Journal of Cellular Biochemistry 71:11–20 (1998)

Prereplicative Increase of Nuclear Matrix-Bound DNA Polymerase-α and Primase Activities in HeLa S3 Cells Following Dilution of Long-Term Cultures

Alberto M. Martelli,[1]* **S. Capitani,**[2] **and Luca M. Neri**[2,3]

[1]Dipartimento di Morfologia Umana Normale, Università di Trieste, Trieste, Italy
[2]Dipartimento di Morfologia ed Embriologia, Sezione di Anatomia Umana Normale, Università di Ferrara, Italy
[3]Istituto di Citomorfologia Normale e Patologica del C.N.R., c/o I.O.R., Bologna, Italy

Abstract We investigated the association of DNA polymerase and DNA primase activity with the nuclear matrix in HeLa S3 cells diluted with fresh medium after having been cultured without any medium change for 7 days. Flow cytometric analysis demonstrated that just before dilution about 85% of the cells were in the G_1 phase of the cycle, whereas 8% were in the S phase. After dilution with fresh medium, 18–22 h were required for the cell population to attain a stable distribution with respect to the cell cycle. At that time, about 38% of the cells were in the S phase. DNA polymerase and DNA primase activity associated with the nuclear matrix prepared from cells just before dilution represented about 10% of nuclear activity. As judged by [^{3}H]-thymidine incorporation and flow cytometric analysis, an increase in the number of S-phase cells was evident at least 6 h after dilution. However, as early as 2 h after dilution into fresh medium, a striking prereplicative increase of the two activitites was seen in the nuclear matrix fraction but not in cytosol or isolated nuclei. Both DNA polymerase and primase activities bound to the matrix were about 60% of nuclear activity. Overall, the nuclear matrix was the cell fraction where the highest induction (about 10-fold) of both enzymatic activities was seen at 30 h after dilution, whereas in cytosol and isolated nuclei the increase was about two- and fourfold, respectively. Typical immunofluorescent patterns given by an antibody to 5-bromodeoxyuridine were seen after dilution. These findings, which are at variance with our own previous results obtained with cell cultures synchronized by either a double thymidine block or aphidicolin exposure, strengthen the contention that DNA replication is associated with an underlying nuclear structure and demonstrate the artifacts that may be generated by procedures commonly used to synchronize cell cultures. J. Cell. Biochem. 71:11–20, 1998.

Key words: nuclear matrix; DNA replication; α-polymerase; confocal microscopy

Our knowledge of how DNA replication takes place in eukaryotes is lagging far behind the outstanding progress that has been made in studying DNA synthesis in prokaryotes [Kornberg and Baker, 1992]. Uncertainity still exists as to whether or not true origins of chromosomal DNA replication exist in mammals [Boulikas, 1995, 1996], and although several proteins involved in DNA duplication have been identified and characterized by biochemical and genetic approaches it is difficult to envision how they can interact to achieve as accurate and well-coordinated round of DNA replication as observed in mammalian cells [Kornberg and Baker, 1992]. Indeed, it is unclear how 3×10^9 base pairs per typical human cell are replicated with a high degree of fidelity in a relatively short time in a process that could involve up to 50,000–60,000 origins of DNA replication.

Given the complexity of the phenomenon, it is likely that DNA replication takes place in tight association with an underlying organizing structure. Evidence suggests that this structure could be the nuclear matrix, scaffold or skeleton, a mainly proteinaceous entity prepared by treating isolated nuclei with nonionic detergents, nucleases, and solutions of high ionic strength [Berezney et al., 1995]. Indeed,

Contract grant sponsors: Associazione Italiana per la Ricerca sul Cancro; PF ACRO; Italian M.U.R.S.T.

*Correspondence to: Dr. Alberto M. Martelli, Dipartimento di Morfologia Umana Normale, Università di Trieste, via Manzoni 16, I-34138 Trieste, Italy. E-mail: martelli@univ.trieste.it

Received 27 February 1998; Accepted 14 April 1998

the nuclear matrix has been shown to be highly enriched in newly synthesized DNA [Berezney and Coffey, 1975]. Subsequent biochemical fractionation experiments have demonstrated that several enzymic activities involved in DNA replication (DNA polymerase α, DNA primase, DNA methyltransferase, RNase H) are tightly bound to the matrix in a cell-cycle dependent fashion [Smith and Berezney, 1982, 1983; Tubo and Berezney, 1987a]. These results have been corroborated by the observation that in mammalian nuclei replication takes place in very distinct foci that can easily be visualized by pulse labeling with either biotinylated d-uridine 5′-triphosphate (dUTP) or 5′-bromodeoxyuridine (5-BrdU) [Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Neri et al., 1992; Hassan and Cook, 1993] and immunofluorescence microscope. In addition to newly synthesized DNA, these foci contain a number of proteins involved in DNA replication such as DNA polymerase α, proliferating cell nuclear antigen (PCNA; the auxiliary protein to DNA polymerase δ), 70-kDa replication factor A (RPA; a single stranded DNA binding protein) [Humbert et al., 1992; Krude, 1995]. Interestingly enough, the granular sites of replication resist DNase digestion and high salt extraction, hinting at the possibility that they may be bound to an underlying insoluble structure [Nakayasu and Berezney, 1989; O'Keefe et al., 1992]. Tubo and Berezney [1987b] proposed that the replication foci correspond to the DNA polymerase α–primase megacomplexes isolated by mild sonication from the nuclear matrix of regenerating rat liver.

In addition, electron microscopic techniques have shown that DNA replication in mammalian cells occurs in distinct sites [Mazzotti et al., 1990], and Hozak et al. [1993, 1994] identified "nuclear factories", i.e., a distinct subset of nuclear inclusions where DNA is replicated and which contain multiple replication factors such as DNA polymerase and PCNA. It has been proposed that newly synthesized DNA moves away from the factories, which would thus represent fixed sites of DNA replication [Hozak et al., 1993], in agreement with the pioneering studies of Pardoll et al. [1980].

