, pH 7.5, at 68 °C, and the radioactivity in the S_1 nuclease resistant duplex was determined (Gadski & Chae, 1978).

cDNA Plasmid. Chicken β cDNA plasmid pHb1001 was obtained from Winston Salser, and a 460-bp *Hpa*II fragment was purified to remove poly(dA·dT) sequences. The *Hpa*II fragment was labeled with [^{32}P]dCTP by nick translation (Rigsby et al., 1977).

Formaldehyde Gel Electrophoresis. Agarose (1.0%) in Mops buffer [1×; 20 mM 3-(*N*-morpholino)propanesulfonic acid (Sigma), 5 mM sodium acetate, and 1 mM EDTA, pH 7.0] was dissolved by heating, and the mixture was cooled to 50 °C. Reagent-grade formaldehyde was added to a final concentration of 6% and ethidium bromide to a final concentration of 1 μg/mL (Saul Silverstein, personal communication). The sample RNA was dissolved in 50% formamide and 1× formaldehyde–Mops buffer with 0.05% bromophenol blue, heated to 68 °C for 5 min, quick cooled, and run at 70 V for 20 h on a 0.6 × 13 × 35 cm gel. The gel was washed for 30 min in 20× SSC and then for 30 min in 10× SSC, photographed, and applied directly to nitrocellulose paper (Schleicher & Schuell; 0.45 μm). The transfer was usually done overnight. The nitrocellulose was baked at 80 °C for 4 h and then prehybridized and hybridized according to Wahl et al. (1979). The hydridization solution contained ^{32}P-labeled globin cDNA in 10% dextran, 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 6.8, 200 μg/mL denatured salmon sperm DNA, 50 μg/mL yeast tRNA, and 5× Denharts buffer. Washing and autoradiography were done according to Wahl et al. (1979) using Kodak RP-5 film at −70 °C.

Labeling of Erythroblast RNA with [^{3}H]Uridine. Erythroblasts were washed 2 times in F-10 medium, and the number of cells was adjusted to 5 × 10^6 cells/mL. The cells were labeled in F-10 medium (no serum added) with 10 μCi/mL [^{3}H]uridine (New England Nuclear; 37 Ci/mmol) for 2 h at 37 °C. After centrifugation and washing at 1000*g*, the cells were suspended in 20 mL of 10 mM Tris, pH 7.5, 30 mM KCl, and 2 mM $MgCl_2$. After 5 min on ice the suspension of lysed cells was made 0.25 M in sucrose and centrifuged at 1000*g*. RNA was extracted from the supernatant and the nuclear pellet, and poly(A)-containing RNA was isolated as described (Gadski & Chae, 1978). The final specific radioactivity of the RNA was 1 × 10^5 cpm/μg.

Assay for the Possible Binding of Isolated RNA to Nuclear Matrix. One microgram of erythroblast nuclear poly(A)-containing [^{3}H]RNA (1 × 10^5 cpm) was mixed with either the erythroblast nuclear matrix (originally from 5 mg of nuclei in DNA) or 1 mg of erythroblast nuclei in 4 mL of 2 M NaCl, 0.2 mM $MgCl_2$, and 10 mM Tris, pH 7.5; after 60 min on ice

the sample was layered on a step gradient consisting of 2 mL of 60% sucrose, 2 M NaCl, and 10 mM Tris, pH 7.5, under 6 mL of 15% glycerol, 2 M NaCl, and 10 mM Tris, pH 7.5, in SW27 rotor tubes and centrifuged for 4 h at 25 000 rpm. Fractions (0.6 mL) were collected from the bottom of the tube.

Nuclei contain 122 μg of RNA/mg of DNA, and the nuclear matrix contains 40 μg of RNA/original mg of DNA. Therefore, a large excess of nuclei or matrix was mixed with the [^{3}H]nRNA for the binding assay.

