INTERMEDIATE FRAGMENTS OF THE NEWLY REPLICATED DNA IN MAMMALIAN CELLS

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SUMMARY Using the new method of sucrose density gradient centrifugation technique for the analysis of intact cell DNA without artificial scission, the discontinuous replication of DNA in cultured mouse mammary carcinoma cells was investigated. The results indicated the sequence of elongation of short fragments in the process of replication and several intermediate fragments were found. Their sizes were 20S, 40S, 70-100S, 280S and the bulk of the cell DNA was 380S.

The discontinuous replication of DNA strand and its elongation by ligation was reported in bacterial cells and phages (1 - 3). Recently, also in mammalian cells, short segments of newly synthesized DNA were shown in some investigations with Chinese hamster cells (4), regenerating rat liver (5), HeLa cells (6), and isolated HeLa cell nuclei (7). But there is some doubt whether degradation of DNA strands occurs during the manipulations to extract DNA (8). For the bulk of the DNA rather small S-values were shown in previous investigations. Terashima and Tsuboi (9) presented a new sucrose density gradient technique for isolation of intact cell DNA with no degradation by shearing and they showed the bulk of the L-cell DNA to be as large as 330S. Here, we present evidence for

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the discontinuous replication of DNA indicating elongation by replication and ligation in mammalian cells and that the intermediate fragments had several states of elongation. Their sizes were 20S, 40S, 70-100S, 280S and the bulk of the cell DNA was 380S.

MATERIALS AND METHODS

The cell line used for this study was FC-1, which had been derived from FM3A line (C3H mouse mammary carcinoma origin) established by Nakano (10) and led to 8-azaguanine resistant cells by Koyama, Yatabe and Ono (11). The cells were maintained in modified Eagle's minimum essential medium (Nissui Pharm. Co.) supplemented with 5% calf serum and 0.1% Bacto-Peptone (Difco Lab.). The cells in log phase were cultivated overnight in the medium without Bacto-Peptone and 2 ml of cultures (1.5 - 3.0 x 10^5 cells/ml) were allowed to incorporate ^{3}H-TdR. In some experiments FUdR (0.1 µg/ml) was added to the culture medium 2 hours before ^{3}H-TdR pulse labeling. ^{3}H-TdR (2-10 µc/ml, 18 c/mmole) was added to 2 ml of culture medium and pulsed a few minutes at 37°C. In the pulse-chase experiments, cells were labeled with ^{3}H-TdR 5 minutes, then transferred to medium containing 10 µg/ml cold TdR and chased for 10-60 minutes. Incubation mixtures were poured into ice-cold 30 ml saline to stop the reaction. Cells were then pelleted, resuspended in 1 ml of ice-cold saline and 0.3-0.5 ml cell suspensions were layered over a layer of 2% SDS on top of a sucrose gradient. The sucrose gradient was made up by the modified method of Terashima and Tsuboi (9). 5-20% alkaline sucrose gradient in 10 mM EDTA, 0.1 M NaCl, 0.02% SDS, NaOH (pH 12.5) was first made up in a centrifugation tube (SW 25.3). Then 0.5 ml of 2% SDS in 4% sucrose, 0.1 M NaCl, 10 mM EDTA, 30 mM Tris-Cl (pH 7.5) was layered on the top of the sucrose gradient. Cell suspension in saline was placed on the SDS layer without mixing of the top fraction. Gradient was centrifuged

21,000 r.p.m. for 90 minutes or the time indicated in the legend of each figure at 20°C. After centrifugation, to each 0.5 ml fraction from the top of the gradient was added 100 μg bovine serum albumine as co-precipitate, 2 ml of 10% ice-cold TCA and the suspension was filtered through the glass fiber filter (Toyo Roshi Ltd.). Filters were washed with 10 ml of cold 5% TCA and ethanol, then dried and counted by Packard liquid scintillation spectrophotometry in toluene-PPO-POPOP system. To estimate the S-value of DNA, λ phage DNA was used as a standard and centrifuged for 18 hours. S-values are calculated following the equation of Abelson and Thomas (12).

RESULTS AND DISCUSSION

The bulk of FM3A cell DNA was labeled overnight with ^{3}H-TdR 20 mμc/ml). Sedimentation profile of this DNA in alkaline sucrose gradient was appeared to show a single peak (Fig. 1-1), indicating that the bulk of cell DNA is very homogenious and no scission is brought about by this method. According to our estimate, the bulk of the DNA was 380S.

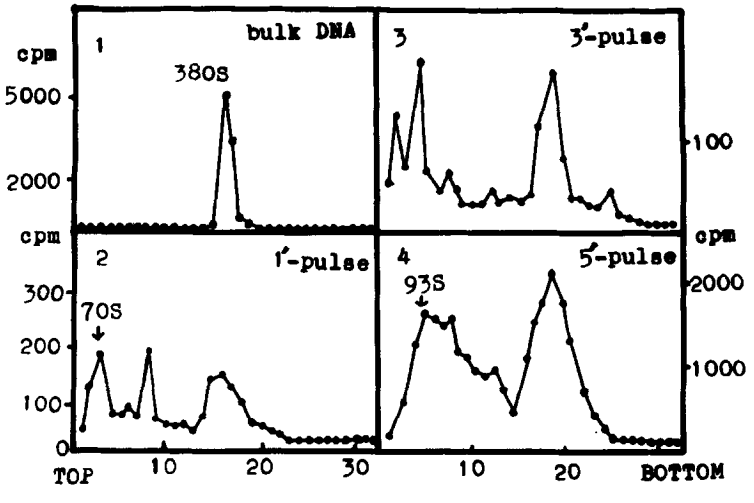

Fig. 1
Sedimentation profiles of cell DNA in alkaline sucrose gradients (5-20%). Cells were labeled with ^{3}H-TdR for overnight or 1-5 minutes and centrifuged for 1.5 h., 21,000 r.p.m. at 20°C.

