Nuclear matrix-DNA complex resulting from *Eco*R1 digestion of HeLa nucleoids is enriched for DNA replicating forks

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ABSTRACT

Upon treatment of HeLa nucleoids with EcoR1 restriction enzyme, a minute fraction of the DNA remains attached to the nuclear matrix. This matrix-DNA complex appears to be composed of a unique set of proteins. Electron microscopic analysis of the DNA fragments present shows an enrichment for HeLa DNA replicating forks.

INTRODUCTION

Direct electron microscopic studies on eucaryotic chromosomal DNA replication have been, for the most part, restricted to early embryionic systems. This is mainly due to the natural enrichment for replicative intermediates that is attained in these systems because of the short duration of both cell division and S phase. This in turn is ahieved by the activation of a large number of origins of replication; hence, at any given time the density of replicating forks is large enough to ensure a good recovery of replicative intermediates. In non-embryionic systems with longer S phases, the actual sites of replication represent a small part of the total DNA. It is not surprising then, that attempts to analyze these latter systems by electron microscopy, have been frustrated by the low recovery of replicative intermediates. This problem is not, however, only confined to eucaryote cells. It is a rather general characteristic of any genome where the number of active origins of replication is relatively small compared to the molecular weight of the DNA. Thus, bacterial systems are also included in this category. Recently we have found a way of circumventing this problem in bacteria, by artificially increasing the concentration of replicative intermediates. This was achieved by exploiting the association between bacterial membranes and replication forks. When such complexes are digested with a restriction endonuclease, the remaining fast sedimenting material is found to be enriched for replicative intermediate fragments.[1,2]

In eucaryote systems, since the pioneering work of Berezney and Coffey[3] the nuclear matrix, a loosely defined entity "derived from proteinaceous components of the in situ interchromatic matrix as well as a residual nucleoli and the pore-complex lamina"[4] has been implicated as the major site for eucaryote DNA replication. Several laboratories

have presented evidence supporting this view[5-7] and more recently it has been proposed that regions of the chromosomes involved in replication may be preferentially bound to the nuclear matrix.[3,6,8-10] Given the striking functional resemblance of these structures to bacterial membranes, we decided to extend to eucaryote systems, the approach described above for the isolation of bacterial DNA replication forks. In this report we show that this approach is applicable to HeLa cells. A minute amount of the total DNA can be isolated as part of a nuclear matrix complex and this DNA appears to be enriched for putative HeLa DNA replicating growing forks.

MATERIALS AND METHODS

(a) Growth and synchronization: HeLa cells were grown in suspension in modified Eagle's medium as described by Mueller et al.[11] Synchronization of DNA synthesis was brought about by double thymidine block which consisted of: (a) subjecting exponentially growing cells (approximately 10^5/ml) to 3 mM thymidine for 16 hrs; (b) washing off the thymidine and allowing one cycle of DNA synthesis and cell division in thymidine-free medium (8 hrs); and (c) blocking DNA synthesis with 10^{-6} M Fluorodeoxyuridine for 16 hrs. These steps achieve a high degree of synchronization at the beginning of the S-phase as indicated by the sharp linear uptake of thymidine for the duration of about 8-10 hrs followed by a doubling of the cell number manifesting synchronized cell division when the block was released by addition of 2 µg/ml thymidine (data not shown).

Cells from three stages of growth were used in the experiments described in this paper. Exponentially growing cells were harvested without any treatment; thymidine-fluorodeoxyuridine blocked cells were harvested without allowing any DNA synthesis; and cells released from the double block with 2 µg/ml bromodeoxyuridine were harvested after allowing 2 hrs of DNA synthesis.

(b) Radioactive and density labelling of the DNA: Total cell DNA was labelled with 0.01 µCi/ml of ^{14}C-thymidine for one cycle of replication between the thymidine and fluorodeoxyuridine blocks. The new synchronously replicated DNA was density labelled with 2 µg/ml bromodeoxyuridine and also with 0.1 µCi/ml ^{3}H-bromodeoxyuridine for 2 hrs. The cells were chilled in ice, harvested by centrifugation, and resuspended in phosphate buffered saline solution.

