DNA REPLICATION AND THE NUCLEAR ENVELOPE

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Upon isolation of the nuclear membrane from cultured mouse leukemia L5178Y cells, approximately 1% of the total nuclear DNA was found to be attached to this structure. After pulse labeling of DNA and isolation of the nuclear membrane, the ratio of labeled DNA in the membrane fraction and in the rest of chromatin was compared. Results indicate that with a 3 min pulse, DNA in the membrane fraction showed slightly higher specific activity, but when the pulse was longer than 5 min there was no difference in the specific activities. Since the DNA fragment associated with the membrane fraction was found to be long enough to contain most of the DNA labeled during a 5 min pulse, the results obtained indicate that there is no preferential association of DNA to the nuclear envelope during initiation or elongation of DNA.

Using electron microscopic autoradiography, Comings and Kakefuda (1) reported that in synchronized human amnion cells, the initiation of DNA replication occurred at the nuclear envelope at the beginning of the S phase. However, this result was not reproducible by other investigations (2,3,4). Recently Comings and Okada (5) showed, in cultured Chinese hamster cells, that only at the end of S phase, when the heterochromatin is replicated, the silver grains are associated with nuclear membrane.

Results of biochemical experiments, however, suggest that newly replicated DNA is attached to some cellular component, presumably nuclear membrane. It has been shown that newly replicated DNA can be found in the interphase protein layer after phenol extraction (3,6,7), in M-band fractions formed by the crystallization of magnesium sarcosinate in sucrose gradients (8,9,10) and in isolated nuclear membrane fractions (7,11).

Thus, the results obtained by electron microscopic autoradiography and biochemical experiments are controversial. We have examined the specific activities of pulse labeled DNA fragments attached to nuclear membranes of mouse leukemia L5178Y cells isolated from log phase and synchronized cells and our results indicate that there is no preferential association of DNA to

the nuclear envelope during the initiation of DNA replication in early S, nor during chain elongation.

MATERIALS AND METHODS

Cells and cell fractionation

Mouse leukemia L5178Y cells were cultured in suspension in Fischer's medium supplemented with 10% horse serum (Grand Island Biol. Co.). Detailed methods of synchronization and cell fractionation will be published elsewhere (12).

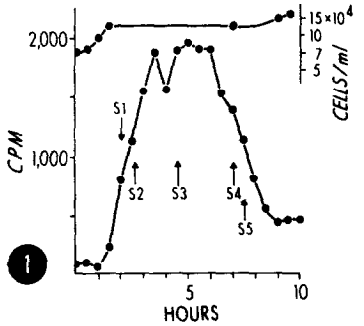
Cells were synchronized by excess thymidine treatment followed by colcemid block to adjust cells at mitotic phase (13). In the synchronized cells, DNA replication starts 2 h after the release of colcemid block as measured by the pulse labeling of the cells at intervals (results shown in Fig. 1). The duration of S phase was about 6 h. The cells were pulse labeled with ^{3}H-thymidine (at 5-10 μCi/ml, sp. act. 6.7 Ci/mmole, New England Nuclear) at log phase or at different times of S phase of synchronized cells. Incorporation was stopped by quickly shaking the culture bottle in an ice water bath. The ^{3}H-thymidine labeled cells were mixed with the cells which were uniformly labeled with ^{14}C-thymidine (labeled for 24 h at 0.05 μCi/ml, sp. act. 51.5 mCi/mmole, New England Nuclear). The nuclear membrane was isolated by the method of Monneron, et al. (14). Isolated nuclei were lysed in 0.5 M $MgCl_2$ in 55% sucrose, 50 mM Tris-Cl pH 7.5. The sheared lysate was overlayered by a discontinuous multistep gradient in which the densities were 1.20, 1.18, 1.17, 1.16 and 1.10. After centrifugation in a SW 5.1 rotor at 43,000 rpm for 6 h at 3°C, a band containing the nuclear membrane was observed at the density of 1.17. This membrane fraction and the remainder of the chromatin fraction of the lysate layers at the bottom of the tube were collected by puncturing the tube with a hypodermic syringe and removing the bands from the side and bottom, respectively. Small portions of both of these fractions were diluted with 1xSSC (SSC; 0.15 M NaCl, 0.015 M Na-citrate) and precipitated with 2 volumes of ice cold 10% TCA. Precipitates were collected on glass fiber filters dried and counted in toluene-Liquiflour (New England Nuclear).

