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Avian Nuclear Matrix Proteins Bind Very Tightly to Cellular DNA of the β-Globin Gene Enhancer in a Tissue-Specific Fashion†

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ABSTRACT: We have previously shown that a cloned 480 bp DNA fragment that spans the 3′-enhancer region of the avian β-globin gene cluster can become very tightly, perhaps covalently, bound to protein in avian nuclear matrices in vitro [Zenk et al. (1990) *Biochemistry 29*, 5221-5226]. This binding was not tissue-specific and was probably not mediated by topoisomerase enzymes. In the present study, we have examined avian nuclear matrices (or scaffolds) for the presence of very tight cellular DNA-protein complexes in the region of the β-globin gene enhancer and of several other avian genes. Nuclear matrices were prepared by both high- and low-salt methods, and protein-DNA complexes were isolated by SDS/K^+ precipitation after restriction enzyme digestion. In adult reticulocytes, up to 30% of the intact 3800 bp *Hin*dIII-*Eco*RI fragment that encompasses the β-globin enhancer element may be very tightly bound to nuclear matrix protein. In adult avian thymus nuclei, the β-globin enhancer is neither matrix-associated nor tightly bound to protein. In contrast, a 5.0-kb *Hin*dIII fragment of the malic enzyme gene is very tightly bound to nuclear matrix-associated protein in thymus cells, but not reticulocytes. The malic enzyme gene is active in thymus cells, and not in reticulocytes. These results suggest that certain regions of avian cellular DNA are very tightly, perhaps covalently, attached to nuclear matrix-associated proteins. Attachment follows a tissue-specific pattern that is associated with transcriptional activity.

Eukaryotic chromatin is organized into large loops of DNA that are associated with histones and a wide variety non-histone proteins, which together are attached to a salt-resistant structure collectively referred to as the nuclear matrix or scaffold (Berezney & Coffey, 1974; Cook & Brazell, 1975; Benyajati & Worcel, 1976). Evidence suggests that both replication (Pardoll et al., 1980; Blow & Laskey, 1988) and transcription (Jackson et al., 1981; Ciejek et al., 1983; Robinson et al., 1985; Keppel, 1986; Buttyan & Olsson, 1986; Roberge et al., 1988) occur at the interface of DNA with the nuclear matrix. Regions of DNA, capable of mediating matrix attachment [matrix attachment regions (MARs)],[1] have been shown to correspond to enhancer regions in several genes (Cockerill & Garrard, 1987; Loc et al., 1990; Mielke et al., 1990) and autonomously replicating sequences in yeast and humans (Amati & Gasser, 1988; Sykes et al., 1988). It appears that the sequence ATATTT is necessary for matrix attachment (Cockerill & Garrard, 1986; Mielke et al., 1990). This sequence is the core of the consensus sequence for the enzyme topoisomerase II, itself a major constituent of the nuclear matrix (NM) (Berrios et al., 1985; Gasser et al., 1986), and recent evidence has suggested that topoisomerase II may be involved in DNA binding to NMs (Cockerill & Garrard, 1986; Sperry et al., 1989; Adachi et al., 1989; Pommier et al., 1990; Mielke et al., 1990). However, this conclusion has not been universally accepted. We have recently shown that a 480 bp fragment that spans the avian β-globin gene enhancer can bind in vitro to NMs prepared from a variety of cell types and that a fraction of this matrix-associated DNA becomes very tightly, perhaps covalently, bound to non-topoisomerase NM-associated proteins (Zenk et al., 1990). These results were somewhat surprising as the β-globin enhancer region contains

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[1] Abbrevations: Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; MAR, matrix attachment region; NM, nuclear matrix.

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several topoisomerase II cleavage sites (Muller & Mehta, 1988; Muller et al., 1988; Reitman & Felsenfeld, 1990), in addition to the MAR core consensus sequence (Muller et al., 1988).