Assay for the Possible Binding of the Globin Genes to the Nuclear Matrix. The nuclear matrices were prepared three different ways. (a) The nuclei washed with 0.35 M NaCl were digested with DNase I for different lengths of time and the final matrix contained 1–20% of DNA. (b) The nuclei suspended in 2 M NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA for 1 h at 0 °C were passed through a 21-gauge needle under maximum thumb pressure 3 times. The sample was layered on a 15% glycerol and 60% sucrose step gradient containing 2 M NaCl and centrifuged for 45 min at 17 000 rpm in a SW27 rotor. Only the structure resembling whole nuclei appeared at the border of 60% sucrose, and most of the DNA and histones appeared at the top of the gradient. On the average about 20% of the original DNA was associated with the nuclear matrix. The whole nuclei in 2 M NaCl (not sheared) remain at the top of the gradient under the centrifugation conditions used. (c) The nuclei suspended in 2 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.5, for 1 h at 0 °C were layered on a 15% glycerol and 60% sucrose step gradient containing 2 M NaCl and centrifuged at 25 000 rpm for 4 h to remove histones. The nuclei accumulated at the 60% sucrose layer still maintain the nuclear morphology when examined under a microscope after staining with 0.1% Coomassie Blue. The nuclei with the associated gelatinous DNA were dialyzed against *Eco*RI buffer (0.1 M Tris, pH 7.2, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 50 mM NaCl) and digested with *Eco*RI (1 unit/μg of DNA) for 2 h at 37 °C. The reaction was stopped by adding 10 mM EDTA; the mixture was centrifuged through a 15% glycerol–60% sucrose step gradient (no NaCl) at 17 000 rpm for 45 min, and the matrix accumulated at the 60% sucrose layer was collected. About 30% of the original DNA was associated with the nuclear matrix.

DNA was isolated from the matrix preparation as described above after digesting with proteinase K or Pronase and treated with RNase and 0.3 N NaOH (68 °C; 30 min) to ensure that no RNA contaminates. The content of globin genes was compared with that in the total DNA, which was directly isolated from whole erythroblast nuclei, by globin cDNA excess hybridization. Hybridization was carried out for 72 h at 68 °C. Hybridization was assayed with S_1 nuclease as above.

Urea (7 M)–Acrylamide (10%) Gels. Urea (7 M)–acrylamide (10%) gel electrophoresis was done according to Maniatis et al. (1975).

Electron Microscopy. The freshly prepared nuclear matrix was fixed in 2.5% glutaraldehyde in 0.1 M phosphate (pH 6.9) for 1 h at 25 °C. The matrix was then washed in phosphate buffer and fixed in 2% osmium tetraoxide in phosphate buffer. After dehydration in ethanol and propylene oxide, the sample was embedded in Maraglas, sectioned, and stained with uranyl acetate and lead acetate. Sections were cut to 600–800 Å and viewed on a JEM 100B electron microscope.

Results

The nuclear matrix was prepared from erythroblasts and erythrocytes of chicken red blood cells by the method of Berezney & Coffey (1975) with slight modifications. The principal modifications were the preliminary wash of the nuclei with 0.35 M NaCl and an RNase inhibitor, aurintricarboxylic acid, and a protease inhibitor, phenylmethanesulfonyl fluoride (PMSF), were included in all solutions. Briefly, the washed nuclei were digested with RNase-free DNase I and subsequently extracted with 2 M NaCl, 0.2 mM $MgCl_2$, and 10 mM Tris, pH 7.5, and then with a solution containing 1% Triton X-100 (see Experimental Procedures). The final nuclear pellet still maintained its original nuclear morphology as examined under a light microscope after staining with Coomassie Blue, and only 1–2% of the original DNA remained as reported by others (Berezney & Coffey, 1975). An electron micrograph of the erythroblast nuclear matrix is shown in Figure 1. The residual structure contains the nuclear lamina as well as various amounts of interior structure on which one can see very darkly staining particles, possibly hnRNP. The variation in the amount of interior structure may occur for several reasons: we are not dealing with a strictly homogeneous cell population; the phenylhydrazine treatment of adult chickens will cause the appearance of all types of precursor erythroid cells. Also, since the micrographs are thin sections, we do not see the three-dimensional network of interior structure. Therefore, the amount of interior structure may depend on where across the nuclear sphere the thin section was prepared.

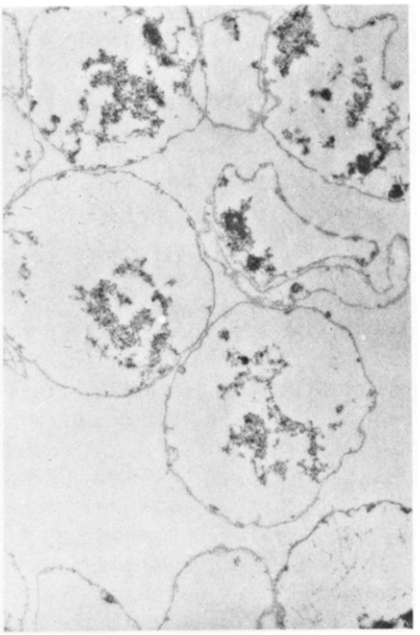

FIGURE 1: Electron micrograph of the erythroblast nuclear matrix. Freshly prepared nuclear matrix was fixed, sectioned, and stained with uranyl acetate. The photograph shows 5500× magnification.