The association of DNA polymerase α and primase activities with the nuclear matrix has been thoroughly studied in regenerating rat liver [Smith and Berezney, 1982, 1983; Tubo and Berezney, 1987a,c]. In this system, the nuclear matrix is the cell domain where the highest induction of DNA polymerase α activity can be observed (over 100-fold over the control). Moreover, the association of these enzymes to the matrix is strictly dependent on the phases of the cell cycle, being observable prereplicatively before the first wave of DNA replication but not when cells stop genome duplication [Smith and Berezney, 1983]. Nevertheless, we have observed somewhat different results by using HeLa S3 cells synchronized either by a double thymidine block or aphidicolin. In that case, 25–30% of nuclear DNA polymerase α activity was always associated with the matrix, even if the cells were not replicating their DNA (i.e., they were in G_1 phase of the cell cycle). We have interpreted those results to mean that the association of DNA polymerase α activity with the matrix was artifactual, especially considering the fact that the matrix had been isolated from nuclei exposed to mild heat in vitro (37°C), a procedure that is considered by several investigators to be a source of artifacts [Fisher et al., 1989; Jack and Eggert, 1992; Martelli et al., 1992].

However, recent evidence provided by Jackson [1995] has shown that when HeLa cells are exposed to aphidicolin for long periods of time (>8 h), as we did in our previous studies, there is a perturbation of the characteristics of DNA replication. Indeed, results indicate that not all the potential origins of replication were activated. Moreover, in vitro labeling with biotinylated dUTP demonstrated in cells synchronized by aphidicolin the presence of changes in the morphology of replication sites. For these reasons, we decided to investigate the asssociation with the nuclear matrix of DNA polymerase α and primase activities by using freshly diluted HeLa S3 cells that were cultured for a prolonged period of time without any medium change [Negri et al., 1996]. In the present study, we demonstrate that the nuclear matrix is the cell domain where the highest induction of the two enzymatic activities could be observed. We have also verified by immunocytochemistry that the replication patterns due to the incorporation of 5-BrdU were typical and not altered by the culture conditions.

Our results strengthen the contention that DNA replication is a process linked to an insoluble nuclear structure and hint at the artifacts that may be induced by procedures commonly used for synchronizing cell cultures.

MATERIALS AND METHODS

Cell Culture

HeLa S3 cells were grown at 37°C as suspension cultures in Joklik's modified minimum essential medium supplemented with 10% fetal calf serum. Cells were seeded at 1×10^5/ml and left to grow for 7 days without any change of medium. They were then diluted in fresh medium to 3×10^5/ml. Cell viability was assessed by the Trypan blue exclusion test, as previously reported [Negri et al., 1993].

Incorporation of [^{3}H]-Thymidine and 5-BrdU

Samples were removed from the cultures and incubated with 10 µCi/ml of [^{3}H]-thymidine (30 Ci/mmol; Amersham International, Buckinghamshire, U.K.) for 30 min at 37°C. Ice-cold medium was added to stop the incorporation, and the cells were centrifuged twice in cold medium. They were then resuspended in cold 5% trichloroacetic acid and collected by filtration onto Whatman GF/C glass fiber filters. Acid-precipitable radioactivity was measured by scintillation counting.

For 5-BrdU incorporation, cells were pulse-labeled with 100 µm 5-BrdU for 5 min. Incorporation was stopped by several washes with ice-cold Dulbecco' s phosphate buffered saline, pH 7.4 (PBS). Cells were then cytocentrifuged to glass slides and fixed as detailed below.

Flow Cytometric Analysis

For detection of cells in the different phases of the cell cycle, the method of Cataldi et al. [1992] was followed. Briefly, cells were incubated with 100 µm 5-BrdU for 30 min, fixed, denatured with 4 N HCl, incubated with fluoresceinated anti-5-BrdU antibody (Becton Dickinson, Milan, Italy), and counterstained with 5 µg/ml propidium iodide. Analyis was carried out by using an Epics XL flow cytometer (Coulter Immunology, Hileah, CA).

Immunofluorescent Staining

Samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature and then treated as previously described [Neri et al., 1992]. Briefly, DNA was denatured in 4 N HCl for 30 min and fixed in a −20°C graded ethanol series to prevent DNA reannealing. Slides were then air dried and incubated for 3 h at 37°C with a fluorescein isothiocyanate (FITC)-conjugated anti-5-BrdU antibody (Becton Dickinson), diluted 1:10 in PBS, 2% bovine serum albumin (BSA), and 3% normal goat serum (NGS). Slides were then washed three times in PBS and reacted with an FITC-conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis MO) diluted 1:45 in PBS, 2% BSA, and 5% NGS for 1 h at 37°C. Samples were subsequently washed three times in PBS and mounted in 20 mM Tris-HCl, pH 8.2, 90% glycerol containing 2.3% of the antifading agent 1,4-diazobicyclo-[2.2.2]-octane.

Confocal Laser Scanning Microscopy (CLSM) Analysis

Samples were imaged by a Zeiss LSM 410 CLSM, coupled with a 25-mW multiline argon ion laser as a light source, tuned at 15 mW. They were observed with a 100×, 1.4 numerical aperture planapochromat objective lens. To block any unwanted contribution signal, when observing FITC, a 515 OG long pass filter was inserted before the photomultiplier (PMT) as a barrier filter. The PMT was set at 823 mV. Settings were rigorously maintained for all experiments. Images were acquired, frame by frame, with a scanning mode format of 512 × 512 pixels and reconstructed as previously described [Neri et al., 1997a]. Digitalized optical sections, i.e., Z series of confocal data ("stacks"), were transferred from the CLSM to the graphics workstation Indy (Silicon Graphics, Mountain View, CA) and stored with a scanning mode format of 512 × 512 pixels and 256 gray levels. The image processing and the volume rendering were performed by using the ImageSpace software (Molecular Dynamics, Sunnyvale, CA), as reported elsewhere [Neri et al., 1997a]. The FITC signal was elaborated to optimize the contrast, the brightness, and the intensity of the images. Photographs were taken with the digital video recorder Focus ImageCorder Plus (Focus Graphics, Foster City, CA) using 100 ASA TMax black-and-white film (Kodak Limited, Rochester, NY).

