

# Analysis of DNA Attached to the Chromosome Scaffold

M. TIEN KUO

*Department of Cell Biology, University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030*

**ABSTRACT** Two different methods have been described to investigate whether any specific DNA sequences are intimately associated with the metaphase chromosome scaffold. The chromosome scaffold, prepared by dehistonization of chromosomes with 2 M NaCl, is a nonhistone protein complex to which many looped DNA molecules are attached (Laemmli et al., 1977, *Cold Spring Harbor Symp. Quant. Biol.* 42:351-360). Chromosome scaffold DNA was prepared from dehistonized chicken MSB chromosomes by restriction endonuclease *EcoRI* digestion followed by removal of the looped DNA by sucrose gradient sedimentation. Alternatively, the scaffold DNA was prepared from micrococcal nuclease-digested intact chromosomes using sucrose gradients containing 2M NaCl. Solution hybridization of the radioactively labeled scaffold DNA with a large excess of total nuclear DNA revealed that, in either case, the scaffold DNA is not a unique sequence class of genomic DNA. Southern-blotting hybridization also showed that the scaffold DNA prepared from *EcoRI*-digested dehistonized chromosomes was not enriched (or depleted) in the ovalbumin gene sequences. The possibility of a dynamic interaction of protein and DNA in the chromosome scaffold and the possibility that the scaffold is a preparative artifact are discussed.

The mitotic chromosome is the highly condensed form of chromatin that appears during cell division. Condensation of chromatin into a chromosome involves the folding and coiling of nucleoprotein fibers, which results in the formation of an ordered structure such that the genetic information can be precisely distributed between the daughter cells.

To explain these processes, models for the higher-order structure of metaphase chromosomes have been proposed. An early model described by Taylor (34) suggested that in metaphase chromosomes there was a ribbon, a two-layered central column, to which DNA was attached. Later, the "epichromatin and core" model, which described a binemic protein core running down the center of chromosomes, was proposed by Stubblefield and Wray (32). Recently, another model that described the presence of a scaffold structure from which the nucleoprotein fibers formed loops emanating in radial fashion was proposed by Laemmli and his associates (1, 2, 18, 19). All these models generally recognize central axial elements in the higher-order structure of metaphase chromosomes.

Laemmli and his associates (1, 2, 18) reported that the scaffold could be isolated by dehistonization of chromosomes with 2 M NaCl or dextran sulfate/heparin. The prepared scaffold contained nonhistone proteins. Electron microscope observations revealed that looped DNA molecules 30-90 kb (kilo-base pairs) in length were attached to the scaffold. Recently, the question of whether the scaffold is really present in

a chromosome or merely represents an experimental artifact generated during preparation of dehistonized chromosomes has been raised (10, 11, 25). Therefore, more studies, especially from a biochemical approach, are needed to further substantiate the chromosome scaffold model. One important question to be asked regarding this model is: Are there any specific DNA-protein interactions in the chromosome scaffold? As a first step in pursuing the answer, the present study was undertaken to determine whether there are any specific DNA sequences, particularly in the chicken ovalbumin gene region, attached to the chromosome scaffold.

## MATERIALS AND METHODS

### *Cell Culture and Isolation of Chromosomes*

The chicken lymphoblastoid cell line MSB-1, which was established from a spleen tumor in a bird having Marek's disease, was used for this study. The karyotype of the cells has been shown to be normal, except for a single translocation (3). The cells were propagated at 41°C in McCoy's 5a medium supplemented with 10% fetal calf serum. The doubling time of cells cultured under these conditions is ~8 h. For the preparation of labeled chromosomes, cells were grown in medium containing 0.2 µCi/ml of [<sup>3</sup>H]thymidine (50 mCi/mmol; New England Nuclear, Boston, MA) for 24 h. Synchronization of cells was achieved by a single mitotic block with Colcemid (0.06 µg/ml) for 6 h. A mitotic index of ~70% was commonly obtained.

All the following procedures were performed at 4°C using plasticware or siliconized glassware unless otherwise indicated. After the Colcemid block, the cells were pelleted by centrifugation (2,000 rpm for 10 min, Sorvall GSA rotor;

DuPont Co., Wilmington, DE) and resuspended in the same culture medium at  $\sim 10^8$  cell/ml. 4 vol of twice-deionized water were added, and the cell suspension was incubated at room temperature for 15 min. The cells were then pelleted and resuspended at  $5 \times 10^7$  cell/ml in chromosome isolation medium, which contained 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 15 mM  $\beta$ -mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA, 0.34 M sucrose (5, 12), and 1 M hexylene glycol (2-methyl-2,4-pentanediol; Eastman, Rochester, NY). After incubation in this chromosome isolation medium for 20 min, the cell suspension was forced through a 20-gauge needle six times to disrupt the cells and release the chromosomes. The lysed cell suspension (5 ml) was layered onto 20 ml of a 20% sucrose solution prepared in chromosomal isolation medium in a Corex tube. Nuclei were pelleted by centrifugation in a Sorvall HB4 rotor at 2,000 rpm for 10 min. The supernatant fluid, which consisted of two layers (cell mixture and sucrose solution layers), was carefully removed one layer at a time and reassembled in another Corex tube. The chromosomes were pelleted by centrifugation at 7,000 rpm for 20 min in the same rotor. Cross-contamination of chromosomes and nuclei in the fractions was monitored by staining the samples with ethidium bromide (20  $\mu$ g/ml) and observing them in a Leitz fluorescence microscope. If necessary, the pelleted chromosomes were resuspended in the chromosome isolation medium and subjected to another differential sedimentation procedure as described above to remove contaminating nuclei. Chromosome preparations usually contained <5% nuclei. This was calculated from the number of nuclei in an aliquot of the isolated chromosomes using light microscopy, on the assumption that 28 chromosomes are equivalent to one nucleus. (Chicken cells contain 28 light-microscopically identifiable macrochromosomes and some 40 microchromosomes that cannot be clearly seen by light microscopy.)

