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Replication forks are associated with the nuclear matrix

J.P.Vaughn, P.A.Dijkwel*, L.H.F.Mullenders[1] and J.L.Hamlin
Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908, USA and [1]Department of Radiation Genetics and Chemical Mutagenesis, Sylvius Laboratoria, State University at Leiden, 2333 AL Leiden, The Netherlands

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ABSTRACT

It has been proposed that DNA in eukaryotic cells is synthesized via replication complexes that are fixed to a proteinaceous nuclear matrix. This model has not been universally accepted because the matrix and its associated DNA are usually prepared under hypertonic conditions that could facilitate non-specific aggregation of macromolecules. We therefore investigated whether different ionic conditions can significantly affect the association of nascent DNA with the nuclear matrix in cultured mammalian cells. Matrices were prepared either by a high salt method or by hypotonic or isotonic LIS extraction. Chromosomal DNA was subsequently removed by digestion with either DNAse I or EcoRI. With all methods of preparation, we found that newly synthesized DNA preferentially partitioned with the nuclear matrix. Furthermore, when the matrix-attached DNA fraction was analyzed by two-dimensional gel electrophoresis, we found that it was markedly enriched for replication forks. We therefore conclude that attachment of DNA to the matrix in the vicinity of replication forks is not induced by conditions of high ionic strength, and that replication may, indeed, occur on or near the skeletal framework provided by the nuclear matrix. From a practical standpoint, our findings suggest a strategy for greatly increasing the sensitivity of two important new gel electrophoretic methods for the direct mapping of replication fork movement through defined chromosomal domains in mammalian cells.

INTRODUCTION

In eukaryotic cells, chromosomal DNA appears to be organized into looped domains by periodic attachment to a proteinaceous nuclear structure that is commonly referred to as the nuclear matrix or scaffold (see Ref. 1 for review). The loops, which are topologically constrained (2,3), are comparable in size to the average eukaryotic replicon (3,4). Both transient and permanent attachment of DNA to the nuclear matrix have been suggested. Transient attachment appears to occur at replication forks, since nascent DNA has been found to be closely associated with the matrix (3,5,6,7). The presence of DNA polymerase α (8), DNA primase (9), and topoisomerase II (10,11) in nuclear matrix preparations further supports this contention. Transient attachment of DNA may also occur during transcription, since nascent RNA is observed to partition with the nuclear matrix (12).

Permanent matrix attachment regions (MARs) may also be involved in different functions. Several lines of evidence suggest that MARs are close to replication initiation sites in mammalian cells (13−16), and in yeast, a subset of autonomously replicating sequence (ARS) elements appear to associate with the matrix (17). A second type of permanent attachment occurs at or near *cis*-acting elements that are involved in the regulation of gene expression. For instance, a MAR was identified directly upstream from the kappa immunoglobulin enhancer in murine cells (18) and in enhancer-like sequences located upstream from three developmentally regulated genes in *Drosophila* (19). Functional diversity of MARs has additionally been suggested to occur within the amplified dihydrofolate reductase (DHFR) domain of the methotrexate-resistant CHO cell line, CHOC 400 (20). In this 270 kb amplicon, permanent attachment sites were identified between two closely spaced replication initiation zones, as well as upstream from the DHFR gene and near a junction between amplified units (20).

The question as to whether MARs actually play functional roles in the cell has been difficult to answer, since matrix isolation procedures differ and investigators do not always agree on the significance of the association between DNA and the nuclear scaffolding. The permanent mode of attachment has been observed in both high salt- and LIS (lithium diiodosalicylate)-extracted matrices (13−17,19−22), arguing that specific MARs may, in fact, play important roles in nuclear metabolism. However, transient attachment of replication forks has essentially been studied only in matrices prepared by the high salt extraction procedure (3,5,6,16). Consequently, it cannot be rigorously excluded that artifactual attachment of the single-stranded regions of replication forks is induced by high ionic strength (although an elegant recent report using cells encapsulated in agarose beads suggests that this is not the case; 7). In the present study, we have therefore asked whether the preferential association of replication forks with the nuclear matrix depends on the isolation procedure employed.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Cell culture and labelling protocols. Diploid normal human fibroblasts were cultured as monolayers on 15 cm plates in Ham's F-10 medium supplemented with 15% fetal calf serum (23). Methotrexate-resistant CHO cells (CHOC 400; 24) were grown as monolayers in Minimal Essential Medium supplemented with non-essential amino acids and 12.5% fetal calf serum. Subconfluent cultures were labelled for 24 hr with 0.01−0.04 uCi/ml ^{14}C-thymidine (50 μCi/mmol) and were then pulse-labelled for 2 min with 25 μCi/ml ^{3}H-thymidine (80 Ci/mmol; New England Nuclear Corp.).