Preparation of Subcellular Fractions

The procedures outlined by Martelli et al. [1992] were followed. Briefly, cells were resuspended to a concentration of 10^7/ml in 10 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of leupeptin and aprotinin (TM-2 buffer, prewarmed at 10°C). After 1 min at room temperature, cells were incubated for 5 min at 0°C.

Triton X-100 was added to 0.5% (w/v), and incubation on ice continued for 5 min. Cells were sheared by three up-and-down passages through a 22-gauge needle fitted to a 30-ml plastic syringe, and nuclei were separated from cytosol by centrifugation at 300*g* for 6 min. The cytosol was saved for measuring enzymic activities. The nuclear pellet was washed twice in TM-2 buffer and resuspended to 2 mg DNA/ml in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$ plus protease inhibitors as above (STM-5 buffer). They were then stabilized for 45 min at 37°C. Nuclei were then diluted with STM-5 buffer to one-tenth of the original concentration and extracted by slowly adding an equal volume of 4 M NaCl. DNase I digestion was then performed (50 U/ml for 30 min at 37°C), and the nuclear matrix was recovered by centrifugation (2,400*g* for 30 min) through an STM-5 buffer cushion containing 2 M NaCl. The final pellet was washed once in STM-5 buffer.

DNA Polymerase and DNA Primase Assays

The activity of DNA polymerase (DNA pol) α was measured by using [α-^{32}P]dATP as the radiolabeled deoxyribonucleoside trisphosphate (20 Ci/mmol, 25 μM; Amersham) in the presence of 50 mM Tris-Cl, pH 7.5, 8 mM $MgCl_2$, 600 μg/ml DNase-free BSA, 15% glycerol, 2 mM dithiothreitol (DTT), and 50 μM each of dGTP, dCTP, dTTP. DNase I-activated calf thymus DNA was present at 300 μg/ml. Incubation was for 30 min at 37°C. DNA poly α activity was distinguished from DNA pol β activity because of its sensitivity to 10 mM *n*-ethylmaleimide [Martelli et al., 1990]. No significant changes in DNA pol β activity were seen in any of the subcellular fractions (data not shown).

For DNA primase activity, assays contained 50 mM Tris-Cl, pH 8.0, 2 mM DTT, 2 mM $MgCl_2$, 100 μM poly(dT) (Pharmacia Biotechnology Italia, Milan, Italy), 300 μM ATP, 100 μg/ml DNase-free BSA, 40 mM of [α-^{32}P]dATP (5 Ci/mmol), and 0.5 unit of nuclease-free *Escherichia coli* DNA polymerase I (Klenow fragment; Sigma). The assays were incubated for 30 min at 37°C [Tubo and Berezney, 1987c; Martelli, 1993].

In both cases, the incorporation was stopped by the addition of ice-cold 10% trichloroacetic acid (TCA) containing 1% sodium pyrophosphate. Acid-precipitable counts were trapped on Whatman GF/C glass fiber filters, washed three times with 5% TCA, once with 70% ethanol, air dried, and counted in a liquid scintillation system.

RESULTS

Cell Proliferation During Long-Term Cell Culture

In Table I we show the number of cells, the cell viability, and the percentage of 5-BrdU-positive cells under our standard culture conditions after being seeded at 1×10^5/ml. During the first 5 days of cultures, the cell number almost doubled every day and then remained constant for the following 2 days. Cell viability, as assessed by the Trypan blue exclusion test, was always more than 87%. During the first 4 days of culture, 5-BrdU-positive cells were always more than 28% of the total; the number of 5-BrdU-positive cells started to decline at day 5 and was reduced to a small fraction (approximately 8%) after 7 days.

DNA pol α and DNA Primase Activities in Subcellular Fractions During Long-Term Cell Culture

In Table II we show DNA pol α and DNA primase activities in the subcellular fractions during the first 7 days of culture. In agreement with previous reports [Martelli, 1993; Foster and Collins, 1985], both enzymatic activities were mainly recovered in the cytosol because they leach out from nuclei during preparation. Thus, nuclear DNA pol α activity represented around 6.8–14.1% of the cytosolic activity, the lowest value observable after 7 seven days of culture, and the same holds true for primase activity, with values ranging from approxi-

TABLE I. Cell Number, Viable Cells, and Cells Positive for 5-BrdU in HeLa S3 Cells Under Our Standard Culture Conditions*

Days of culture	Cell number	%Viable cells	%Cells incorporating 5-BrdU
1	1.0×10^5	94	38
2	2.2×10^5	90	33
3	4.3×10^5	92	35
4	8.9×10^5	90	28
5	1.5×10^6	90	13
6	1.7×10^6	87	9
7	1.8×10^6	84	8

*Results are the mean of three separate experiments (SD < 15%). To determine percentage of viable cells as well as of 5-BrdU-positive cells a minimum of 300 cells were counted for each preparation.

TABLE II. DNA pol α and DNA Primase Activities in the Cellular Fractions During the First 7 Days of Culture*

Day of culture	DNA pol α			DNA primase		
	Cytosol	Nucleus	Matrix	Cytosol	Nucleus	Matrix
1	600.5	83.4	20.8	25.3	5.2	1.3
2	592.1	83.7	21.3	25.6	5.4	1.3
3	574.5	80.4	19.7	24.9	5.1	1.3
4	550.9	63.3	16.4	23.4	4.0	1.1
5	372.5	40.2	5.8	19.6	2.9	0.5
6	340.9	29.6	3.9	13.2	1.4	0.2
7	280.2	19.2	2.1	11.4	1.0	0.1

*Results refer to cytosol, nuclei, and nuclear matrix prepared from 10^6 cells and are expressed as pmol [^{32}P]dAMP incorporated. Data are the mean from three different preparations (SD < 13%).