(c) Isolation of nuclear matrix DNA complex: About 2 ml of a HeLa cell suspension (10^7 cells/ml) either from synchronized or exponential cultures was mixed with 6 ml of a lysis mix containing 2.6 M NaCl, 0.13 M Tris pH 8.0, 2.6 mM EDTA and 0.67% Triton X-100.[12] After incubation at 4° for 15 minutes, the lysate was applied to a sucrose step gradient containing from bottom to top, 3 ml CsCl, (density 1.67); 5 ml 30% sucrose and 20 ml 15% sucrose. Sucrose solutions contained 1.95 M NaCl, 0.01 M Tris pH 8.0, and 1 mM EDTA. After centrifugation in a SW27 rotor for 20 minutes at 15,000 rpm

at 5°C, fractionation was performed from top to bottom. Fractions containing HeLa nucleoids were pooled and dialyzed for 3 hrs at 4°C against 4 liters of a solution containing 0.02 M Tris pH 8.0, 0.002 M EDTA (TE). The suspension was then treated with EcoR1 restriction enzyme for about 30-40 minutes at 37°C. After this time the nucleoid precipitate became homogeneous and was then applied to a second sucrose step gradient, centrifuged and fractionated as described above.

(d) Isolation of HeLa DNA fragments and electron microscopy: Fractions of the second sucrose gradient centrifugations containing DNA for both slow and fast sedimenting components were pooled separately and dialyzed against 4 liters of TE buffer. The suspensions were then concentrated to about 3 ml with carbowax C-6000 and then treated with 0.1 ml 20 mg/ml pronase at room temperature for 60 minutes, followed by incubations with 40 µl of 30% sarkosyl (15 minutes at room temperature) and additional 0.1 ml pronase (3 hrs at 37°C). The solution was adjusted to a density of 1.67 with solid CsCl and centrifuged at 30,000 rpm for 3 days at 10°C in a 60Ti rotor. The gradient was fractionated from top to bottom, at a rate of 0.35-0.70 ml/min into 0.2 ml fractions. The resulting DNA fractions with densities between light-light and heavy-light were inspected in the electron microscope by the standard conditions described elsewhere.[13]

(e) SDS-PAGE for the Nuclear Matrix Proteins: Fractions from sucrose density gradient or direct nuclear lysates were prepared for electrophoresis by precipitating the proteins with 4 volumes of acetone at -20°C for 30 minutes to 1 hr. The precipitates were pelleted either in Sorvall or in Beckman microfuge and dried under vacuum. The pellets were dissolved in SDS sample buffer (1% SDS, 10 mM Tris-HCl, pH 7.5; 2 mM EDTA; 1% mercaptoethanol) and boiled at 100°C for 5 minutes. Stacking SDS-polyacrylamide gel electrophoresis was performed following Laemmli.[14] The separating gel consisted of a 7.5%-20% acrylamide gradient (13 cm x 9 cm x 0.15 cm). Electrophoresis was run at a constant current of 20 mA for approximately 4.5 hrs with water cooling. Polypeptides used as standards included myosin, ß-galactosidase, phosphorylase A, bovine serum albumin, ovalbumin, carbonic anhydrase and haemoglobin (200,000; 116,000; 94,000; 68,000; 43,000; 30,000; and 16,000 daltons molecular weight respectively).

RESULTS

Isolation of HeLa Matrix-DNA Complex

About 10^7 HeLa cells from synchronized or exponential cultures were gently lysed by the procedure developed by Cook et al.[12] in the presence of 1.95 M NaCl. The lysate was then applied to a 15-30% sucrose step gradient containing 1.95 M NaCl and a cushion of CsCl solution at the bottom of the tube. After centrifugation, most of the DNA was recovered as a fast sedimenting material at the 15-30% sucrose interphase

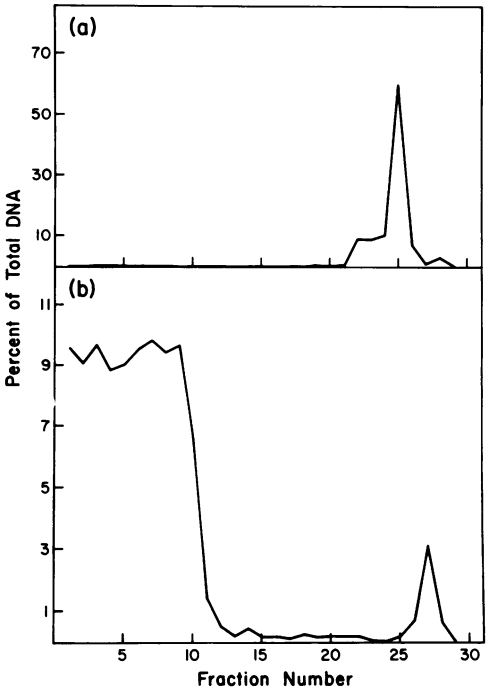

Figure 1. (a) Sedimentation analysis of HeLa nucleoids in a 15-30% sucrose step gradient containing a CsCl shelf. Sedimentation is from left to right. (b) The fast sedimenting material from (a) was treated with EcoR1 and rerun under similar conditions as in (a).

