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DNA fragments associated with chromosome scaffolds

Brian C.Bowen*

Department of Biochemical Sciences, Princeton University, Princeton, NJ 08544, USA

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ABSTRACT

Following extensive digestion of HeLa metaphase chromosomes with either Hae III endonuclease or micrococcal nuclease, nonhistone protein scaffolds may be isolated. Scaffolds isolated after Hae III digestion have about 1.5% of the chromosomal DNA attached to them. This DNA is heterogeneous in size, ranging from about 0.2 to 20 kbp. It can be cleaved with either Eco RI or Hae III - Eco RI, producing a series of repeated fragments, of which the most abundant is 1.7 kbp in length. The 1.7-kbp fragment is tandemly repeated and is enriched (about 50-fold) in the scaffold-associated DNA. It is located primarily on human chromosome 1 and is probably a component of human satellites II and III. Scaffolds isolated after micrococcal nuclease digestion have about 0.1% of chromosomal DNA attached. This DNA is present in two size classes - fragments larger than 10 kbp and fragments approximately 0.2 kbp long. Restriction enzyme digestion of this DNA gives no prominent repeated fragments. Its reassociation kinetics are similar to those of total DNA, indicating that it is not enriched in either highly repetitive or middle repetitive sequences.

INTRODUCTION

Metaphase chromosomes from HeLa cells remain compact after removal of the histones with 2 M NaCl or polyanions (1). In this compact structure, loops of DNA at least 30-90 kbp long appear to emanate from a central core, or scaffold (2). The chromosome scaffold is not sensitive to DN'ase or RN'ase and is essentially composed of nonhistone proteins (3). Laemmli et al. (4,5) have proposed that the nonhistone "scaffolding" proteins are associated with DNA sequences at the base of each loop. Such a model suggests that these DNA sequences may be distinct from the bulk of the DNA in the loops. The purpose of this communication is to test this hypothesis by characterizing the DNA fragments which co-sediment with scaffolds produced by either Hae III or micrococcal nuclease digestion of HeLa chromosomes.

Several other groups have presented evidence from studies of chromosomes (6,7,8) and nuclei (9,10,11) which suggests that eukaryotic DNA is organized

into long loops, and that these are attached to nuclear substructures (12,13,14,15,16). Two groups have purified metaphase chromosomes (or metaphase plates), digested them with micrococcal nuclease, and isolated 2 M NaCl - insoluble protein cores. Georgiev and co-workers (6,12), using mouse L cells, and Jeppesin and Bankier (7,13), using Don Chinese hamster cells, found a slight (about twofold) enrichment in repetitive DNA sequences associated with the cores. Rattner et al. (8) have argued, however, that micrococcal nuclease digests both the euchromatin and heterochromatin of mouse chromosomes equally.

In nuclei, an alternative method of determining whether certain DNA sequences are associated with 2 M NaCl-insoluble proteins has been used by Cook and Brazell (14) and Kuo and Chambon (17). Each group digested nuclear chromatin with restriction enzymes, purified the 2 M NaCl insoluble structures, and then probed the associated DNA with cloned genes. Based on their results, Cook and Brazell claim that specific sequences are associated with the "nuclear cage" in HeLa cells. Kuo and Chambon, though, find no significant evidence for the specific association of any region of the ovalbumin, X, and Y genes (75 kbp total) with 2 M NaCl-insoluble proteins in chick nuclei.

The results presented in this communication indicate that scaffolds from chromosomes digested with Hae III have about 1.5% of the total DNA associated, while scaffolds from chromosomes digested with micrococcal nuclease have about 0.1%. The micrococcal nuclease-generated scaffold DNA is not enriched in either highly repetitive or middle repetitive sequences. The Hae III-generated scaffold DNA is enriched in a set of highly repeated sequences, which can be cleaved by Eco RI, or Hae III-Eco RI. The most abundant restriction fragment produced is 1.7 kbp long and is homologous to the other prominent, repeated fragments. It is tandemly repeated, and by *in situ* hybridization, appears to be located primarily in the centromeric region of human chromosome 1. Thus, the highly repeated sequences in the Hae III-generated scaffold DNA are probably not scaffold-specific sequences present on all chromosomes, but instead are components of satellites II and III (18,34) present on chromosome 1.