The RNA in each fraction of the nuclear matrix preparation was quantitated by hydridization analysis for globin RNA content (Figures 2 and 3 and Table I), stained for snRNA (Figure 4) and large RNA species (Figure 5), and finally blot hybridized for analysis of the β globin RNA size (Figure 6). Quantitation of RNA at each step in the preparation of the nuclear matrix shows that 1–2% of the total erythroblast nuclear RNA appears in the 0.35 M NaCl wash and 70% is released during the DNase I digestion, 2 M NaCl wash, and subsequent washes (most of the RNA is released in the DNase I digestion). The final nuclear matrix contains about 28–30% of the total nuclear RNA. The content of globin RNA in these nuclear fractions was determined by RNA excess hybridization with globin [^{3}H]cDNA (a mixture of α and β globin sequences). A value of 7.5×10^{-4} mol·s/L was used as the $C_0t_{1/2}$ for hybridization between the cDNA and globin mRNA

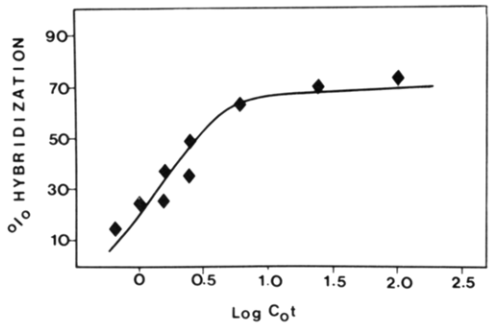

FIGURE 2: Hybridization analysis of the erythroblast RNA in the DNase I plus 2 M NaCl wash. RNA from the DNase I plus 2 M NaCl washes of erythroblasts was hybridized in vast excess to ^{3}H-labeled globin cDNA. A log $C_0t_{1/2}$ of 0.3 mol·s/L was obtained.

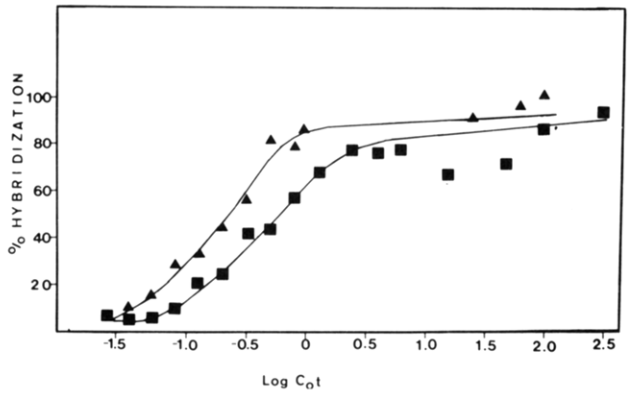

FIGURE 3: Hybridization analysis of erythroblast nuclear RNA. ^{3}H-Labeled globin cDNA was hybridized to total nuclear matrix RNA (▲) and total nuclear RNA (■) from erythroblasts by using RNA in vast excess. The log $C_0t_{1/2}$ for nuclear matrix RNA is −0.9 mol·s/L while the log $C_0t_{1/2}$ for total nuclear RNA is −0.47 mol·s/L, indicating a 3× enrichment of globin RNA sequences in the preparation of the nuclear matrix.

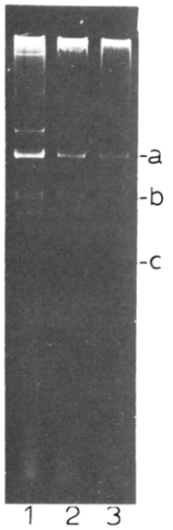

FIGURE 4: Electrophoresis of small nuclear RNA of the erythroblast nuclear matrix. (1) The nuclear matrix; (2) total nuclear RNA; (3) DNase I plus 2 M NaCl wash. 20 μg of RNA from each fraction was run on a 7 M urea–10% acrylamide gel according to Maniatis (1975) and stained with ethidium bromide. RNA bands a–c are discussed in the text.

(Hastie & Bishop, 1976; Reynaud et al., 1980). In erythroblasts the RNA in 0.35 M NaCl wash contains 1–2% of the total RNA of which 0.2% is globin RNA (Table I); it is possible that this RNA is derived from slight cytoplasmic contamination or broken nuclei. Due to the low amount of

Table I: Number of Globin RNA Molecules per Cell[a]

cell fraction	erythroblast	erythrocyte
cytoplasm	1.5×10^5[a]	
0.35 M NaCl wash	20	15
2 M NaCl wash–DNase I wash	132	<1
total nuclei (after 0.35 M NaCl wash)	1100	<1
nuclear matrix	1000	<1

[a] A value of 7.5×10^{-4} was used for the $C_0t_{1/2}$ for the hybridization of globin cDNA and RNA based on the reports by Hastie & Bishop (1976) and Reynaud et al. (1980). The salt concentration used for hybridization was similar. A molecular weight average of 6.2×10^5 was used to calculate globin RNA per cell. [b] From Hunt (1974).

