Localization of the replication point of mammalian cell DNA at the membrane

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SUMMARY: The replication site of DNA in HeLa S3 cells has been studied with "M-band" technique after Tremblay and others. When the cells are mixed with sodium lauroyl sarcosinate and Magnesium ion directly on the sucrose gradient and spun, virtually all the DNA is caught in the M-band, suggesting that mammalian cell DNA is attached to the nuclear membrane. After being treated with sodium lauroyl sarcosinate and sheared by a vortex-mixer, the DNA is found to have moved from the M-band to the supernatant. Pulse-labeled DNA is more resistant to shearing as compared with the bulk of DNA and chased away from the M-band during a subsequent growth. These results indicate that, as in the case of bacteria, the replication points of mammalian cell DNA are on the membrane.

The attachment of the chromosomal DNA to the cell membrane or the nuclear membrane has recently been reported in a variety of organisms from bacteria to mammals (1-5). With bacteria, it has been indicated that this attachment occurs at the point at which replication takes place: Ganesan and Lederberg (6) and Smith and Hanawalt (7) demonstrated, by sedimentation analysis of *B. subtilis* and *E. coli*, respectively, that newly synthesized DNA was associated with the rapidly sedimenting membrane fraction. After being chased, the DNA behaves in the same manner as the bulk of DNA, moving towards the slowly sedimenting fraction. Sueoka and Quinn (8) have shown the membrane attachment of the replication origin as well as of the replication point in *B. subtilis*, using genetic and biochemical analyses combined. More recently, Tremblay and others (9,10) have devised an isolation technique of cell membrane-DNA complex of bacteria, by which membrane-bound, nascent DNA was shown to be co-sedimented with sodium lauroyl sarcosinate-Mg crystals. The results of these researches confirm Jacob's model that bacterial genomes or replicons are attached to the bacterial membrane

at the replication site (11,12).

As to the mammalian cell DNA, on the contrary, Comings and Kakefuda (5) have shown, by autoradiographic studies on thin-sectioned, cultured human amnion cells, that the replication is initiated on the nuclear membrane and nucleolus at the commencement of the DNA synthetic phase, but the growing point is not definitely on the membrane and migrates from the initiation site on the membrane towards the center of the nuclei. With their experimental results, they suggest that a strict extension of the bacterial model would not fit in mammalian cells and that, in mammalian cells, DNA polymerase progresses along a fixed DNA than DNA moves along a fixed DNA polymerase.

We have re-examined on the replication site of the mammalian cell DNA using sedimentation analysis after "M-band" technique, the result of which will be described in this report.

Materials and Methods: HeLa S3 cells were grown in 5 ml of Eagle's MEM medium supplemented with 10% calf serum in Petri dishes. Incubation of the culture in a CO_2 cabinet at 37° yielded a doubling time of 20 to 24 hours.

The DNA in exponentially-growing cells was uniformly labeled by allowing two or more generations of growth with ^{14}C-thymidine at 0.2 μCi/ml (50 mCi/m mole, The Radiochemical Centre, Amersham, England). The replication site was selectively labeled with a short pulse of ^{3}H-thymidine at 10 μCi/ml (24 Ci/m mole, The Radiochemical Centre). After stopping the growth by addition of sodium azide (final concentration: 50 μg/ml), the cells were harvested from the glass surface with 0.05% trypsin (1:300, Nutritional Biochemical Corp., Bethesda, Md., U.S.A.), washed, and suspended in Ca- and Mg-free phosphate buffered saline (13). A final cell suspension was made by mixing of pulse- and uniformly-labeled cells at a final concentration of 1.2×10^5 cells/ml and 4×10^4 cells/ml, respectively.