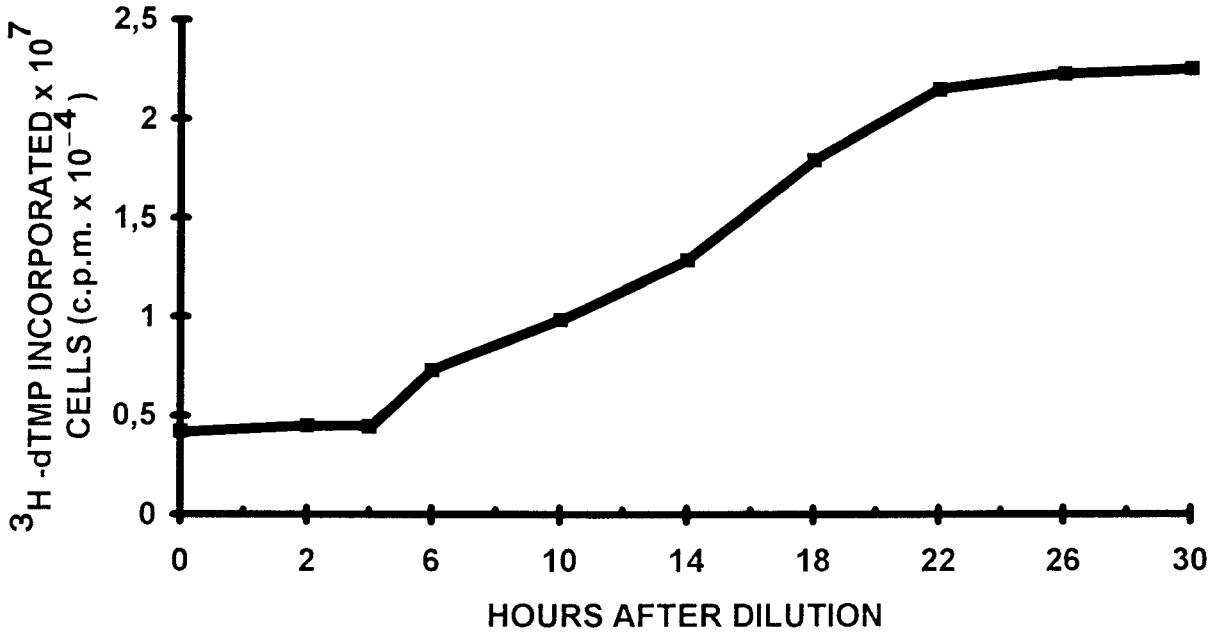

Fig. 1. Incorporation of [^{3}H]-thymidine in HeLa cells after dilution into fresh medium. Aliquots of cells at different times after dilution were pulsed for 30 min with [^{3}H]-thymidine, and acid-insoluble counts were then measured by scintillation counting. Results are the average from three separate preparations (SD > 16%).

mately 9% to 21%. During the first 4 days of culture, matrix-bound DNA pol α activity was about 25% of nuclear activity, in agreement with our previous findings for exponentially growing cells [Martelli et al., 1990]. However, at day 5 the high salt resistant activity started to decline and after 7 days it was reduced to approximately 11%. On the whole, nuclear matrix-bound DNA primase activity showed a similar behavior.

Cell Proliferation After Dilution With Fresh Medium

As shown in Figure 1, the incorporation of [^{3}H]-thymidine was unchanged for 4 h after dilution of the cultures with fresh medium, started to rise, and reached a plateau at 22 h after medium change.

By flow cytometric analysis we studied the incorporation of 5-BrdU and propidium iodide just before and at different times after dilution of cells into fresh medium. As shown in Table III, no changes were seen in the distribution of the cells in the different phases of the cell cycle up to 4 h. Then a progressive increase in the number of the cells in the S phase was observable starting at 6 h and a plateau was reached around 22 h, when a stable distribution of the cells in the different phases of the cell cycle was reached. A concomitant decrease in the percentage of cells in the G_1 phase was also observed.

TABLE III. Flow Cytometric Analysis of HeLa Cells Immediately Before and at Different Times After Dilution in Fresh Medium*

Time (h)	G_1	S	G_2/M
0	84.8	8.3	6.9
2	84.3	8.0	7.7
4	83.8	8.5	7.7
6	80.6	13.2	6.2
10	76.2	18.7	5.1
14	68.8	24.9	6.3
18	57.5	33.8	8.7
22	50.4	38.8	11.6
26	48.0	39.2	12.8
30	48.3	38.7	13.0

*Results are the mean of three different preparations (SD < 13%).

The increase of cells in the G_2M phase of the cell cycle was clearly recognized 22 h after dilution.

Visualization of DNA Synthesis After Dilution With Fresh Medium

After 5-BrdU pulse labeling of HeLa cells after dilution, CLSM analysis demonstrated various labeling patterns. On the basis of several reports concerning the spatial arrangement in the interphase nucleus of the DNA replication sites [Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Neri et al., 1992; Hassan and Cook, 1993; Hozak et al., 1993], the S phase was divided into early, middle, and late phases, each composed of two different stages.

