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Nucleoskeleton and initiation of DNA replication in metazoan cells

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SUMMARY

To determine whether or not initiation sites for DNA replication in mammalian cells are defined by association with nuclear structure, attachments between the nucleoskeleton and the hamster DHFR gene initiation zone were examined. Nucleoskeletons were prepared by encapsulating cells in agarose and then extracting them with a nonionic detergent in a physiological buffer. The fraction of DNA that remained following endonuclease digestion was resistant to salt, sensitive to Sarkosyl, and essentially unchanged by glutaraldehyde crosslinking. Although newly replicated DNA was preferentially attached to the nucleoskeleton, no specific sequence was preferentially attached within a 65 kb locus containing the DHFR gene, two origins of bi-directional replication and at least one nuclear matrix attachment region. Instead, the entire region went from preferentially unattached to preferentially attached as cells progressed from G_1 to late S-phase. Thus, initiation sites in mammalian chromosomes are not defined by attachments to the nucleoskeleton. To further assess the relationship between the nucleoskeleton and DNA replication, plasmid DNA containing the DHFR initiation region was replicated in a *Xenopus* egg extract. All of the DNA associated with the nucleoskeleton prior to S-phase without preference for a particular sequence and was released upon mitosis. However, about half of this DNA was trapped rather than bound to the nucleoskeleton. Thus, attachments to the nucleoskeleton can form in the absence of either DNA replication or transcription, but if they are required for replication, they are not maintained once replication is completed.

Key words: Nucleoskeleton, Nuclear matrix, Nuclear scaffold, Origin of DNA replication, DNA replication, Mammalian cell, *Xenopus* egg

INTRODUCTION

Nuclear structure has long been implicated as an important factor in replicating eukaryotic genomes. An intact nucleus is generally required to observe initiation of DNA replication in extracts from *Xenopus* eggs (Dimitrova and Gilbert, 1998) human cells (Krude et al., 1997) and yeast cells (Pasero et al., 1997), demonstrating that nuclear structure plays one or more critical roles in regulating eukaryotic DNA replication. One role is regulating the access of replication factors to the DNA substrate. For example, replication licensing factor activity is absent from nuclei during the G_2-phase of the cell cycle, but can be introduced by permeabilizing the nuclei with a detergent and then incubating them in *Xenopus* egg extract (Laskey et al., 1996). Recently, a single round of ORC-dependent DNA replication has been achieved in a *Xenopus* egg extract in the absence of nuclear structure by substituting a concentrated nuclear extract (Walter et al., 1998). This result suggests that the primary role of the nucleus in DNA replication is to concentrate replication factors, and implies that any role for nuclear structure in establishing replication forks or in selecting initiation sites will be facilitative rather than obligatory, because these functions can be achieved in the apparent absence of nuclear structure. In fact, the ability of SV40 chromosomes to complete replication outside the nucleus (Su and DePamphilis, 1976) and the ability to assemble active DNA replication forks from soluble factors (Stillman, 1989) demonstrates that nuclear structure is not required for replication fork activity. Nevertheless, the evidence is compelling that nuclear structure is involved either in the assembly or maintenance of cellular replication forks.

Several studies have shown that newly synthesized DNA is preferentially associated with nuclear structure in the form of ‘nuclear matrix’, ‘nuclear scaffold’ and ‘nucleoskeleton’ (experimental definitions of a network of filaments within the nucleus), and that replication forks are co-localized in ‘replication factories’ or ‘replication foci’ distributed throughout the nucleus (reviewed by Hozák et al., 1996; Laskey and Madine, 1996). Although formation of replication foci does not appear to require specific DNA sequences, it does require a specific protein activity (Yan and Newport, 1995). A functional requirement for replication foci is suggested by the fact that DNA must first be assembled into chromatin, and then into nuclei, before DNA replication can begin in *Xenopus* eggs or egg extracts. If nuclear assembly is prevented by omitting the vesicular fraction from the extract, then the ability of

Xenopus egg extracts to replicate DNA is lost (reviewed by Laskey and Madine, 1996). More specifically, formation of a nuclear lamina is required for DNA replication. Nuclei assembled in lamin-B3 depleted *Xenopus* egg extracts do not assemble a nuclear lamina (a network of filaments underneath the nuclear membrane) and do not replicate DNA (Meier et al., 1991; Newport et al., 1990), but the ability to replicate DNA can be rescued by restoring lamin B3 to the depleted extract (Goldberg et al., 1995). Moreover, perturbation of nuclear lamina organization by introduction of truncated lamin proteins also inhibits DNA replication (Ellis et al., 1997; Spann et al., 1997). Nuclei assembled in the absence of lamin B3 still contain nuclear pores and continue to accumulate a variety of karyophylic proteins, but do not form replication factories (Jenkins et al., 1993). Thus, nuclear lamina is required for DNA replication, because it may be required for correct assembly of a nuclear matrix (Zhang et al., 1996).

Another role suggested for nuclear structure is in establishing initiation sites. This hypothesis is based on the observation that site-specific initiation of DNA replication can be achieved in a frog egg extract if intact nuclei are used as the substrate rather than DNA (Gilbert et al., 1995; Dimitrova and Gilbert, 1998). When either sperm chromatin or DNA is added to *Xenopus* egg extracts, replication is initiated at many sites along the DNA molecule, regardless of whether or not it contains specific prokaryotic or eukaryotic replication origins. However, when intact nuclei are isolated from differentiated mammalian cells in G_1-phase of their cell cycle and then incubated in a *Xenopus* egg extract, DNA replication is initiated at or close to the same replication origins normally utilized by this cell in vivo. Initiation under these conditions does not require either the vesicular fraction or the *Xenopus* origin recognition complex, but does require a nucleus from late G_1-phase cells that has not been permeabilized and a protein kinase activity. Nuclei from early G_1-phase can also initiate DNA replication under these conditions, but initiation occurs 'randomly' throughout the genome. Therefore, establishment of specific initiation sites requires both nuclear structure and a cell cycle dependent event (the 'origin decision point'; Wu and Gilbert, 1996).

The frequent appearance of matrix attachment regions (MAR) and scaffold attachment regions (SAR) at eukaryotic replication origins has led to the hypothesis that nuclear/DNA attachment sites may define chromosomal domains where initiation can occur (Dijkwel and Hamlin, 1995b; Hyrien et al., 1997). These linkages between DNA and nuclear structure are defined operationally as resistant to high salt or strong detergents. However, some studies have suggested that these linkages actually form in vitro as a result of experimental conditions (Jack and Eggert, 1992; Neri et al., 1997), and do not exist in vivo (Eggert and Jack, 1991; Hempel and Stratling, 1996). Therefore, in order to avoid these potential artifacts, we examined the relationship between the 'nucleoskeleton' described by Jackson and Cook (1988) and the ori-β locus (Kobayashi et al., 1998), a well characterized replication origin located ~17 kb downstream of the DHFR gene in Chinese hamster ovary (CHO) cells.

Nucleoskeleton is prepared by encapsulating mammalian cells in agarose and then extracting them with a nonionic detergent in a 'physiological buffer'. The nuclear structure that remains encapsulated in agarose retains replicative DNA polymerase activity (Jackson and Cook, 1986a), and can continue DNA replication and transcription (Jackson and Cook, 1985, 1986b) at rates close to those observed in vivo. Both DNA replication (Hozák et al., 1993, 1994) and transcription (Iborra et al., 1996) occur at morphologically discrete intranuclear 'factories' associated with the nucleoskeleton. Only a fraction of the DNA remains associated with the nucleoskeleton following digestion of chromosomal DNA by endonucleases. This treatment reveals a network of intranuclear filaments approximately 10 nm wide with an axial repeat of 23 nm, characteristic of intermediate filaments (Jackson and Cook, 1988). Lamin A, one of the constituents of intermediate filaments and a component of the nuclear envelope, exists in the interior as well as the periphery of nuclei (Hozák et al., 1995). Since lamin proteins must be present in the nucleus in order to initiate DNA replication and since initiation sites are not restricted to the nuclear periphery, but are distributed throughout the interior of the nucleus, initiation sites for DNA replication may require interaction with intermediate filaments.