[Fig. 1(a)]. Upon treatment of this material with EcoR1 restriction enzyme and subsequent centrifugation under similar conditions as above, the bulk of the DNA is now recovered at the top of the gradient whereas about 5% retained the fast sedimenting behavior [Fig. 1(b)]. This latter component will be referred to as the matrix-DNA complex in the remaining text.

Proteins Attached to the Matrix-DNA Complex

The proteins, present in the matrix-DNA complex after nuclease digestion, were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 2 shows the comparison of the protein composition of the whole nuclei (N) with that of the matrix-DNA complexes isolated from exponential and synchronously replicating cultures (M_E & M_S respectively.) It can be seen that the histones as well as a large number of non-histone proteins are released from the nuclei as a result of the salt extraction and digestion with restriction enzyme. Matrix DNA complexes from both exponential and synchronous cultures show very similar band patterns. Prominent proteins in the matrix-DNA complex can be grouped according to their molecular weights into 4 or 5 strong bands in the range between 42,000-60,000 daltons and some weaker bands above 200,000 daltons. These bands are also present in nuclear preparations (N), indicating that they are indeed a

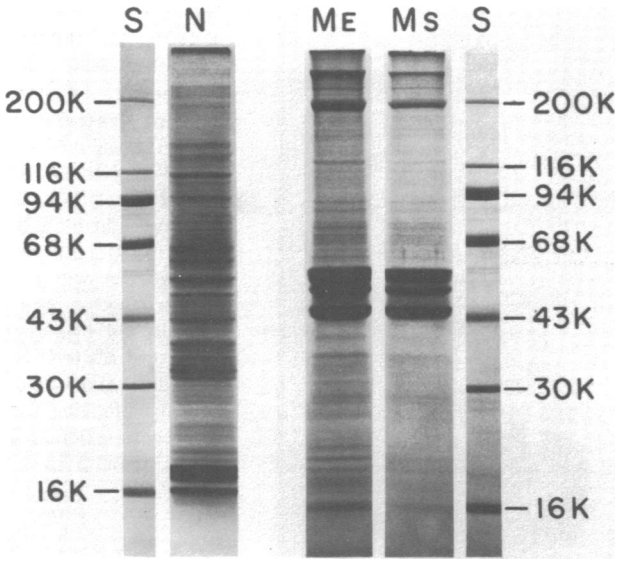

Figure 2. Electrophoretic pattern of proteins of the nuclear matrix DNA complex. (lane S) Standard polypeptide markers, with corresponding molecular weights shown adjacent. (lane N) Whole nuclei extracted from synchronized HeLa cells. Matrix DNA complex isolated from exponentially growing HeLa cells (lane M_E) or from synchronously replicating cells (lane M_S).

subset of the nuclear proteins. The strongest bands correspond to polypeptides of molecular weights 43,000, 49,000, 52,000 and 55,000 daltons respectively. Of these, the 43,000 and the 55,000 dalton proteins are found to comigrate with actin and tubulin respectively (data not shown).

Electron Microscope Analysis of DNA Fragments Present in Matrix-DNA Complex

Matrix DNA complexes were extensively treated with sarkosyl and pronase in order to digest proteins and lipids present, and the remaining DNA was further purified through a CsCl equilibrium gradient centrifugation. Fractions from the CsCl gradient with densities higher than light-light DNA were then inspected in the electron microscope. About 4% of the DNA fragments obtained from the matrix-DNA complex isolated from the synchronized culture were branched. Three types of branched structures were found and are presented in Fig. 3(a-c). The most predominant of them corresponded to forked molecules Fig. 3(a), and about 5% of the remaining branched molecules were either eye structures [Fig. 3(b)] or multiply branched molecules [Fig. 3(c)]. In all cases, however, two of the branches had the same length. Branched DNA fragments appear to be particularly enriched in the matrix-DNA complex since when the same analysis is performed on the slow sedimenting DNA obtained after EcoR1 digestion or in total