In early S phase (stage 1) the replication sites, visualized as small fluorescent points, were distributed homogeneously throughout most of the nucleus, but a low density was observed close to the nuclear membrane (Fig. 2A). In stage 2 the cells showed small, discrete granules, closely spaced and dispersed in the nucleus with a uniform distribution (Fig. 2B). Nuclei of mid S-phase cells (stage 1) displayed the labeling of the perinucleolar chromatin, detectable as large and intensely fluorescent areas (Fig. 2C). The nucleoplasm showed a relevant decrease of staining that was enhanced at the nuclear periphery, assuming the aspect of a continuous ring. However, large clusters of fluorescence were closely associated with the negative nucleolar area. Moreover, other labeled structures were visible inside the nucleus, appearing as dots clustered into ring- or horseshoe-like arrays. Stage 2 was characterized by nuclei containing a limited number of dots of variable size, sometimes clustered together. The smaller ones were usually distributed mainly at the nuclear periphery, and a few large clustered granules were located preferentially in the inner regions of the nucleoplasm (Fig. 2D). The first stage of late S phase was characterized by large replication clusters, observable as large fluorescent spots, irregularly shaped, and preferentially located close to the nuclear boundary, partly separated from each other (Fig. 2E). In the inner nucleoplasm rare ring- or horseshoe-like arrays were visible together with few small replication spots. Late S phase (stage 2) was characterized by replicating DNA confined mainly to the nuclear periphery. This immunostaining featured large spots of replicating DNA that spanned portions of the nuclear periphery and fewer areas of the interior. These areas were in general smaller than those observed in the correspondent stage 1 and less numerous. In fact, the nuclear edge had large portions lacking any fluorescent labeling, i.e., the spots were well spaced one from each other. Furthermore, the nuclear interior in this case showed the sparsest labeling observable (Fig. 2F).

In Table IV we report the relative abundance of the six patterns during the first round of DNA replication after diluting the cells with fresh medium. It is evident that the fluorescent patterns typical of early S phase predominated up to 10 h. At this time stage 2 was the most represented. Immunostaining typical of mid S phase was preferentially detected after 14 h. At 18 h the mid-S-phase fluorescent patterns were reduced in favor of a high presence of late-S-phase immunistainings. Starting at 22 h and even more at 26 h, the distribution throughout the different phases of the cell cycle was typical of exponentially growing cells. It should be emphasized that mid S phase and in particular its stage 1 were the most represented replicon cluster pattern and was accompanied in decreasing order by early- and late-S-phase immunostainings.

DNA pol α and DNA Primase Activities in Subcellular Fractions After Cell Dilution

As shown in Table V, soluble DNA pol α activity remained stable up to 6 h after serum dilution and changed relatively little, being doubled after 30 h, in agreement with previous data [Foster and Collins, 1985; Fry and Loeb, 1986; Vishwanatra et al., 1986; Collins and Chu, 1987; Wang, 1991], and conceivably representing the effect of de novo synthesis of the enzyme [Miyazawa et al., 1993]. Nuclear DNA

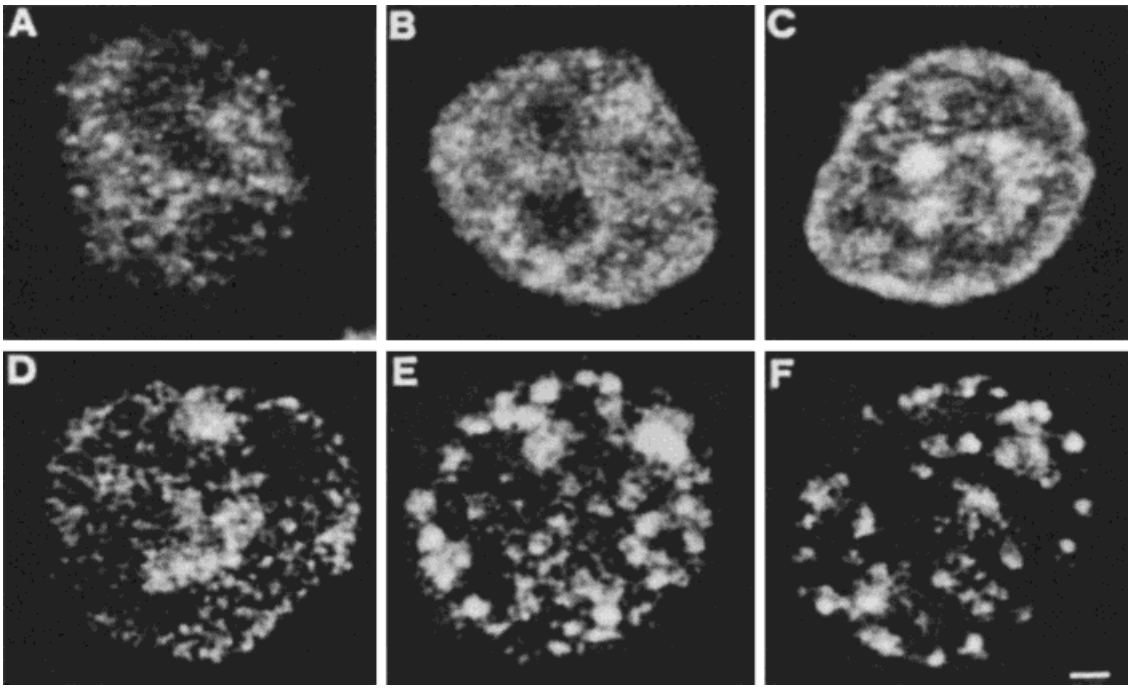

Fig. 2. Distinctive patterns of DNA synthesis as demonstrated by 5-BrdU immunofluorescent staining. Early S phase, stages 1 (**A**) and 2 (**B**). These two stages are characterized by the presence of hundreds of distinct replication granules distributed all over the nucleoplasm except for the nucleoli. As the early S phase proceeds, the nuclear periphery also becomes more stained. Mid S phase, stages 1 (**C**) and 2 (**D**). In stage 1, replication granules are clustered along the nuclear periphery. Stage 2 is characterized by a less intense fluorescence at the periphery plus fewer and more loosely spaced granules in the nuclear interior. Late S phase, stages 1 (**E**) and 2 (**F**). In stage 1, individual granules of replication sites appear as large aggregates that outline the periphery of the nucleus and that are also dispersed in some regions of the nuclear interior. Stage 2 is characterized by fewer and smaller aggregates located in similar regions.