The present study was undertaken to determine if cellular DNA formed associations with the NM similar to those detected in vitro using cloned DNA and high-salt-extracted nuclei. To do this, NMs with intact cellular DNA have been prepared from avian reticulocytes and thymus gland cells and subjected to restriction enzyme digestion. DNA very tightly bound to NM-associated protein has been isolated and identified by Southern hybridization. Our results indicate that a significant fraction of cellular DNA sequences that contain the β-globin enhancer are very tightly bound to NM-associated protein in reticulocytes. The overwhelming majority of the protein-bound DNA is not cleaved. This region of DNA is neither NM-associated nor very tightly bound to protein in thymus cells. Interestingly, a region of the malic enzyme gene is very tightly bound to protein in thymus NM, but not reticulocytes. This gene is active in thymus cells, and inactive in reticulocytes. These results suggest that the formation of β-globin enhancer DNA–NM protein complexes, resistant to dissociation by SDS, is tissue-specific in vivo and that topoisomerase II is not the protein(s) to which the DNA is principally bound.

Materials and Methods

Adult reticulocytes were obtained from 1.8–2-kg white leghorn chickens made anemic with acetylphenylhydrazine (20 mg/kg for 4–5 days). Adult avian thymus glands were commercially obtained (Pel-Freeze). Both the high- and low-salt methods for NM isolation described elsewhere were employed (Mirkovitch et al., 1984; Zenc et al., 1990). Briefly, nuclei were made by lysing cells or tissue in a Dounce homogenizer (or Waring blender for frozen tissue) in 0.25 M sucrose, 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 0.5 mM PMSF (RSB) with 0.025% NP-40. After being filtered through three layers of cheesecloth, nuclei were washed 2 additional times, with homogenization, in this same buffer and then twice more in sucrose–RSB without NP-40. For high-salt extraction, 20–200 A_{260} units of nuclei were incubated in sucrose–RSB with 0.5 mM $CuSO_4$ for 10 min at 4 °C. Nuclei were resuspended in 25 mL of sucrose–RSB, to which was added 25 mL of 4 M NaCl, 20 mM Tris-HCl, pH 7.4, and 20 mM EDTA, and incubated 30 min at 4 °C. Disrupted reticulocyte nuclei were sheared by passage through a 18G needle 5 times and then through a 21G needle 5 times. As an alternative to shearing, reticulocyte nuclei were predigested with 1.2 units of micrococcal nuclease/A_{260} unit for 1 min at 37 °C just prior to high-salt extraction. It should be noted that neither shearing nor predigestion were required to make NM from thymus nuclei. The high-salt-extracted nuclei were collected by centrifugation (2000*g*, 10 min), and the pellet was washed several times with 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.25 mg/mL BSA, and 0.5 mM PMSF. High-salt-extracted NMs were washed 3 times with 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 0.25 mg/mL BSA, and 0.5 mM PMSF. NMs were stored at −20 °C in this same buffer with glycerol (50% v/v) at 1 mg/mL. For restriction digestion, 1–2 A_{260} units of NM were washed and resuspended in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mg/mL BSA, and 0.5 mM PMSF. Enzyme (100 units/A_{260} unit) was added, and samples were digested for 1 h at 37 °C. Matrix-associated DNA was separated from matrix-released DNA by centrifugation. NM-associated DNA, very tightly bound to protein, was prepared by washing NM in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA (TE) and then resuspending in TE made 1% with SDS and 10 mg/mL with BSA. The solution was incubated at 65 °C until the NM pellet was completely solubilized, when KCl was added to 100 mM. The solution was then incubated on ice for 10 min. SDS/K^+ and precipitated NM-associated protein and DNA were recovered by centrifugation at 12000*g* for 5 min at 4 °C. The precipitate was washed once on ice with 100 mM KCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. DNA was recovered from all fractions by incubation with proteinase K (1 μg/0.02 A_{260} unit) in 0.5% SDS and 0.5 M NaCl in TE at 37 °C for 2 h, followed by phenol and chloroform extraction and ethanol precipitation. Hot phenol extraction of DNA–protein complexes was performed as described previously (Brotherton & Zenk, 1990). NMs were also made from 0.5 mM $CuSO_4$-treated nuclei by the low-salt method (Mirkovitch et al., 1984). Nuclei (20 A_{260} units) were washed in 3.75 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 1% (v/v) thiodiglycol, 20 mM KCl, 0.1% (w/v) digitonin, and 0.5 mM PMSF, then resuspended in 250 μL of this same buffer, and extracted by slowly mixing with 20 mL of 5 mM HEPES/KOH, 0.25 mM spermidine, 2 mM EDTA/KOH, 2 mM KCl, 0.1% (w/v) digitonin, and 25 mM lithium diiodosalicylate. The mixture was incubated at room temperature for 15–30 min and then spun 5000*g* for 10 min. The pellet was washed 3 times and resuspended in 10 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.1% digitonin, and 0.5 mM PMSF. Restriction enzyme was added at 100 units/A_{260} unit, and the sample was incubated 2–12 h. After restriction digestion, samples were treated similarly to high-salt-extracted and digested NM. In some experiments, samples were prepared from NM by alternative methods; details are given in the appropriate text and figure legends.