Nuclear matrix isolation

Protocol A: 2 M NaCl extraction. After the pulse, cells were immediately transferred to 0°C, and all subsequent steps up to the addition of nucleases were carried out in the cold. Five ml of Triton X-100 (0.1%) in 10 mM NaCl/25 mM Tris-HCl, pH 8.0 were added to each plate. Nuclei and cytoplasmic debris were scraped from the plate with a plastic policeman and nuclei were liberated by forcing the suspension three times through a 21-gauge hypodermic needle. The nuclei were collected by centrifugation (800×g for 5 min) and were resuspended in 10 mM NaCl/25 mM Tris-HCl, pH 8.0. Histones and soluble nuclear proteins were extracted by the addition of one volume of 4 M NaCl (final vol 10 ml 2 M NaCl/plate), incubation on ice for five min, and centrifugation (20 min at 8,000×g in a Sorvall HB-4 rotor at 4°C). The matrix pellet was either resuspended in DNaseI buffer (100 mM NaCl/5 mM $MgCl_2$/25 mM Tris-HCl, pH 8.0), or in EcoRI buffer (70 mM NaCl/30 mM KCl/10 mM $MgCl_2$/20 mM Tris-HCl, pH 7.4). Aliquots were digested at 37°C with increasing concentrations of DNAseI (0−10 ug/ml) for 10 min, or with 25 U/ml EcoRI for increasing time periods (0−120 min). Digestion was terminated by adding EDTA to 20 mM and transferring the aliquots to ice. Matrix-attached DNA was separated from detached DNA by centrifugation (10 min at 8,000×g in a Sorvall HB-4 rotor at 4°) and the TCA-precipitable radioactivity was determined in both the matrix pellet and in the supernatant.

Protocol B: Hypotonic LIS extraction. Nuclei were isolated as described above and were resuspended in cold 10 mM NaCl/0.05 M spermine/0.25 M spermidine/25 mM Tris-HCl, pH 8.0. Lithium diiodosalicylate (LIS) was added to 25 mM, and the samples were incubated at room temperature for five min. Matrices were collected by centrifugation at room temperature, washed twice with the appropriate cold digestion buffer, and digested either with EcoRI or DNAseI as described above.

Protocol C: Isotonic LIS extraction. After pulse-labelling with ^{3}H-thymidine, cells were washed twice with ice-cold cell wash buffer (CWB; 50 mM KCl/0.5 mM EDTA/0.05 mM spermine/0.125 mM spermidine/0.5% thiodiglycol/5 mM Tris-HCl, pH 7.4). Nuclei were isolated by lysing the cells in cold CWB containing 0.1% digitonin and forcing the suspension three times through a 21-gauge hypodermic needle. Nuclei were collected by centrifugation, washed once in cold CWB/digitonin, and incubated for 20 min at 37°C in CWB/digitonin in which EDTA had been replaced by 0.5 mM $CuSO_4$. The nuclei were extracted at room temperature with 10 mM LIS/100 mM lithium acetate/0.05% digitonin/0.05 mM spermine/0.125 mM spermidine/20 mM HEPES-KOH, pH 7.4. Matrices were collected by centrifugation (20 min at 2,500×g in a Sorvall HB-4 rotor at 21°), washed once with cold EcoRI digestion buffer containing 0.1% digitonin, and twice with EcoRI digestion buffer. Finally, the matrices were resuspended in an appropriate volume of cold EcoRI buffer, and were digested with EcoRI as described above.