MATERIALS AND METHODS

Preparation of chromosomes and chromosome scaffolds: HeLa S-3 cells were grown in suspension culture in the presence of [^{3}H] thymidine as previously described (1). Metaphase chromosomes were prepared by the hexylene glycol

(2-Me-2, 4 pentanediol) method of Wray and Stubblefield (19) with some modifications (2). Chromosomes were released from cells by dounce homogenization, purified by 2 cycles of differential centrifugation at 4°C, and finally resuspended in nuclease digestion buffer (10mM Tris-H 1, 1 mM $MgCl_2$, 10 mM NaCl, 0.5 mM PMSF, pH 7.4). Endonuclease Hae III (New England Biolabs) was added (100 units/ml chromosome suspension with A_{260} = 7), and the suspension incubated at 30°C for an appropriate length of time. Alternatively, micrococcal nuclease was added (600 units/ml chromosome suspension with A_{260} = 7), and suspension incubated at 0°C. Reactions were terminated by adding an equal volume of high salt buffer, HSB (20 mM Tris HCl, 4 M NaCl, 20 mM Na-EDTA, 0.2% ammonyx LO, 1 mM PMSF, pH 9) at 0°C. These conditions result in the solubilization of histones and most nonhistone proteins following digestion of HeLa metaphase chromosomes with micrococcal nuclease (1).

Scaffolds were purified by centrifugation of samples through a 3-ml cushion containing 10% sucrose in 0.5 x HSB at 4°C. Centrifugation was for 15 min at 1500 rpm and then 45 min at 12,000 rpm in the Beckman SW 50.1 rotor. Large samples (4 to 6 ml) were layered onto 15-ml cushions and centrifuged in the Beckman SW27 rotor at an equivalent centrifugal field strength. After centrifugation, the supernatants and cushions were removed by pipet. Pellets were washed once with 0.5 x HSB, and resuspended in protease digestion buffer (see below). In preliminary experiments, the protein composition of the pellets was determined by SDS gel electrophoresis (21) and was found to be the same as that of the nonhistone protein scaffolds described by Adolph et al. (1). Thus, the pellet fraction is also termed the "scaffold" fraction.

Scaffold-associated DNA fragments used as probes in Figures 3 and 5 (see Results) were purified from chromosomes which underwent two cycles of Hae III digestion, high salt treatment, and centrifugation, before protease digestion.

Purification of DNA fragments associated with chromosome scaffolds: The pellet, or scaffold, fraction was incubated overnight at 37°C with 1 mg/ml pronase ("nuclease-free" Calbiochem) in protease digestion buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM Na-EDTA, 0.2% SDS, pH 8). The corresponding supernatant fraction or the unfractionated total mixture were also diluted into protease buffer and treated similarly. At this stage, aliquots from both fractions and the total mixture were removed; the ^3H cpm of each were determined by liquid scinitillation counting, in order to calculate scaffold DNA recovery. After protease treatment, each sample was purified by phenol extraction and ethanol precipitation. Samples were finally resuspended in TEN_5 (10 mM Tris-HCl, 1 mM Na-EDTA, 5 mM NaCl, pH 7.4).

Analysis of DNA fragments by electrophoresis and hybridization: Purified DNA and RNA fragments were digested with restriction enzymes under conditions suggested by the supplier (New England Biolabs). 40 µg/ml RN'ase was included in each reaction. After digestion, DNA fragments were re-purified, loaded onto neutral agarose gels, and separated by electrophoresis (22). Fragments were recovered from preparative agarose gels by a modification (23) of the "freeze-squeeze" method (24), followed by chromatography over DE52 cellulose (Whatman).

Southern blotting and hybridization conditions (25) are indicated in the appropriate figure legends. DNA probes were labelled with ^{32}P-dCTP by "nick translation" synthesis (26). Blots were immersed in hybridization solution (27) containing approximately 30 ng/ml ^{32}P-labelled probe (specific activity $\sim 10^7$-10^8 cpm/µg), and incubated at 65°C for 24 hours.

In situ hybridization: Metaphase chromosomes from HeLa S-3 cells or HT1080 cells (generously provided by Dr. Raju Kucherlapati) were prepared for hybridization by a modification (28) of the method of Gall and Pardue (29). The hybridization procedure, including [^{3}H] cRNA synthesis, was that of Gall and Pardue. Autoradiography was performed using Ilford L.4 emulsion.

DNA-DNA renaturation: Scaffold-associated [^{3}H] DNA fragments (labelled *in vivo*) were purified as described above, resuspended in 0.2 mM EDTA, 10 mM PIPES, pH 6.8, and then sonicated (Microsonicator, Kontes) so that the average fragment length was between 250 and 400 nucleotides upon denaturation (see Discussion). Samples (100 µl) were denatured at 100°C, cooled to 0°C, and brought to 0.3M NaCl. They were then placed at 65°C to allow renaturation. 5 µl aliquots were removed from each sample, and duplex formation was assayed by S1 nuclease resistance (30). S1 digestion solutions were incubated at 37°C for 30 min. Each contained a total of 50 µg/ml of denatured DNA, representing the sum of scaffold [^{3}H] DNA and unlabelled *E. coli* DNA carrier, and 100 units/ml of S1 nuclease (sigma). S1 resistant [^{3}H) DNA was detected by its adsorption to DEAE-cellulose filter discs (DE81, Whatman), as described by Maxwell *et al.* (31).