TABLE IV. Relative Abundance of the Six Replication Patterns After Dilution of Cells With Fresh Medium*

	Early S		Mid S		Late S	
Hours after dilution	% Type 1 cells	%Type 2 cells	% Type 1 cells	%Type 2 cells	% Type 1 cells	%Type 2 cells
0	76	10	5	3	3	3
6	75	18	3	1	2	1
10	28	38	19	7	5	3
14	15	22	32	15	9	7
18	12	14	19	20	23	12
22	10	16	30	18	15	12
26	11	18	32	16	12	9
30	12	19	31	16	12	8

*For each time point, at least 200 positive cells were scored. Results are the mean of three different preparations (SD $<$ 15%).

pol α activity started to rise at 6 h after dilution and then gradually increased, reaching a plateau value at 22 h. Overall, this activity showed a fourfold increase at 30 h. A striking increase in nuclear matrix-bound DNA pol α activity was seen as early as 2 h after dilution. Indeed, the activity already showed a fivefold increase over the basal value, and it represented about 57% of nuclear activity and about 10% after 7 days. Matrix-bound DNA pol α activity continued to increase, albeit at a slower rate, up to 22 h, when a plateau was reached. When the cells attained a stable distribution with respect to the cell cycle (22 h), the nuclear matrix-bound activity was about 25% of nuclear activity, as seen in exponentially growing cells (see Table II). Overall, after 30 h,

TABLE V. DNA pol α Activity in Subcellular Fractions Immediately Before and After Dilution of Cells With Fresh Medium*

Hours after dilution	Cytosol	Nucleus	Nuclear matrix	%Nuclear activity recovered in nuclear matrix
0	283.2	19.0	2.0	10.5
2	278.7	19.1	10.9	57.1
6	295.9	29.4	14.5	49.3
10	374.4	45.3	17.2	38.0
14	426.2	60.7	18.6	30.1
18	487.5	71.5	19.9	27.8
22	592.5	82.6	20.5	24.8
26	601.4	81.9	20.6	25.2
30	590.6	83.9	20.9	24.9

*Results refer to activity detected in cytosol, nuclei, and nuclear matrix obtained from 10^6 cells and are expressed as pmol [^{32}P]dAMP incorporated. They are the average from three different preparations (SD > 13%).

TABLE VI. DNA Primase Activity in Subcellular Fractions Immediately Before and After Dilution of Cells With Fresh Medium*

Hour after dilution	Cytosol	Nucleus	Nuclear matrix	%Nuclear activity recovered in nuclear matrix
0	11.3	1.0	0.10	10.0%
2	11.3	1.0	0.65	65%
6	11.2	1.6	0.75	46.9
10	15.1	2.3	0.89	38.7
14	17.3	3.3	0.97	29.4
18	19.8	3.8	1.09	28.7
22	23.1	4.4	1.20	27.3
26	24.2	4.5	1.22	27.1
30	23.9	4.5	1.25	27.7

*Results refer to activity detected in cytosol, nuclei, and nuclear matrix obtained from 10^6 cells and are expressed as pmol [^{32}P]dAMP incorporated. They are the average from three different preparations (SD > 16%).

nuclear matrix-bound DNA pol α activity increased over 10-fold with respect to the basal levels.

As shown in Table VI, DNA primase activity displayed a similar behavior, with the exception that after 2 h the increase in matrix-bound activity was even more marked.

DISCUSSION

To study variations in activity of replicative enzymes during the cell cycle, cells are usually synchronized by means of a double thymidine block [e.g., Chiu and Baril, 1975], aphidicolin [e.g., Pedrali-Noy et al., 1980; Cordeiro-Stone and Kaufman, 1985], a single thymidine block followed by nitrous oxide under high presssure [e.g., Jackson and Cook, 1986a; 1986b], mitotic selection [e.g., Petersen et al., 1968], or other inhibitors of DNA synthesis, such as hydroxyurea and cytosine arabinoside [Jackson, 1995]. Although widely employed, these methods are far from being completely satisfactory. For example, CH3 10T1/2 cells have been reported as being able to enter S phase even in the presence of aphidicolin [Cordeiro-Stone and Kaufman, 1985]. As far as the double thymidine block is concerned, this method does not achieve an arrest of cells at the G_1–S border as often supposed but causes them to accumulate in the early part of the S period [e.g., Petersen et al., 1968; Bostock et al., 1971].

Very recently, Jackson [1995] showed that prolonged (8–12 h) exposure to aphidicolin, a procedure often employed to synchronize HeLa cells at the G_1–S border [e.g., Pedrali-Noy et al., 1980; Martelli et al., 1992], causes dramatic perturbations in DNA synthesis when the block is relieved, including a progressive reduction in DNA polymerase activity and characteristic morphological changes in the organization of replication clusters.

These findings prompted us to reinvestigate the association of DNA pol α activity with the nuclear matrix prepared from HeLa S3 cells because our previous data [Martelli et al., 1992], obtained in cells synchronized by either a double thymidine block or aphidicolin treatment, showed a behavior of the matrix-associated activity very similar to that detected in isolated nuclei, which is at variance with the results reported by Berezney and coworkers in regenerating rat liver [Smith and Berezney, 1982, 1983; Tubo and Berezney, 1987a]. To this end, we took advantage of the fact that HeLa cells can be cultured for a relatively long period of time without any medium change, as demostrated by Negri et al. [1996]. In addition, we analyzed the association of DNA primase with the nuclear matrix in the same experimental model.