Experiments described here show that a fraction of hamster DNA is attached to the nucleoskeleton by salt resistant bonds that are sensitive to Sarkosyl. This fraction is enriched for newly replicated DNA, but no preferential attachments were detected between the nucleoskeleton and either the transcriptionally active DHFR gene that is devoid of replication initiation sites, or origins of bi-directional DNA replication, or MAR sites. The frequency of nucleoskeleton attachment sites increased throughout this locus as cells progressed from G_1 to late S-phase. Plasmid DNA replication in a *Xenopus* egg extract revealed that while nucleoskeleton attachments could form in the absence of either DNA replication or transcription, they were not maintained once replication was complete and were lost completely during mitosis. We conclude that while nucleoskeleton attachments may facilitate either the assembly or activity of replication forks in metazoan nuclei, they do not appear to be involved in the initiation of DNA replication.

MATERIALS AND METHODS

Cell culture and synchronization

Chinese hamster ovary cell lines CHO K1 and CHO C400 were synchronized at the beginning of S-phase by first depriving them of isoleucine for 3 days to arrest them in G_1-phase and then releasing them into normal culture medium containing 10 μg/ml aphidicolin to arrest them as they enter S-phase (Burhans et al., 1990). Alternatively, cells were synchronized in mitosis by incubating them briefly in nocodazole (Gilbert et al., 1995). Mitotic cells were collected, washed free of nocodazole and cultured under normal conditions. Cells in G_1-phase were collected 3 hours after release, and cells in S-phase were collected 12 hours after release. The fraction of cells undergoing DNA synthesis was monitored by incorporation of BrdU followed by staining cells with anti-BrdU antiserum (Gilbert et al., 1995). FACS analysis (Gilbert et al., 1995) confirmed that ~98% of CHO cells were in G_1-phase 3 hours after mitosis, and >90% were in S-phase 12 after mitosis.

Preparation of nucleoskeleton from hamster cells

Nucleoskeletons were prepared as previously described by Jackson et al. (1988). In brief, 50 μl containing 5×10^5 hamster cells in phosphate buffered saline (PBS) was mixed with 12.5 μl of 2.5% low melting agarose at 37°C in PBS (InCert, FMC Inc.) to produce a single 0.5%

agarose plug of 62.5 μl by incubating on ice for 30 minutes. All buffers contained the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pA-PMSF, 5 μg/ml aprotinin (Trasylol), and 1 μg/ml pepstatin (Boehringer-Mannheim). Agarose plugs were then washed for 20 minutes at 4°C in 20 volumes of PBS, then twice in 0.5% Triton X-100 in 'physiological buffer' (130 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM dithiothreitol that was adjusted to pH 7.4 with 100 mM KH_2PO_4), and then twice in physiological buffer only. The washed plugs were incubated with the indicated concentrations of either *Hae*III restriction endonuclease or micrococcal nuclease (MNase) in physiological buffer for 1 hour at 4°C to allow enzyme to diffuse into the plug and then either for 1 hour (*Hae*III) or 0.5 hour (MNase) at 33°C to digest chromatin. MNase reactions included 0.2 mM $CaCl_2$. Plugs were sealed into the slots at the top of 1% agarose gels and subjected to electrophoresis at 2 V/cm at 4°C in physiological buffer containing 40 mM Tris-acetate (pH 7.4) and only 90 mM KCl. Plugs were recovered from the gel and incubated for 10 minutes at 100°C in 0.3 N NaOH in order to melt the agarose and denature the encapsulated DNA. One aliquot was used to quantify the amount of ^{14}C- and ^{3}H-radioactivity in the plug. A second aliquot was transferred to a Zetaprobe membrane using a slot-blot device, washed with 0.5 ml 0.3 M NaOH, dried in vacuum, UV cross-linked (Stratalinker, Stratagene), washed with 2× SSC, baked in vacuum for 30 minutes and stored dry. Blotted DNA samples were then hybridized with sequence specific ^{32}P-DNA probes as described by Gilbert et al. (1995).

Preparation of nucleoskeletons from *Xenopus* egg extract

Plasmid pneoS13 DNA (200 ng) was incubated with 20 μl of *Xenopus* egg extract supplemented with DNA replication reaction mix as described by Gilbert et al. (1995). Agarose plugs were formed and treated as described above by adding 1.5 ml of 0.5% agarose in physiological buffer, and then dispensing 62.5 μl aliquots.

RESULTS

Newly synthesized DNA is preferentially associated with the nucleoskeleton

Previous studies have reported that newly synthesized mammalian DNA is preferentially attached to the nucleoskeleton (Jackson and Cook, 1986b). Since this experiment was done with HeLa cells, a human transformed cell line that undergoes unrestrained proliferation, it was repeated here using CHO cells, a stable cell line that contains a well characterized DNA replication initiation locus. CHO cells were used in later experiments to determine the relationship between an initiation locus and the nucleoskeleton.

Following the protocol of Jackson and Cook (1986b, 1988), exponentially proliferating CHO cells were first incubated with ^{14}C-Thd to uniformly label their DNA, then encapsulated in agarose plugs and briefly incubated with ^{3}H-Thd to pulse-label newly synthesized DNA in vivo. Agarose plugs were incubated with Triton X-100 in the presence of salt, pH and Mg^{2+}ATP levels that approximate normal intracellular conditions in order to permeabilize cells under conditions that preserved gross structure and allowed a continuation of DNA replication and transcription at pre-existing sites (see Introduction). Plugs were then incubated with *Hae*III restriction endonuclease to digest chromosomal DNA and then subjected to electroelution to remove detached DNA fragments. The amount of DNA remaining within the plug (i.e. attached to the nucleoskeleton) was quantified.

In the absence of an endonuclease, all of the ^{14}C and ^{3}H-labeled DNA remained in the agarose plug. Digestion with increasing amounts of *Hae*III released chromatin fragments that migrated in the range of 2 to 20 kb DNA fragments; after treatment with Sarkosyl, they migrated as 0.1 to 2 kb DNA fragments (data not shown). About 70% of the bulk [^{14}C]DNA and 40% of the newly synthesized [^{3}H]DNA were released as the digestion approached completion (Fig. 1A). This preferential attachment of newly synthesized DNA to the nucleoskeleton was eliminated when newly synthesized ^{3}H-DNA was 'chased' into bulk DNA (Fig. 1B). When synchronized cells were used, enrichment for newly synthesized DNA attached to the nucleoskeleton increased from early S-phase cells to exponentially proliferating cells to late S-phase cells (Fig. 1C), suggesting that the frequency of nucleoskeleton attachments to nascent DNA increased during S-phase. When newly synthesized DNA was radio-labeled in

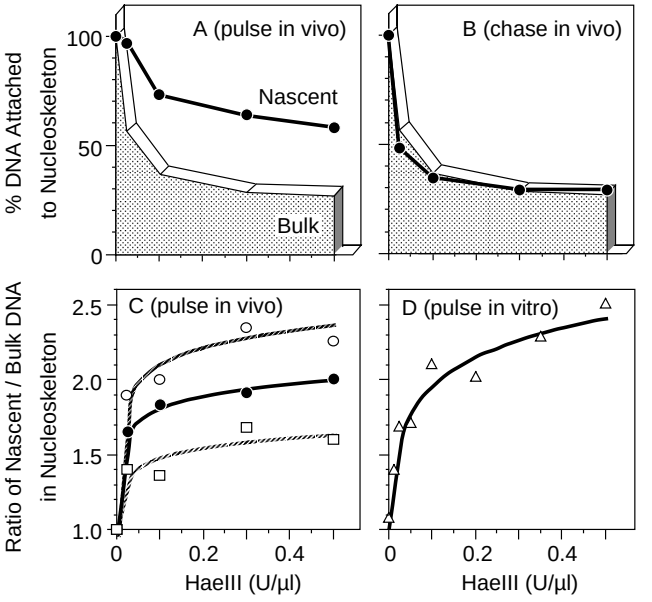

Fig. 1. Attachment to the nucleoskeleton of newly synthesized hamster DNA. (A) Exponentially proliferating CHO cells (50% confluent) were incubated in the presence of [^{14}C]thymidine (0.01 μCi/ml; 60 mCi/mmol) for 20 hours to label bulk DNA. Newly synthesized DNA was radio-labeled by incubating cells encapsulated in agarose plugs in the presence of DMEM supplemented with [^{3}H]thymidine (100 μCi/ml; 50 Ci/mmol) at 37°C for 3 minutes (●). Plugs were then treated with Triton X-100 in a 'physiological' buffer, incubated with *Hae*III restriction endonuclease, and then electroeluted to remove the soluble chromatin. The fraction of [^{14}C]DNA and [^{3}H]DNA remaining in the plug was measured. In the absence of *Hae*III, >99% of all radiolabeled DNA was retained by the plug. (B) Cells were treated the same as in A except that they were washed free of [^{3}H]Thd and then incubated for an additional 1 hour before lysis. (C) The ratio of nascent [^{3}H]DNA to bulk [^{14}C]DNA was determined at each *Hae*III concentration for the cells shown in A (●), and for other cells that were arrested at their G_1/S-phase boundary by aphidicolin and then released into S-phase for 3 hours (□) or 6 hours (○) before pulse-labeling their DNA. (D) Nucleoskeletons were incubated for 3 minutes in a replication cocktail containing 20 μCi/ml [α-^{32}P]TTP (Jackson and Cook, 1986b) before the ratio of nascent [^{32}P]DNA to bulk DNA was determined. In this case, bulk DNA was labeled with 0.5 μCi/ml [^{3}H]Thd to allow complete separation of the two isotopes.