RESULTS

DNA associated with scaffolds from HAE III-treated chromosomes: Scaffolds were isolated from chromosomes which had been digested with Hae III (Figure 1A). The percentage of total DNA associated with the scaffold was reduced to about 1.5% by increasing the digestion time to 180 minutes.

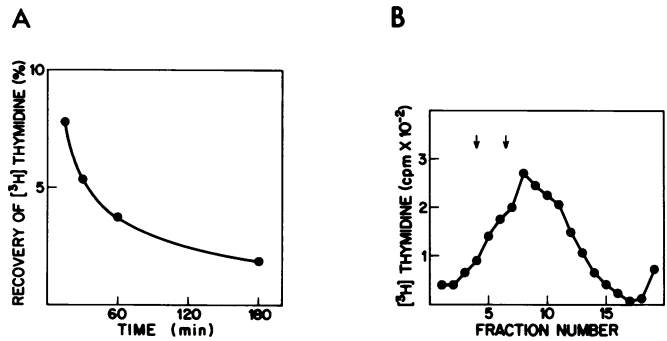

Figure 1.A. Recovery of [^{3}H] thymidine-labelled DNA associated with Hae III scaffolds. HeLa metaphase chromosomes were digested with Hae III for 15, 30, 60 and 180 min. Scaffolds were isolated as described in Materials and Methods. For each sample, the percentage of total [^{3}H] DNA co-sedimenting with the scaffold is presented.

B. Fractionation of DNA associated with Hae III scaffolds by sedimentation through a sucrose density gradient. Scaffolds were isolated from ^{3}H-labelled chromsomes which had been digested with Hae III for 180 min. The scaffold-associated DNA representing about 1.5% of the total DNA, was purified by protease digestion, and phenol and ether extractions with mild agitation. This DNA was applied to a 5-20% sucrose gradient containing 10mM Tris·HCl, 0.2M NaCl, 1mM Na-EDTA, 0.1% sarkosyl, pH8, and centrifuged for 12 hours at 35,000 rpm in a Beckman SW50.1 rotor. The position of SV40 DNA form I(21 S) and form II(16 S) in a parallel gradient are indicated by the left and right arrows, respectively.

This DNA was heterogeneous in size (Figure 1B). Its sedimentation profile exhibited a peak of 14.5S, or 5.4 kbp (32), while the largest fragments were about 20 kbp in length.

In order to identify repetitive DNA sequences associated with scaffolds, the scaffold DNA isolated at each time point in Figure 1A was digested with restriction enzymes. As shown in Figure 2, several bands were produced by the enzyme combination Hae III-Eco RI. Two of these bands, at 2.1 and 1.7 kbp, were noticably enriched in scaffolds, i.e., their fluorescence is approximately constant in lanes c (15 min digestion) through f (180 min digestion) while the background fluorescence has dropped. In lane f, the 2.1 and 1.7 kbp fragments represent about 15% of the scaffold DNA, based on ^{3}H cpm in the excised gel slices. For comparison, DNA in the supernatant fraction of chromosomes digested for 180 min was also treated with the Hae III-Eco RI combination. The resulting pattern (lane a, Figure 2) exhibited no prominent bands. The 2.1- and 1.7-kbp bands in lanes c through f were not produced by digestion of the scaffold DNA with Hae III alone (see below), although

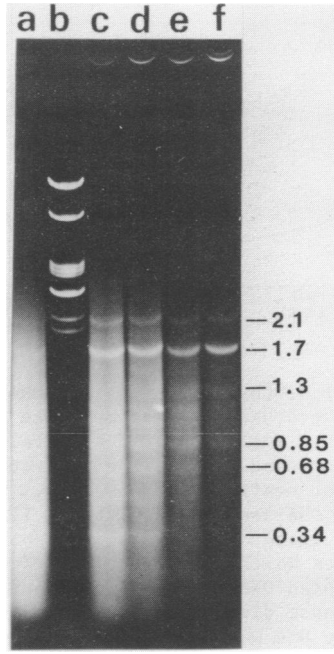

Figure 2. Agarose gel electrophoresis of fragments produced by Hae III and Eco RI digestion of scaffold DNA and supernatant DNA. Scaffold and supernatant DNA corresponding to each point in Figure 1 were redigested with Hae III and Eco RI, and loaded onto a 1% agarose gel. Scaffold DNA represents (c) 8.0% (d) 5.3%, (e) 3.7%, and (f) 1.7% of total DNA. (a) Supernatant DNA represents 98.3% of total DNA. (b) Molecular weight standards were generated by Hind III digestion of Ad2 DNA.

a small amount of DNA of this size is generated by Hae III digestion of the total DNA (33).

