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Termination Sites of Replication Are Anchored to the Nuclear Matrix during S Phase in Mouse LP1-1 Cells

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The association of replication origins/termini with nuclear matrix during S phase was investigated by DNase digestion of halo structures in synchronized mouse LP1-1 cells. The binding of parental DNA to nuclear matrix was constant throughout S phase. When nuclear matrix was isolated from the cells pulse-labeled with ^{3}H-thymidine at various stages of S phase, total ^{3}H-labels associated with nuclear matrix were specifically higher at S_0, S_4 and S_8 stages than other stages of S phase, suggesting that the newly synthesized DNAs at those stages are not excluded out of nuclear matrix. Similar patterns were observed from the pulse-chase experiments, in which cells were pulse-labeled at each stage of S phase and further incubated for 1 hr. These results suggest that the replication origins and termini are fixed at the nuclear matrix, and that the nuclear matrix binding fractions of DNA at 3C-pause may contain a large population of replication origins and termination sites.

KEY WORDS: Termination site of replication, replication origin, nuclear matrix

The DNA replicating S phase in varoius cell lines exhibits two waves of DNA synthesis rate, termed 'early' and 'late' DNA replication. The period between the two peaks of DNA replication, the 3C-pause, has been suggested to the time at which dissociation of replication machinery bound to 'early DNA' and subsequent translocation to the 'late DNA' occur (Holmquist *et al.*, 1982; Foster and Collins, 1985). Activities of DNA polymerase and DNA polymerase α-DNA primase complex at nuclear matrix during S-phase also showed the biphasic nature similar to DNA synthesis rate (Foster and Collins, 1985; Collins and Chu, 1987, Lee *et al.*, 1990).

Current evidences indicate that multienzyme complex tightly associated with nuclear matrix can be a structural and functional unit for nuclear processes, such as transcription and DNA replication (Jackson and Cook, 1985; Kornberg, 1988; Pardoll *et al.*, 1980; Vander Velden and Wanka, 1987). The supercoiled DNA loops as structures of arranged bulky DNA molecules, also, are attached to the nuclear matrix by specific sequences of the loops (Nelson *et al.*, 1986). The supercoiled loops are reeled through nuclear matrix-bound replication complexes in order to be duplicated (Carri *et al.*, 1986; Pardoll *et al.*, 1980).

The specific attachment site of the loop to nuclear matrix might consist of the origin or origin-related sequences (Amati and Gasser, 1988; Goldberg *et al.*, 1983). Carri *et al.* (1986) proposed that the replication origins bind permanently to the nuclear matrix during cell cycle and that replicon might be a double-looped structure of which origin and terminus bind to

nuclear matrix. However, it is not still clear whether the termini of replication units bind to nuclear matrix. We tested whether the termini of replication as well as the origin act as permanent anchorage of the loops, and we analyzed the relationship between the DNA fragments synthesized at 3C-pause and nuclear matrix. For these purposes we analyzed primarily the DNA synthetic pattern of S phase in LP1-1 cells. To elucidate the relationship between the origins/termini of replication and nuclear matrix, cells were pulse-labeled or pulse-chased at various stages of S phase, the halo structures were isolated and digested with DNase I and radioactivities associated with nuclear matrix were measured.

Materials and Methods

1. Cell culture and synchronization

Mouse LP1-1 cells (Lee *et al.*, 1990) were cultured as monolayers in Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% (v/v) bovine calf serum (Hyclone), 100 units/ml penicillin G (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were synchronized at G1/S border by culturing the cells in the serum-deprived medium (0.3% (v/v) serum) for two days, normal growth medium for 6 hr, and finally in the normal growth medium containing hydroxyurea (1.5mM) for 14 hr. Cells were then allowed to enter S phase synchronously by replenishing with the normal growth medium.

2. Cell cycle analysis

Cells were prelabeled with ^{14}C-thymidine (0.02 μCi/ml; specific activity 50-60 mCi/mM, Amersham) for 2-3 days and synchronized at G1/S border. Throughout 45 hours, newly synthesized DNA was labeled for 30 min with ^{3}H-thymidine (2 μCi/ml, specific activity 40-60 mCi/mM, Amersham) at every hour. Cells were harvested immediately after labeling, suspended in 1X SSC and fixed overnight in 4% perchloric acid. The acid-precipitated DNA was filtered onto GF/C glass fiber filters and its ^{3}H/^{14}C ratios were determined by liquid scintillation counting. These values were taken as the DNA synthesis rate.