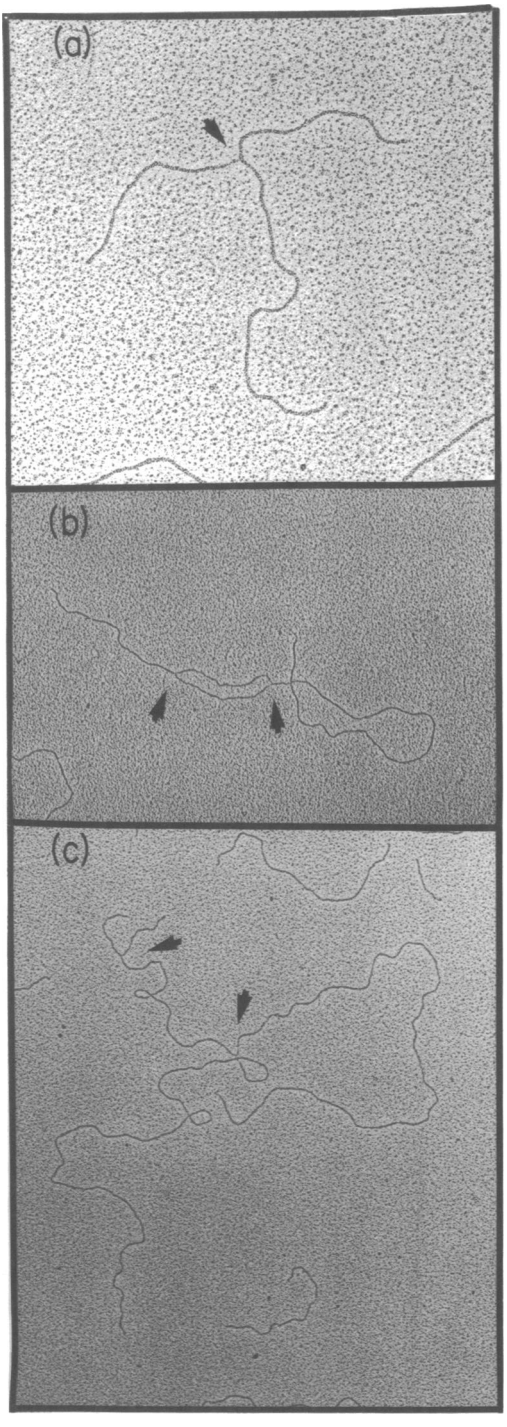

Figure 3. Electron micrographs of types of branched HeLa DNA fragments observed after purification of the fast sedimenting material obtained following digestion of HeLa nucleoids with EcoR1. Arrows point to branch junctions. (a) Single branched fragment or fork; (b) Double branched fragment in which two branches are forming an eye structure; (c) Multiply branched fragment consisting of two forks. All fragments show two arms of equal length projecting away from a common junction. Size of fragments ranges from 2 to 7 microns.

Table 1
Electron Microscope analysis of DNA fragments present after EcoR1 Treatment of HeLa Nucleoids

Cell Culture	DNA Component	Number of Molecules	% of Branched Fragments
Synchronized (2 hr replication)	Matrix	335	3.9
	Slow	850	0.7
	Total	400	0.7
Exponential	Matrix	200	1.5
	Slow	300	0.3
Synchronized (no replication)	Matrix	400	1.5
	Slow	400	0.5

EcoR1 digested DNA from synchronized cells, the proportion of branched DNA fragments is around 0.7% for both samples. The size of the DNA fragments obtained is about 3-7 μ for the matrix DNA complex and 2-5 μ for the slow sedimenting DNA. This size range varies with the extent of EcoR1 digestion, but overall the proportion of types of branched structures found is about the same. Table 1 shows a summary of our electron microscope analysis of DNA fragments obtained from synchronized and exponential cultures and for a synchronized culture which was extracted before the 2 hr replication pulse. It can be seen that, (a) in all samples matrix DNA complexes are enriched for branched molecules compared to their slow sedimenting counterparts; (b) Synchronized cultures which have been allowed to replicate for 2 hrs yielded the highest proportion of branched DNA fragments; and (c) both exponential and synchronized (prior to the 2 hr replication pulse) cultures contain about the same proportion of branched DNA fragments, suggesting that synchronization may not have been 100% efficient.