Two-dimensional agarose gel electrophoresis. Matrices were isolated by the isotonic LIS extraction procedure (protocol *C*), and were digested to completion with EcoRI. Digestion was terminated by the addition of EDTA to 20 mM and the matrices were placed on ice. Matrix-attached DNA was separated from detached DNA by centrifugation (10 min at 2,500×g in a Sorvall HB-4 rotor at 4°). The matrix pellet was resuspended in cold EcoRI buffer containing 20 mM EDTA. This suspension, as well as one-tenth of the sample containing the detached DNA fraction, were adjusted to 1.0% sarkosine/500 mM NaCl/25 mM EDTA/40 mM Tris-HCl, pH 7.9 (2 ml final volume/15 cm plate of cells). The samples were then digested with 2 mg/ml Proteinase K (American Research Co.) at room temperature for 4 hr. CsCl was added to a final refractive index of 1.4 and centrifugation was performed at 30,000 RPM for 48 hr at 21°C in a Beckman Ti50.2 rotor. The gradients were fractionated and the three or four peak fractions containing the majority of the DNA were pooled and dialyzed for 12 h at 4°C versus 1,000 volumes of 200 mM NaCl/20 mM Tris-HCL, pH 7.9/1 mM EDTA/0.05 mM phenylmethanesulfonyl fluoride. After dialysis, DNA was precipitated by adding two volumes of absolute ethanol and was redissolved in 70 μl of cold 10 mM Tris/0.1 mM EDTA, pH 7.9 (TE). After five min, each sample was adjusted to 200 mM NaCl and was separated by two-dimensional gel electrophoresis essentially by the method of Brewer and Fangman (25). Each sample (equivalent to 3−4 plates of cells) was loaded into one well of a 0.4% agarose gel. The gel was run for 36 hr at room temperature in TBE buffer (89 mM Tris-HCl, pH 8.0/89 mM borate/200 mM EDTA) at 0.3 V/cm. The gel was stained for 30 min in 0.3 ug/ml ethidium bromide dissolved in TBE buffer, and individual lanes (10 cm by 1.5 cm) were excised, turned through a 90° angle, and placed at the top of a 1.0% agarose gel. Electrophoresis in the second dimension was performed for 12 hr at 4°C and 3.5 V/cm in TBE buffer containing 0.3 ug/ml ethidium bromide. The gel was photographed and the DNA was transferred to GeneScreen (New England Nuclear Corp.) by an alkaline blotting technique (26). Membranes were hybridized to appropriate probes as described previously (20).

RESULTS AND DISCUSSION

Since nascent DNA is found to be closely associated with the nuclear matrix, replication has been suggested to occur at the matrix (3,5,6,7). However, it has not been ruled out that some of the methods used to prepare nuclear matrices might induce binding of the single-stranded regions of replication forks to this residual nuclear structure. We therefore prepared matrices by extracting nuclei by several different methods, and assessed the position of newly synthesized DNA relative to the nuclear matrix.

Replicating DNA partitions with matrices prepared by both high salt and hypotonic LIS extraction methods. In the first experiment, exponentially growing human primary fibroblasts (23) were pre-labelled with ^{14}C-thymidine for 24 hr, and were then pulse-labelled with ^{3}H-thymidine for 2 min. Uptake of isotope was rapidly quenched and nuclei were isolated by the addition of ice-

cold hypotonic medium containing 0.1% Triton X-100. Half of the sample was extracted with 2 M NaCl (protocol *A*, Materials and Methods), while the other half was treated with 25 mM LIS under hypotonic conditions (protocol *B*). Matrices were collected by centrifugation and aliquots were digested with increasing concentrations of DNaseI.

When matrices are prepared by the high salt extraction procedure, approximately 60% of the pre-labelled DNA remains attached to the matrix (the rest is lost during subsequent isolation and washing procedures). As increasing amounts of DNA are released from the matrix by the action of DNAseI (i.e., as the amount attached to the matrix decreases), the $^{3}H/^{14}C$ ratio of the matrix-associated DNA progressively increases (Fig. 1). This value goes from slightly above parity to approximately six when all but the last 1% of the pre-labelled DNA is removed. Since the probability of a DNA segment being released from the nuclear matrix by DNAseI is lowest when it is situated close to a matrix attachment site, we conclude that DNA in the process of replicating is located close to or is actually attached to the nuclear matrix under the high salt conditions used to isolate matrices in this experiment. This result agrees with previous studies in our own and other laboratories (3,5,6).