3. Labeling of parental DNA and newly synthesized DNA

To analyze the parental DNA binding to nuclear matrix, the cells were prelabeled with ^{14}C-thymidine (0.02 μCi/ml) for 2-3 days, synchronized at G1/S border, allowed to enter the S phase, their halo structures were isolated at various stages of S phase. To analyze the binding of newly synthesized DNA to nuclear matrix, the cells were synchronized at G1/S border, allowed to enter the S phase, pulse-labeled for 10 min with ^{3}H-thymidine (10 μCi/ml) at various stages of S phases and then their nuclear matrices were isolated. In the pulse-chase experiments, the pulse-labeled cells were further incubated for 1 hr in fresh medium before the isolation of nuclear matrix.

3. Nuclear matrix isolation

Nuclei and nuclear matrix were isolated as previously described (Mirkovitch *et al.*, 1984) with minor modifications. The synchronized cells were washed three times with isolation buffer (3.75 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA/KOH, pH 7.4, 1% (v/v) thiodiglycol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM dithiothreitol (DTT) and 20 mM KCl). The cells were resuspended in the ice-cold isolation buffer with 0.1% digitonin (Sigma) and immediately homogenized in a Dounce homogenizer with about 15 strokes. Nuclei were collected by repeated washing and centrifugation (10 min, 900g), and incubated in the isolation buffer with 0.1% digitonin without EDTA at 37°C for 20 min. 5 ml of extraction buffer (5 mM HEPES-NaOH, pH 7.4, 0.25 mM spermine, 2 mM EDTA/KOH, pH 7.4, 2 mM KCl, 0.1% digitonin and 25 mM 3,5-diiodosalicylic acid, lithium salt (LIS)) was slowly added at room temperature. After 5 min at RT, the histone-depleted nuclei were recovered by centrifugation at 250g for 30 min. The pellet was then washed three times with DNase I-digestion buffer (5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4). DNase I was added to the pellet (halo structure) at a final concentration of 5 μg/ml and

digestion was allowed to proceed for 1 hr at 37°C. Solubilized DNA was separated from nuclear matrix by centrifugation at 2,400g for 20 min at 4°C, and the radioactivities of the pellet (nuclear matrix) and the supernatant were measured by liquid scintillation counting.

Results

When LP1-1 cells synchronized at G1/S border by serum deprivation and hydroxyurea treatment were released by addition of fresh medium, over 90% of cells began to enter S phase synchronously (Lee *et al.*, 1990) and S phase lasted for 8 hr (Fig. 1). The next S phase occurs with only a trace of the original synchrony, that is, the synchrony decayed during the traverse of cell cycle. The decay of synchrony is a common phenomenon in eukaryotic cells. In the first S phase, there are apparently two peaks termed the early and the late replication and 3C-pause (Fig. 1).

In terms of good synchrony and apparent biphasic pattern of DNA synthesis rate, the LP1-1 cell is a good model system for analyzing the dynamic behavior of origin/terminus of replication during S phase. To examine the parental DNA binding to nuclear matrix during S phase, we performed the following experiment. The cells pre-labeled with ^{14}C-thymidine were synchronized at G1/S boundary, allowed to enter the S phase, their halo structures were isolated at various stages of S phase, digested by DNAse I and ^{14}C radioactivities tightly attached to nuclear matrix were measured. Figure 2 shows that radioactivities of ^{14}C were roughly constant throughout the S phase except S_3 stage, indicating that parental DNA binding to nuclear matrix was constant throughout S phase.

To examine whether newly replicated DNA binds to nuclear matrix during S phase, the cells were synchronized at G1/S boundary, allowed to enter S phase, pulse-labeled with ^{3}H-thymidine for 10 min at various stages of S phase, their halo structures were isolated, digested by DNAse I and radioactivities of ^{3}H associated with nuclear matrix were measured. Different from parental DNA binding, the percentages of total ^{3}H radioactivity on nuclear matrix were specifically higher at S_0

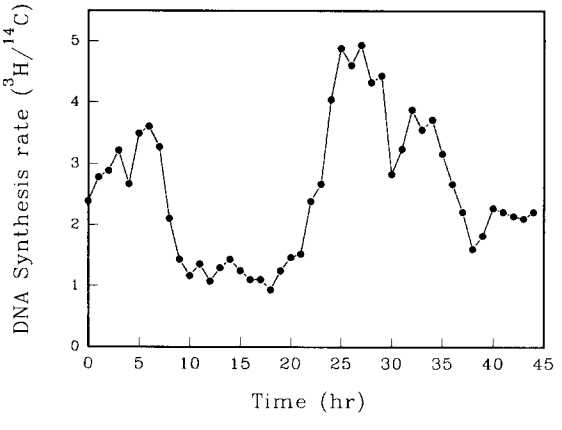

Fig. 1. Cell cycle analysis of mouse LP1-1 cells. Cells were synchronized and DNA synthesis rates were measured as described in Materials and Methods.