The cloned DNA probes used are described elsewhere (Zenk et al., 1990; Brotherton et al., 1990) and are shown in Figure 1. Probe fkk22 is a 500 bp fragment of the β-globin gene cluster located just upstream from the 5′-DNase I hypersensitive site (Stadler et al., 1980). Probe β480 is a 480 bp fragment that spans the 350 bp region identified as a tissue-specific enhancer situated 2000 bp 3′ to the β-globin cap site (Choi & Engel, 1986; Hesse et al., 1986). The probe C4-malic is a 1-kb *Pst–Eco* fragment that spans a unique sequence in the second exon of the avian malic enzyme gene (Winberry et al., 1983). The chicken thymidine kinase (cTK) probe is a 2.9-kb *Hin*dIII fragment isolated from a recombinant DNA plasmid (Perucho et al., 1980; Groudine & Casimir, 1984).

Results

β-Globin Enhancer DNA Is NM-Associated in Reticulocytes and Very Tightly Bound to Protein. We have previously shown that reticulocyte DNA in the region of the β-globin gene enhancer is matrix-associated in vivo (Bennett et al., 1989) and that cloned fragments that span the β-globin gene enhancer can become very tightly bound to NM-associated protein in vitro (Zenk et al., 1990). To establish that this region of cellular DNA is also very tightly bound to NM-associated protein, DNA associated with reticulocyte NM after restriction enzyme digestion was subjected to fractionation by SDS/K^+ precipitation. It has been documented that DNA precipitated in the presence of SDS/K^+ is very tightly bound to protein (Muller, 1984). Results of one experiment are shown in Figure 2. The 3.8-kb *Hin*dIII–*Eco*RI fragment that contains the β-globin enhancer is enriched in the NM-associated DNA fraction precipitated by SDS/K^+. In contrast, the 4.6-kb *Hin*dIII fragment containing the ρ-globin gene

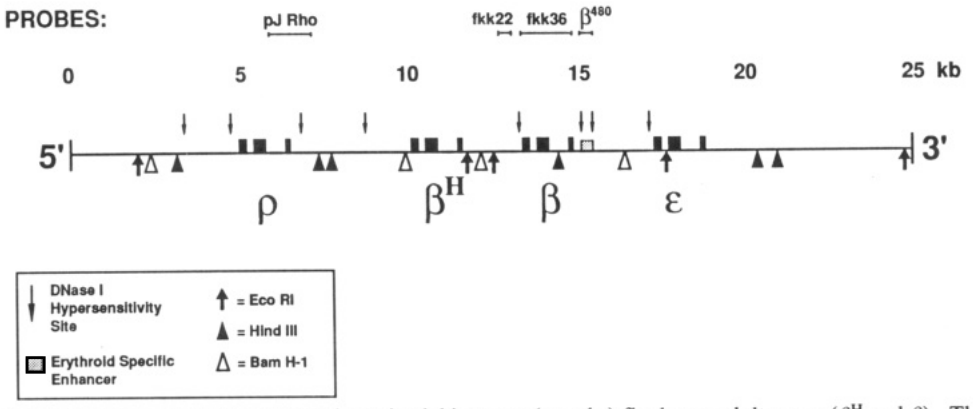

FIGURE 1: Map of avian β-globin gene cluster. Two embryonic globin genes (ρ and ϵ) flank two adult genes (β^H and β). The size and location of the cloned DNA probes used in this study are shown. The position of the erythroid-specific β-globin enhancer is indicated by the gray box and of known DNase I hypersensitive sites (Stalder et al., 1980; G. Ginder, unpublished results) near the β-globin gene by arrows above the line.