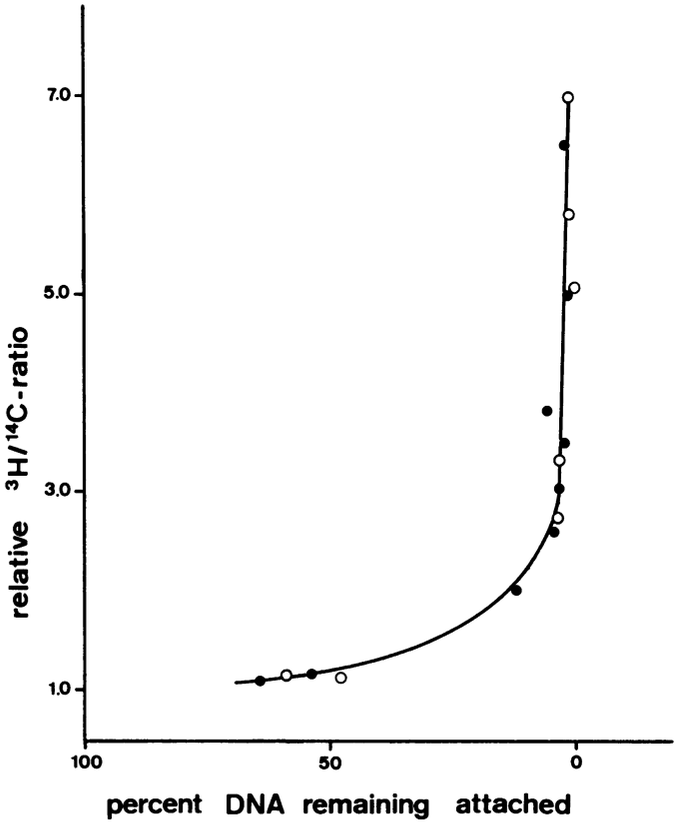

Figure 1. Association of newly synthesized DNA with the nuclear matrix in human fibroblasts. Primary human fibroblasts were prelabelled for 24 hr with ^{14}C-thymidine, and were pulse-labelled with ^{3}H-thymidine for 2 min. Nuclei were isolated, half of the nuclear suspension was extracted with 2 M NaCl (protocol *A*), and the other half was made 25 mM in LIS (protocol *B*). Both preparations were then divided into aliquots and matrices were collected by centrifugation. After incubation with increasing amounts of DNaseI, the aliquots were centrifuged to separate matrix-attached DNA from detached DNA, and TCA-precipitable radioactivity was determined in both fractions. The graph plots the $^{3}H/^{14}C$ ratio of the matrix-attached DNA as a function of the proportion of DNA remaining attached to the matrix. ●---●, matrices extracted with 2 M NaCl (protocol *A*); ○---○, matrices extracted in hypotonic LIS (protocol *B*).

However, when DNAseI was used to progressively detach DNA from matrices that were prepared with LIS under hypotonic conditions, a steady increase in the $^{3}H/^{14}C$ ratio of DNA partitioning with the matrix was also observed (Fig. 1). In fact, the two curves in Fig. 1 are essentially superimposable. This result suggests that replicating DNA is associated in a comparable way with matrices that are prepared either by extraction with high salt or with hypotonic LIS.

Replicating DNA is enriched in the matrix fraction regardless of the enzyme used to remove the DNA loops. In the majority of studies aimed at mapping the location of permanent MARs within a specific chromosomal domain, restriction enzymes have been used to detach DNA from the nuclear matrix. We therefore repeated the experiments described above to assess whether attachment at replication forks would also be observed if a sequence-specific restriction enzyme such as EcoRI were used to remove DNA from the matrices instead of DNAseI, which is more or less sequence-independent. CHOC 400 cells (24) were pre-labelled with ^{14}C-thymidine and were pulse-labelled with ^{3}H-thymidine for two min. Uptake was quenched and nuclei were isolated in ice-cold hypotonic buffer containing 0.1% Triton X-100. Half of the nuclear suspension was extracted with 2 M NaCl (protocol *A*) and the other half with 25 mM LIS under hypotonic conditions (protocol *B*). Matrices were then collected by centrifugation, washed, and digested with EcoRI for various time intervals.