### Treatment of Chromosomes with 2 M NaCl and Sucrose Gradient Sedimentation Analysis

$^3\text{H}$ -labeled chromosomes, isolated as described above, were resuspended in chromosome isolation medium at  $10 A_{260}$  units/ml (10  $\mu$ l was diluted to 1 ml with 2 M NaCl for absorbance measurement). To 0.3 ml of chromosome suspension, 0.2 ml of 5 M NaCl was added dropwise. The mixture was gently shaken for 5 min and then layered onto 16 ml of a 5–45% sucrose gradient that was prepared in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA (NTE buffer). The gradients were centrifuged in a Beckman SW 27.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 6,000 rpm for 45 min and fractionated from the top, using a Buchler Autodensiflow connected to a Buchler pump (Buchler Instruments, Inc., Fort Lee, NJ). The radioactive material in each fraction (1 ml) was precipitated by 5% ice-cold TCA, collected on a glass fiber filter, and counted in scintillation fluid.

To prepare 2 M NaCl-treated chromosomes for subsequent restriction enzyme digestion, 3 ml of chromosome suspension at the same concentration given above was gently mixed with 2 ml of 5 M NaCl. The mixture was layered onto a sucrose gradient consisting of (from the bottom) 2 ml of 2.4 M and 26 ml of 0.5 M sucrose solutions, both prepared in NTE buffer. The gradients were centrifuged in a Beckman SW 27 rotor at 22,000 rpm for 45 min and fractionated (2 ml/fraction). The last four fractions, which contained the dehistonized chromosomes, were pooled.

### Digestion of the Dehistonized Chromosomes with *EcoRI* Restriction Endonuclease and Sucrose Gradient Sedimentation Analysis

Dehistonized chromosomes were dialyzed against a solution containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA for 4 h (two changes, 1,000 ml each). The sample was then digested with the restriction enzyme *EcoRI* in the presence of 10 mM  $\text{MgCl}_2$  at 37°C. The *EcoRI* enzyme was purified according to the procedure described by Sümegei et al. (33). Enzyme digestion was terminated by the addition of 0.2 M EDTA to a final concentration of 15 mM.

*EcoRI*-digested, dehistonized chromosomes (2 ml) were layered onto a sucrose gradient consisting of (from the bottom) 1 ml of 2.4 M sucrose and 13 ml of 0.5 M sucrose prepared in NTE buffer. The gradients were centrifuged in a SW 27.1 rotor at 22,000 rpm for 2 h and fractionated as described above (1 ml/fraction). For quantitative analyses, labeled chromosomes were used as starting material. The DNA in each fraction was precipitated with 5% ice-cold TCA, collected on a glass fiber filter, and counted in scintillation fluid. The protein content of each fraction was determined by a colorimetric method as described by McKnight (22). For preparative purposes, a slight modification of the above gradient was used. 10 ml of the chromosome sample was layered onto a sucrose gradient containing 2 ml of 2.4 M sucrose (bottom) and 22 ml of 0.5 M sucrose

solution. The sucrose gradient was made in the NTE buffer and centrifuged as described above in an SW 27 rotor.

### Extraction of DNA, Labeling DNA with $^{32}\text{P}$ by Nick Translation, and DNA Annealing

The DNA was purified from the fractions of sucrose gradients described in the preceding section by the procedure described previously (15). The DNA concentration was measured by a fluorometric method (13).

Purified DNA was labeled with  $^{32}\text{P}$  by the nick-translation procedure described by Rigby et al. (28) with slight modification. The reaction mixture (35  $\mu$ l) contained 50 mM Tris-HCl, pH 7.6, 5 mM  $\text{MgCl}_2$ , 5 mM  $\beta$ -mercaptoethanol, 50 ng of DNA, 0.3 mg/ml each of unlabeled deoxyribonucleotides (dGTP, dATP, TTP), 10  $\mu$ Ci  $\alpha$ - $^{32}\text{P}$ -dCTP (3,000 Ci/mmol; Amersham, Arlington Heights, IL), and 1 U of DNA polymerase I (grade I; Boehringer Mannheim, Indianapolis, IN; this enzyme contains a trace amount of DNase). The reaction mixture was incubated at 14°C for 30 min and terminated by the addition of 100  $\mu$ l of 10 mM Tris-HCl, pH 7.6, and 10 mM EDTA. The labeled DNA was separated from the unincorporated nucleotides by Sephadex G-50 column chromatography and precipitated with ethanol in the presence of *Escherichia coli* carrier DNA (10  $\mu$ g/ml). The labeled DNA gave a consistent single-strand length of  $\sim 600$  nucleotides (ranging from 200 to 1,000 nucleotides). This was determined by agarose gel (1.6%) electrophoresis under alkaline conditions (30 mM NaOH, 2 mM EDTA) using  $^{32}\text{P}$ -end-labeled *HindIII*-digested simian virus 40 (SV40) DNA fragments as marker. The procedure for annealing the nick-translation-labeled DNA with a large excess of sonicated total chicken genomic DNA was described previously (17).

### Preparation of Micrococcal Nuclease-Digested Chromosome Scaffolds by Sucrose Gradient Sedimentation and Analysis of Sequence Complexity in the Scaffold DNA

The isolated metaphase chromosomes were resuspended in 1 ml of the chromosome isolation medium at  $10 A_{260}$  units/ml in which 0.5 mM  $\text{CaCl}_2$  was substituted for EDTA and EGTA. 40  $\mu$ l of micrococcal nuclease (1,000 U/ml; Worthington Biochemical Corp., Freehold, NJ) was added, and the digestion was carried out at 37°C for 60 min. The digestion was stopped by the addition of 0.2 M EDTA to a final concentration of 15 mM. Chromosome scaffolds were prepared by sedimenting the nuclease-digested chromosome sample through a sucrose gradient as described above for the preparation of the scaffolds from the *EcoRI*-digested dehistonized chromosomes.