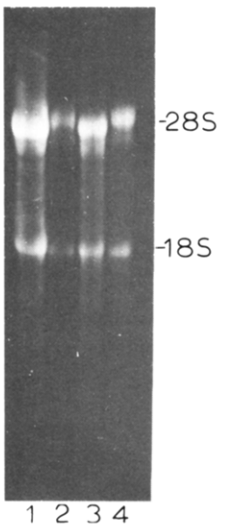

FIGURE 5: Formaldehyde–agarose gel electrophoresis of RNA from the erythroblast nuclear matrix. (1) 0.35 M NaCl wash; (2) DNase I plus 2 M NaCl wash; (3) the nuclear matrix; (4) total nuclear RNA. 10 μg of RNA from each fraction was run on a formaldehyde–agarose gel and stained with ethidium bromide. 18S and 28S ribosomal RNAs are indicated.

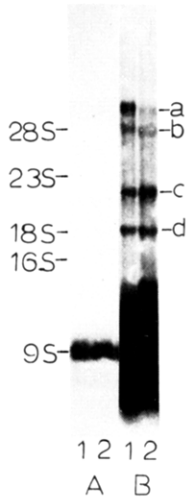

FIGURE 6: Southern blot hybridization of the nuclear matrix and total nuclear RNA with a β globin cDNA. (1) The nuclear matrix or (2) total nuclear RNA was run on a 1% agarose–formaldehyde gel, transferred to nitrocellulose, hybridized to a ^{32}P-labeled 460-bp *Hpa*II fragment from a β globin cDNA clone (pHb1001), and autoradiographed. (A) Short exposure; (B) long exposure. Bands a–d are discussed in the text and Figure 7. Positions of molecular weight markers used (ribosomal RNA) are indicated. 9 S indicates the position of mature globin RNA.

RNA in this fraction it was not extensively explored. There is 0.03% globin RNA in the DNase I and 2 M NaCl washes

of erythroblast nuclei (which contain 70% of the total RNA) (Figure 2). Finally, as shown in Figure 3, the globin RNA content in total nuclei is 0.2%. However, the amount of globin RNA in the nuclear matrix is 0.6%, suggesting that the globin RNA is about 3 times more enriched in the matrix compared to the total RNA in erythroblast nuclei (Figure 3). This is due to the release of substantial amounts of nonglobin RNA (70%) during the preparation of the nuclear matrix. When the amount of globin RNA was calculated in terms of the number per cell (see Table I), it is clear that practically all of the globin nuclear RNA is tightly associated with the nuclear matrix. Much of the poly(A+) nuclear matrix RNA is globin RNA as it has a similar $C_0t_{1/2}$ to that of cytoplasmic poly(A+) RNA (data not shown).

Erythrocytes maintain the chromatin of the globin genes in an active state (Weintraub & Groudine, 1976). Gariglio et al. (1981) have shown that RNA polymerase is present on the β globin gene in erythrocytes. Since secondary mechanisms may be used to deactivate the globin genes in erythrocytes, we investigated whether the globin RNA attached to the matrix was affected. Essentially no globin RNA is present in the erythrocyte matrix (data not shown). The small amount of globin RNA per cell present in the erythrocyte matrix fraction (less than one copy per cell) could be accounted for by the low level of circulating erythroblasts in normal chicken.

The size of RNA in various fractions obtained during the preparation of matrix was analyzed either by 7 M urea–10% acrylamide gel electrophoresis for the snRNAs or by formaldehyde–agarose gel for large RNAs. snRNA appears in all fractions as shown in Figure 4. However, the snRNAs "a" and "b" appear to be enriched in the matrix but the RNA "c" (probably tRNA) seems to be depleted in the matrix.

Figure 5 shows a formaldehyde–agarose gel of the RNA in washes and nuclear matrix preparation. It is immediately evident that 18S and 28S ribosomal RNAs (rRNAs) are present in all fractions. One can obtain an approximate quantitation of the amount of rRNA in each fraction by scanning the gel and determining the peak area. Since an equal amount of RNA was applied to each lane, by knowing the total amount of RNA in each fraction, one can obtain an estimate of the relative amount of rRNA in each fraction. The amount of rRNA in the washes is approximately twice that in the nuclear matrix. This is in contrast to the globin RNA which remains with the matrix (Table I). Although the analysis of rRNA is more crude than the hybridization studies, it provides evidence that not all types of RNA are selectively bound to the erythroblast nuclear matrix. The same is true for most of the snRNA.