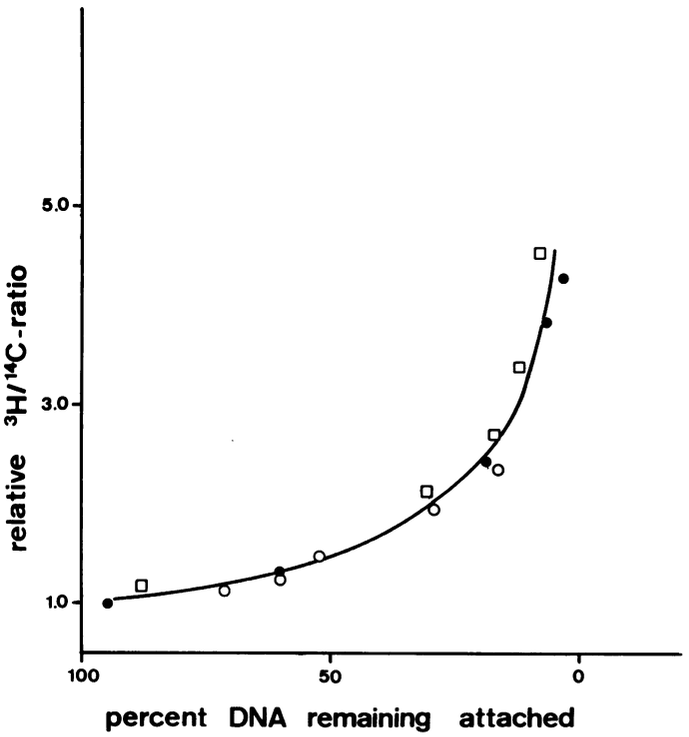

Figure 2. Association of newly synthesized DNA with the nuclear matrix in CHOC 400 cells. Methotrexate-resistant CHO cells (CHOC 400; 24) were labelled and nuclear matrices were isolated by the same two protocols as in Fig. 1. The matrix preparations were digested with EcoRI for increasing periods of time (up to two hr), after which matrix-attached and -detached DNA were separated and TCA-precipitable radioactivity was determined. In one experiment, an alternative LIS-extraction method was used (protocol *C*); nuclei were stabilized at 37°C in a solution containing copper ions, after which they were extracted with 10 mM LIS in 100 mM lithium acetate. ●–●, matrices extracted with 2 M NaCl (protocol A); ○---○, hypotonic 25 mM LIS (protocol B); □---□ isotonic 10 mM LIS (protocol C).