End-labeling of DNA was performed as follows. The DNA was treated with alkaline phosphatase in a mixture (15  $\mu$ l) containing 6 mM Tris-HCl, pH 7.9, 6 mM  $\text{MgCl}_2$ , 100 mM NaCl, 6 mM  $\beta$ -mercaptoethanol, 1  $\mu$ g of DNA, and 4 U of the enzyme (Worthington Biochemical Corp.). The reaction mixture was incubated at 37°C for 1 h and terminated by the addition of 2  $\mu$ l of a solution containing 100 mM  $\beta$ -mercaptoethanol, 500 mM Tris-HCl, pH 7.5, and 70 mM  $\text{K}_2\text{HPO}_4$ . The reaction mixture was then transferred to another tube which contained flash-evaporated  $\gamma$ - $^{32}\text{P}$ -ATP (10  $\mu$ Ci, 800 Ci/mmol) and polynucleotide kinase (1 U in 1  $\mu$ l; Worthington Biochemical Corp.). The mixture was incubated at 37°C for another 30 min. After the addition of 50  $\mu$ l of 10 mM EDTA, the reaction mixture was passed through a Sephadex G-50 column. Fractions in the first peak, which contain the end-labeled DNA, were pooled and precipitated with ethanol in the presence of *E. coli* carrier DNA (10  $\mu$ g/ml). The end-labeled DNA was annealed with sonicated total chicken DNA according to the procedure described previously (17).

### Analysis of Ovalbumin Gene Sequences in the Scaffold DNA

DNA purified from fractions of sucrose gradients containing *EcoRI*-digested dehistonized chromosomes as described above were digested again with the *EcoRI* enzyme to ensure that all sites were cleaved. The DNA was separated by 0.7% agarose gel electrophoresis, transferred to a nitrocellulose filter by the procedure described by Southern (30), and hybridized to  $^{32}\text{P}$ -labeled recombinant DNA probes specific for sequences in the chicken ovalbumin gene region.  $^{32}\text{P}$ -labeled DNA probes were prepared by the nick-translation procedure as described above, except that the incubation time was 2 h. Two recombinant DNA clones containing the inserts for the sequences within and around the ovalbumin gene region (8, 29) were used for preparation of the  $^{32}\text{P}$ -labeled DNA probes. The locations of these inserts and restriction enzyme *EcoRI* cleavage sites in the ovalbumin gene region are shown in Fig. 6B.

## RESULTS

### Dehistonization of Isolated Chromosomes and Preparation of Scaffold DNA

It has been reported by Laemmli and his associates (1, 2) that most, if not all, of the histones can be dissociated from HeLa cell chromosomes by sedimenting the chromosomes through a sucrose gradient containing either 2 M NaCl or dextran sulfate/heparin. We have tested this using isolated chicken MSB-1 chromosomes. Fig. 1 shows the sedimentation behavior of [<sup>3</sup>H]thymidine-labeled MSB-1 chromosomes under the conditions described in Materials and Methods. The sedimentation coefficient of MSB-1 chromosomes is calculated to be ~4,000S, using the relationship established by McEwen (21) and the manufacturer's specification for the rotor we used. This value is in agreement with that reported for the dehistonized HeLa chromosomes (i.e., 4,000–7,000S, [1]). Using this information, we designed a preparative scheme to isolate dehistonized MSB-1 chromosomes.

Isolated chromosomes were dehistonized with 2 M NaCl as described in Materials and Methods. The chromosomes that sedimented onto the sucrose cushion contained very few, if any, histones. This was determined by SDS PAGE (not shown). Virtually 100% of the total chromosomal DNA but only ~10% of the total proteins was found in dehistonized chromosomes. Dehistonized chicken MSB-1 chromosomes had a DNA: protein ratio of ~5:1, which is in close agreement with the 6:1 ratio reported by Adolph et al. (1) for the dehistonized HeLa chromosomes.

Dehistonized chromosomes were digested with *Eco*RI endonuclease, followed by sedimentation through a sucrose gradient to separate the scaffolds from free DNA. Fig. 2 shows the results of a typical experiment. Without enzyme digestion, >90% of the DNA sedimented to the bottom two fractions of the gradient (Fig. 2A, upper panel). However, in the enzyme-digested sample (Fig. 2A, bottom panel), only about 10% of the DNA sedimented to the same position, with the majority of the DNA remaining on the top of the gradient. In both cases, >90% of the proteins were found in the bottom two fractions of the gradients (Fig. 2B). The DNA in the bottom

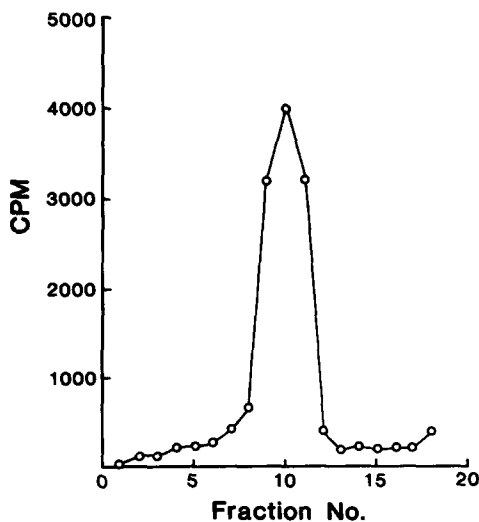


FIGURE 1 Sedimentation of the chromosomes through a sucrose gradient containing 2 M NaCl. Sedimentation conditions are described in Materials and Methods. Sedimentation is from left to right.

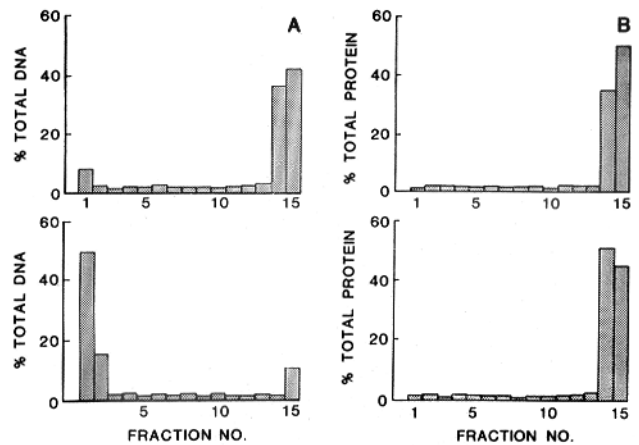


FIGURE 2 Sucrose gradient sedimentation of the *Eco*RI-digested dehistonized metaphase chromosomes. Distributions of DNA (A) and protein (B) in different fractions across the gradients were determined as described in the Materials and Methods. Upper panels: control, no *Eco*RI was used; lower panels: *Eco*RI-digested sample. Sedimentation is from left to right.

two fractions of the gradient is therefore tentatively referred to as scaffold DNA; and the DNA in the top fraction of the gradient is referred to as loop DNA.