Size of the Matrix-Bound Globin RNA. The RNA from total erythroblast nuclei or from the nuclear matrix was run on formaldehyde–agarose gels. The RNA was transferred to nitrocellulose paper and hybridized to a β globin cDNA clone (pHb1001; kindly provided by W. Salser). The cDNA was cut with *Hpa*II to remove the poly(dA·dT) regions at both 5′ and 3′ ends of the molecule. Parts A and B of Figure 6 show the resultant autoradiograms. The majority of the globin RNA is in the 9S mature form. There are, however, four precursor RNAs which are evident after longer exposure (Figure 6B), and Figure 7 provides a molecular weight estimate for the four precursors. The size of a precursor which contains the coding and intervening regions is about 1600 bases, and at least three precursors are much larger than this: 2600, 4400, and 5300 bases. The 1800-base precursor probably corresponds to the actual gene size of 1600 bases with poly(A) added. Two of the β globin precursors (4400 and 5300 bases) could encompass

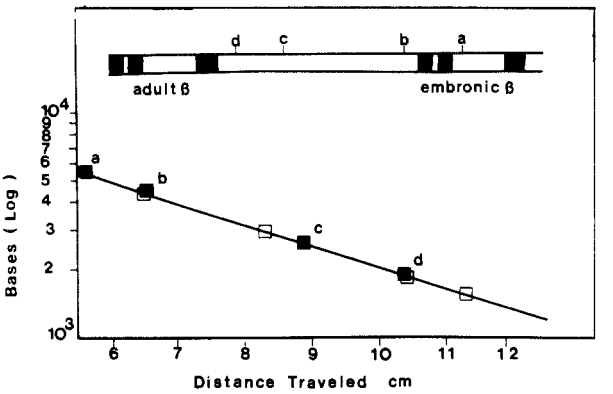

FIGURE 7: Size determination of β globin precursor RNA. The results shown in Figure 6 were plotted. Sizes of standards used were from Carmichael & McMaster (1980). RNA standards (□): 28 S, 4400 bases; 23 S, 2904 bases; 18 S, 1873 bases; 16 S, 1541 bases. 18S and 28S RNAs were chicken rRNA, and 16S and 23S rRNAs were from *Escherichia coli*. The approximate positions of termination sites a–d of the precursor β globin RNAs are shown on the scale map of genomic chicken β globin genes (adult and embryonic) including two introns (Dodgson et al., 1979). It was assumed that the transcription initiation site corresponds to the 5′ end of the gene (Day et al., 1981), and no corrections were made for the presence of the poly(A) tail. Dark areas indicate coding blocks of β globin genes.

all of the space between adult and embryonic β globin gene since the distance between the two genes is about 3000 bp (Dodgson et al., 1979). Also, the 5300-base precursor may include a part of the embryonic β globin gene sequences since the gene is located at the 3′ side of the adult β gene (Dodgson et al., 1979). This possibility is being investigated. Very large precursors of β globin RNA have also been seen in the mouse; they are probably poly(A–) (Hofer & Darnell, 1981; Bastos & Aviv, 1977). The existence of very large precursors is consistent with the DNase I sensitivity analysis of the β globin chromatin (Stalder et al., 1980): the DNase I sensitivity extends well past the 3′ poly(A) addition site of the adult β globin gene.

Nuclear RNA by Itself Does Not Bind to Nuclear Matrix. Pogo et al. have shown that nuclear RNA by itself does not bind to the nuclear matrix (Miller et al., 1978a). In order to confirm their conclusion that the binding of nuclear RNA to matrix is not an experimental artifact, we mixed the erythroblast nuclear matrix with highly labeled poly(A)-containing erythroblast nuclear RNA. We believe that the amount of matrix used was in large excess over the amount of RNA which would normally be present in the nuclei. No detectable amount of labeled RNA migrates with the matrix during centrifugation (data not shown). Under the conditions used, the nuclear matrix accumulates at the border of 60% sucrose and 15% glycerol. Nuclei were also mixed with the labeled nuclear RNA in 2 M NaCl and centrifuged. Under this condition DNA uncoils and leaks out of the nucleus. However, as reported by Georgiev and co-workers (Razin et al., 1979), the nucleus still maintains its overall morphology when observed under a microscope; this is presumably due to the nuclear matrix. Only a small amount of radioactive RNA (0.5%) cosediments with this structure. We feel that this small amount of RNA was trapped in the DNA gel during centrifugation. Therefore, the results suggest that nuclear RNA itself does not have any binding affinity for the nuclear matrix nor does it become entangled in the fibrous network of the nuclear matrix. Perhaps specific RNA binding proteins allow RNA to bind to the nuclear matrix as suggested by Hardy et al. (1978). Indeed, van Eekelen & van Venrooij (1981) have shown that the 41 500- and 43 000-dalton hnRNP proteins are involved in attaching the nuclear RNA to the nuclear matrix