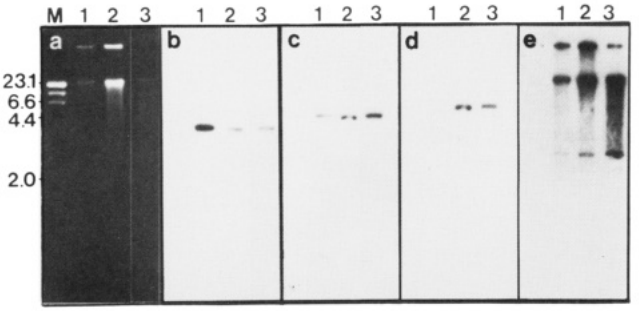

FIGURE 2: β-Globin enhancer region DNA very tightly bound to protein in reticulocyte NM made by 2 M NaCl extraction. NMs (100 μg) were prepared from avian reticulocytes after preincubation with 0.5 mM $CuSO_4$ and micrococcal nuclease by high-salt extraction (see Materials and Methods) and subjected to restriction digestion with *Hin*dIII and *Eco*RI. After digestion, NM-associated and -released DNAs were separated by centrifugation, and NMs were washed once with 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. NM-associated DNA was solubilized with 1% SDS at 68 °C and subjected to fractionation by SDS/K^+ precipitation, as described under Materials and Methods. Samples were deproteinized and submitted to electrophoresis in 1% agarose and alkaline Southern transferred. (a) Ethidium bromide stained gel; (b) autoradiogram of hybridization to probe β480; (c) autoradiogram of hybridization to probe ρJrho; (d) autoradiogram of hybridization to probe C4-malic; (e) autoradiogram of hybridization to probe cTK. Lane 1, NM-associated DNA precipitated by SDS/K^+ (1.5 μg); lane 2, NM-associated DNA soluble in SDS/K^+ (12 μg); lane 3, NM-released DNA soluble in SDS/K^+ (2.5 μg). A total of 60 μg DNA was present in this fraction; the other lanes are the total amount of DNA recovered in each fraction. M, *Hin*dIII–λ DNA marker (2 μg).

(Figure 2c) and the 2.0-kb *Eco*RI–*Hin*dIII fragment containing the 5′-upstream regions of the β-globin gene (data not shown) are enriched in the matrix-released DNA and depleted in the matrix-associated DNA precipitated by SDS/K^+. A 5.0-kb *Hin*dIII fragment, containing the second exon of the malic enzyme gene (Winberry et al., 1983), is enriched in the matrix-released DNA and the matrix-associated fraction of DNA soluble in SDS/K^+, but greatly depleted in the SDS/K^+-precipitated fraction of DNA (Figure 2d). The malic enzyme gene is not expressed in avian reticulocytes (A. Goodridge, personal communication). A 2.2-kb *Hin*dIII–*Eco*RI fragment of the thymidine kinase gene, that includes a nuclease-hypersensitive site, is enriched in matrix-released DNA but also detectable in matrix-associated DNA fractions. An unidentified ≥10-kb band is also invariably detected with the cTK probe. This band is well represented in all lanes in Figure 2. The thymidine kinase gene is reported to be transcriptionally engaged in adult avian reticulocytes (Groudine & Casimir, 1984). Results similar to those in Figure 2 were obtained if hot phenol extraction replaced SDS/K^+ precipitation as a method to isolate DNA very tightly bound to protein (data not shown). In addition, experiments similar to that shown in Figure 2, but with DNA analyzed by alkaline denaturing gels, fail to show additional single-strand DNA cleavages in the fraction of NM DNA precipitated by SDS/K^+ (data not shown).