Laemmli and his associates (1, 2) also utilized dextran sulfate/heparin to prepare histone-depleted chromosomes. Dehistonized chromosomes prepared by this method showed the same general characteristics as described above, except that the DNA in dehistonized chromosomes prepared by this method was not cleaved by subsequent *Eco*RI digestion, even when a 100-fold excess of the enzyme was used. We therefore routinely used 2 M NaCl to dehistonize chromosomes for preparation of scaffold DNA and loop DNA.

### Sequence Complexity of Scaffold DNA Isolated from *Eco*RI-digested Dehistonized Chromosomes

We utilized a liquid hybridization technique to determine whether the scaffold DNA represents a subset of the genomic DNA sequences. If the scaffold DNA consists of a unique sequence class, one would expect to observe distinct hybridization kinetics when the labeled scaffold DNA is annealed with a large excess of unlabeled genomic DNA. The scaffold DNA, loop DNA, and total chicken nuclear DNA were labeled with <sup>32</sup>P by nick translation. The labeled DNA was annealed with a large excess of unlabeled, sonicated chicken nuclear DNA (~500 nucleotides long). Shown in Fig. 3 are hybridization curves for these experiments. All three hybridization reactions reached a similar plateau value of ~65% at a Cot of 10,000 (Cot is the initial concentration of DNA [moles of nucleotide/liter] × time [seconds]). Furthermore, all three hybridization curves were more or less superimposable, except at lower Cot values (<10) where the scaffold DNA shows a slightly greater percent of reassociated material than do the other two. In the three hybridization reactions, ~40% of the labeled DNA annealed to the total genomic DNA in a manner resembling that for nonrepetitive sequences. These results suggest that scaffold DNA, like loop DNA and total genomic DNA, contains at least 60% of the single-copy sequences (after normalization of hybridization to 100% completion). In agreement with the previous reports (14, 27), these results suggest

that scaffold DNA is slightly enriched in repetitive DNA sequences (see Discussion).

### Sequence Complexity Analysis of Scaffold DNA Isolated from Micrococcal Nuclease-Digested Chromosomes

Adolph et al. (2) reported that the protein scaffold of chromosomes can be isolated free of DNA loops by treating HeLa chromosomes with micrococcal nuclease followed by the removal of histones with high salt treatment. We have also used this method to prepare scaffold DNA from chicken MSB-1 chromosomes. Isolated chromosomes were extensively digested with micrococcal nuclease; ~60% of chromosomal DNA became acid-soluble. The digested chromosomes were dehistonized by centrifugation through a sucrose gradient containing 2 M NaCl as described in Materials and Methods. Less than 0.5% of the total DNA was found in the bottom of the gradient (Fig. 4, upper panel), whereas ~20% of the total chromosomal proteins was present in this fraction (Fig. 4, lower panel). SDS PAGE of the proteins showed that very few, if any, histones

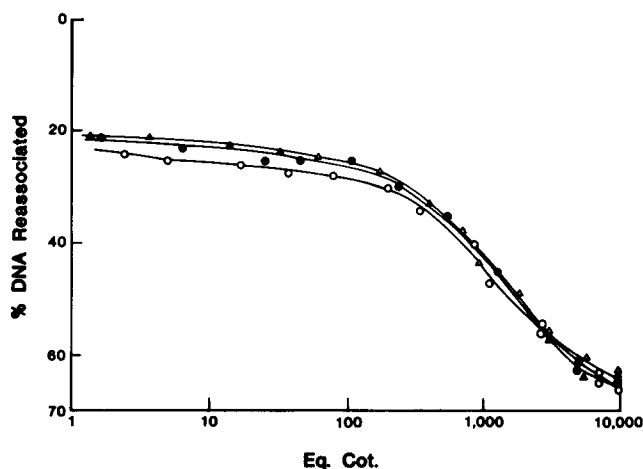


FIGURE 3 Time course of annealing of  $^{32}\text{P}$ -labeled DNA with sonicated total MSB-1 nuclear DNA. The  $^{32}\text{P}$ -labeled DNA was prepared by nick translation. (○) scaffold DNA; (△) loop DNA; (●) total unfractionated DNA.

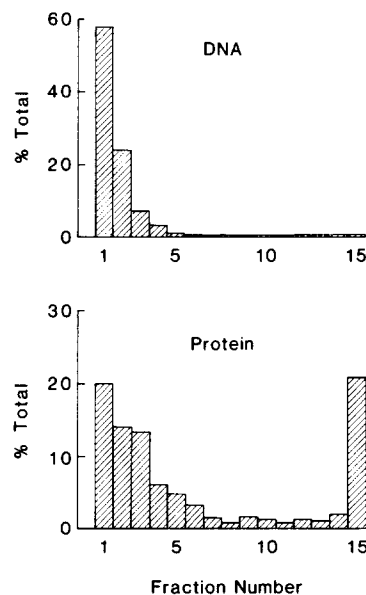


FIGURE 4 Sucrose gradient sedimentation of the micrococcal nuclease-digested chicken metaphase chromosomes. The procedures for digestion of chromosomes with enzyme and sucrose gradient sedimentation are described in Materials and Methods. The distributions of DNA (upper panel) and proteins (lower panel) in the gradients are shown. Sedimentation is from left to right.