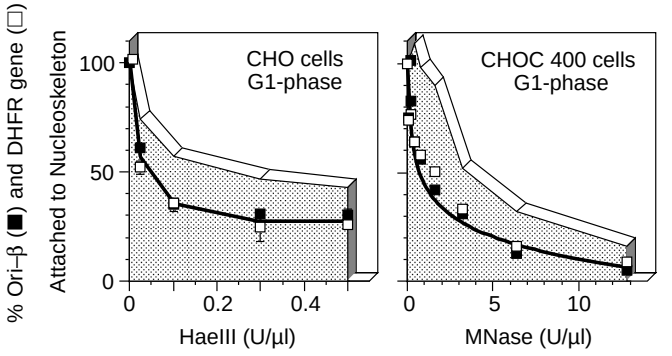

Fig. 2. Attachment to the nucleoskeleton of specific hamster ori-β and DHFR gene sequences as a function of their copy number and the endonuclease used to digest DNA. CHO and CHOC 400 cells whose DNA had been uniformly radio-labeled with [^{14}C]Thd were synchronized in mitosis and then released into G_1-phase. Nucleoskeletons were prepared and incubated with either *Hae*III or MNase. The fraction of DNA remaining in the agarose plug (i.e. attached to the nucleoskeleton) after electroelution was quantified. Bulk [^{14}C]DNA (shaded area) was measured by liquid scintillation counting. The % DHFR gene (□) and % ori-β (■) were measured by blotting-hybridization with probes A and C+D, respectively (see Fig. 4). The amount of DNA remaining in the undigested sample was defined as 100%.

vitro after nucleoskeletons were prepared, it was preferentially attached to the nucleoskeleton (Fig. 1D), confirming that the DNA replication machinery was preserved during nucleoskeleton preparation.

These results were in excellent agreement with those reported by Jackson and Cook (1986b). They observed a 2- to 2.5-fold enrichment of pulse-labeled [^{3}H]DNA in the nucleoskeleton when ~70% of bulk [^{14}C]DNA was released (see Fig. 3b in Jackson and Cook, 1986b) and found that the fraction of newly synthesized DNA attached to the nucleoskeleton was greatest (~4-fold) when labeled for the shortest period of time (0.5 minutes) and digested until ~90% of the bulk DNA was released. We occasionally observed up to a 4-fold enrichment with shorter labeling periods and release of 80% of the bulk DNA (data not shown), but most of our *Hae*III digestions were limited to ~70% release of bulk DNA. The reason for this apparent reduction in the accessibility of *Hae*III sites in hamster nucleoskeletons prepared in agarose plugs is not clear. However, it was not a concern, because later experiments showed that our conclusions were independent of the extent of digestion.

Replication origins are not defined by attachments to the nucleoskeleton

A well characterized initiation zone for DNA replication lies between the DHFR gene and the 2BE2121 gene in CHO cells (see Fig. 4, top) that is active at the beginning of S-phase. Replication bubbles have been detected throughout the initiation zone (Dijkwel and Hamlin, 1995a), although most initiation events in this region occur at the three primary (high frequency) origins of bi-directional replication known as ori-β, β′ and γ (Kobayashi et al., 1998). Initiation events have never been detected within the 25 kb DHFR gene transcription unit. Three MARs have been reported in this locus, one at the 5′-end of the DHFR gene, one within the DHFR gene, and one between ori-β′ and ori-γ (Dijkwel and Hamlin, 1988; Kas and Chasin, 1987). Are specific attachments to the nucleoskeleton involved in defining these initiation sites (see Introduction)?

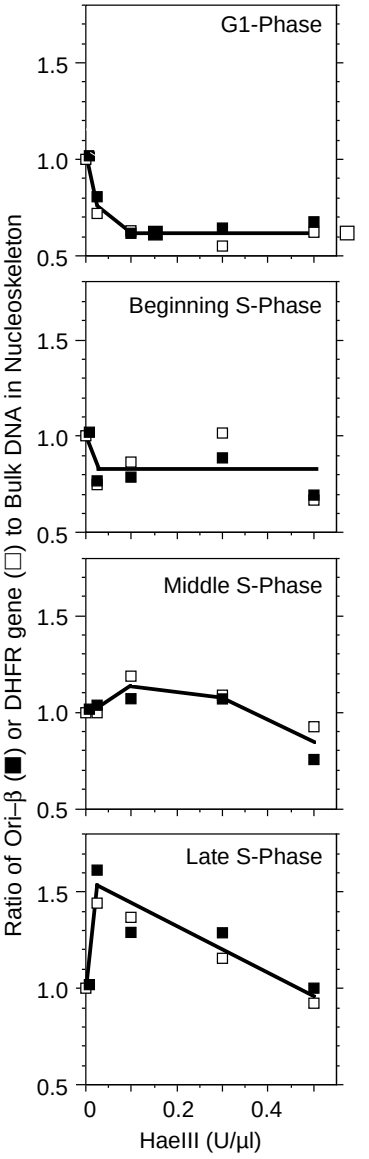

Fig. 3. Attachment to the nucleoskeleton of the hamster ori-β and DHFR gene sequence regions as a function of the cell division cycle. CHO cells were synchronized in mitosis. Nucleoskeletons were prepared at 3 hours (G_1-phase) and 12 hours (6 hours into S-phase; 'Late S-phase') after release from mitosis. A separate pool of cells was also synchronized at their G_1/S-phase boundary and then released into S-phase for either 5 minutes ('Beginning S-phase') or 3 hours ('Middle S-phase') before preparing nucleoskeletons. One aliquot of each sample was used to measure the fraction of bulk DNA that remained in the plug. DNA remaining in the agarose plug (i.e. attached to the nucleoskeleton) was transferred to a membrane and hybridized with probe A (DHFR gene, □), or probe C+D (ori-β, ■) (see Fig. 4). The fraction attached to the nucleoskeleton at each *Hae*III concentration was determined relative to the amount of probe that hybridized to the undigested sample. At each *Hae*III concentration, the ratio of % probe to % bulk DNA in the agarose plug was calculated.

Nucleoskeleton attachments at the DHFR gene locus were investigated in CHO cells that contain a single copy of the DHFR

gene per haploid genome, and in CHOC 400 cells that contain two segments of ~500 tandemly repeated copies of a 230 kb repeat containing the DHFR gene/2BE2121 gene region. Both cell lines were synchronized in mitosis, and nucleoskeletons were prepared from cells in G_1-phase. Sequences containing ori-β were only 60% as frequently attached to the nucleoskeleton relative to bulk DNA, regardless of whether they were in CHO or CHOC 400 cells (Fig. 2). This preferential release of ori-β did not depend on either the endonuclease used to release unattached DNA or the extent of digestion. Micrococcal nuclease (MNase), a nonspecific endonuclease that digests chromatin into nucleosomes containing 100 to 200 bp of DNA, released 80% to 90% of the DNA. Nevertheless, ori-β was still released at about twice the rate as bulk DNA at low as well as high concentrations of either *Hae*III or MNase (Fig. 2). Therefore, in late G_1-phase, origins that will activate at the beginning of S-phase are less frequently associated with nucleoskeleton than bulk DNA. Moreover, the DHFR gene (a region devoid of initiation events) was indistinguishable from ori-β in its relationship to the nucleoskeleton. Therefore, preferential release of DNA from the nucleoskeleton in G_1-phase cells was not specific for replication origins.