The distribution of scaffold DNA sequences in total DNA was studied by Southern blotting and hybridization. In Figure 3, total genomic DNA was digested with various restriction enzymes and then probed with either scaffold DNA (lanes a through e) or the purified 1.7-kbp fragment (lanes f through h). The scaffold DNA hybridized to a similar series of bands in the Hae III-Eco RI (lane b), Eco RI (lane c), and Alu I (lane d) digests. The most prominent bands corresponded to 1.3, 1.7, and 2.1 kbp. When identical digests (lanes f through h) were probed with purified 1.7-kbp DNA, the same series of bands was observed. These results showed that a major component of the scaffold DNA is a set of sequences which are homologous to

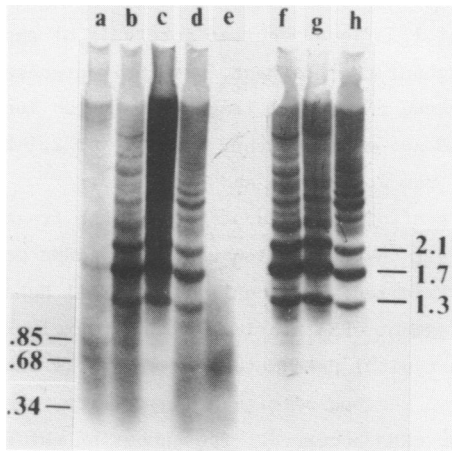

Figure 3. Detection of scaffold DNA sequences in HeLa chromosomal DNA. Total HeLa DNA was digested with various restriction enzymes: (a) Hae III; (b), (f) Hae III and Eco RI; (c), (g) Eco RI; (d), (h) Alu I; (e) Hinf I. Fragments were separated on 1% agarose gels and then blotted onto nitrocellulose. Lanes (a) through (e) were hybridized to ^{32}P-labelled, scaffold DNA from Hae III - treated chromosomes. Lanes (f) through (h) were hybridized to ^{32}P-labelled 1.7-kbp fragment (indicated in Figure 3).

the 1.7-kbp fragment. These sequences contain Eco RI sites, but no Hae III sites. Also, the Eco RI sites and Alu I sites are probably tandemly repeated, if, as shown below, the 1.7-kbp Eco RI fragment contains a single Alu I site. Lane a, Figure 3, shows that the scaffold DNA probe hybridized to short (less than 1 kbp) and very large Hae III fragments, and that only a faint band was observed at 1.7 kbp.

The major difference between the blot probed with scaffold DNA and that probed with the 1.7-kbp fragment is in the low molecular weight (less than 1 kbp) region. Hae III-Eco RI (lanes b, f) and Alu I (lanes d, h) digests showed significant hybridization to scaffold DNA, but very little to 1.7-kbp DNA. Faint bands were detected at approximately 0.34, 0.68, and 0.85 kbp in the Hae III (lane a) and Hae III-Eco RI (lane b) samples. While the scaffold DNA probe hybridized to short Hinf I fragments (lane e), particularly at 0.68 kbp, the 1.7-kbp probe gave no detectable hybridization to an identical digest (data not shown). Thus, these low molecular weight fragments probably represent a component(s) of scaffold DNA, which is different from the 1.7-kbp homologues.

In additional experiments, a preliminary restriction map of the 1.7-kbp

Eco RI fragment was constructed (Figure 4). Not indicated on the map are cleavage sites for Hinf I. These occur at intervals of approximately 50, 75, 100, 125. . . bp throughout the fragment, but their precise location was not determined. Alu I cleaved the Eco RI fragment into two lengths, 1.0 and 0.7 kbp. Enzymes which did not cleave either the 1.7- or 2.1-kbp fragments were Eco RII, Hpa I, Hha I, Bam I, Bgl II, and Hind III.