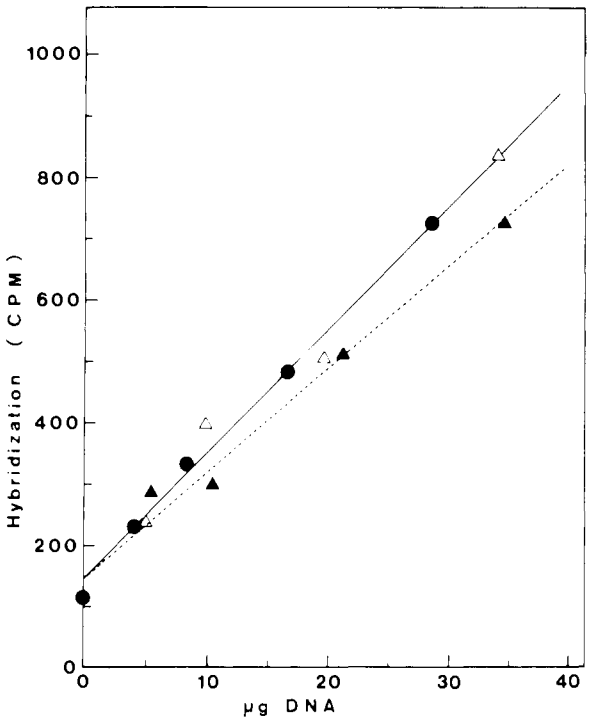

FIGURE 8: Lack of globin gene enrichment in the erythroblast nuclear matrix. The nuclear matrix was prepared from the nuclei suspended in 2 M NaCl either by shearing the DNA under pressure (▲) or by *Eco*RI digestion after removal of histones (△). The content of the globin genes was compared with that of total chicken DNA (●) by hybridization with an excess of ^{3}H-labeled globin cDNA.

in vivo. This was shown by UV cross-linking the proteins to RNA in whole cells.

Globin Genes Are Not Bound to the Nuclear Matrix. Since some of the matrix-bound globin RNA appears to be a large precursor RNA, we tested the possibility that the binding of the globin genes to the nuclear matrix could occur through nascent globin RNA. The first approach we took was mild shearing of the DNA released from the erythroblast nuclei in 2 M NaCl and subsequent isolation of nuclear matrix by step glycerol–sucrose gradient centrifugation. In one case the DNA was sheared by passing the nuclei in 2 M NaCl through a 21-gauge needle. In the other case, histones were first removed from the nuclei in 2 M NaCl and the DNA was then digested with *Eco*RI restriction endonuclease. In both cases the final nuclear matrix still maintained the nuclear morphology and contained 20–30% of the DNA. The DNA prepared from the two matrix preparations contains the same representative amount of the globin genes as the total DNA directly extracted from erythroblast nuclei (see Figure 8), suggesting that there is no enrichment of the globin genes in the matrix. Shearing the DNA by passing through a 21-gauge needle results in DNA fragments with an average size greater than 10 000 bp which is larger than the structural globin genes (1600 bp) (Dodgson et al., 1979). Nuclear matrix prepared by digesting the exposed DNA with *Eco*RI endonuclease should also contain intact α and β globin genes in chicken (Dodgson et al., 1979; Engle & Dodgson, 1980). Therefore, the matrix-bound DNA should have contained intact structural globin genes if linkage occurred between the active globin genes and the nuclear matrix within the *Eco*RI sites. In no case did the globin gene DNA show specific association with the matrix.

Since DNA uncoils from histones in 2 M NaCl, there is the possibility that the bonds between the globin genes and the nuclear matrix may be broken during the swelling of DNA in 2 M NaCl. Therefore, the possible enrichment of the globin genes in the matrix was tested with the matrix prepared after DNase I digestion. The active genes are usually more sensitive to DNase I than inactive genes (Weintraub & Groudine, 1976). However, extraction of nuclei with 0.35 M NaCl abolishes the DNase I sensitivity of active genes (Weisbrod & Weintraub, 1979). Therefore, erythroblast nuclei were washed in 0.35 M NaCl and digested with DNase I for varying lengths of time. The final nuclear matrix containing 1–20% DNA was tested for the possible enrichment of the globin genes. However, the amount of globin genes in the matrix-bound DNA was the same as in the total DNA (not shown here). Partial digestion of the globin genes by DNase I would not affect the binding of the genes to the matrix through nascent RNA since many copies of RNA are attached to the actively transcribing genes (McKnight & Miller, 1979), and internal digestion of the genes by DNase I may not affect the gene segments bound to the matrix by nascent RNAs.