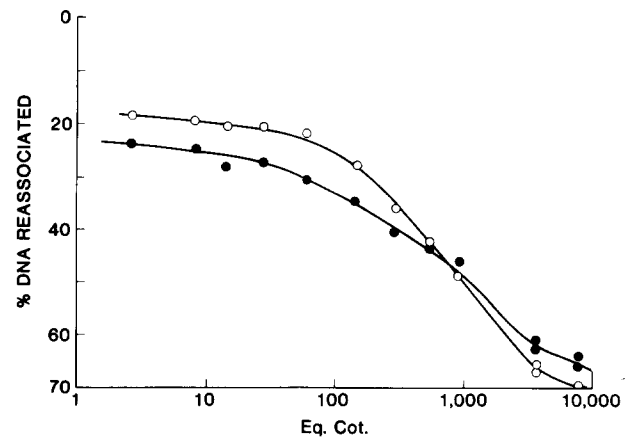


FIGURE 5 Annealing of the  $^{32}\text{P}$  end-labeled scaffold DNA isolated from the micrococcal nuclease-digested chromosomes with sonicated chicken nuclear DNA. (●) end-labeled DNA; (○) total DNA.

were present in the bottom fraction (not shown). This result is in agreement with that published by Adolph et al. (2). These authors reported that ~0.1–1% of DNA was associated with the scaffold prepared from micrococcal nuclease-digested HeLa chromosomes. According to these authors, this DNA may directly interact with the chromosomal scaffold proteins. We have used a solution hybridization technique to determine the sequence complexity of this DNA.

DNA was isolated from the bottom fraction of the gradient and end-labeled with  $^{32}\text{P}$ . The labeled DNA was ~140 bp (base pairs) in length as analyzed by electrophoresis in 2% agarose gels (not shown). This value is in good agreement with that reported by Jeppesen and Bankier (14), who reported that the scaffold DNA isolated from Chinese hamster chromosomes was 140 bp long.

The  $^{32}\text{P}$ -end-labeled DNA was used for hybridization with a large excess of total sonicated nuclear DNA from MSB-1 cells. As shown in Fig. 5, ~25% of the labeled DNA was hybridized at a Cot of 10, while only ~20% of the driver DNA had reassociated at this point. A further 40% of the labeled scaffold DNA was annealed at a Cot of 10,000 with a  $\text{Cot}_{1/2}$  of 800. The  $\text{Cot}_{1/2}$  of the nonrepetitive component in the reassociation of total chicken nuclear DNA is very close to this value. These results suggest that the scaffold DNA contains at least 60% of the nonrepetitive sequences and that it is slightly enriched in repetitive sequences.

### Analyses of Ovalbumin Gene Sequences in the Scaffold DNA Isolated from EcoRI-digested Dehistonized Chromosomes

We also utilized radioactively labeled DNA probes to investigate whether the scaffold DNA is enriched (or depleted) in the ovalbumin gene sequences. Loop DNA and scaffold DNA were isolated from the appropriate fractions of the gradient (Fig. 2A, lower panel), fractionated by agarose gel electrophoresis, transferred to a nitrocellulose filter, and hybridized with the  $^{32}\text{P}$ -labeled probes containing the ovalbumin gene and its neighboring DNA sequences (Fig. 6B). The result of this experiment (Fig. 6A) shows that neither the loop DNA (lanes 3 and 4) nor the scaffold DNA (lanes 5 and 6) contains a significant enrichment (or depletion) of ovalbumin or its related gene sequences (i.e., X and Y genes) (Fig. 6B), compared with unfractionated total chicken DNA (lanes 1 and 2).

## Effects of Hexylene Glycol on the Chromosome Scaffolds

Hexylene glycol was initially included in the Wray and Stubblefield chromosome isolation medium (35). Laemmli and his associates used it in the medium to prepare chromosomes for their chromosome scaffold studies. For this reason, we included hexylene glycol in our chromosome isolation buffers. We have studied the effects of hexylene glycol on chromosomes analyzed as described above. MSB chromosomes prepared in the absence of hexylene glycol showed the same sedimentation velocity (4,000S) in the sucrose gradients containing 2 M NaCl as chromosomes prepared in its presence. Similarly, most of the chromosomal proteins (>90%) can be removed from these chromosomes by 2 M NaCl. However, when dehistonized chromosomes prepared in this way were digested with *Eco*RI endonuclease and centrifuged through a sucrose gradient, we consistently (four experiments) found only 2% of the DNA in the bottom fraction, rather than the 10% found with chromosomes isolated in the presence of hexylene glycol. This highly reproducible result, although still preliminary, indicates that the presence of hexylene glycol during chromosome preparation can affect the protein:DNA ratio in the chromosome scaffolds.