Membrane fractions were isolated by means of a modified M-band technique as described by Tremblay et al.(9,10). Nine-tenth ml of the cell suspension was mixed gently with 0.1 ml of 1% sodium N-lauroyl sarcosinate (Sarkosyl, the gift of the Kao Soap Co., Ltd., Tokyo, Japan) and made to stand for 2 min in an ice-water bath. After magnesium acetate was added up to 0.01 M, the mixture was

allowed to stand in an ice-water bath for another 5 min so as to facilitate the formation of Mg-Sarkosyl crystals. A vortex-mixer (Thermonics Co., Ltd., Tokyo, Japan) was used to obtain mild shear of the lysate. Three-tenth ml of the lysate-crystal mixture was layered on the top of 4.7 ml of 15-47% (w/v) linear sucrose gradient in Tris-Mg-K (10) and the tubes were centrifuged at 15,000 rev/min for 20 min in the RPS 40 swinging-bucket rotor of a Hitachi Model 55P ultracentrifuge at 4°. Under these conditions the position of the M-band is near the middle of the gradient. After centrifugation, the topmost 1 ml fraction and the next one below it were collected by a bent-tip capillary pipette (fractions 5 and 4). Then the bottom fraction and the next one were collected by thrusting a straight capillary pipette through the M-band (fractions 1 and 2). Finally, the middle 1 ml was transferred to a test tube (fraction 3).

The radioactivity was assayed as follows: 0.5 ml of 1 mg/ml cod sperm DNA was added at 4° to each fraction (1 ml) and subsequently 2 ml of cold 10% trichloroacetic acid was added. Acid precipitable material was collected on glass filter paper (Whatman GF83), washed 4 times with 3 ml of cold 5% trichloroacetic acid, dried and placed in glass counting vial containing 10 ml toluene, 40 mg PPO, and 3 mg dimethyl-POPOP. The Packard Tri-Carb liquid scintillation spectrometer was adjusted for two channel simultaneous assay of ^{3}H and ^{14}C. Overlap correction was made by reference to freshly prepared standards. Recovery of the labeled material from the sucrose gradients varied from 70% to greater than 95% of input acid-insoluble radioactivity.

Results and Discussion: As shown in Fig. 1, virtually all of the DNA is sedimented with Mg-Sarkosyl crystals when the cell suspension is mixed gently with Sarkosyl on the sucrose gradient as described originally by Tremblay et al.(10). In Fig. 1a, a result is shown when drops were collected through a needle pierced at the bottom of the tube. The M-band is viscous and in some cases crystals are caught by the needle, resulting in an incomplete recovery. So we fractionated the gradient by capillary pipette method as described in Materials and Methods. Sometimes unbroken cells are contained in fraction 1 or 2, so we call fraction 3 as the "M-band fraction" and the combined fractions 4 and 5 "the supernatant."

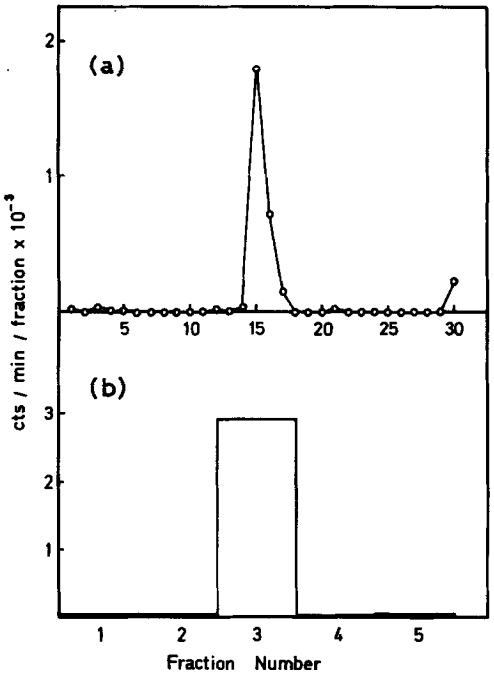

Fig. 1. Zone sedimentation of uniformly-labeled DNA in a sucrose density gradient. HeLa S3 cells, uniformly-labeled with ^{14}C-thymidine, were suspended in Tris-Mg-K at a concentration of 1.5 x 10^5 cells/ml. The cell suspension (0.27 ml) was layered over 4.7 ml of sucrose gradient and 0.03 ml of 1% Sarkosyl was layered on the top of the gradient. The cells and detergent were mixed gently with a glass rod on the top of the gradient in an ice-water bath. Centrifugation was done as described in Materials and Methods. Ten drops were collected from the bottom of the tube (Fig. 1a) or 1 ml fractions were withdrawn by capillary pipettes (Fig. 1b) and radioactivity was assayed as described in Materials and Methods.