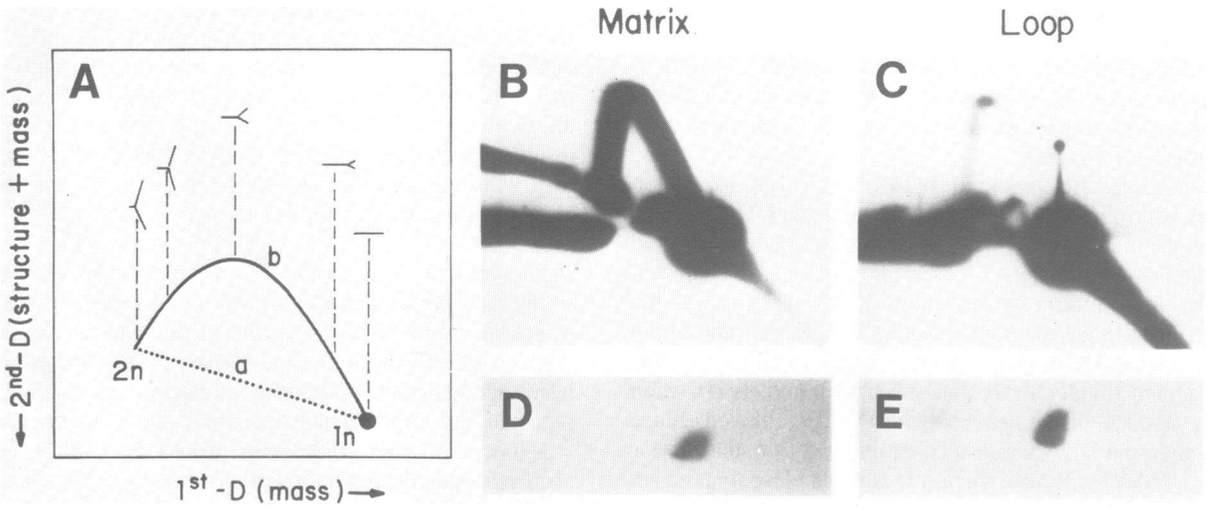

Figure 3. Matrix-associated DNA is highly enriched in replication forks. Matrices were prepared from exponentially growing CHOC 400 cells. After digestion with EcoRI, matrix-attached DNA was separated from detached DNA by centrifugation. Both DNA fractions were purified and subjected to two-dimensional agarose gel electrophoresis, as described by Brewer and Fangman (24). DNA was transferred to a nylon membrane by an alkaline transfer method (26) and the blot was probed for a 5.1 kb fragment located approximately 60 kb downstream from the DHFR gene in the DHFR amplicon. Panel *A*: diagram of the pattern obtained from a two-dimensional gel separation of a fragment that is replicated passively (i.e., does not contain an origin of replication); curve *a* represents the diagonal, of all non-replicating fragments in the genome, and curve *b* represents the simply Y arc obtained from forked structures. *B,D*: matrix-associated DNA. *C,E*: DNA detached from the matrix by EcoRI. Film exposure time for *B* and *C* was seven days, and for *D* and *E*, one hour. Note that the two dark horizontal streaks migrating at greater than 2*n* in the mass dimension are artifacts of this particular gel, and probably represent trailing of the sample in the first dimension.

Fig. 2 shows that with progressive detachment of DNA from the matrices with EcoRI, the relative $^{3}H/^{14}C$ ratio of the DNA in the matrix-associated fraction rises steadily. Again, plots of $^{3}H/^{14}C$ ratio versus the proportion of DNA remaining with the high salt-and LIS-extracted matrices are virtually superimposable. Furthermore, the profiles very much resemble the curves generated in the experiments in which DNaseI was used to remove loop DNA (Fig. 1).

Nascent DNA also partitions preferentially with matrices prepared in isotonic LIS extraction buffer containing copper ion. In the studies above, attachment of nascent DNA to the nuclear scaffolding was observed in matrices prepared with both high salt and hypotonic LIS extraction methods, regardless of the cell line used or the enzyme employed to detach DNA from the nuclear scaffolding. In several recent studies designed to map specific, permanent MARs relative to functional elements in the eukaryotic genome (17,20,22), the following modification of the hypotonic LIS extraction procedure has been employed in an attempt to stabilize the matrices during isolation: nuclei are extracted in 0.1% digitonin instead of Triton X-100, nuclei are stabilized with Cu^{++} for 20 min at 37°C prior to extraction with LIS, and the LIS concentration in the extraction buffer is reduced from 25 mM to 10 mM and 100 mM lithium acetate is added to render the extraction buffer essentially isotonic. We therefore determined whether nascent DNA would also partition with the nuclear matrices if they were prepared by this modified LIS extraction method.

Matrices were prepared from CHOC 400 cells by protocol *C* (Materials and Methods), and the attachment of nascent DNA to the proteinaceous matrix was monitored by digestion with EcoRI. It can be seen in Fig. 2 that in this preparation as well, the progressive detachment of DNA resulted in an increase in the $^{3}H/^{14}C$ ratio of the DNA remaining attached to the matrix. Therefore, unlike the attachment of specific sequences that map near enhancer elements (19,21,22), attachment of DNA to the matrix in the vicinity of the replication fork does not appear to be dependent on prior stabilization.

Two-dimensional gel electrophoretic analysis demonstrates the preferential association of replication forks with the nuclear matrix. The results of the isotopic labelling studies above therefore suggested that nascent DNA is preferentially associated with the nuclear matrix regardless of the procedure used to isolate either the nuclei or the nuclear matrices themselves. To assess the relationship of replicating DNA to the matrix by a different approach, we have utilized a two-dimensional gel electrophoretic method that allows the direct analysis of replication intermediates in any region of the genome for which there are probes (25). This method takes advantage of the altered mobility of branched DNA molecules in agarose gels under certain conditions (Fig. 3A). CHOC 400 cells were used as the source of replication intermediates for this experiment since they contain 1,000 copies of the dihydrofolate reductase locus, facilitating analysis of replication intermediates in this defined chromosomal region.