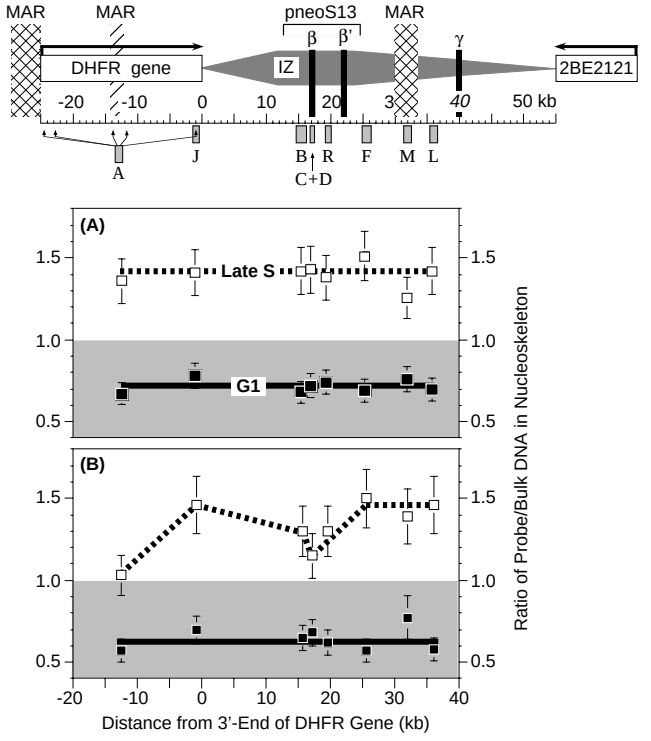

Fig. 4. Attachment to the nucleoskeleton of sequences within and around ori-β. The experiment described in Fig. 3 was carried out with probes A (the six DHFR gene exons), J (includes DHFR gene exon 6), B, C+D (ori-β), R, F, and L (Gilbert et al., 1995). The two MARs detected by Dijkwel and Hamlin (1988) are indicated by cross-hatched bars, and a new probe (M) was constructed from the sequence in cosmid cScZb1/2R that corresponds to the indicated MAR. Two closely spaced MARs detected by Kas and Chasin (1987) are indicated by a single stripped bar. Also indicated are the sequences represented in the plasmid pneoS13 (Gilbert et al., 1995), the DHFR and 2BE2121 gene transcription units, the origins of bi-directional replication at ori-β, ori-β′, and ori-γ (solid vertical bars), and the 55 kb intergenic, initiation zone (IZ) (Kobayashi et al., 1998). The fraction of each of the indicated probes remaining attached to the nucleoskeleton after digestion with *Hae*III divided by the fraction of bulk DNA remaining attached was plotted. (A) The average (± s.e.m.) for 0.025 and 0.1 U/μl *Hae*III in which 40% of the bulk chromatin was digested. (B) The average of 0.3 and 0.5 U/μl *Hae*III in which 60% of the bulk chromatin was digested. G_1-phase cells (■) and late S-phase (□) cells are plotted separately. Shaded area denotes ratios of probe DNA to bulk DNA of less than one (preferentially unattached).

To determine whether or not this relationship endured after DNA replication began, CHO cells were synchronized either at their G_1/S-phase boundary (aphidicolin arrested) or at mitosis and then released into their cell division cycle. Both methods gave the same results. Ori-β and the DHFR gene were indistinguishable in their attachment to the nucleoskeleton; they changed steadily from preferentially released to preferentially retained as cells progressed from G_1 to late S-phase (Fig. 3). Thus, while association between the nucleoskeleton and specific DNA sequences changed during the cell division cycle, origin and non-origin sites behaved the same.

The same DNA samples were also analyzed using six other probes that together with probes A (DHFR gene cDNA) and C+D (ori-β) spanned about 65 kb of DNA and included the DHFR transcription unit, two mapped replication origins, two mapped MARs, and about 65% of the initiation zone (Fig. 4, top). The results with either low (Fig. 4A) or high (Fig. 4B) *Hae*III concentrations gave essentially the same result, suggesting that attachment sites are randomly distributed throughout this genomic region. At high *Hae*III concentrations, where the frequency of cleavage events was greatest, the DHFR coding sequences and ori-β were more easily released, consistent with nucleoskeleton attachments that lie outside of these sequences. Thus, the entire region changed from preferentially unattached to preferentially attached as cells went from G_1-phase to late S-phase, with no obvious preferences for attachments to a transcribed gene, replication origins, or nuclear matrix attachment regions.

Stability of nucleoskeleton attachments

Nucleoskeleton preparations were treated with various reagents to determine the nature of nucleoskeleton attachments and whether or not the stability of these attachments to specific DNA sequences were equivalent. Nucleoskeletons were prepared from proliferating CHOC 400 cells and then treated with MNase. Treatment of these samples with 2 M NaCl did not alter the fraction of bulk [^{14}C]DNA attached to the nucleoskeleton (Fig. 5A, bulk DNA). Therefore, since 2 M salt removes most of the histones from chromatin, attachment of DNA to the nucleoskeleton did not result from an association between histones and the nucleoskeleton. However, the link between DNA and the nucleoskeleton did appear to involve protein:protein interactions, because it could be disrupted by 1% Sarkosyl (Fig. 5A, bulk DNA). Nevertheless, the frequency of these links was increased only marginally by incubation of nucleoskeleton preparations in the presence of the cross-linking reagent, 1% glutaraldehyde (Fig. 5A, bulk DNA). The fraction of bulk DNA remaining in the nucleoskeleton after glutaraldehyde treatment was essentially unchanged relative to untreated samples (Fig. 5B, ± glutaraldehyde). Therefore, since glutaraldehyde crosslinking stabilizes protein-protein interactions during agarose gel electrophoresis (Sedman and

Stenlund, 1995), the fraction of bulk DNA attached to the nucleoskeleton was not under-estimated due to the lability of these attachments during electroelution.

The same DNA samples also were analyzed using probes directed against ori-β, the DHFR gene and the MAR located between ori-β and ori-γ (see Fig. 4, top). All three sequences were preferentially unattached to the nucleoskeleton (Fig. 5A). For example, ~5 times more MNase was required to release 50% of bulk DNA than ori-β DNA (Fig. 5B, Control), regardless of whether or not digested nucleoskeletons were subsequently treated with 2 M salt. Moreover, the ratio of either ori-β, DHFR gene, or MAR sequences to bulk DNA in nucleoskeletons were essentially the same throughout the digestion profile (Fig. 5B, Enrichment). At the highest concentration of MNase, >90% of these sequences were released from the nucleoskeleton at about twice the rate of bulk DNA. As with bulk DNA, high salt did not alter the fraction of each sequence released from the nucleoskeleton, but Sarkosyl dramatically increased it (Fig. 5A). After Sarkosyl treatment, all three probes exhibited the same sensitivity as bulk DNA, a sensitivity only 1.2 times less than bulk DNA (Fig. 5B, + Sarkosyl). However, the frequency of the nucleoskeleton attachment to these sequences could be increased by treatment with glutaraldehyde, but only when most of the chromatin was digested (Fig. 5B, ± glutaraldehyde). This would occur if a few random nucleoskeleton attachments existed throughout this region, preventing release of the larger DNA fragments produced at low MNase levels.

Assembly of the nucleoskeleton in *Xenopus* egg extract

DNA added to *Xenopus* egg extracts is first assembled into chromatin and then into nuclear structures referred to as