The method of SDS/K^+ precipitation is inefficient; typically, half of the protein-bound DNA is lost with each wash step (Zenk et al., 1990). Selective loss of material in each wash step may lead to erroneous estimation of the proportion of DNA sequences in the SDS/K^+-precipitated DNA fractions. In addition, in our experience, reticulocyte nuclei need to be vigorously sheared, or briefly digested with nuclease, in order to make NM. These treatments may degrade the DNA sequences of greatest interest, as MARs are often associated with nuclease-sensitive enhancer regions (Cockerill & Garrard, 1986). To overcome these problems, an alternative method for identifying very tight DNA–protein complexes was employed. Reticulocyte NMs were prepared by the low-salt method (see Materials and Methods), followed by incubation with *Hin*dIII and *Eco*RI. In these experiments, no attempt was made to separate NM-associated from NM-released DNA, so that the potential loss of protein–DNA complexes was minimized. NM-associated proteins, and associated DNA, were solubilized in SDS and either directly submitted to gel electrophoresis or treated with proteinase K prior to electrophoresis. As shown in Figure 3, the 3.8-kb fragment that hybridizes to the probe β480 and the 2.0-kb fragment that hybridizes to the probe fkk22 can be identified, despite the incomplete restriction cutting that occurred in the experiment. After digestion, there is a 30% increase in the amount of DNA in the 3.8-kb band, but no change in the quantity of DNA detected in the 2.0-kb band (Figure 3b). This is documented by scanning densitometry (Figure 3f,g). In addition, a 550 bp fragment can be seen in the densitometrogram of the proteinase-treated sample (Figure 3g). This sub-band was detected by the β480 probe alone (data not shown). It is likely that this band arises from star activity of *Eco*RI (Gardner et al., 1982), as this band was not detected with *Bam*HI was substituted in *Eco*RI in these experiments (data not shown), and was inconsistently detected in other experiments using *Eco*RI (Figure 2). No increase in the relative intensity of the 5.0-kb *Hin*dIII band of the malic enzyme gene (Figure 3c), or the 2.2-kb *Hin*dIII–*Eco*RI band of the thymidine kinase gene (Figure 3d), was detected after proteinase treatment. To confirm that the increase after proteinase digestion of the 3.8-kb band detected with the probe β480 was due to inter-