The locations of scaffold DNA and of the 1.7-kbp fragment in chromosomes were determined by in situ hybridization using [^{3}H] cRNA probes (Figure 5). While scaffold cRNA hybridized almost uniformly to all HeLa chromosomes (not shown), the 1.7-kbp fragment cRNA hybridized almost exclusively to a subset of the chromosomes. A typical HeLa metaphase spread is shown in Figure 5A. Arrows indicate the five regions of high grain density, where hybridization to the 1.7-kbp fragment cRNA occurred. The number of chromosomes in all spreads varied between 62 and 68, of which 4 to 6 chromosomes exhibited grain clusters. In Figure 5A, four of the five grain clusters were obviously located over centromeric regions of the chromosomes. This experiment was repeated using chromosomes from a diploid, fibrosarcoma cell line (HT 1080). As shown in Figure 5B, the grain clusters were located over the centromeric regions of only two chromosomes. These are the two largest metacentric chromosomes, and thus are easily identified as the chromosome 1 pair. In Figure 5A and 5B, individual grains were located over other chromosomes; however, the limited number of grains and the apparent randomness of these locations from experiment to experiment ruled out any other obvious location for the 1.7-kbp fragment.

DNA associated with scaffolds from micrococcal nuclease-treated chromosomes: Scaffolds were also isolated from chromosomes which had been digested with micrococcal nuclease. After extensive digestion at 0°C, about 0.1% of the total DNA remains associated with the scaffolding proteins. As in

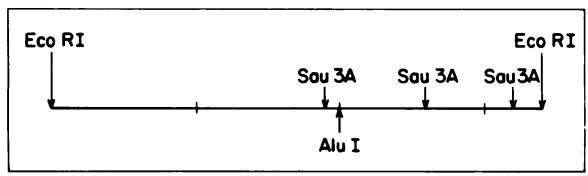

Figure 4. Preliminary restriction map of the 1.7-kbp fragment. Short bars on the map are separated by 0.5 kbp. The 2.1-kbp fragment has the same map except that the distance between the left Eco RI site and the left Sau 3A site is increased from 0.95 kbp to 1.35 kbp.

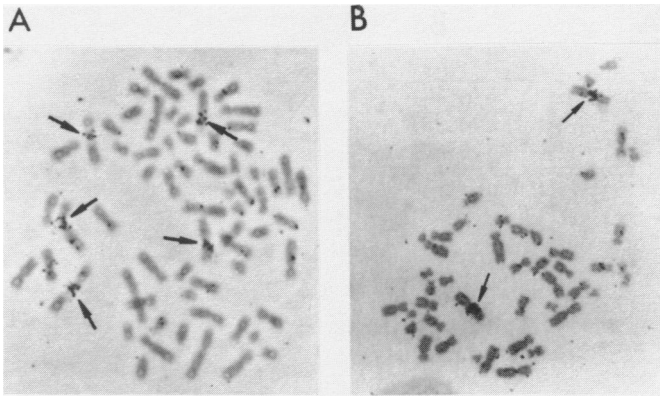

Figure 5. Location of the 1.7-kbp fragment in human metaphase chromosomes
A. In situ hybridization to HeLa chromosomes.
B. In situ hybridization to HT 1080 chromosomes. In each experiment, the probe was [^{3}H]RNA transcribed in vitro (cRNA) from the 1.7-kbp fragment. Arrows indicate regions of high grain density.

the case of Hae III scaffolds this DNA was heterogeneous in size. When fractionated on a 1% agarose gel, the DNA appears to have two components (lane c, Figure 6A) - fragments larger than about 10 kbp or fragments approximately 0.2 kbp in length. Digestion of the DNA with Hae III removes the high molecular weight component (lane b). Eco RI cleaves some of the high molecular fragments (lane a), but does not produce the prominent 1.7 and 2.1 kbp bands seen in Figure 2 (the bands observed in lane a are λ DNA fragments included as internal standards).

When the distribution of scaffold sequences in total HeLa DNA was examined by Southern blotting (lanes a through e, Figure 6B), the 1.3, 1.7, and 2.1 kbp bands were still observed, yet they were considerably less prominent than in the blots probed with the Hae III-generated scaffold DNA (Figure 3). In fact, the patterns in Figure 6B, lanes a through e, were very similar to those produced by hybridization to a total DNA probe (lanes f through j, Figure 6B). This result implies that DNA associated with micrococcal nuclease-generated scaffolds contains repetitive sequences, but that these sequences are not significantly enriched over their abundance in total DNA. In order to determine whether repetitive sequences, as a class, partition with the scaffold, the reassociation kinetics of the scaffold, supernatant, and total DNA's were measured (Figure 7).