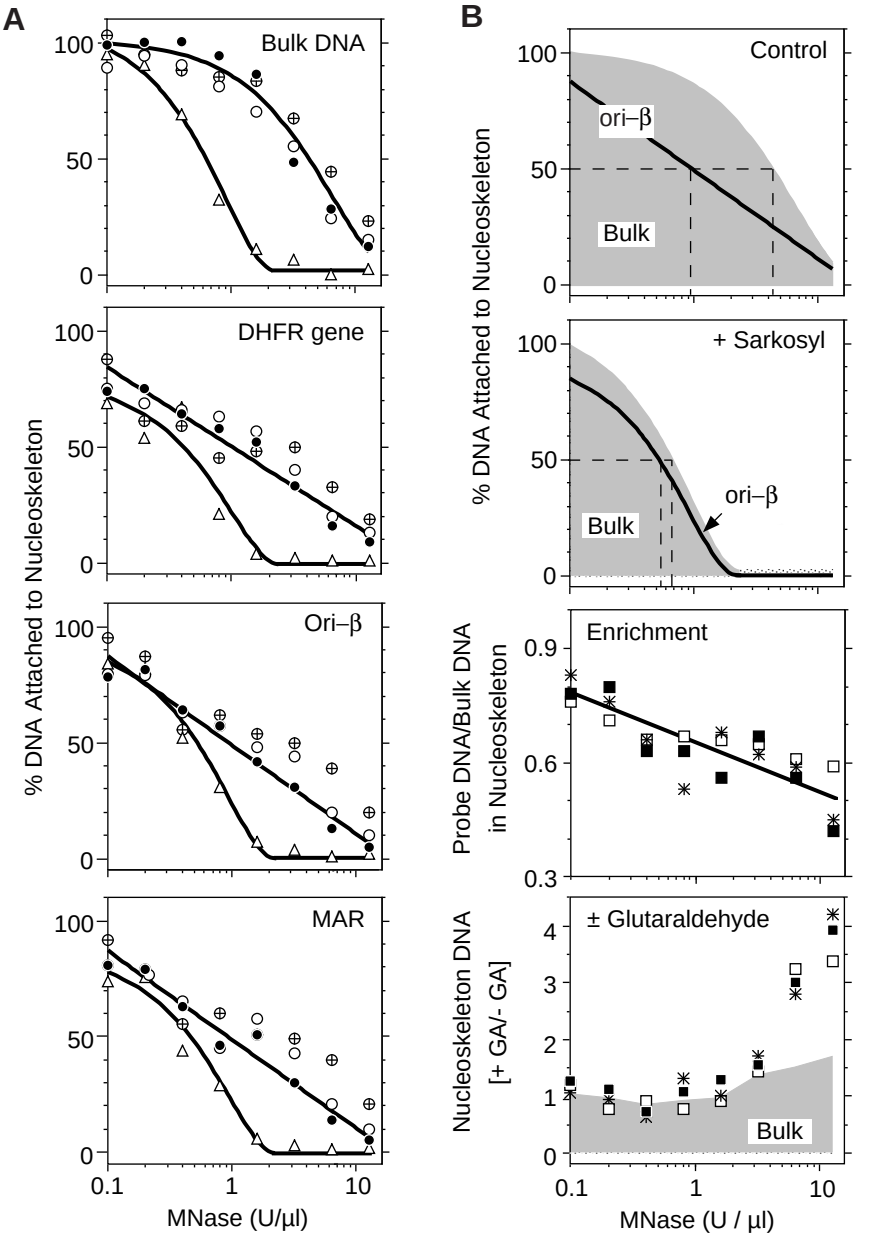

Fig. 5. Effect of various treatments on the release of DNA within and around ori-β.
(A) Nucleoskeletons were prepared from exponentially proliferating CHOC 400 cells and incubated with MNase. The agarose plugs were then incubated for 30 minutes at 4°C in 1 ml physiological buffer (●), or physiological buffer with either 2 M NaCl (○), or 1% Sarkosyl (△), or 1% glutaraldehyde (⊕) before washing plugs in 1 ml physiological buffer and electroeluting DNA. The fraction of each sequence retained in the agarose plug was determined as in Fig. 2. MAR was detected with probe M (Fig. 4). (B) 'Control' compares the average of physiological and 2 M NaCl treated ori-β results (solid line) with those from bulk DNA (shaded area). '+ Sarkosyl' compares Sarkosyl treated ori-β (solid line) with Sarkosyl treated bulk DNA (shaded area). 'Enrichment' shows the amount of ori-β (■), DHFR gene (□) and MAR (✳) released by MNase relative to bulk DNA in samples washed in physiological buffer alone. '± glutaraldehyde' shows the amount of ori-β, DHFR gene, MAR and bulk DNA (shaded area) released after treatment with glutaraldehyde (+GA) relative to the amount released in the absence of glutaraldehyde (-GA). Ratios of +GA/-GA greater than one indicate retention within the nucleoskeleton by glutaraldehyde cross-linking.

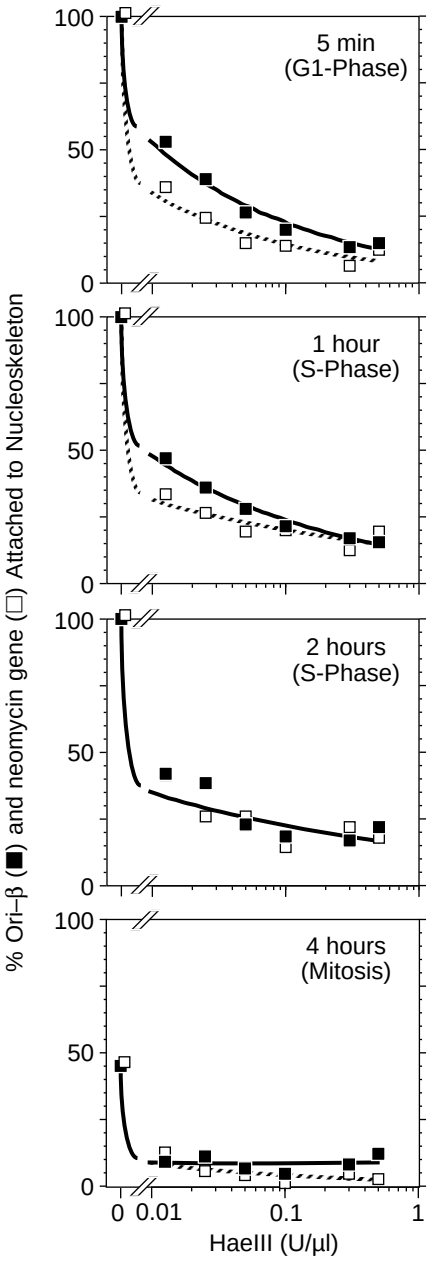

Fig. 6. Attachment of both hamster and bacterial DNA sequences to nucleoskeletons assembled in *Xenopus* egg extract. Plasmid pneoS13 (see Fig. 4, top) was incubated in activated *Xenopus* eggs extract for the time indicated before encapsulating the entire extract in agarose plugs, incubating with Triton X-100 in physiological buffer, digesting with *Hae*III restriction endonuclease, and subjecting the agarose plugs to electroelution. Agarose plugs were then recovered and their contents hybridized either with probe specific for the ori-β (■) present in the hamster DNA insert, or with probe specific for the neomycin gene (□) present in the plasmid vector sequences. The fraction retained in the agarose plug was calculated from the data.

'pseudonuclei' before it undergoes replication (see Introduction). However, pseudonuclei initiate replication at many different sites within the same genomic region, even when the DNA contains the ori-β region (Gilbert et al., 1995). This is in marked contrast to the site-specific initiation of DNA replication observed at ori-β in vivo (Kobayashi et al., 1998), or when late G_1-nuclei are incubated in a *Xenopus* egg extract in vitro (Gilbert et al., 1995; Wu and Gilbert, 1996). One possibility is that pseudonuclei lack a nucleoskeleton. To address this question, pneoS13, an 18 kb plasmid containing a 12 kb *Xho*I fragment encompassing the ori-β OBR (Fig. 4, top), was incubated in *Xenopus* egg extract under conditions that allowed efficient replication of this plasmid (Gilbert et al., 1995). The reaction mixture was then encapsulated in agarose, extracted with Triton X-100 in physiological buffer, digested with *Hae*III restriction endonuclease, and the non-attached DNA electroeluted from the agarose plugs.

DNA synthesis did not begin until 15 to 20 minutes after addition of pneoS13 and replication was essentially completed at between 1 and 2 hours (Gilbert et al., 1995). Yet the same fraction of DNA was retained by the nucleoskeleton in the first 5 minutes as in the first 2 hours (Fig. 6). Therefore, plasmid DNA rapidly attached to the nucleoskeleton prior to the onset of DNA synthesis. Ori-β was attached about 20% more frequently than the plasmid's neomycin gene 6 kb away, suggesting a slight preference for eukaryotic sequences. However, at least half of the DNA was released by digestion at the lowest *Hae*III concentration, suggesting that at least half of the DNA was not actually attached to the nucleoskeleton during the first two hours, but simply trapped within the nucleoskeleton.