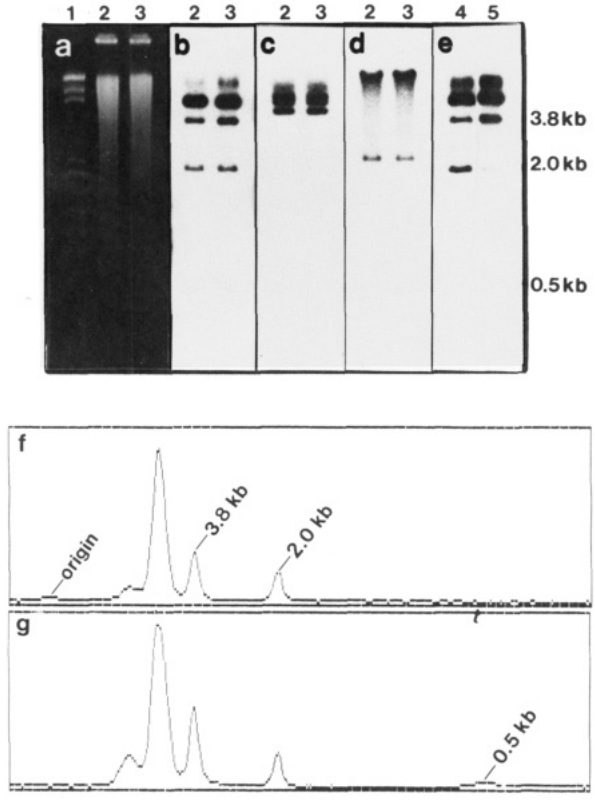

FIGURE 3: β-Globin enhancer region is very tightly bound to protein in reticulocyte NM made by low-salt extraction. Avian reticulocyte nuclei (1 mg) were extracted with lithium diiodosalicylate and solubilized in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 1% (w/v) SDS. After precipitation in ethanol, the DNA–protein pellet was resuspended in 1 mL of 20 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.1% (w/v) digitonin, and 0.2 mM PMSF with 1000 units of each *Hin*dIII and *Eco*RI. After 2 h at 37 °C, the sample was ethanol-precipitated and resuspended in buffer with restriction enzymes. This process was repeated until the ethanol-pelleted material uniformly resuspended for a total of four digestion/precipitation steps. The material was then either directly submitted to electrophoresis or incubated with proteinase K (20 $\mu g/A_{260}$ unit, 2 h at 50 °C) and phenol–chloroform extracted prior to electrophoresis. A sample was also fractionated by SDS/K^+ precipitation (see Materials and Methods); these samples were deproteinized prior to electrophoresis. After electrophoresis, DNA was alkaline Southern transferred to nylon. (a) Ethidium bromide stained gel; (b) autoradiogram of hybridization to probes fkk22 and β480; (c) autoradiogram of hybridization to the probe C4-malic; (d) autoradiogram of hybridization to the probe cTK; (e) autoradiogram of hybridization to probes fkk22 and β480; (f) densitometric scan of lane 2 in panel b; (g) densitometric scan of lane 3 in panel b. Lane 1, *Hin*dIII–λ DNA marker; lane 2, DNA directly submitted to electrophoresis; lane 3, DNA incubated with proteinase K prior to electrophoresis; lane 4, DNA (5 μg) soluble in the presence of SDS/K^+; lane 5, DNA (5 μg) precipitated by SDS/K^+.

actions with protein that were resistant to dissociation by protein denaturation, DNA was also subjected to SDS/K^+ precipitation prior to protease digestion. Results after hybridization with the probe β480 and fkk22 are shown in Figure 3e. Reticulocyte DNA not tightly bound to protein is slightly depleted in the 3.8 bp fragment that hybridizes to β480 (Figure 3e, lane 4). In contrast, DNA precipitated by SDS/K^+, and thus very tightly bound to protein, is enriched in the 3.8-kb fragment. Of note, the 550 bp fragment is detected only in the SDS/K^+-precipitated fraction, although it is markedly depleted compared to unfractionated, proteinase K digested reticulocyte DNA, and is only detected after prolonged exposure of the autoradiogram (data not shown). Neither the 2.2-kb *Hin*dIII–*Eco*RI band that hybridizes to the probe cTK nor the 5.0-kb *Hin*dIII band that hybridizes to the probe

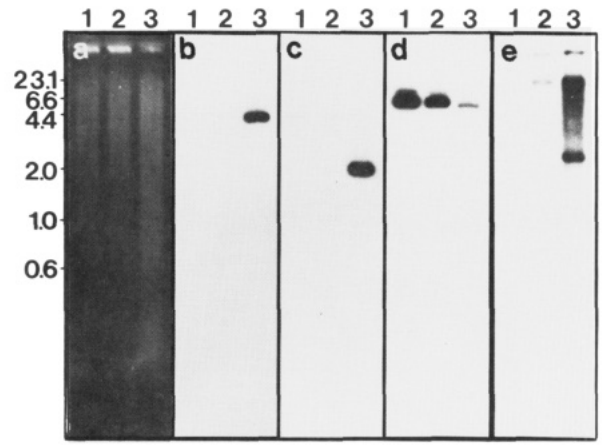

FIGURE 4: β-Globin enhancer region is neither NM-associated nor very tightly bound to protein in thymus gland nuclei. NM were prepared by the low-salt, lithium diiodosalicylate method (Mirkovitch et al., 1984). After digestion with *Hin*dIII–*Eco*RI, NM-associated and -released DNAs were separated by centrifugation. NMs were then washed twice with digest buffer and then dissolved in 1% SDS, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. Dissolved material was fractionated by SDS/K^+ precipitation and deproteinized prior to electrophoresis. After electrophoresis, the gel was transferred by alkaline Southern to nylon. (a) Ethidium bromide stained gel; (b) autoradiogram of hybridization to probe β480; (c) autoradiogram of hybridization to probe fkk22; (d) autoradiogram of hybridization to probe C4-malic; (e) autoradiogram of hybridization to probe cTK. Lane 1, NM-associated DNA precipitated by SDS/K^+; lane 2, NM-associated DNA soluble in SDS/K^+; lane 3, NM-released DNA.

C4-malic is enriched in the SDS/K^+-precipitated DNA fraction (data not shown).