Discussion

In this report we present evidence for the following: (a) practically all of the globin nuclear RNA in chicken erythroblasts is associated with the nuclear matrix preparation. (b) There is a 3-fold enrichment of globin RNA in the matrix-bound RNA. (c) There is also a slight enrichment of some snRNAs in the matrix. (d) In contrast, the majority of the rRNA is lost during the preparation of the erythroblast nuclear matrix along with 70% of the nuclear RNA. (e) The nuclear matrix globin RNA contains both 9S mature globin RNA and four precursors of the β globin RNA. Two of the globin precursors are large enough to extend to the embryonic β globin gene. (f) There is little if any globin RNA in the erythrocyte nuclear matrix. (g) The globin genes are probably not attached directly or indirectly to the nuclear matrix.

The digestion of erythroblast nuclei with DNase I and subsequent extraction with high salt yield a structure which morphologically resembles the cell nucleus (Figure 1). We have shown that practically all of the globin nuclear RNA remains bound to this nuclear matrix structure. Although it is difficult to say conclusively whether globin nuclear RNA is bound in vivo to the nuclear matrix as we see it in vitro, there is little evidence to support a rearrangement or nonspecific association of the globin RNA with the matrix. Added ^{3}H-labeled poly(A) nuclear RNA does not bind to the matrix after it has been incubated with either the matrix or intact nuclei from which the matrix is subsequently purified. A stronger argument develops from the actual enrichment (3×) of globin nuclear RNA in the matrix as compared to that of the total nuclear RNA. If any kind of nonspecific binding occurred, this enrichment would be unlikely. The globin RNA and other matrix-bound hnRNA can be released by using protein denaturing agents (Long et al., 1979). The RNA–protein complex (hnRNP) or a protein component thereof may have a strong affinity for the nuclear matrix proteins, and the hnRNP as a whole or in part could remain bound to the matrix in 2 M NaCl. Indeed, van Eekelen & van Venrooij (1981) have shown that the hnRNP proteins of 41 500 and 43 000 daltons may be responsible for binding hnRNA to the nuclear matrix in vivo. We can rule out the possible entanglement and trapping of globin RNA in the matrix sphere during the exposure to 2 M NaCl since nuclei lysed in a hypotonic medium and subsequently exposed to 2 M NaCl still contain all of the globin RNA bound to an insoluble structure, presumably a remnant of the nuclear matrix (not shown here).

It is not clear why other RNAs such as rRNA and snRNA are not quantitatively bound to the matrix erythroblast. The tetrahymena nuclear matrix also shows an enrichment of some

snRNAs (Herlan et al., 1979). It is possible that the partial binding of these RNAs or the complete binding of the globin RNA reflects the functional state of RNA. For example, it is possible that the bulk of rRNA is being assembled into ribosomes and these are washed away during the preparation of the matrix. A similar situation may exist with the snRNAs in which the slight enrichment in the nuclear matrix of a particular snRNA may reflect the particular usage of the RNA. Such enrichments therefore may vary widely, depending on the cell type. As mentioned above, the type of proteins bound to RNA, but not the RNA itself, may determine the binding of RNA to the nuclear matrix. In this regard it is interesting that the nuclear matrix (or matrix-bound hnRNP proteins) protects specific regions of the globin RNA against micrococcal nuclease (D. A. Ross and C.-B. Chae, unpublished experiments) and thus strongly suggests specific interaction of the globin RNA and the matrix.

Wunderlich et al. (1976) and recently Herlan et al. (1979) have suggested that RNA processing and transport may occur in association with the nuclear matrix. In a pulse–chase experiment, Herlan et al. found that label first appears in the matrix-bound precursor ribosomal RNA and then is gradually released. Since we find both the 9S and precursor forms of β globin RNA attached to the nuclear matrix, we feel that globin nuclear RNA may also be processed while attached to the nuclear matrix. Three of the β globin RNA precursors are larger than the genomic β globin gene (Dodgson et al., 1979; Engle & Dodgson, 1980); it is probable that additional 3′ sequences are transcribed but are removed during processing of the RNA. The 1800-base precursor probably corresponds to the size of the DNA gene with poly(A) added. The large precursors would extend to the embryonic β globin gene which are located on the 3′ side of the adult β globin gene (Dodgson et al., 1979). Hofer & Darnell (1981) have also shown for the mouse β globin system that a very large β globin precursor exists which has 1000 bases 3′ to the poly(A) addition site. Bastos & Aviv (1977) have shown that the 27S globin precursor is not polyadenylated, while the 15S precursor is polyadenylated. Indeed, the event which removes the extra 3′ sequence of RNA from the β globin precursor may be concomitant with the addition of the poly(A) tail (Fraser et al., 1979; Nevins & Darnell, 1978). The 5′ end of β globin RNA has been mapped at +29 bases from the "TATA" box although the RNA used was probably a mature form of globin RNA (Day et al., 1981).