DNA was isolated from scaffolds containing 3%, 0.9%, and 0.1% of the

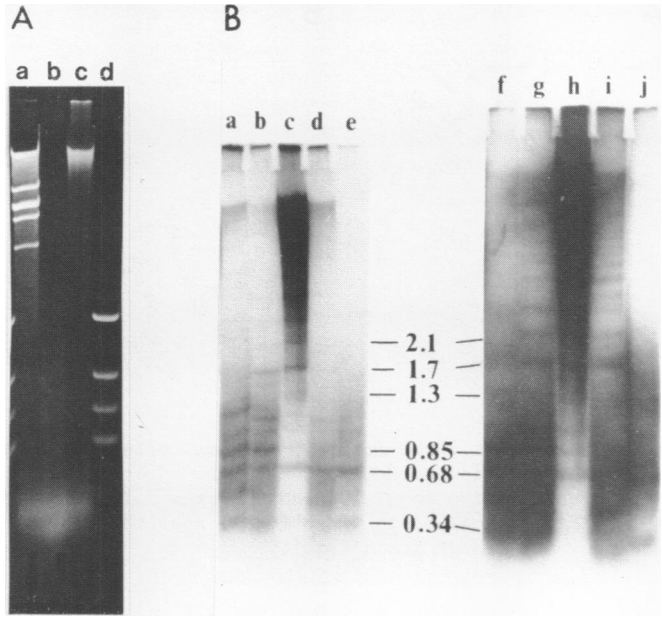

Figure 6. A. Agarose gel electrophoresis of scaffold DNA from micrococcal nuclease-treated chromosomes. Chromosomes were digested with micrococcal nuclease at 0°C. The scaffolds had 0.1% of the total DNA associated with them. This DNA was purified and then digested with (a) Eco RI and (b) Hae III. (c) Undigested scaffold DNA. Eco RI-treated λDNA was included in (a) to provide internal molecular weight standards. (d) Hinf I digestion of SV40 DNA.

B. Detection of scaffold DNA sequences in HeLa chromosomal DNA. Total DNA was digested with the same enzymes as in Figure 3: (a), (f) Hae III; (b), (g) Hae III and Eco RI; (c), (h) Eco RI; (d), (i) Alu I; (e), (j) Hinf I. After blotting, lanes (a) through (e) were hybridized to ^{32}P-labelled, scaffold DNA (representing 0.1% of total DNA from micrococcal nuclease-treated chromosomes. Lanes (f) through (j) were hybridized to ^{32}P-labelled, total DNA. The positions of bands which were identified in Figures 2 and 3 are indicated.

total DNA. The supernatant and total (digested) DNA's were also isolated, and diluted to the same concentration as the corresponding scaffold DNA for reassociation reactions. Figure 7 shows that scaffold DNA representing 3% (closed circles) of the total DNA had the same rate of reassociation as the supernatant (closed triangles) and total (closed squares) DNA's. Furthermore, scaffold DNA representing 0.9% and 0.1% (open circles) give a similar result. Thus reducing the percentage of scaffold DNA from 3% to 0.1% does not cause a significant enrichment in either highly repetitive or middle

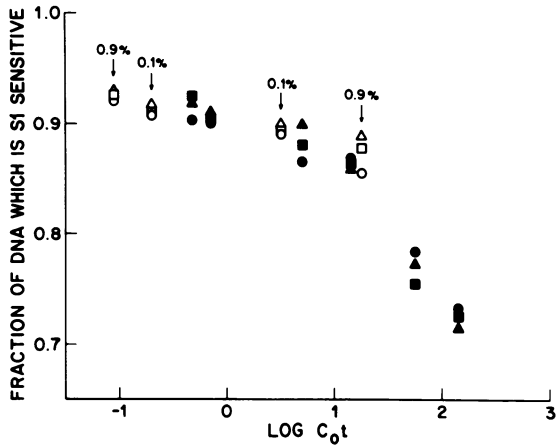

Figure 7. Reassociation kinetics of scaffold, supernatant, and total DNA. Chromosomes were digested with micrococcal nuclease to the extent that 3.0% (closed symbols), 0.9% and 0.1% (open symbols) of the total DNA remained associated with the scaffolds. Total DNA (◘) was obtained from chromosomes before separation into supernatant (▲) and scaffold (O) fractions. Each synbol represents the average of three measurements.

repetitive sequences. Data at higher C_0t values were not obtained; thus, any enrichment in a subset of single copy sequences cannot be entirely ruled out. The conclusion from these results is that no obvious partitioning of repetitive sequences comparable to the Hae III-generated scaffolds occurs with micrococcal nuclease-generated scaffolds. In Table 1, the fractions of S1-sensitive DNA before and immediately after denaturation are presented. Once again, no significant differences between scaffold, supernantant, or total DNA's were observed.