Analysis of DNA in membrane fraction

The DNA which was isolated with the nuclear membrane fraction was analyzed by sucrose and CsCl gradient centrifugation. In preparation for sucrose gradient centrifugation, the membrane fraction was dialysed against 1xSSC to remove sucrose and then deproteinized by 2% sodium dodecyl sulfate (SDS). The lysate was placed over the 2% SDS layer at the top of the neutral 5-20% sucrose gradient in 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-Cl, pH 7.0. The tubes were centrifuged in a SW 50.1 rotor at 30,000 rpm for 4 h at 20°C. Ribosomal RNA was run as a reference to estimate the S value of DNA. Fractionation was accomplished by an ISCO density gradient fractionator Model 640. For the CsCl gradients, the dialysed membrane fractions were incubated with 200 μg/ml pronase and 1% SDS for 4 h at 37°C. The fraction was then extracted with 1xSSC saturated phenol twice at room temperature and dialysed against 1xSSC to remove the phenol. Pre-formed, 2-layer CsCl gradients (15) were made in SW 50.1 tubes and centrifuged at 25,000 rpm for 20 h at 20°C. After centrifugation, saturated CsCl was introduced from the bottom of the tube, using an ISCO fractionator, and 0.1 ml fractions were collected from the top of the tube. To each fraction was added 1 ml of 5% ice cold TCA and precipitates were collected on glass fiber filters.

RESULTS AND DISCUSSION

In order to determine whether newly replicated DNA in mouse leukemia



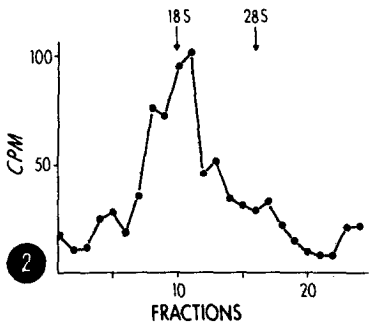


Fig. 1. Cell Synchrony. Cells were synchronized with successive excess thymidine and colcemid blocks. After the release of the block, small aliquots of culture were taken at intervals and labeled with 1 μCi/ml ^{3}H-thymidine for 10 min. Increase in cell numbers indicated that synchrony was 75 to 100%. Arrows (S1-S5) indicate the time of pulse labeling with ^{3}H-thymidine for the analysis of DNA in nuclear membrane fraction. Results are shown in Table II.

Fig. 2. Sedimentation Profile of DNA. To estimate its length, DNA released from nuclear membrane fraction was centrifuged in neutral 5-20% sucrose gradient in a SW 50.1 rotor at 30,000 rpm for 4 h at 20°C. Arrows indicate the positions of 18S and 28S ribosomal RNA prepared as references.

L5178Y cells is preferentially associated with the nuclear envelope, cells were pulse labeled for various durations with ^{3}H-thymidine and mixed with cells uniformly labeled with ^{14}C-thymidine. The cells were fractionated, the nuclear envelope isolated, and the ratios of the specific activities (^{3}H/^{14}C) of the DNA in membrane fraction was divided by the ratio of the specific activity of DNA in the bottom chromatin fraction of the same tube. Table I shows the results of such experiments for log phase cells pulse labeled for 3 to 30 min. Table II shows the results with synchronized cells which were pulse labeled at different times in S phase. These results indicate that in log phase cells, for a 3 min pulse, the ratio was slightly higher than 1.0, but for a 5 min pulse, the ratio was 1.0. In synchronized cells, the same higher ratio was observed at early S phase (S1) for the 3 min pulse, but ratios were 1.0 at middle and late S phase.

Since these results depend on the relationship between the length of DNA labeled during a short pulse and length of DNA which is attached to the isolated nuclear membrane after the shearing of the fractionation procedure, we analyzed the length of DNA in nuclear membrane fraction by sucrose density

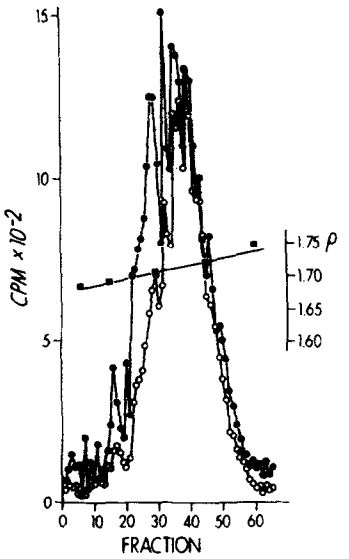


Fig. 3. Buoyant Density Profile of Extracted DNA. DNA was extracted from the nuclear membrane fraction of ^{3}H-thymidine labeled cells (●——●) and chromatin lysate fraction of ^{14}C-thymidine labeled cells (○——○). Mixtures os these DNAs were centrifuged in 2-layer CsCl gradient in a SW 50.1 rotor at 25,000 rpm for 20 h at 20°C.

gradient centrifugation. From the result shown in Fig. 2, the size of DNA associated with the membrane fraction as estimated from the S values of ribosomal RNA was 15 to 20 S. From the Studier's equation (16), the molecular weight of this DNA corresponds to 2.79×10^6 to 6.42×10^6. And the length of this DNA is 1.46 to 3.37 μ, assuming the molecular weight of 1 μ of DNA to be 1.90×10^6.