DISCUSSION

HeLa nucleoids were isolated using a standard technique developed by Cook and collaborators.[12] Briefly, the technique consists of a gentle lysis of HeLa cells with a non-ionic detergent, in the presence of 1.95 M NaCl. When this lysate is applied toa step sucrose gradient, most of the DNA is recovered as a fast sedimenting material near the bottom of the tube. Nucleoids appear in the electron microscope as compact structures containing most of the DNA bound to a common cage or matrix (unpublished, reference 6). Upon treatment of these nucleoids with a restriction enzyme, only a minute amount of the DNA remains fast sedimenting. When this DNA is further purified

and analyzed in the electron microscope, we found it to be enriched for branched DNA fragments. By three independent criteria we would like to argue that branched DNA fragments were produced by the action of the restriction enzyme on putative HeLa DNA replicative intermediates. First, the highest proportion of these molecules is found in fractions of the gradient that have shifted towards a heavier density indicating that they have incorporated BUdR present during the 2 hr pulse; second, synchronously replicating cultures show a higher proportion of branched structures compared to exponential cultures; and third, inspection of the most predominant of the branched DNA fragments (i.e. forked molecules) shows that two arms of the fork have similar lengths. Assuming that the number of functional origins at the beginning of S phase in HeLa cells is similar to that present in cultured cells of Chinese hamster ovary, which has been calculated to be about 1000 per haploid chromosome,[15] and if we take 10^{12} Daltons as an average molecular weight for HeLa chromosomes, and $7 \cdot 10^{6}$ Daltons for the average DNA fragment obtained after complete EcoR1 digestion (under the conditions of our experiments), then the matrix DNA complex from synchronized cultures is found to have about a 40-fold enrichment for DNA growing forks. Functional origins in DNA from HeLa cells must be very well spaced along the whole chromosome rather than being clustered since multiple branched DNA molecules [Fig. 3(c)] are not commonly found, even if the extent of EcoR1 digestion is such that the average molecular weight DNA fragment obtained is about 10^{7} Daltons.

The matrix-DNA complex that we have isolated seems to have a unique and reproducible polypeptide composition (Fig. 2) similar to that reported by other investigators.[16,17] This composition is unaffected by RNaseA or DNaseI treatment (data not shown) indicating that the integrity of the matrix structure per se is not dependent on the association between the protein scaffold and DNA and/or RNA although there might exist points of strong attachments between them.[17-19] Despite the similarity in the polypeptide profiles of the matrix-DNA complexes from synchronously replicating and exponentially growing cells, we noticed that prior to restriction enzyme digestion there was a distinct difference among them. The former appeared to yield more compact matrix structures as judged by their sedimentation rates and from the amount of nuclear proteins released after restriction enzyme digestion (data not shown). This is consistent with a previous finding that a number of the major proteins retained by the nuclear matrix from mid-S phase cells, after extensive DNaseI digestion, are not present in similar preparations from synchronous, non-replicating, G1 phase cells.[16]

The prominent protein bands present in the matrix DNA complex, viz., the 43K, 49K, 52K and 55K polypeptides, differ from those isolated by Berezney and Coffey[4] from rat liver. This is not unusual in view of the tissue- and species-related differences in nuclear matrix composition observed by other investigators.[18,20,21] The slightly

different size range in the previously reported HeLa nuclear matrix proteins (49K-65K) by Hodge et al.[15] as opposed to 43K-60K in the present report, could be easily explained on the basis of the different gel systems used. Furthermore, the molecular weight estimates from one dimensional gel electrophoresis alone should be interpreted with caution since many of the proteins could be glycoproteins, which would not yield accurate size on SDS-acrylamide gels.[16,22] In view of these uncertainties, the co-migration of actin and tubulin with some of the matrix-DNA complex proteins does not necessarily prove their presence in the matrix complex. However, actin, tubulin and myosin have been detected in nuclear proteins from mouse liver,[23] physarum[24] and in the nuclear matrix from bovine lymphocyte.[25] More recently Nakayasu et al.[26] have also identified the 43K protein in the bovine lymphocyte nuclear matrix as actin on the basis of its amino acid composition and its interaction with DNaseI.

Whether the matrix complex is a passive scaffolding for the nuclear processes or whether it has a functional role, especially in chromatin replication, cannot be answered directly from the results presented in this report. However, the association of the replication, transcription and protein kinase activities with the nuclear matrix in a variety of systems[4,6,20,27,28] seem to suggest that a large number of nuclear processes may be taking place in the vicinity of the matrix. Our data on the enrichment of the matrix-associated DNA with replication forks provides strong support for a functional role of the nuclear matrix in DNA replication. Recently, it has been shown that the DNA polymerase activity is strongly attached to the matrix isolated from regenerating rat liver.[23]

In conclusion, we have shown that due to the striking similarity between bacterial membranes and eucaryotic nuclear matrices, the same approach that we have used to isolate membrane-associated replication intermediates from bacteria[1,2] can, in practice, be extended to eucaryote systems.

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