DISCUSSION

Laemmli and co-workers have proposed a "radial loop" model for the structure of metaphase chromosome (4,5). An important question raised by the model concerns the loop attachment sites - do the central scaffolding proteins bind to an identifiable class of DNA sequences within each loop? I have attempted to answer this question by analyzing the DNA fragments which co-purify with scaffolds after either restriction endonuclease Hae III or micrococcal nuclease digestion of HeLa metaphase chromosome. In neither case, do I find evidence for an abundant class of sequences (≥0.1% of total DNA) common to all chromosomes.

TABLE 1

Reassociation of scaffold, supernatant, and total DNA

Scaffold DNA*	Fraction of DNA which is S1 sensitive immediately after denaturation (t=0) scaffold	supernatant	total
3.3%	0.98(0.16)+	0.99(0.21)+	0.98(0.12)+
0.9%	0.98(0.20)	0.98(0.11)	0.99(0.16)
0.1%	0.98(0.13)	0.98(0.13)	0.97(0.07)

*Percentage of total DNA co-sedimenting with scaffolds.

+Number in parenthesis refers to fraction of DNA which was sensitive to S1 before denaturation, and is therefore in theory, the lowest attainable value after renaturation.

When chromosomes are digested with Hae III, the purified scaffolds are enriched in a class of DNA sequences homologous to a 1.7-kbp Eco RI fragment. Rather than being distributed throughout the genome, the fragment appears to be located primarily on chromosome 1 - although some homologous sequences may be present on the other chromosomes at a copy number too low to be detected by in situ hybridization. Since the fragment is tandemly repeated (see below), it is probably a satellite DNA component. Of the human satellites (I, II, III, and IV), both satellite II and III have been shown to contain a component which resides primarily on chromosome 1 (17,34). Thus, the 1.7-kbp fragment (and its homologues in Figure 3) is likely to be a major component of satellite II and III.

Recently, a fragment which is located on chromosome 1 and which hybridizes to satellite II was cloned by Cooke and Hindlay (34). The fragment was originally generated by Eco RI digestion of human satellite III, and is 1.77 kbp long. (In order to avoid confusion, the reader should be aware that the 1.77-kbp fragment may be a component of both satellites II and III, as long as the sequences to which it is linked are different for the two satellite bands; see reference 34 for a detailed discussion). It possesses a single Alu I site and two Mbo I sites. The Alu fragments are 1.06 and 0.71 kbp. My results (Figure 4) agree closely with theirs. Furthermore, the positions of the two Mbo I sites are the same ($\pm$50 bp) as the two central Sau 3A (an isoschizomer of Mbo I) sites in Figure 4. An Mbo I site corresponding to the Sau 3A site 100 bp from the right end of

the fragment was not reported. Additional evidence that the 1.7-kbp fragment is a satellite II component comes from Hinf I digestion. Hinf I cleaves the 1.7-kbp fragment into an arithmetic series of short pieces varying in length by 25 bp, the shortest piece being about 50 bp, (data not shown). This result is in agreement with observations by Cooke and Hindley and by Manuelidis (35).

Given that the 1.7-kbp Eco RI fragment possesses one Alu I site, it is easy to demonstrate tandem repetition of the fragment. Digestion of total DNA with Eco RI (lane g) or Alu I (lane h) in Figure 3 gives the same 1.7-kbp band. Thus, this fragment (and apparently its longer homologues) is tandemly repeated. The abundance (∿10%) of the 1.7-kbp fragment in scaffold DNA implies that there are over 1000 copies of it in the human genome. If these are tandemly repeated, they cover at least 1700 kbp of DNA. This estimate is relevant to the radial loop model, which proposes an average value of 70 kbp for the loop length. Because the region of satellite II DNA, which lacks Hae III sites, can be much longer than several loops, the enrichment of the 1.7-kbp (and 2.1-kbp) fragment in Hae III scaffolds may be explained merely by the absence of Hae III sites in the centromeric region of chromosome 1. It is not possible to argue that the 1.7-kbp fragments are partitioning with the scaffold solely because of their proximity to or identity with, proposed scaffold attachment sites. In fact, the 1.7- and 2.1 kbp fragments can be released from scaffolds by digestion with Hinf I. The residual scaffold DNA shows no enrichment for any particular class of sequences by gel electrophoresis or by reassociation kinetics (unpublished results).