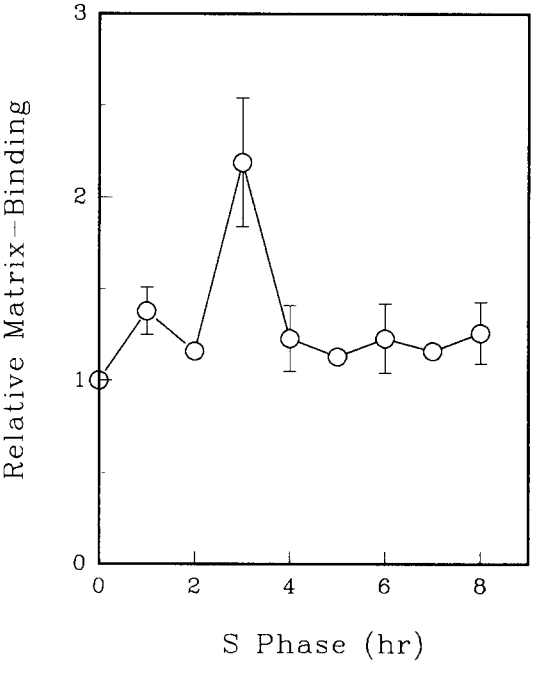

Fig. 2. The matrix binding pattern of parental DNA. LP1-1 cells were prelabeled with ^{14}C-thymidine and synchronized at G1/S boundary. At various stages of S phase, the halo structures of the cells were isolated by LIS-method and digested with DNAse I for 1 hr. ^{14}C-radioactivity associated with nuclear matrix was measured.

and S_8 stages and slightly higher at S_4 stage than those of the rest of the S stages (Fig. 3). Because only currently replicating parts of DNA are engaged with matrix-bound replication complexes, the replicated segments are continuously excluded out of matrix during the pulse labeling and then digested by DNase I later. As DNA replication begins at S_0 stage and terminates at S_8 stage (Fig. 1), the sequence pulse-labeled at S_0 and S_8 stages may contain the large populations of the origins and termination sites, respectively. The fact that sequences labeled at S_0 and S_8 stages are resistant to DNase digestion indicates that the sequences of origins and termini of replication units are fixed at nuclear matrix.

This result was confirmed by pulse-chase experiment, in which the synchronized cells were pulse-labeled with hourly time intervals and further incubated in the fresh medium for 1 hr, which was enough time to complete the duplication of a single replicon. The patterns of ^{3}H-radioactivity associated with nuclear matrix showed apparent 3 peaks at S_0, S_4 and S_8 stages (Fig. 4), indicating that the sequences pulse-labeled at these stages are not excluded out of nuclear matrix during replication process.

Discussion

The present study strongly suggests that the termini of replication as well as origins act as anchorage to nuclear matrix during S phase. The DNA fragments synthesized at the start point and termination point of S phase such as S_0 and S_8 stages were relatively resistant to DNase I-digestion in pulse-labeling and pulse-chase experiment (Fig. 3, 4). The origins and termini of

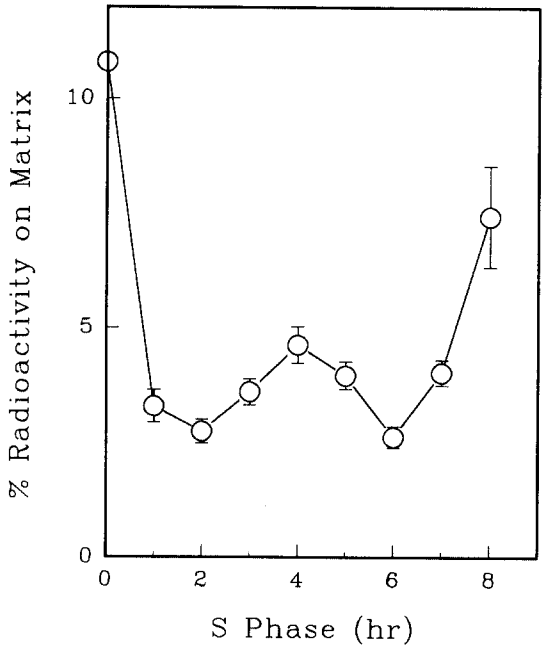

Fig. 3. Percent of ^{3}H-radioactivity associated with nuclear matrix in pulse-labeling experiment. LP1-1 cells were synchronized at G1/S boundary and pulse-labeled with ^{3}H-thymidine at various stages of S phase. Halo structures of the cells were isolated by LIS-method and digested with DNase I for 1 hr. The ^{3}H-radioactivity associated with nuclear matrix was measured.