β-Globin Enhancer Region Is neither NM-Associated nor Tightly Bound to Protein in Avian Thymus. Experiments similar to those described above were repeated using nuclei obtained from frozen adult avian thymus glands. Results are shown in Figure 4. It can be seen that the restriction fragments that contain the 5′-upstream region of the β-globin gene and the β-globin enhancer are neither NM-associated nor very tightly bound to NM protein in these cells. Likewise, the 2.2-kb *Hin*dIII–*Eco*RI fragment of the thymidine kinase gene is neither NM-associated nor very tightly bound to NM protein. The thymidine kinase gene is reported to be transcriptionally active in thymus cells (Groudine & Casimir, 1984). In contrast, matrix attachment, and very tight binding to protein, is documented for the 5.0-kb *Hin*dIII fragment from the second exon of the malic enzyme gene. Malic enzyme activity in extracts of adult thymus glands is equivalent to that in extracts from the livers of fed birds (data not shown). Whether all or only a subpopulation of the cell types present in thymus glands express the malic enzyme gene remains to be determined. In addition, it should be noted that the C4-malic probe fails to bind to NMs in an in vitro assay (Cockerill & Garrard, 1986) commonly used in identify MARs (data not shown). We have not yet determined if other sequences in the 5.0-kb *Hin*dIII fragment of this gene can act as MARs in the in vitro assay. However, these results do indicate that transcriptional activity of the malic enzyme gene and very tight binding of a region of it to an NM-associated protein occur together in thymus cells.

Discussion

The principal findings of this paper are the following. First, DNA in the region of the β-globin enhancer is NM-associated in reticulocytes, but not in thymus cells. Second, DNA in the region of the β-globin enhancer is very tightly bound to protein in reticulocyte, but not thymus, NMs. DNA in the region of

the second exon of the malic enzyme gene is very tightly bound to protein in thymus, but not reticulocyte, NMs. Malic enzyme activity is detectable in thymus extracts, but not in reticulocyte extracts. The bond between these DNA sequences and protein is called very tight as it is resistant to dissociation by the detergent SDS and phenol extraction at 65 °C. Although the exact nature of this bond is unknown, the extreme stability to dissociation suggests that it may be covalent (Muller, 1984). Third, the restriction-digested DNA recovered from protein–DNA complexes is mostly, if not all, without internal cleavages. These findings lead us to conclude that very tight binding to NM protein is a characteristic of certain regions of actively transcribed genes and that it is unlikely that the protein to which these DNA sequences bind is a known topoisomerase.

Previous studies of matrix attachment regions have given conflicting results regarding the tissue specificity of matrix association (Cockerill & Garrard, 1988; Gasser & Laemmli, 1986; Phi-Van & Stratling, 1988). It has been proposed that several types of NM–DNA interactions may exist, with some being ubiquitous and others being tissue-specific (Razin et al., 1988). Our own studies on the β-globin enhancer element failed to show tissue specificity using an in vitro assay of matrix attachment (Zenk et al., 1990). In contrast, the studies presented in this paper clearly document tissue specificity for binding of the β-globin enhancer region, and the second exon of the malic enzyme gene, to the NM in vivo. The factors that regulate tissue-specific NM attachment are unknown, but must be relatively dominant as it has been estimated that up to 10000 potential NM binding sites are present in each eukaryotic nucleus (Cockerill & Garrard, 1986). In addition, it has been shown that matrix attachment regions from widely diverse sources in nature can confer elevated, less position-dependent expression on heterologous promoters after transfection into cells of different species (Phi-Van et al., 1990; Mielke et al., 1990). Taken together, these observations suggest that NM association, in conjunction with soluble trans-acting factors, may be an important determinant of tissue-specific gene regulation.