It is intriguing that there is an actual enrichment of globin nuclear RNA when the nuclear matrix is prepared. This suggests an interesting possibility that active gene transcripts are selected by binding to the nuclear matrix. Recently Britten and Davidson and others (Davidson & Britten, 1979; Chikaraishi et al., 1978) have shown that a selection process for cell-specific RNA occurs in the nucleus. That is, the nuclear RNA from many tissues is very similar in sequence composition, while the cytoplasmic RNA of various tissues is more divergent in sequence composition (Wold et al., 1978; Lev et al., 1980). It will be of interest to determine whether all cytoplasmic RNA precursor (hnRNA) is selectively associated with the nuclear matrix in their respective cell types while those RNAs not utilized in the cytoplasm do not bind to the nuclear matrix.

In contrast to specific globin RNA attachment to the matrix, our attempt to show the possible association of transcriptionally active globin gene DNA directly or indirectly with the matrix was unsuccessful. The active gene DNA could have been either directly linked to the nuclear matrix through protein or connected by a linkage between the nascent RNA–protein complex and the nuclear matrix. If either of these attachments were occurring, the globin gene DNA should have been enriched by one or more of the matrix isolation procedures used. Although we do not see globin gene DNA preferentially bound to the matrix, further study is needed to completely rule out the attachment of transcriptionally active genes to the matrix.

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Solubilization of the Carcinogen Nickel Subsulfide and Its Interaction with Deoxyribonucleic Acid and Protein†

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ABSTRACT: Significant concentrations (1–10 mM) of nickel(II) were found in solution after incubation of the potent carcinogen nickel subsulfide in 0.05 M Tris-HCl, pH 7.4, solutions containing DNA, rat liver microsomes, and NADPH. The presence of NADPH decreased the rate of solubilization of nickel subsulfide. The solubilized nickel exhibited electronic absorption spectra and magnetic moments characteristic of octahedral nickel(II). The solubilized nickel(II) bound to DNA with an apparent equilibrium constant of 730 M^{-1} and with a saturation binding value of one nickel per 2.4 nucleotides. Microsomes lowered the saturation binding of nickel to DNA but dramatically increased the amount of nickel–DNA complex stable to precipitation with salt and poly(ethylene glycol). The amount of protein associated with DNA precipitated from protein-extracted solutions correlated with the amount of nickel bound to DNA. These results suggest that microsomes mediate the binding of nickel to DNA by forming a stable ternary protein–nickel(II)–DNA complex.

Epidemiological evidence has implicated nickel compounds as the causative agents in human respiratory and renal cancers in nickel refinery workers worldwide (Doll et al., 1977; Pedersen et al., 1973; Lessard et al., 1978). Inhalation of nickel refinery dust appears to be the major route of exposure to particulate nickel compounds, which include nickel subsulfide (Ni_3S_2) (Sunderman, 1976). Nickel subsulfide induced renal carcinomas (Jasmin & Riopelle, 1976; Sunderman et al., 1979) following intrarenal (ir) injection in rats, and primary rhabdomyosarcomas following intramuscular (im) injection (Sunderman et al., 1976; Yamashiro et al., 1980). Ocular tumors were induced in rats following a single intraocular injection of nickel subsulfide (Albert et al., 1981).

Cellular uptake and solubilization of particulate nickel compounds are important to the mechanism of nickel-induced carcinogenesis. Crystalline nickel subsulfide, but not amorphous nickel sulfide, was actively phagocytized by cultured mammalian cells (Costa & Mollenhauer, 1980), resulting in an increased frequency of morphological transformation. Phagocytized nickel particles were observed in the cytoplasm where they were solubilized to a form capable of entering the nucleus and interacting with nuclear macromolecules (Costa et al., 1981). The rate of nickel subsulfide solubilization in vitro in rat serum was found to be dependent upon molecular oxygen and was enhanced by the presence of albumins and amino acids (Kasprzak & Sunderman, 1977). Solubilization of nickel compounds in vivo in the rat occurred after intraperitoneal (ip) injection of nickel carbonate, which was shown to interact with cellular macromolecules in kidney, inducing DNA single-strand breaks and DNA–protein cross-links (Ciccarelli et al., 1981).

The present investigation is concerned with the solubilization of the carcinogen nickel subsulfide in vitro in the presence of calf thymus DNA, rat liver microsomes, and reduced nicotinamide adenine dinucleotide phosphate (NADPH).[1] The

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[1] Abbreviations: BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMG, dimethylglyoxime; DPA, diphenylamine; PEG, poly(ethylene glycol); NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

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