Thus, it appears that satellite DNA sequences are enriched in Hae III scaffolds. While I have not identified components of satellites I and IV in the scaffold DNA, they may be present because several satellite DNA's studied by Manuelidis (36) were Hae III resistant. Manuelidis also identified highly repeated sequences in the human genome which contain Hae III sites at at 0.17, 0.34, 0.68,. . . kbp. I have observed similar repeats in both the scaffold (Figures 2 and 3) and supernatant DNA fractions. These sequences are located in the centromeric regions of several chromosomes (36), indicating that centromeric DNA is accessible to Hae III.

A second approach to the isolation of hypothetical attachment sites is to digest chromosomes with a nonspecific nuclease, such as micrococcal nuclease. In this case, satellite DNA's should be cleaved as readily as other sequence classes of DNA, and only those sequences protected by non-

histone proteins will remain associated with the scaffold. Approaches similar to this have been taken by Jeppesen and Bankier (13), using Chinese hamster chromosomes, and Razin et al. (12), using mouse chromosomes. In contrast to the results presented in Figure 7, each of these groups observes some enrichment in repeated DNA sequences associated with 2M NaCl - insoluble proteins. The results of Razin et al. indicated that scaffolds having about 2% of the total DNA attached are enriched in middle repetitive DNA. The enrichment represents approximately a 2-fold increase in middle repetitive mouse DNA. Evaluation of their reassociation kinetics data is difficult because Razin et al. failed to include such information as zero-time reassociation and the percentage of renaturation before normalization.

Jeppesen and Bankier found that 0.1-0.5% of the total DNA was protected from degradation by micrococcal nuclease and was recovered as 140-bp fragments. When these fragments were nick-translated and then annealed with total DNA, the reassociation kinetics suggested an enrichment in rapidly reassociating sequences. The data, however, were variable; for example, the enrichment varied between 5% and 70% at $C_o t = 1$. Because the authors failed to indicate the zero-time reassociation values, it is difficult to determine the contribution of snapback sequences, which can be generated by nick translation (see reference 37, for example), to the overall enrichment.

Jeppesin and Bankier found that a pyrimidine tract "fingerprint" of the protected DNA sequences was indistinguishable from a "fingerprint" of a random set of sequences. I have obtained a similar result - the scaffold DNA and total DNA have identical pyrimidine tract patterns (unpublished results).

There appears to be no significant sequence differences between scaffold, supernatant, and total DNAs from HeLa chromosomes digested with micrococcal nuclease. In Figure 7, the fraction of DNA which is S1 sensitive, uncorrected for zero-time S1 resistance (Table 1) is reported. Nick-translated probes were not used for this experiment because they often contain snapback regions, as mentioned above. One difficulty with the kinetics experiment is that, after sonication, the average scaffold fragment length is about 400 nucleotides while the supernatant DNA is about 250 nucleotides. However, this length difference would account for only a 20% difference (38) in the rate of reassociation of supernatant DNA versus scaffold DNA - a value too small to be detected in my measurements.

At the present time I attribute the discrepancy between my results in Figure 7 and those of Razin et al., and Jeppesen and Bankier, to their use of nick-translated DNA probes, and possibly to differences in the dis-

tribution of highly repeated sequences in the mouse and hamster chromosomes versus human chromosomes. In this last respect, it is significant that no rapidly reassociating sequences were found to be associated with 2M NaCl-insoluble proteins isolated from sheared, chicken chromatin (15). The possibility that artifacts of association between protein and DNA occur during different isolation procedures cannot be ruled out, and may account for the discrepancies between the results of different groups studying scaffold protein-DNA interactions.

The major conclusions to be drawn from the results in this paper are: (1) the DNA fragments associated with scaffolds from Hae III-treated chromosomes are enriched in highly repeated sequences which are located primarily on chromosome 1 and which represent a component of satellites II and III DNA; and (2) the DNA fragments associated with scaffolds from micrococcal nuclease-treated chromosomes are not enriched in these sequences, nor in the broad class of middle repetitive sequences. These conclusions do not support the idea of a uniform class of sequences which bind scaffolding proteins, nor do they disprove it. Such sequences may exist if they are very short, on the order of 9 bp, as Jeppesen and Bankier (13) have suggested.

The most promising approach to the question of chromosomal scaffold attachment sites seems to be the use of recombinant DNA techniques. To date no specific attachment sequences in nuclei or chromosomes have been identified. The first step in doing so will probably require the clear partitioning of a gene with the scaffold and then "chromosome walking" to identify an attachment sequence. Such a sequence could then be assayed for its abundance and location throughout the genome.

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*Present Address: Laboratory of Genetics, University of Wisconsin, Madison, WI 53706, USA

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