In the experiments reported in this paper, DNAs containing both the embryonic ρ-globin gene and the thymidine kinase gene appear to be matrix-associated and very tightly bound to NM protein, when nuclear matrices were made by high-salt extraction of reticulocytes. The proportion of total DNA present in the fraction of DNA precipitated by SDS/K^+ for these two genes is 3–5-fold less than that for the β-globin enhancer. It has been reported that an MAR is present in the 4.6-kb *Hin*dIII fragment that spans the ρ-globin gene (Bennett et al., 1989). Thus, the association of the 2.2-kb fragment of the thymidine kinase gene with reticulocyte NM-associated protein would suggest that an MAR is present in this DNA region as well. However, this gene is neither NM-associated nor very tightly bound to NM-associated protein in thymus nuclei, even though the gene is reported to be transcriptionally active in thymus, as well as in reticulocytes (Groudine & Casimir, 1984). Furthermore, none of the regions of DNA tested, with the exception of the β-globin enhancer region, appeared to be very tightly protein-bound as assayed by the proteinase release studies of reticulocyte NM. Therefore, we suspect that the trace levels of ρ-globin and thymidine kinase gene detected in the NM protein-associated fraction of reticulocyte DNA may be contaminants. It is not entirely clear why contamination is a problem in reticulocyte NM preparations, and not in thymus cell preparations, but this may be another manifestation of the reported difficulties in making NM from reticulocytes (Lafond & Woodcock, 1983). We have been unable to make NM from these terminally differentiated cells without using measures that partially degrade nuclear DNA, and are currently exploring agarose encapsulation methods (Jackson & Cook, 1985) to overcome this problem. Until better NM preparations are available for reticulocytes, we must leave unanswered the question of whether or not very tight binding to NM-associated protein is occurring in a small fraction of DNA sequences containing the ρ-globin and thymidine kinase genes.

The identity of the NM proteins that bind very tightly to DNA that contains the β-globin enhancer and the malic enzyme gene remains elusive. In our previous studies using an in vitro assay, DNA that was very tightly bound to NM protein contained neither single nor double-strand cuts (Zenk et al., 1990). This was interpreted as indicating that topoisomerase II was not the protein to which DNA was bound. However, these studies had to be performed in the absence of divalent cations, due to residual DNase I activity in the NM prepared for use in the in vitro assay (Brotherton & Zenk, 1990); topoisomerase II activity, which requires divalent cations (Lee et al., 1989; Wang, 1985), may have been overlooked. Therefore, it is significant that the vast majority of cellular DNA that is very tightly bound to protein is also not cleaved, as this lends support to the hypothesis that topoisomerase II is not the NM protein to which this region of DNA is bound. It is possible that the 550 bp fragment that hybridizes to the β480 probe and that was found in some experiments using *Eco*RI (Figure 3) was not due to star activity but rather to endogenous topoisomerase II activity. However, this seems unlikely, as topoisomerase II mediated P4 unknotting activity is very low, or absent, in adult reticulocytes, and etoposide-inducible cleavages cannot be detected in these cells (Brotherton et al., 1989; Reitman & Felsenfeld, 1990). Furthermore, etoposide-induced DNA cleavage cannot be reconstituted in reticulocyte nuclei by the addition of ATP (Brotherton, unpublished results). Finally, careful exam of total adult avian reticulocyte DNA has failed to detect any spontaneous cleavages in this region (Reitman & Felsenfeld, 1990).

Previously published reports have supported the hypothesis that a non-topoisomerase NM-associated protein covalently binds to eukaryotic cellular DNA (Avramova & Tsanev, 1987; Avramova et al., 1989) by tyrosine–phosphate linkage (Neuer et al., 1983). Several studies have purported to identify multiple NM-associated proteins of molecular weight below 200K, including vimentin, that are covalently bound to DNA (Razin et al., 1981, 1988; Bodnar et al., 1983; Cress & Kurath, 1988). Recently, a ubiquitous 95-kDa protein, isolated first from chicken oviduct NM, has been identified as a matrix attachment region binding protein (von Kries et al., 1991). However, the protein that mediates NM binding of the avian β-globin enhancer is not likely to be very similar to these previously described proteins as it fails to enter 1% agarose gels in the presence of SDS (Figure 3, and data not shown). We have previously shown that the major DNA–protein complex in avian reticulocyte nuclei isolated by phenol–SDS extraction does not enter SDS–acrylamide stacking gels (Brotherton & Zenk, 1990). A similar conclusion has been reached in other studies using different methods and nonavian cell lines (Avramova & Tsanve, 1987). On the basis of these observations, we conclude that the non-topoisomerase NM protein that tightly binds to the β-globin gene enhancer region is either relatively insoluble, very large, or a large complex of smaller proteins held together by bonds resistant to SDS, phenol, disulfide-reducing agents, and boiling (Zenk et al.,

1990). Identification of the protein(s) that mediate(s) NM association of the β-globin enhancer, and of the process by which this association is made tissue-specific in vivo, await further work.

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