To test this hypothesis, pneoS13 was incubated in *Xenopus* egg extract for 1 hour in either the presence or absence of [α-^{32}P]dATP. Nucleoskeletons were prepared, digested with *Hae*III, and electroeluted. Newly synthesized [^{32}P]DNA was detected by autoradiography (Fig. 7A), and total DNA by blotting-hybridization of the unlabeled samples with pneoS13 [^{32}P]DNA (Fig. 7B). Greater than 95% of the plasmid DNA was retained in the agarose plug ('untreated' lanes). Consistent with the effect of Sarkosyl in previous experiments (Fig. 5A), incubation of the agarose plugs with lithium 3,5-diiodosalicylate (LIS), a detergent that strips off histones quantitatively from the genome (Izaurralde et al., 1989), released all of the DNA as either covalently closed, superhelical DNA (Form I) or relaxed circular DNA (Form II, 'LIS' lanes). Incubation of the agarose plugs with *Xho*I restriction endonuclease released about 30% of the newly synthesized DNA and 70% of the total DNA ('*Xho*I' lanes). Surprisingly, most of the released DNA had been cut at only one of the two *Xho*I sites present in the plasmid to produce linear DNA (Form III); a small fraction had been cut at both sites to release the hamster DNA insert. Subsequent incubation with LIS released the remaining plasmid DNA as either Form I or Form II molecules ('*Xho*I, LIS' lanes). These results, together with the *Hae*III digestion profiles (Fig. 6), revealed that only about half of the plasmid DNA was actually bound to nucleoskeleton during the first two hours of incubation. At least half of the DNA was easily released by *Hae*III, and at least half of the DNA was released by a single double strand cut that converted circular molecules into linear molecules. Since most of the plasmid DNA migrated as full length circular or linear molecules, the fraction of replicating intermediates in these gels is small. The [^{32}P]DNA that remains at the top of the gel even after treatment with LIS (Fig. 7A) does not represent DNA remaining in the agarose plug (which was removed from these gels), but most likely represents

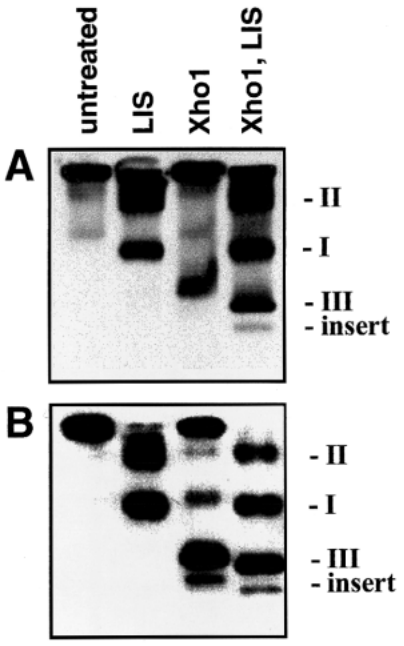

Fig. 7. Requirements for release of plasmid DNA from nucleoskeletons assembled in *Xenopus* egg extract. Plasmid pneoS13 was incubated in activated *Xenopus* egg extracts for 1 hour either in the presence (A) or absence (B) of [α-^{32}P]dATP (Gilbert et al., 1995) before encapsulating the extracts in agarose plugs. Some agarose plugs were incubated with 0.1 U/μl of *Xho*I restriction endonuclease under the conditions used for *Hae*III, and some were incubated with 6 mM lithium 3,5-diiodosalicylate (LIS). Agarose plugs were then electroeluted in the presence of 0.5 μg/ml ethidium bromide. Nascent [^{32}P]DNA (A) was detected by autoradiography, while total DNA (B) was transferred to a Zetaprobe membrane and hybridized with pneoS13 [^{32}P]DNA (Gilbert et al., 1995). pneoS13 DNA and the *Xho*I hamster DNA insert were run in parallel to mark the positions of circular, covalently closed, superhelical plasmid DNA (I), circular relaxed plasmid DNA (II), linear plasmid DNA (III), and the hamster DNA insert (insert). From 12 to 16% of the plasmid DNA had replicated in 1 hour.

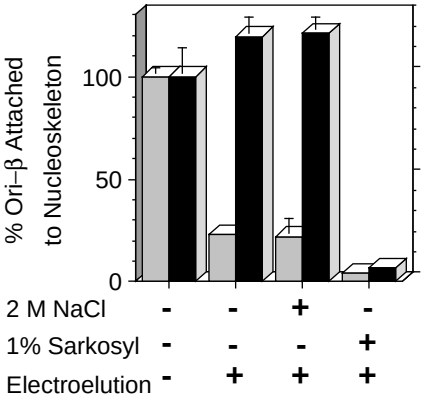

Fig. 8. Attachment to the nucleoskeleton in *Xenopus* egg extract requires formation of nuclei in activated egg extract. Plasmid pneoS13 was incubated for 5 minutes in a *Xenopus* eggs extract that had been either previously activated for 10 minutes (solid bars) by addition of 1 mM $CaCl_2$ or one that had not (speckled bars). Unactivated extracts are arrested in metaphase II. Aliquots of the extracts were then encapsulated in agarose, incubated with Triton X-100 in physiological buffer and then treated with either 2 M NaCl or 1% Sarkosyl prior to electroelution, as in Fig. 5. Agarose plugs were recovered, and their contents hybridized with a ^{32}P-probe for ori-β.

concatenated molecules or rolling circle replication products that commonly appear during in vitro plasmid DNA replication. They constitute a minor fraction of the total DNA, because they are absent in B. Thus, attachments to the nucleoskeleton can form in the absence of DNA replication, but if they are required for replication, they are not maintained once replication is completed.

By 4 hours of incubation, many of the nuclei had undergone mitosis. At least 50% of the DNA was electroeluted from the agarose plug without *Hae*III digestion, and the remainder was rapidly released with minimum *Hae*III digestion (Fig. 6, 4 hours). Therefore, a nuclear structure apparently is required to retain plasmid DNA inside the agarose plug. This hypothesis was tested by preparing extract from unfertilized *Xenopus* eggs in the absence of calcium. Since these cells are arrested in metaphase II, they do not assemble nuclei when DNA is added, but they can be activated to initiate DNA replication by addition of calcium (Lawlis et al., 1996). When pneoS13 DNA was incubated for 5 minutes in an unactivated, mitotic *Xenopus* egg extract, and then encapsulated in agarose and treated with Triton X-100 in physiological buffer, at least 80% of the DNA could be electroeluted (Fig. 8). In contrast, when the same DNA was added to a Ca^{2+} activated *Xenopus* egg extract, it was retained by the nucleoskeleton. Release of plasmid DNA from the nucleoskeleton was not facilitated by 2 M salt, but was completely released by 1% Sarkosyl (Fig. 8), characteristic of cellular DNA attached to the nucleoskeleton (Fig. 5). Thus, nucleoskeleton attachments are also lost when mitosis occurs.

DISCUSSION

Nucleoskeleton and replication forks

The ability of replicating SV40 chromosomes to complete replication outside the nucleus (Su and DePamphilis, 1976), and the ability to assemble active DNA replication forks from soluble factors (Stillman, 1989) demonstrates that attachment to a nuclear structure is not required either for replication fork activity or for separation of sibling molecules when two replication forks meet. Nevertheless, attachments to the nucleoskeleton may facilitate replication either by facilitating the assembly of replication forks, the coordination of bi-directional replication, or the rate of fork progression. Our observations on the attachment of newly synthesized DNA to the nucleoskeleton are in agreement with those of Jackson and Cook (1986b): mammalian replication forks are preferentially attached to the nucleoskeleton. These and similar results with nuclear matrix preparations (discussed by Jackson and Cook, 1986b) support a model in which the sites where DNA synthesis occurs are attached to the nucleoskeleton, and the DNA template is spooled through it. The feasibility of such a model has been demonstrated with the SV40 T-antigen DNA helicase (Wessel et al., 1992).

Nucleoskeleton and replication origins

DNA replication begins at specific genomic loci in the cells of

adult flies, frogs and mammals (DePamphilis, 1996, 1998; Kobayashi et al., 1998). These replication origins consist of one or more high frequency initiation sites and perhaps several low frequency ones. Distinguishing individual initiation sites depends on the limits of resolution and quantification inherent in the assay used. One way in which nuclear structure could define initiation sites is through specific attachments to DNA within or around a replication origin. In fact, 'matrix attachment regions' (MAR) or 'scaffold attachment regions' (SAR) are frequently associated with eukaryotic replication origins (Dijkwel and Hamlin, 1995b; Maric and Hyrien, 1998). MARs are experimentally defined as sequences that remain insoluble after nuclei are extracted with 2 M NaCl and then digested with endonucleases. SARs are defined as DNA sequences that remain insoluble after nuclei are 'stabilized' with $CuSO_4$ at 37°C, extracted with LIS and then digested with endonucleases. Both definitions appear to identify the same sites; a series of short A tracts that can bind specific proteins such as topoisomerase II (discussed by Strick and Laemmli, 1995). However, the significance of these attachments to initiation of DNA replication is questionable. For exmple, selection of specific MAR/SAR DNA binding sites cannot account for the appearance of specific DNA replication initiation sites following the midblastula transition in *Xenopus* development (Maric and Hyrien, 1998). We conclude from the results presented here that replication origins are not defined by specific attachments to the nucleoskeleton. Although newly replicated DNA was preferentially attached to the nucleoskeleton, no specific sequence was preferentially attached within a 65 kb locus containing the DHFR gene, two origins of bi-directional replication and at least one nuclear matrix attachment region. Instead, the entire region went from preferentially unattached to preferentially attached as cells progressed from G_1 to late S-phase.