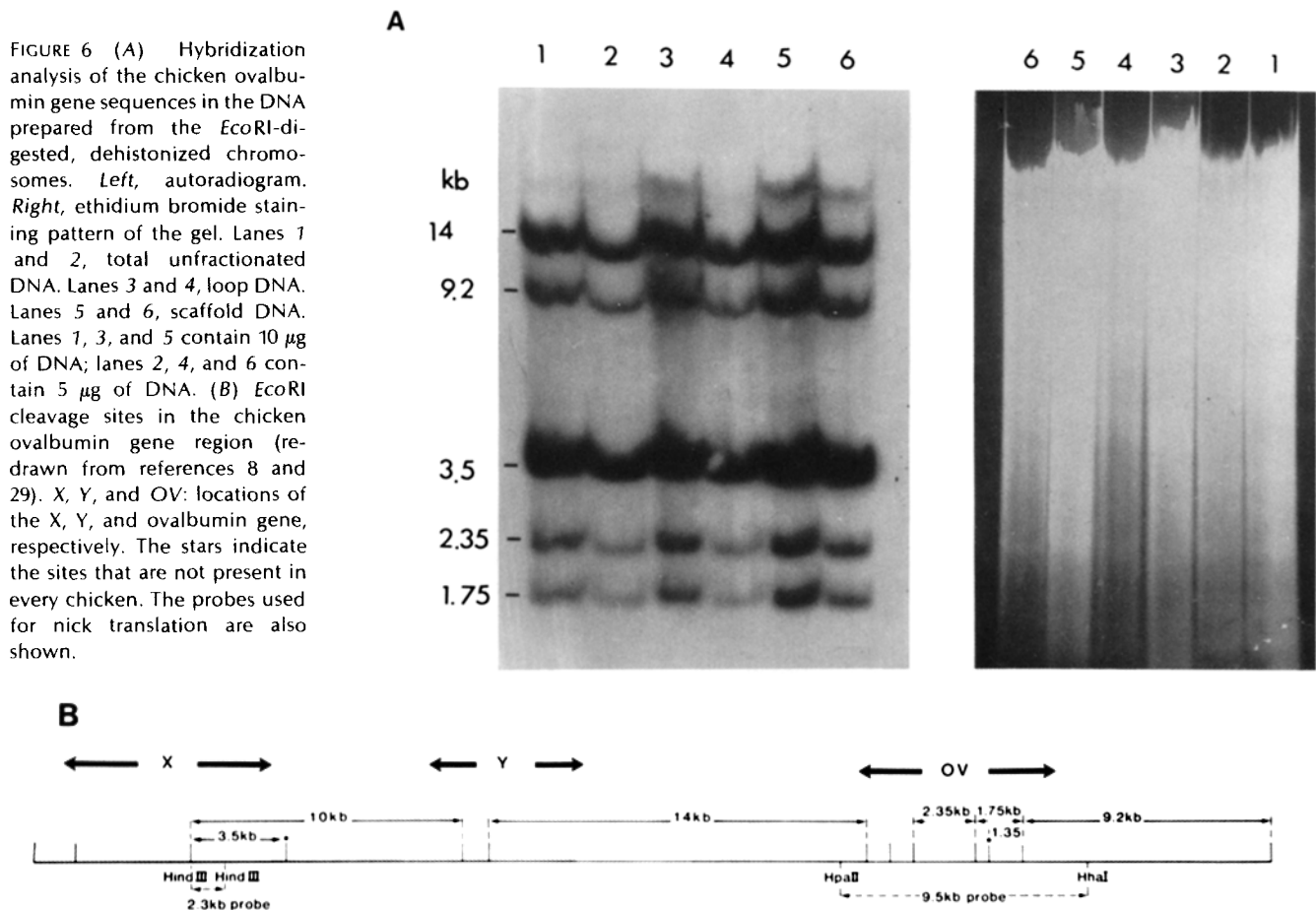
Very recently, Mullinger and Johnson (23) reported that, when hexylene glycol was used in the isolation medium to prepare chromosomes as described by Laemmli et al. (18), a typical Laemmli scaffold structure was observed under electron microscopy for the dehistonized chromosomes. However, when chromosomes were prepared with medium that did not contain hexylene glycol, typical Laemmli's scaffolds were not observed

in the dehistonized chromosomes. Instead, straight parallel DNA fibers were seen. These results, in agreement with our finding, suggest that hexylene glycol can change the chromosomal scaffold structure. It is noteworthy that the content of the ovalbumin gene sequences in *Eco*RI-digested scaffold DNA prepared from chromosomes isolated with medium containing no hexylene glycol was again found to be no different from that in the total unfractionated genome of chicken MSB-1 cells (not shown).

## DISCUSSION

In this paper, we describe the results of our investigation on DNA sequence complexity in chromosome scaffolds prepared from chicken MSB-1 chromosomes. Chicken MSB-1 cells are particularly suited for this study, since these cells grow very rapidly (8-h cell doubling time) and can be synchronized readily. Also, they grow in suspension at a high cell density ( $2 \times 10^6$  cell/ml).

Isolation of chromosomes that maintain good structural integrity was very important for the present study. Laemmli and his associates (1, 2, 18) used the chromosome isolation medium of Wray and Stubblefield (35) to prepare chromosomes for their chromosome scaffold studies. However, we found that this was not suitable for the present study because MSB chromosomes prepared in this medium exhibited extensive degradation of chromosomal DNA. Instead, we used the medium that was originally reported by Hewish and Burgoyne (12) and subsequently adapted by Blumenthal et al. (5), to prepare chicken chromosomes. Blumenthal's medium contains spermine and spermidine as well as EDTA and EGTA. Sperm-



ine and spermidine maintain chromosomes in a condensed structure, while EDTA and EGTA inhibit endogenous nuclease activities. DNA purified from the isolated chromosomes prepared with Blumenthal's medium is >50 kb in length, in contrast to <10 kb for the chromosomes prepared with the medium of Wray and Stubblefield (M. T. Kuo, unpublished results). The chromosomes isolated in the Blumenthal medium contain no detectable alteration of nucleosomal structure as determined by digesting chromosomes with micrococcal nuclease and analysis of the chromosomal DNA by agarose gel electrophoresis (M. T. Kuo, unpublished result).

One major point worthy of mention is that a structural gene (i.e., glyceraldehyde-3-phosphate dehydrogenase, GPD) in metaphase chromosomes isolated by the Blumenthal method remains preferentially sensitive to digestion by DNase I (Kuo and Schwartz, manuscript in preparation). In addition, DNase I site-specific cleavages (16, 31, 36) are also observed in the GPD gene regions in the isolated chromosomes. The locations of these cleavage sites are not the same as those found in the interphase nuclei. These characterizations strongly suggest that chromosomes isolated by the Blumenthal method maintain a good degree of structural integrity.

The isolated MSB chromosomes show a sedimentation coefficient (4,000S) similar to that of HeLa chromosomes in the sucrose gradients containing 2 M NaCl. In *EcoRI*-digested dehistonized MSB chromosomes, ~10% of total DNA was found to be associated with the nonhistone protein complex that was referred to as the chromosome scaffold (Fig. 2A). We consider the presence of 10% of the *EcoRI* fragments in the scaffold to be reasonable according to the following calculation. Assuming that the average size of the DNA loops attached to the scaffold is 60 kb (range 30–90 kb, [18]) and that the average size of *EcoRI* fragments in the chicken genome is 4 kb (our unpublished data), one would expect that at least 6.6% of DNA would be associated with the chromosome scaffold ( $(4/60) \times 100\% = 6.6\%$ ). These observations suggest that chicken chromosomes contain a scaffold structure with biochemical characteristics similar to those of HeLa chromosomes.