Tremblay et al.(9,10) have shown that the surface of the crystals of Mg-Sarkosyl is hydrophobic and some fractions of the bacterial cell membrane (or phospholipids) attached to the M-band. In mammalian cells, also DNA would be involved in the M-band fraction in the form of the nuclear membrane-DNA complex, since virtually all of the DNA is found in the M-band when the cells are treated with Sarkosyl and Mg directly on the sucrose gradient. Biochemical analysis of the M-band fraction will be published elsewhere.

Sarkosyl-treated cell lysates were vortex-mixed in order to separate the membrane-attached DNA from the bulk of DNA. The effect of shearing with vortex-mixing on the retention of pulse- and uniformly-labeled DNA in the M-band fraction is illustrated in Fig. 2. Both pulse- and uniformly-labeled DNA are released from

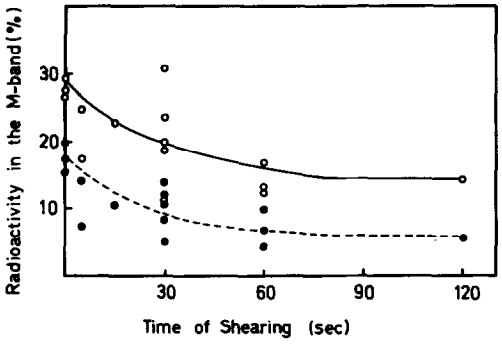

Fig. 2. Effect of vortex-mixing on the retention of pulse- and uniformly-labeled DNA in the M-band fraction. Both pulse- and uniformly-labeled HeLa S3 cells were lysed as described in Materials and Methods, and the lysate was sheared with vortex-mixing for various times.

○ ^{3}H; pulse-label, ● ^{14}C; uniform-label.

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the M-band as the time of shearing becomes longer, but pulse-labeled DNA is left in the M-band in a higher proportion. This result suggests the special interaction between the nuclear membrane and the replicating point of chromosomes.

If the replicating points were attached to specific sites on the nuclear membrane, it should be possible to chase the pulse-label from the M-band by a subsequent growth with non-radioactive thymidine. Fig.3 shows such experiments. When pulse-label is chased with non-radioactive thymidine, the ratio of $\%^{3}H$ to $\%^{14}C$ in the M-band decreases gradually and finally, about 3 hours later, reaches to the same level as the ratio in the supernatant fraction. When pulse-label was chased in the presence of fluorodeoxyuridine (5×10^{-4}M), an inhibitor for DNA synthesis, the initial ratio was found to have been kept at least for 3 hours. These results confirm, even in mammalian cells, the prediction that the replicating points are attached to specific sites on the nuclear membrane.

Our present data on the replication point of mammalian DNA conflict with those reported by Comings and Kakefuda (5). As noted above, they indicated by autoradiographic studies that the replication points is restricted on the nuclear membrane at the inception of S phase, but the figure becomes more obscure as the incubation time becomes longer. Pulse-labeled, unsynchronously growing cells, in particular, revealed grains homogeneously distributed in the nucleus. Based