In G_1-phase cells, all of the sites examined were attached only 60% to 70% as frequently as bulk DNA, regardless of whether the extent of *Hae*III digestion was 40% (Fig. 4A) or 60% (Fig. 4B). As cells progressed from G_1 to late S-phase, the frequency of attachment increased (Fig. 3) until by late S-phase, the same sites were attached 140% to 150% compared to bulk DNA (Fig. 4A). However, with more extensive digestion ori-β and the DHFR gene were released more easily (Figs 3, 4B, late S-phase). Therefore, attachment sites are located at some distance from these sites, because they were released after multiple *Hae*III restriction sites were cleaved. To circumvent the potential problem that all *Hae*III restriction sites may not be equally accessible to *Hae*III endonuclease (Tack et al., 1981), a similar experiment was carried out using the non-sequence specific MNase. Again, no difference was detected in the frequency of nucleoskeleton attachment to these three sites during digestion (Figs 2, 5B). MNase digested hamster chromatin to ~90% completion at which point the DHFR gene, ori-β and MAR sites were each attached only 30% as frequently as bulk DNA. The absence of site specific nucleoskeleton attachments did not result from their dissociation during electroelution, because treatment of nucleoskeletons with glutaraldehyde increased retention of the DHFR gene, ori-β, and the MAR to the same extent as chromatin was digested into nucleosomes (Fig. 5B). Glutaraldehyde cross-linking has been shown to stabilize protein-protein interactions during agarose gel electrophoresis (Sedman and Stenlund, 1995). Thus, the DHFR gene (a region in which initiation events have never been detected either by 2-D gel electrophoresis analysis of replication bubbles and forks or by analyses of newly synthesized DNA strands (DePamphilis, 1996; Kobayashi et al., 1998) and ori-β (the highest frequency initiation site in this region) were indistinguishable in their relationship to the nucleoskeleton.

The absence of preferential attachment sites appeared to extend over at least a 65 kb region, because all eight probes behaved in a similar manner, and probe A included all six DHFR gene exons. Moreover, chromatin fragments that remain attached to HeLa nucleoskeleton after 60% *Hae*III digestion are 5 to 125 kb and those remaining after 90% digestion are 5 to 20 kb (Jackson et al., 1990). In contrast, 90% digestion by MNase routinely reduces chromatin to mononucleosomes (140 bp) by randomly cutting the internucleosomal regions. Thus, no preferential attachments to the nucleoskeleton were detected over a broad range of DNA digestion products. This would occur if a small number of nucleoskeleton attachments were randomly distributed throughout this genomic region, as has been suggested for MAR sites (Basler et al., 1981). The frequency of such attachments in HeLa cells is once every 80 to 90 kb (Jackson et al., 1990), or about one attachment per DHFR initiation locus.

Nucleoskeleton and matrix attachment regions

The MAR reported between ori-β and ori-γ (probe M) and the MAR reported within the DHFR gene (probe A) were attached to the nucleoskeleton with the same frequency as non-MAR sites, consistent with previous reports that MARs in chicken and *Drosophila* cells are not preferentially retained by the nucleoskeleton (Eggert and Jack, 1991; Hempel and Stratling, 1996). We also found that the constitutive MAR adjacent to the tissue-specific enhancer of the mouse IgG kappa gene (Cockerill and Garrard, 1986) was not preferentially attached to the nucleoskeleton (data not shown). Therefore, DNA attachments to the nucleoskeleton do not occur specifically at MARs, even though both attachments are similar in their resistance to salt and sensitivity to Sarkosyl. The only demonstrable function of MARs has been their ability to stimulate promoter activities (Jenuwein et al., 1997), and there is no evidence that this stimulation is related to their ability to bind nuclear matrix. Apparently, attachment to MARs is strongly dependent on experimental conditions. This dependence is evident in the hamster DHFR gene region where two independent investigations led to identification of different MARs (Fig. 4; Dijkwel and Hamlin, 1988; Kas and Chasin, 1987).

Nucleoskeleton and the cell division cycle

Nucleoskeleton attachments also exist prior to DNA synthesis in hamster cells and the frequency of these attachments in the DHFR gene locus increased about 3-fold as cells progressed from G_1-phase to late S-phase. This likely resulted from an increase in the number of replication forks and transcripts in this region, both of which are preferentially attached to nucleoskeleton. DNA replication begins in a fraction of the DHFR initiation zones during the first 3 hours of S-phase while the remaining cells replicate this region passively throughout S-phase (Dijkwel and Hamlin, 1995a), and the rate of DHFR gene transcription increases at the beginning of S-phase (Farham and Schimke, 1985).

Analysis of plasmid DNA replication in *Xenopus* egg extract confirmed that attachments to the nucleoskeleton do not require DNA replication, but form as soon as nuclei are assembled. Since *Xenopus* eggs are transcriptionally inactive, these nucleoskeleton attachments do not result from formation of transcription foci. Little, if any, sequence specificity was detected in this process. However, if all the molecules associated with the nucleoskeleton, then these attachments were not maintained once plasmid DNA molecules had completed replication, because at least half of the full length molecules were not attached to the nucleoskeleton (Fig. 7). Furthermore, all nucleoskeleton attachments were lost once nuclei underwent mitosis (Figs 7, 8). This implies that new nucleoskeleton attachments must be assembled during each G_1-phase of the cell cycle. Whether or not these attachments are required for initiation of DNA replication remains to be determined. Nevertheless, the appearance of a large fraction of unattached DNA before and after replication is completed suggests that initiation of replication does not require attachment to the nucleoskeleton, in which case, replication forks would attach to the nucleoskeleton at some point after DNA synthesis begins. This hypothesis is consistent with the recent report that DNA replication can occur in the absence of nuclear structure if it is replaced by a concentrated nuclear extract and *Xenopus* egg cytosol (Walter et al., 1998). The explanation for why an intact nuclear structure is required for site specific initiation of DNA replication when G_1-nuclei are incubated in a *Xenopus* egg extract also remains to be determined (Dimitrova and Gilbert, 1998).

REFERENCES

Basler, J., Hastie, N. D., Pietras, D., Matsui, S. I., Sandberg, A. A. and Berezney, R. (1981). Hybridization of nuclear matrix attached deoxyribonucleic acid fragments. *Biochemistry* **20**, 6921-6929.

Burhans, W. C., Vassilev, L. T., Caddle, M. S., Heintz, N. H. and DePamphilis, M. L. (1990). Identification of an origin of bidirectional DNA replication in mammalian chromosomes. *Cell* **62**, 955-965.

Cockerill, P. N. and Garrard, W. T. (1986). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**, 273-282.

DePamphilis, M. L. (1996). Origins of DNA replication. In *DNA Replication in Eukaryotic Cells* (ed. M. L. DePamphilis), pp. 45-86. Cold Spring Harbor Laboratory Press, New York.

DePamphilis, M. L. (1999). Replication origins in metazoan chromosomes: fact or fiction? *BioEssays* (in press).

Dijkwel, P. A. and Hamlin, J. L. (1988). Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. *Mol. Cell Biol.* **8**, 5398-5409.

Dijkwel, P. A. and Hamlin, J. L. (1995a). The Chinese hamster dihydrofolate reductase origin consists of multiple potential nascent-strand start sites. *Mol. Cell Biol.* **15**, 3023-3031.

Dijkwel, P. A. and Hamlin, J. L. (1995b). Origins of replication and the nuclear matrix: the DHFR domain as a paradigm. *Int. Rev. Cytol.* **162A**, 455-484.

Dimitrova, D. S. and Gilbert, D. M. (1998). Regulation of mammalian replication origin usage in Xenopus egg extract. *J. Cell Sci.* **111**, 2989-2998.

Eggert, H. and Jack, R. S. (1991). An ectopic copy of the Drosophila ftz associated SAR neither reorganizes local chromatin structure nor hinders elution of a chromatin fragment from isolated nuclei. *EMBO J.* **10**, 1237-1243.