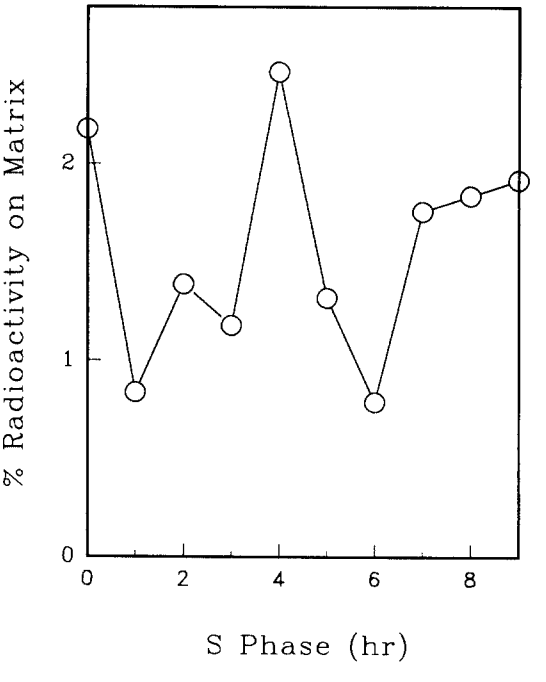

Fig. 4. Percent of ^{3}H-radioactivity associated with nuclear matrix in pulse-chase experiment. LP1-1 cells were synchronized at G1/S boundary, pulse-labeled with ^{3}H-thymidine at various stages of S phase and further incubated in the normal medium for 1 hr. Halo structures of the cells were isolated by LIS-method and digested with DNase I. The ^{3}H-radioactivity associated with nuclear matrix was measured.

replication, therefore, seem not to be excluded out but rather fixed at nuclear matrix during S phase. This concept is supported by the result that bindings of parental DNA to nuclear matrix at S_0 and S_8 stages were not larger than those of other stages (Fig. 2). In other words, DNA synthesis starts and terminates at prefixed DNA of loops in the S_0 and S_8 stages without the requirement of the additional binding of parental DNA. Our results supports the model proposed by Carri *et al.* (1986) that each replicon may form a double loop by binding to nuclear matrix at three points, one origin and two termini. This concept is in good agreement with the report that there is 2:1 ratio between the average replicon size and the average loop size (Buongiorno-Nardelli *et al.*, 1982).

The high resistancy to DNase digestion of the DNA fragment at S_4 stage suggests that the majority of the DNA sequences duplicated at 3C pause act as origins and/or termini of replication. Based on the report that dissociation and reassociation of the replication machinery to the early and the late replicated DNA occur at 3C pause (Foster and Collins, 1985), the majority of DNA synthesized by nuclear matrix-bound replication complexes at 3C pause can be divided into two groups such as the termination sites of the early replicated DNA and the origins of the late replicated DNA.

The reduction in the number of functional replicon during the progress of developmental stages (Blumenthal *et al.*, 1974) may be paralleled by the loss of many anchorages of the loops, such as the origins and termini of replication, to nuclear matrix. It was regarded that the dormant origins which lost the function of origin could be activated by the DNA damages which block the progression of replication fork (Griffiths and Ling, 1987; Lee *et al.*, 1990; Taylor, 1976). The characteristics of the origin and terminus of replication, however, remains to be elucidated.

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생쥐 LP1-1 세포에서 S phase동안 nuclear matrix에 고정되어 있는 복제 끝점

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복제원점/끝점과 nuclear matrix와의 결합을 동시화된 생쥐 LP1-1 세포의 halo 구조를 DNase로 분해하는 방법으로 조사하였다. S 시기의 각 단계별로 세포를 ^{3}H-thymidine으로 순간표지(pulse labeling)하고 nuclear matrix를 분리했을 때, nuclear matrix와 결합한 ^{3}H의 양은 S_0, S_4, S_8 시기에 특히 높게 나타났는 데, 이는 이 시기에 새로 합성된 DNA가 nuclear matrix 밖으로 빠져 나가지 않았음을 시사한다. 순간표지-추적 실험(pulse-chase experiment: S 시기 각 단계에서 세포를 순간표지한 후 1 시간동안 더 배양한 실험)에서도 비슷한 양상이 관찰되었다. 이러한 결과들은 복제원점과 끝점이 nuclear matrix에 고정되어 있고, 3C pause 시기에 nuclear matrix에 결합하는 DNA 부분은 많은 복제원점과 끝점을 가지고 있음을 시사한다.