The rate of DNA replication in mammalian cells has been measured by different methods giving the range of 0.5 to 1.0 μ per min (17-20). Thus, during the 3 min pulse, 1.5 to 3.0 μ of DNA is synthesized and at 5 min, 2.5 to 5.0 μ. From this estimation, the DNA fragments recovered with the membrane fraction should contain the entire length of DNA synthesized during a 3 min pulse. Since it is not likely that during a 5 min pulse the full length of 5μ of DNA will be labeled because of the pool size of the precursor in the cell, most of label from the 5 min pulse should also be present in this sized piece. These results indicate that, first, it is unlikely that the site of chain

TABLE I

Pulse (min)	Membrane ^3H	Membrane ^{14}C	(1) $^3H/^{14}C$	Chromatin ^3H	Chromatin ^{14}C	(2) $^3H/^{14}C$	Ratio (1)/(2)
3	468	306	1.529	1931	1831	1.054	1.450
	551	364	1.513	1971	2027	0.972	1.556
	280	467	0.599	1134	2606	0.435	1.377
	277	455	0.609	1192	2161	0.551	1.105
5	580	419	1.384	2034	1633	1.245	1.111
	694	3606	0.192	1128	5679	0.198	0.969
	953	1004	0.949	2110	2645	0.798	1.189
8	331	2232	0.148	750	5414	0.138	1.072
10	1408	422	3.336	7822	2611	2.996	1.113
30	2656	420	6.328	1217	2024	6.011	1.052

Log phase cells were pulse labeled with ^3H-thymidine and mixed with ^{14}C-thymidine uniformly labeled cells. Ratio was obtained by dividing $^3H/^{14}C$ of membrane fraction by $^3H/^{14}C$ of chromatin fraction.

TABLE II

Pulse	Ratio of $^3H/^{14}C$ of nuclear membrane fraction to chromatin fraction S1 2 h	S2 2.5 h	S3 4.5 h	S4 7 h	S5 7.5 h
3 min	2.10		0.85	0.93	
	1.34		0.99	1.12	
5 min	1.14	1.20	0.94	1.10	1.05
			1.06	0.97	0.86

After the release of colcemid block (time indicated in Table) cells were pulse labeled at different time in S phase (as indicated in Fig. 1) and ratios were calculated as in Table I.

elongation of DNA is the attachment site of chromatin to the nuclear envelope, since at the middle and late S phase, the ratios of the specific activities of the DNA is 1.0. Then secondly, the possibility of initiation of DNA replication at the nuclear membrane also is not likely. Although the ratio of the specific activities for a 3 min pulse is significantly higher in early S phase and in log phase cells, they become 1.0 for a 5 min pulse where the most of the synthesized DNA is still contained in the length of the DNA fragment which is recovered with the nuclear membrane. Rather, a possible interpretation of this higher specific activity might be that during the short pulse of 3 min, the concentration of radioactive precursor may be higher at the surface of

the nuclear envelope than inner area, which then equilibrates within the next 2 min. Also, if all the DNA which were attached to the nuclear membrane *in vivo* were preserved in the membrane fraction, the ratio of ^{3}H counts associated with nuclear membrane to the ^{3}H counts associated with the chromatin portion in pulse label experiments indicate only about 2% of the newly replicated DNA appears to be attached to the nuclear membrane. From these results, we conclude that both initiation and chain elongation of the DNA in these mammalian cells are not localized preferentially at the nuclear envelope.

Yamada and Hanaoka (8,10) reported that newly replicated DNA is associated with the M-band fraction. But, as they have shown, their M-band fraction contained 47% of total cell protein and 67% of total lipid even after the shearing, which indicates that the M-band fraction may contain other proteins in addition to the nuclear membrane itself. And also there is a possibility that newly replicated DNA is contained in the M-band fraction because of its particular structure, either single-strandedness or different amount or species of proteins per unit DNA at the replication site. Fakan, *et al.*, (3) clearly showed from their experiment that single stranded DNA has greater affinity for the interphase protein layer after phenol extraction than native DNA, and this might be the reason for the observation that a newly replicated DNA is rich in this interphase layer.

Because of the reports that at the end of S phase when the heterochromatin is replicated, label was associated with the nuclear membrane (2,5), which may suggest that DNA of nuclear membrane fraction might have different properties from the rest of the chromatin, the density of the DNA which is attached to the nuclear membrane fraction was also analyzed by CsCl gradients. Our preliminary analysis, as shown in Fig. 3, indicates that part of the DNA from the membrane fraction appears to band at lower densities than the bulk chromatin, indicating that perhaps this part may represent some fraction of the DNA with higher AT ratios or poorly separated mouse satellite DNA.

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