By culturing the cells for 7 days without any medium change, we were able to decrease the number of cells in the S phase of the cycle to about 10%, thereby maintaing a high number of viable cells. Matrix-bound DNA pol α and DNA primase activities showed marked variations during the transition from the first 4 days of culture to the last day. Less dramatic variations were seen in cytosol and nuclear fractions.

With dilution of cultures with fresh medium, an increase in the number of S-phase cells was clearly detected at least 6 h after addition of medium. It is clear that HeLa cells behave differently from other cells, e.g., mouse 3T3 fibroblasts, in which a long-term culture in the same medium produces an almost complete arrest of the cells in the G_0/G_1 phases of the cycle and refeeding with fresh medium causes a good degree of synchronization [see Nakayasu and Berezney, 1989].

Nevertheless, we were able to measure a striking prereplicative increase in the amount of nuclear matrix-bound DNA pol α and DNA primase activities. Because this increase precedes any substantial increase in both nuclear and cytosolic activities, it is conceivable that it is due to a shift of the two enzyme activities from a soluble form to a matrix-associated form. In this sense, our results are very similar to the findings reported by Berezney and coworkers in regerating rat liver not only for the two same activities but also for other enzymes involved in DNA replication such as DNA methylase, RNase H, and 3′-5′ exonuclease [Smith and Berezney, 1983; Tubo and Berezney, 1987a].

The present findings are markedly different from previous data obtained with HeLa cells synchronized by either a double thymidine block or aphidicolin treatment [Martelli et al., 1992] and hint at possible artifacts created by commonly used cell synchronization procedures as far as subcellular distribution of enzymes involved in DNA replication is concerned. An issue that remains unresolved is the influence that the heat stabilization of the nuclear matrix may have had on the results because this form of treatment is absolutely necessary to recover substantial amounts of DNA pol α activity in the matrix fraction [Martelli et al., 1990]. Indeed, we have demonstrated that several nuclear matrix proteins change their subnuclear localization after heat treatment in vitro [Neri et al., 1994, 1997b]. However, there are matrix proteins such as topoisomerase II and 240-kDa NuMA that are unaffected by such a stabilizing treatment [Neri et al., 1997c]. Therefore, we are currently trying to establish by means of immunostaining techniques whether or not heat stabilization changes the subnuclear distribution of DNA pol α. Nevertheless, it should also be noted that a change in the spatial distribution does not necessarily involves a functional change.

In conclusion, we feel that our data strengthen the contention that DNA replication and the enzymes involved are tightly bound to an underlying nuclear structure and that the association is strictly dependent on the phases of the cell cycle.

ACKNOWLEDGMENTS

We thank S. Lach and A. Valmori for technical assistance and Dr. C. Celeghini for helpful collaboration. This work was supported by A.I.R.C. (Associazione Italiana per la Ricerca sul Cancro) 1996 and 1997 grants to Alberto M. Martelli, grant PF ACRO, and from Italian M.U.R.S.T. 60% grants to Università di Trieste e Ferrara.

REFERENCES

Berezney R, Coffey DS (1975): The nuclear protein matrix: Association with newly synthesized DNA. Science 189: 291–293.

Berezney R, Mortillaro MJ, Ma H, Wei X, Samarabandu J (1995): The nuclear matrix: A structural milieu for genomic function. Int Rev Cytol 162A:1–65.

Bostock CJ, Prescott DM, Kirkpatrick JB (1971): An evaluation of the double thymidine block for synchronizing mammalian cells at the G_1-S border. Exp Cell Res 68:163–168

Boulikas T (1995): Chromatin domains and the prediction of MAR sequences. Int Rev Cytol 162A:279–388.

Boulikas T (1996): Common structural features of replication origins in all life forms. J Cell Biochem 60:297–316.

Cataldi A, Santavenere E, Vitale M, Trubiani O, Lisio R, Tulipano G, Di Domenicantonio L, Zamai L, Miscia S (1992): Interferon affects growth progression by modulating DNA polymerases activity. Cell Prolif 25:225–231.

Chiu W, Baril EF (1975): Nuclear DNA polymerases and the HeLa cell cycle. J Biol Chem 250:7951–7957.

Collins JM, Chu AK (1987): Binding of the DNA polymerase α-DNA primase complex to the nuclear matrix in HeLa cells. Biochemistry 26:5600–5607.

Cordeiro-Stone M, Kaufman DG (1985): Kinetics of DNA replication in CH3 10T1/2 cells synchronized by aphidicolin. Biochemistry 24:4815–4822.

Fisher PA, Lin L, McConnell M, Greenleaf A, Lee J-M, Smith DE (1989): Heat shock-induced appearance of RNA polymerase II in karyoskeletal protein-enriched (nuclear "matrix") fractions correlates with transcriptional shutdown in *Drosophila melanogaster*. J Biol Chem 264:3464–3469.

Foster KA, Collins JM (1985): The interrelation between DNA synthesis rates and DNA polymerases bound to the nuclear matrix in synchronized HeLa cells. J Biol Chem 260:4229–4235.

Fry M, Loeb LA (1986): "Animal Cell DNA Polymerases." Boca Raton: CRC Press.

Hassan BA, Cook PR (1993): Visualization of replication sites in unfixed human cells. J Cell Sci 105:541–550.

Hozak P, Hassan BA, Jackson DA, Cook PR (1993): Visualization of replication factories attached to a nucleoskeleton. Cell 73:361–373.

Hozak P, Jackson DA, Cook PR (1994): Replication factories and nuclear bodies: The ultrastructural characterization of replication sites during the cell cycle. J Cell Sci 107: 2191–2202.

Humbert C, Santisteban MS, Usson Y, Robert-Nicoud M (1992): Intranuclear co-location of newly replicated DNA and PCNA by simultaneous immunofluorescent labelling and confocal microscopy in MCF-7 cells. J Cell Sci 103:97–103.

Jack RS, Eggert H (1992): The elusive nuclear matrix. Eur J Biochem 209:503–509.