Matrices were isolated by extracting the nuclei of exponentially growing CHOC 400 cells with isotonic LIS buffer (protocol C). The matrices were digested with EcoRI, and matrix-attached DNA and the detached DNA fraction were purified on CsCl gradients (to eliminate the possibility that fragments might migrate anomalously in agarose gels due to bound protein). Each sample was then subjected to two-dimensional gel electrophoresis according to the method of Brewer and Fangman (25). In this procedure, DNA molecules are separated in the first dimension according to their molecular mass and in the second dimension by a combination of both mass and shape (Fig. 3A). If a region is replicating at the time of sampling, then restriction fragments from that region will contain replication forks. The shape of those with a fork in or near the middle of the fragment will deviate most from a simple linear molecule, and will migrate most slowly

in the second dimension. If the fork is situated closer to an end, deviation from the linear form will be less pronounced and the mobility of the fragment will be less affected. These forked, replicating restriction fragments will migrate in a retarded arc (termed a simple Y arc) above the positions of non-forked molecules of equal mass (Fig. 3A).

When a transfer of matrix-attached CHOC 400 DNA was hybridized with a probe for a 5.1 kb EcoRI fragment located ~60 kb downstream from the promoter of the DHFR gene, an arc of restriction fragments containing replication forks was clearly discernable (Fig. 3B). The arc originated at the site to which non-replicating, linear fragments migrate (the 1*n* spot) and returned to the arc of linears at the 2*n* site. However, when the detached DNA fraction was hybridized with the same probe (Fig. 3C), the arc of replicating intermediates was judged to be ~10-fold less intense (by visual inspection), even though the signal at the 1*n* spot was approximately twice as great as the 1*n* signal in the matrix-attached fraction (compare Figs. 3D and 3E).

This result independently supports the conclusions drawn from the pulse-labelling studies (Figs. 1 and 2) that DNA is associated with the nuclear matrix close to replication forks *in situ*. Since the simple Y arc in the matrix-attached fraction contains fragments that have just begun to replicate (i.e., those whose mass is only slightly greater than 1*n*), it can be inferred that attachment actually has to occur within a few hundred base pairs of the replication fork. Furthermore, since the matrix-attached DNA fraction appears to be enriched ~20 fold in replication intermediates relative to the detached DNA fraction, and since it might be feasible to further enrich the replicating fraction by BND-cellulose chromatography (27), it may be possible to analyze single copy sequences in a complex mammalian genome by the two-dimensional gel electrophoretic methods of Brewer and Fangman (25) and Nawotka and Huberman (28). This would be an important advance, since both methods presently appear to be limited to simpler genomes such as yeast by the amount of DNA that can be loaded into the well of an agarose gel.

It should be pointed out that attachment to the matrix at replication forks implies that every DNA sequence in the genome will be associated transiently with the matrix at some time during the S period. However, some sequences (e.g. enhancers or sequences near origins of replication) may be permanently attached to the matrix throughout the cell cycle (13–16,18–20,22). Consequently, when specific regions of the genome are analyzed for the distribution of MARs in an exponentially growing population of cells, only the permanent attachment sites will be detected, since only a small fraction of any particular sequence will be in the act of replicating at the time of sampling.

Since both replication forks and origins of replication have been shown to be attached, or situated very close, to the nuclear scaffolding (13,14,16), it is possible that both initiation and elongation may proceed at fixed sites in the nucleus, as has been suggested (29,30,31). Since topoisomerase II has been suggested to mediate anchorage of DNA to the nuclear matrix (10,11,18), it is tempting to speculate that this protein may recognize DNA sequences involved in the initiation of DNA replication (17,20). The topoisomerase II/origin complex at an initiation site could then serve as a nucleation center for the formation of a larger multienzyme complex which then carries out the elongation phase of DNA replication (32).

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