Although there is no conclusive evidence concerning how the chromosome scaffold is organized in an interphase nucleus, it is thought to be related to nuclear matrix (4, 6) because both can be prepared by high molar salt treatments. Several reports have recently appeared in the literature reporting on DNA sequences found to be associated with the nuclear matrix or chromosome scaffold. Jeppesen and Bankier (14) and Razin et al. (27) reported that scaffold DNA prepared from either Chinese hamster or mouse chromosomes was enriched in middle repetitive DNA sequences. We have also found a slight enrichment of the repetitive DNA sequences in the chicken chromosome scaffold DNA (Figs. 3 and 5). The significance of such an enrichment is unclear, especially if one considers the fact that their data, like those presented in this report (Figs. 3 and 5), also showed a significant amount of single-copy DNA sequences in the scaffold DNA.

Cook and Brazell (7) reported that the human  $\alpha$  globin gene, but not the  $\beta$  and  $\gamma$  globin genes, is specifically associated with the "nuclear cage," which was isolated by centrifuging intact HeLa cells through a sucrose gradient containing 2 M NaCl. We have utilized their method to analyze whether the DNA sequences associated with the MSB nuclear cage are enhanced in ovalbumin gene content. Our result showed that the ovalbumin gene copy number in the cage DNA is similar to that in the total genomic DNA (M. T. Kuo, unpublished data). We suspect that the sedimentation conditions they used might

allow a cosedimentation of the  $\alpha$  globin gene fragment with the *EcoRI*-digested nuclear cage, since in humans the  $\alpha$  globin gene is located in a 23-kb *EcoRI* fragment and the  $\beta$  and  $\gamma$  are located in 6.7- and 7.4-kb *EcoRI* fragments, respectively.

Another paper by Nelkin et al. (24) showed a threefold to sevenfold enrichment of SV40 DNA relative to the total cellular DNA in the nuclear matrix DNA prepared from SV40-infected 3T3 cells. These authors also demonstrated that the mouse  $\alpha$  and  $\beta$  globin genes are evenly distributed in the nuclear matrix and nonmatrix DNA. Another way of interpreting their data, however, is that the enrichment of SV40 sequences in the matrix proteins is due to a copurification of the transcribing gene sequence in the nuclear matrix, since the transcriptional complex is resistant to high salt treatment (9). We have found that the nuclear matrix isolated from chicken oviduct is enriched in the ovalbumin gene but not the globin gene sequences (M. T. Kuo, unpublished data).

Our results using ovalbumin gene probes show that the scaffold DNA (Fig. 6A) and nuclear matrix DNA (M. T. Kuo, unpublished result) are not enriched (or depleted) in these specific gene sequences. Similar results have been obtained by other investigators using chicken globin gene probes (H. Weintraub, personal communication). These data suggest that it is presently premature to conclude that a specific DNA sequence is associated with the nuclear matrix or chromosome scaffold. Either of two interpretations would account for these results. One interpretation is that the interaction between DNA and scaffold (or matrix) proteins may be a transient phenomenon. For example, Pardoll et al. (26) and McCready et al. (20) reported that the DNA replication complex is anchored to the nuclear matrix and that the DNA is reeled through the complex as it is replicated. These observations support the notion of a dynamic interaction of the matrix protein and DNA. Therefore, the matrix or scaffold proteins may be more mobile than one might have thought. The second interpretation is that the chromosome scaffold could be an experimental artifact. This issue has been discussed in the literature recently (10, 11, 25). The major objections to the chromosome scaffold model is that the scaffold may represent an incomplete dispersion of chromatin in the center of chromosomes (25). Alternatively, the scaffold may merely represent an aggregate of residual proteins in the dehistonized chromosomes (10, 11). If this is the case, it would not be surprising to find that specific DNA sequences are not associated with the chromosome scaffold.

Finally, we would like to point out that, although the results presented here do not lend strong support for a chromosome scaffold model, we do favor the notion that a highly ordered structure is present in an intact metaphase chromosome. This is strongly suggested from the results of our experiments using DNase I as a probe to investigate the higher-order chromatin structure in metaphase chromosomes as mentioned above. We think that, at present, the use of intact chromosomes and appropriate probes is still a useful approach to study the highly ordered structure of metaphase chromosomes.

I would like to thank Prof. P. Chambon, Strasbourg, France, for his many helpful suggestions and for allowing me to use the ovalbumin gene clones; Professor T. C. Hsu for his encouragement; Ms. Mildred Lai and Bhanumati Iyer for their technical assistance; and Mrs. Ruby Kirkpatrick for her help in the preparation of the manuscript.

This research was supported in part by grants from the Robert A. Welch Foundation (G-831) and the National Institutes of Health (GM 28573).

Received for publication 13 July 1981, and in revised form 7 December 1981.