Jackson D (1995): S-phase progression in synchronized human cells. Exp Cell Res 220:62–70

Jackson DA, Cook PR (1986a): A cell-cycle-dependent DNA polymerase activity that replicates intact DNA in chromatin. J Mol Biol 192:65–76.

Jackson DA, Cook PR (1986b): Different populations of DNA polymerase in HeLa cells. J Mol Biol 192:77–86.

Kornberg A, Baker TA (1992): "DNA Replication," 2nd ed. New York: W.H. Freeman and Company.

Krude T (1995): Chromatin assembly factor 1 (CAF-1) colocalizes with replication foci in HeLa cell nuclei. Exp Cell Res 220:304–311.

Martelli AM (1993): On the association of DNA primase activity with the nuclear matrix in HeLa S3 cells. Cell Biochem Funct 11:287–290.

Martelli AM, Gilmour RS, Falcieri E, Manzoli FA, Cocco L (1990): Temperature-dependent association of DNA polymerase activity with the nuclear matrix. Exp Cell Res 190:227–232.

Martelli AM, Gilmour RS, Bareggi R, Cocco L (1992): The effect of in vitro heat exposure on the recovery of nuclear matrix-bound DNA polymerase activity during the different phases of the cell cycle in synchronized HeLa S3 cells. Exp Cell Res 201:470–476.

Mazzotti G, Rizzoli R, Galanzi A, Papa S, Vitale M, Falconi M, Neri LM, Zini N, Maraldi NM (1990): High-resolution detection of newly synthesized DNA by anti-bromodeoxyuridine antibodies identifies specific chromatin domains. J Histochem Cytochem 38:13–22.

Miyazawa H, Izumi M, Tada S, Takada R, Masutani M, Ui M, Hanoka F (1993): Molecular cloning of the cDNAs for the four subunits of mouse DNA polymerase α-primase complex and their gene expression during cell proliferation and the cell cycle. J Biol Chem 268:8111–8122.

Nakayasu H, Berezney R (1989): Mapping replicational sites in the eucaryotic cell nucleus. J Cell Biol 108:1–11.

Negri C, Bernardi R, Braghetti A, Astaldi Ricotti GCB, Scovassi AI (1993): The effect of chemotherapeutic drug VP-16 on poly(ADP-ribosylation) in apoptotic HeLa cells. Carcinogenesis 14:2559–2564.

Negri C, Bernardi R, Donzelli M, Prosperi E, Scovassi AI (1996): Sequence of events leading to apoptosis in long term cultured HeLa cells. Cell Death Diff 3:425–430.

Neri LM, Mazzotti G, Capitani S, Maraldi NM, Cinti C, Baldini N, Rana R, Martelli AM (1992): Nuclear matrix-bound replicational sites detected in situ by 5-bromodeoxyuridine. Histochemistry 98:19–32.

Neri LM, Santi S, Marugg RA, Riederer BM, Capitani S, Cataldi A, Martelli AM (1994): In vitro heat exposure induces a redistribution of nuclear matrix proteins in human K562 erythroleukemia cells. Exp Cell Res 213: 275–285.

Neri LM, Martelli AM, Maraldi NM (1997a): Distribution of DNA topoisomerase II after in vitro stabilization of human erythroleukemic nuclei by heat or Cu^{++} by confocal microscopy. Microsc Res Tech 36:179–187.

Neri LM, Riederer BM, Valmori A, Capitani S, Martelli AM (1997b): Different concentrations of Mg++ ions affect nuclear matrix protein distribution during thermal stabilization of isolated nuclei. J Histochem Cytochem 45: 1317–1328.

Neri LM, Fackelmayer FO, Zweyer M, Kohwi-Shigematsu T, Martelli AM (1997c): Subnuclear localization of S/MAR-binding proteins is differently affected by in vitro stabilization with heat or Cu^{2+}. Chromosoma 106:81–93.

O'Keefe RT, Henderson SC, Spector DL (1992): Dynamic organization of DNA replication in mammalian cell nuclei: Spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. J Cell Biol 116:1095–1110.

Pardoll DM, Vogelstein B, Coffey DS (1980): A fixed site of DNA replication in eucaryotic cells. Cell 19:527–536.

Pedrali-Noy G, Spadari S, Miller-Faurès A, Miller AOA, Kruppa J, Koch G (1980): Synchronization of HeLa cell cultures by inhibition of DNA polymerase with aphidicolin. Nucleic Acids Res 8:377–387.

Petersen DF, Anderson EC, Tobey RA (1968): Mitotic cells as a source of synchronized cultures. Methods Cell Physiol 3:347–370.

Smith HC, Berezney R (1982): Nuclear matrix-bound deoxyribonucleic acid synthesis: An in vitro system. Biochemistry 21:6751–6761.

Smith HC, Berezney R (1983): Dynamic domains of DNA polymerase in regenerating rat liver. Biochemistry 22: 3042–3046.

Tubo RA, Berezney R (1987a): Pre-replicative association of multiple replicative enzyme activities with the nuclear matrix during rat liver regeneration. J Biol Chem 262: 1148–1154.

Tubo RA, Berezney R (1987b): Identification of 100 and 150 S DNA polymerase α-primase megacomplexes solubilized from the nuclear matrix of regenerating rat liver. J Biol Chem 262:5857–5865.

Tubo RA, Berezney R (1987c): Nuclear matrix-bound DNA primase. Elucidation of an RNA priming system in nuclear matrix isolated from regenerating rat liver. J Biol Chem 262:6637–6642.

Vishwanatha JK, Coughlin SA, Wesolowski-Owen M, Baril EF (1986): A multiprotein form of DNA polymerase from HeLa cells. J Biol Chem 261:6619–6628.

Wang TS (1991): Eukaryotic DNA polymerases. Annu Rev Biochem 60:513–552.