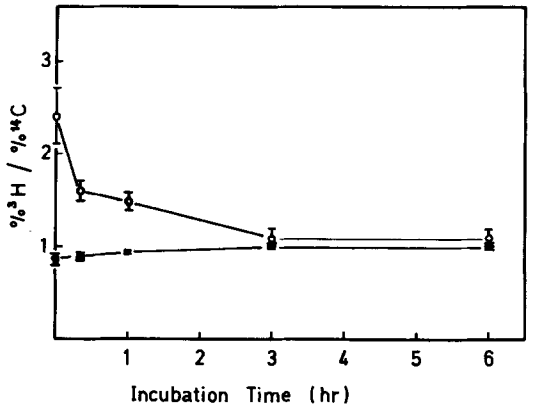

Fig. 3. Effect of thymidine chase on the resolution of pulse-labeled DNA from uniformly-labeled DNA. The culture was pulse-labeled for 3 min with ^{3}H-thymidine, radioactive medium was removed, washed twice with the growth medium, and incubated with the fresh growth medium with non-radioactive thymidine (10^{-5}M). At various times thereafter the cells were harvested. The lysate was sheared for 1 min with vortex-mixing, layered on a sucrose gradient and then centrifuged as described in Materials and Methods. In order to minimize the differential shearing effect on each tube in the course of the lysate formation, the results were indicated as the ratio of %^{3}H to %^{14}C in the M-band or the supernatant fraction. Experiments were carried out in triplicate; the mean and range of standard deviation are shown.

○ M-band fraction, ● Supernatant fraction.

on the rate of replication of a single replicon (14,15), they concluded that replication point would not always be on the nuclear membrane. Huberman and Riggs (15) showed in mammalian cells, however, many independently replicating units jointed together in tandem array. If a number of replicating units as a group were participated in the formation of autoradiograph, their estimation would not be valid. In fact, our preliminary experiment with sedimentation analysis has shown that newly synthesized DNA at the inception of the S phase converts, when chased, from the M-band to the supernatant fraction, suggesting that the multiple replicating origin of mammalian DNA seems to be detached from the membrane.

Ben-Porat and others (16) and recently Friedman and Mueller (17) reported that replicating DNA of cultured cells differed from non-replicating DNA by its partition to the interphase fraction during the extraction with some organic solvents. It was suggested that mammalian DNA attached to some other cellular

component at or near the site of replication. These results coincide with ours though the exact nature of the attachment will be solved in future.

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REFERENCES

1. Ryter, A. and Jacob, F., Compt. Rend. Acad. Sci. (Paris) 257, 3060 (1963); 264, 2254 (1967)
2. Ryter, A., Bacteriol. Rev. 32, 39 (1968)
3. Gall, J.G., J. Biophys. Biochem. Cytol. 5, 295 (1959)
4. Pawlowski, P.J. and Berlowitz, L., Exptl. Cell Res. 56, 154 (1989)
5. Coming, D.E. and Kakefuda, T., J. Mol. Biol. 33, 225 (1968)
6. Ganesan, A.T. and Lederberg, J., Biochem. Biophys. Res. Commun. 18, 824 (1965)
7. Smith, D.W. and Hanawalt, P.C., Biochim. Biophys. Acta 149, 519 (1967)
8. Sueoka, N. and Quinn, W.G., Cold Spring Harbor Symp. Quant. Biol. 33, 695 (1968)
9. Earhart, C.F., Tremblay, G.Y., Daniels, M.J. and Schaechter, M., Cold Spring Harbor Symp. Quant. Biol. 33, 707 (1968)
10. Tremblay, G.Y., Daniels, M.J. and Schaechter, M., J. Mol. Biol. 40, 65 (1969)
11. Jacob, F., Brenner, S. and Cuzin, F., Cold Spring Harbor Symp. Quant. Biol. 28, 329 (1963)
12. Jacob, F., Ryter, A. and Cuzin, F., Proc. Roy. Soc. (London), Ser. B 164, 267 (1966)
13. Dulbecco, R. and Vogt, M., J. Exptl. Med. 99, 167 (1954)
14. Cairns, J., J. Mol. Biol. 15, 372 (1966)
15. Huberman, J.A. and Riggs, A.D., J. Mol. Biol. 32, 327 (1968)
16. Ben-Porat, T., Stere, A. and Kaplan, A.S., Biochim. Biophys. Acta 61, 150 (1962)
17. Friedman, D.L. and Mueller, G.C., Biochim. Biophys. Acta 174, 253 (1969)