## REFERENCES

1. Adolph, K. W., S. M. Cheng, and U. K. Laemmli. 1977. Role of nonhistone proteins in metaphase chromosome structure. *Cell* 12:805-816.
2. Adolph, K. W., S. M. Cheng, J. R. Paulson, and U. K. Laemmli. 1977. Isolation of a protein scaffold from mitotic HeLa cell chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* 74:4937-4941.
3. Aikya, Y., and S. Kato. 1974. Two cell lines from lymphomas of Marek's diseases. *Biken J.* 17:105-116.
4. Berezney, R., and D. S. Coffey. 1975. Nuclear protein matrix: association with newly synthesized DNA. *Science (Wash. D.C.)* 189:291-293.
5. Blumenthal, A. B., J. D. Dieden, L. N. Kapp, and J. W. Sedat. 1979. Rapid isolation of metaphase chromosomes containing high molecular weight DNA. *J. Cell Biol.* 81:255-258.
6. Comings, D. E. 1976. Compartmentalization of nuclear and chromatin proteins. In *The Cell Nucleus*. H. Busch, editor Academic Press, Inc., New York. 4:345-371.
7. Cook, P. R., and I. A. Brazzell. 1980. Mapping sequences in loops of nuclear DNA by their progressive detachment from the nuclear cage. *Nucleic Acids Res.* 8:2895-2906.
8. Gannon, F., K. O'Hare, F. Perrin, J. P. LePennec, C. Benoist, A. Cochet, R. Breathnach, A. Royal, A. Garapin, B. Cami, and P. Chambon. 1979. Organization and sequences at the 5' end of a cloned complete ovalbumin gene. *Nature (Lond.)* 278:428-434.
9. Garioglio, P., R. Llopis, P. Oudet, and P. Chambon. 1979. The template of the isolated native simian virus 40 transcriptional complexes in a minichromosome. *J. Mol. Biol.* 131:75-105.
10. Goyanes, V. J., S. I. Matsui, and A. A. Sandberg. 1980. The basis of chromatin fiber assembly within chromosome studies by histone-DNA cross-linking followed by trypsin digestion. *Chromosoma (Berl.)* 78:123-135.
11. Hadlaczy, G., A. T. Sumner, and A. Ross. 1981. Protein-depleted chromosomes. II. Experiments concerning the reality of chromosome scaffolds. *Chromosoma (Berl.)* 81:557-567.
12. Hewish, D. R., and L. A. Burgoyne. 1973. Chromatin substructure. The digestion of chromatin DNA at regular spaced sites by nuclear deoxyribonucleases. *Biochem. Biophys. Res. Commun.* 52:504-518.
13. Hinegardner, R. T. 1971. An improved fluorometric assay for DNA. *Anal. Biochem.* 39:197-201.
14. Jeppesen, P. G. N., and A. T. Bankier. 1979. A partial characterization of DNA fragments protected from nuclease degradation in histone depleted metaphase chromosomes of the Chinese hamster. *Nucleic Acids Res.* 7:49-67.
15. Kuo, M. T., and T. C. Hsu. 1978. Bleomycin causes release of nucleosomes from chromatin and chromosomes. *Nature (Lond.)* 271:83-84.
16. Kuo, M. T., J. L. Mandel, and P. Chambon. 1979. DNA methylation: correlation with DNase I sensitivity of chicken ovalbumin and conalbumin chromatin. *Nucleic Acids Res.* 7:2105-2113.
17. Kuo, M. T., C. G. Sahasrabudde, and G. F. Saunders. 1976. Presence of messenger specifying sequences in the DNA of chromatin subunits. *Proc. Natl. Acad. Sci. U. S. A.* 73:1572-1575.
18. Laemmli, U. K., S. M. Cheng, K. W. Adolph, J. R. Paulson, J. R. Brown, and W. R. Baumbach. 1977. Metaphase chromosome structure: the role of nonhistone proteins. *Cold Spring Harbor Symp. Quant. Biol.* 42:351-360.
19. Marsden, M. P. F., and U. K. Laemmli. 1979. Metaphase chromosome structure: evidence for a radial loop model. *Cell* 17:849-858.
20. McCready, S. J., J. Godwin, D. W. Mason, I. A. Brazzell, and P. R. Cook. 1980. DNA is replicated at the nuclear cage. *J. Cell Sci.* 46:365-386.
21. McEwen, C. R. 1967. Tables for estimating sedimentation through linear concentration gradients of sucrose solution. *Anal. Biochem.* 20:114-149.
22. McKnight, G. S. 1977. A colorimetric method for the determination of submicrogram quantities of protein. *Anal. Biochem.* 78:86-92.
23. Mullinger, A. M., and R. T. Johnson. 1980. Packing DNA into chromosomes. *J. Cell Sci.* 46:61-86.
24. Nelkin, B. D., D. M. Pardoll, and B. Vogelstein. 1980. Localization of SV40 genes within supercoiled loop domains. *Nucleic Acids Res.* 8:5623-5633.
25. Okada, T. A., and D. E. Comings. 1980. A search for protein cores: is the scaffold an artifact? *Am. J. Hum. Genet.* 32:814-832.
26. Pardoll, D. M., B. Vogelstein, and D. S. Coffey. 1980. A fixed site of DNA replication in eukaryotic cells. *Cell* 19:527-536.
27. Razin, S. V., V. L. Mantieva, and G. P. Georgiev. 1979. The similarity of DNA sequences remaining bound to scaffold upon nuclease treatment of interphase nuclei and metaphase chromosomes. *Nucleic Acids Res.* 7:1713-1735.
28. Rigby, J. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nicktranslation with DNA polymerase I. *J. Mol. Biol.* 113:237-252.
29. Royal, A., A. Garapin, B. Cami, F. Perrin, J. L. Mandel, M. LeMeur, F. Brégegere, F. Gannon, J. P. LePennec, P. Chambon, and P. Kourilsky. 1979. The ovalbumin gene region: common features in the organization of three genes expressed in chicken oviduct under hormonal control. *Nature (Lond.)* 279:125-132.
30. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
31. Stalder, J., A. Larsen, T. D. Engel, M. Dolan, M. Groudine, and H. Weintraub. 1980. Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNase I. *Cell* 20:451-460.
32. Stubblefield, E., and W. Wray. 1971. Architecture of the Chinese hamster metaphase chromosomes. *Chromosoma (Berl.)* 32:262-294.
33. Sümegi, J., D. Breedveld, P. Hossenlopp, and P. Chambon. 1977. A rapid procedure for purification of EcoRI endonuclease. *Biochem. Biophys. Res. Commun.* 76:78-86.
34. Taylor, J. T. 1967. Duplication of chromosomes. *Sci. Am.* 198:36-42.
35. Wray, W., and E. Stubblefield. 1970. A new method for the isolation of chromosomes, mitotic apparatus, or nuclei from mammalian fibroblasts at near neutral pH. *Exp. Cell Res.* 59:469-478.
36. Wu, C., Y.-C. Wong, and S. C. R. Elgin. 1979. The chromatin structure of specific genes. II. Disruption of chromatin structure during gene activity. *Cell* 16:807-814.