Ellis, D. J., Jenkins, H., Whitfield, W. G. and Hutchison, C. J. (1997). GST-lamin fusion proteins act as dominant negative mutants in Xenopus egg extract and reveal the function of the lamina in DNA replication. *J. Cell Sci.* **110**, 2507-2518.

Farnham, P. J. and Schimke, R. T. (1985). Transcription regulation of mouse DHFR in the cell cycle. *J. Biol. Chem.* **260**, 7675-7680.

Gilbert, D. M., Miyazawa, H. and DePamphilis, M. L. (1995). Site-specific initiation of DNA replication in Xenopus egg extract requires nuclear structure. *Mol. Cell Biol.* **1555**, 2942-2954.

Goldberg, M., Jenkins, H., Allen, T., Whitfield, W. G. and Hutchison, C. J. (1995). Xenopus lamin B3 has a direct role in the assembly of a replication competent nucleus: evidence from cell-free egg extracts. *J. Cell Sci.* **108**, 3451-3461.

Hempel, K. and Stratling, W. H. (1996). The chicken lysozyme gene 5′ MAR and the Drosophila histone SAR are electroelutable from encapsulated and digested nuclei. *J. Cell Sci.* **109**, 1459-1469.

Hozák, P., Hassan, A. B., Jackson, D. A. and Cook, P. R. (1993). Visualization of replication factories attached to nucleoskeleton. *Cell* **73**, 361-373.

Hozák, P., Jackson, D. A. and Cook, P. R. (1994). Replication factories and nuclear bodies: the ultrastructural characterization of replication sites during the cell cycle. *J. Cell Sci.* **107**, 2191-2202.

Hozák, P., Sasseville, A. M., Raymond, Y. and Cook, P. R. (1995). Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. *J. Cell Sci.* **108**, 635-644.

Hozák, P., Jackson, D. A. and Cook, P. R. (1996). Role of nuclear structure in DNA replication. In *Eukaryotic DNA Replication. Frontiers in Molecular Biology*, vol. 15 (ed. J. J. Blow), pp. 124-142. IRL Press, Oxford.

Hyrien, O., Maric, C. and Lucas, I. (1997). Role of nuclear architecture in the initiation of eukaryotic DNA replication. *Biochimie* **79**, 541-548.

Iborra, F. J., Pombo, A., Jackson, D. A. and Cook, P. R. (1996). Active RNA polymerases are localized within discrete transcription 'factories' in human nuclei. *J. Cell Sci.* **109**, 1427-1436.

Izaurralde, E., Kas, E. and Laemmli, U. K. (1989). Highly preferential nucleation of histone H1 assembly on scaffold-associated regions. *J. Mol. Biol.* **210**, 573-585.

Jack, R. S. and Eggert, H. (1992). The elusive nuclear matrix. *Eur. J. Biochem.* **209**, 503-509.

Jackson, D. A. and Cook, P. R. (1985). Transcription occurs at a nucleoskeleton. *EMBO J.* **4**, 919-925.

Jackson, D. A. and Cook, P. R. (1986a). Different populations of DNA polymerase alpha in HeLa cells. *J. Mol. Biol.* **192**, 77-86.

Jackson, D. A. and Cook, P. R. (1986b). Replication occurs at a nucleoskeleton. *EMBO J.* **5**, 1403-1410.

Jackson, D. A. and Cook, P. R. (1988). Visualization of a filamentous nucleoskeleton with a 23 nm axial repeat. *EMBO J.* **7**, 3667-3677.

Jackson, D. A., Yuan, J. and Cook, P. R. (1988). A gentle method for preparing cyto- and nucleo-skeletons and associated chromatin. *J. Cell Sci.* **90**, 365-378.

Jackson, D. A., Dickinson, P. and Cook, P. R. (1990). Attachment of DNA to the nucleoskeleton of HeLa cells examined using physiological conditons. *Nucl. Acids Res.* **18**, 4385-4393.

Jenkins, H., Holman, T., Lyon, C., Lane, B., Stick, R. and Hutchison, C. (1993). Nuclei that lack a lamina accumulate karyophilic proteins and assemble a nuclear matrix. *J. Cell Sci.* **106**, 275-285.

Jenuwein, T., Forrester, W. C., Fernandez-Herrero, L. A., Laible, G., Dull, M. and Grosschedl, R. (1997). Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature* **385**, 269-272.

Kas, E. and Chasin, L. A. (1987). Anchorage of the Chinese hamster dihydrofolate reductase gene to the nuclear scaffold occurs in an intragenic region. *J. Mol. Biol.* **198**, 677-692.

Kobayashi, T., Rein, T. and DePamphilis, M. L. (1998). Identification of primary initiation sites for DNA replication in the hamster dihydrofolate reductase gene initiation zone. *Mol. Cell Biol.* **18**, 3266-3277.

Krude, T., Jackman, M., Pines, J. and Laskey, R. A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* **88**, 109-119.

Laskey, R. and Madine, M. (1996). Roles of nuclear structure in DNA replication. In *DNA Replication in Eukaryotic Cells* (ed. M. L. DePamphilis), pp. 119-130. Cold Spring Harbor Laboratory Press, New York.

Laskey, R. A., Gorlich, D., Madine, M. A., Makkerh, J. P. S. and Romanowski, P. (1996). Regulatory roles of the nuclear envelope. *Exp. Cell Res.* **229**, 204-211.

Lawlis, S. J., Keezer, S. M., Wu, J.-R. and Gilbert, D. M. (1996). Chromosome architecture can dictate site-specific initiation of DNA replication in Xenopus egg extracts. *J. Cell Biol.* **135**, 1207-1218.

Maric, C. and Hyrien, O. (1998). Remodeling of chromatin loops does not account for specification of replication origins during Xenopus development. *Chromosoma* **107**, 155-165.

Meier, J., Campbell, K. H., Ford, C. C., Stick, R. and Hutchison, C. J. (1991). The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of Xenopus eggs. *J. Cell Sci.* **98**, 271-279.

Neri, L. M., Fackelmayer, F. O., Zweyer, M., Kohwi-Shigematsu, T. and Martelli, A. M. (1997). Subnuclear localization of S/MAR-binding proteins is differently affected by in vitro stabilization with heat or Cu2+. *Chromosoma* **106**, 81-93.

Newport, J. W., Wilson, K. L. and Dunphy, W. G. (1990). A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* **111**, 2247-2259.

Pasero, P., Braguglia, D. and Gasser, S. M. (1997). ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei. *Genes Dev.* **11**, 1504-1518.

Sedman, J. and Stenlund, A. (1995). Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro. *EMBO J.* **14**, 6218-6228.

Spann, T. P., Moir, R. D., Goldman, A. E., Stick, R. and Goldman, R. D. (1997). Disruption of nuclear lamin organization alters the distribution of replication factors and inhibits DNA synthesis. *J. Cell Biol.* **136**, 1201-1212.

Stillman, B. (1989). Initiation of eukaryotic DNA replication in vitro. *Annu. Rev. Cell Biol.* **5**, 197-245.

Strick, R. and Laemmli, U. K. (1995). SARs are cis DNA elements of chromosome dynamics: synthesis of a SAR repressor protein. *Cell* **83**, 1137-1148.

Su, R. T. and DePamphilis, M. L. (1976). In vitro replication of simian virus 40 DNA in a nucleoprotein complex. *Proc. Nat. Acad. Sci. USA* **73**, 3466-3470.

Tack, L. C., Wassarman, P. M. and DePamphilis, M. L. (1981). Chromatin assembly: relationship of chromatin structure to DNA sequence during SV40 replication, *J. Biol. Chem.* **256**, 8821-8828.

Walter, J., Sun, L. and Newport, J. (1998). Regulated chromosomal DNA replication in the absence of a nucleus. *Molecular Cell* **1**, 519-529.

Wessel, R., Schweizer, J. and Stahl, H. (1992). Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. *J. Virol.* **66**, 804-815.

Wu, J. R. and Gilbert, D. M. (1996). A distinct G_1 step required to specify the Chinese hamster DHFR replication origin. *Science* **271**, 1270-1272.

Yan, H. and Newport, J. (1995). FFA-1, a protein that promotes the formation of replication centers within nuclei. *Science* **269**, 1883-1885.

Zhang, C., Jenkins, H., Goldberg, M. W., Allen, T. D. and Hutchison, C. J. (1996). Nuclear lamina and nuclear matrix organization in sperm pronuclei assembled in Xenopus egg extract. *J. Cell Sci.* **109**, 2275-2286.