When the cell protein was labeled with radioactive amino acids and centrifuged, no significant radioactivity was found with the DNA peak (9, 13). So the possibility that this peak was the aggregate of DNA and protein could be excluded.

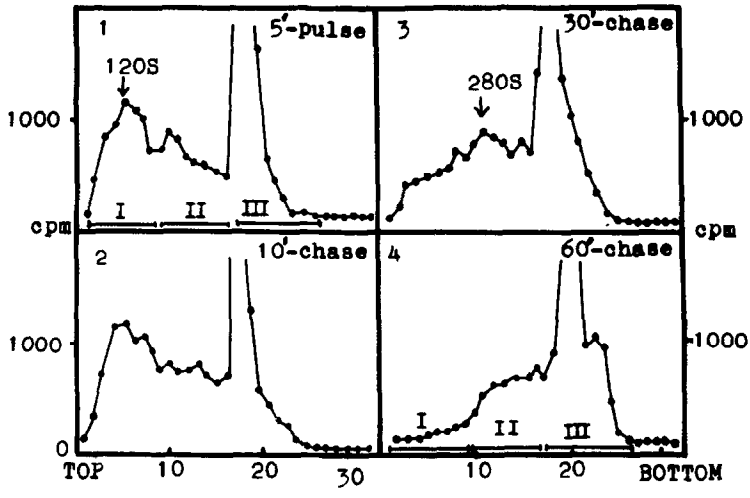

Fig. 2
5 minutes pulse labeled cells were chased in cold TdR medium for 10, 30, 60 minutes and sedimented for 1.5 h. 21,000 r.p.m. at 20°C.

In 1 minute pulse labeled cells (Fig. 1-2), it appeared that at very early time equal amounts of ^{3}H-TdR were incorporated into the bulk DNA and in the short segment found at the top fraction. In 5'pulse labeled cells, labeled DNA showed a broad slow sedimenting peak (around 100S) and was heterogeneous.

In the pulse-chase experiments (Fig. 2) the cells were labeled for 5 minutes with ^{3}H-TdR and chased in a cold medium for 10-60 minutes. Two large peaks were found in this experimental condition, that is, the peak I which appeared at 5 minutes pulse at about 100S (Fig. 2-1) and the peak II which remained after 30-60 minutes chase around 280S (Fig. 2-4). The proportion of radioactivity in each peak fraction was calculated and shown in Table 1. The total radioactivity recovered at each time of chase was almost constant as

Table 1
The amount of radioactivities in the peaks indicated in Fig. 2 against the total radioactivity.

Chased time	Peak I	Peak II	Peak III	Recovered total radioactivities
0 min.	29%	26	44	215.346
10 min.	27	24	46	237.502
20 min.	15	26	58	233.880
60 min.	5	20	75	230.193

shown in the last column of Table 1, thus the existence of the precursor-product relation among each peak was demonstrated.

In these experiments, the estimation of S-value was rather difficult, because a shift of one fraction corresponded to the change of 23S. Moreover, the first and second 0.5 ml fractions from the top were saline in which the cells were suspended, and 2% SDS (4% sucrose) layer, respectively. So, the peak 70S in Fig. 1-2 may contain the fragments of 0-70S length. This fraction was further investigated in detail below.

Recently Schandl and Taylor (4) reported that the newly synthesized short DNA segment is 4-5S and the template DNA is 70-75S in cultured Chinese hamster cells. To isolate this short segment, pulse labeled cells were centrifuged for 7 hours at 21,000 r.p.m. (Fig. 3, 1-3). It was found that the "70S" peak in Fig. 1 was indeed a mixture of short fragments and contained at least 20S, 40S, and 70-95S peaks.

The sequence of elongation of short fragments from 20S to about 100S was clearly demonstrated in this result. But a discrepancy with the results of Schandl and Taylor still remains and there may have been some artificial degradations in their results, since the template DNA has rather smaller S-value.

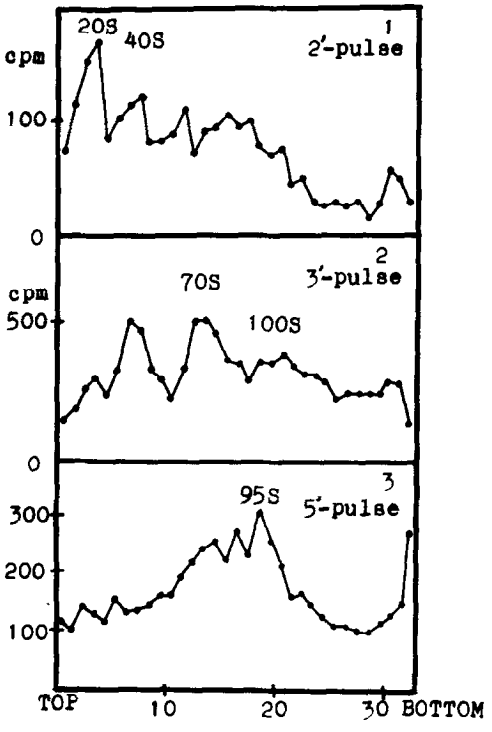

Fig. 3
Sedimentation profiles of pulse labeled cells.
Centrifuged for 7 h. 21,000 r.p.m. at 20°C.

The possibility that the shorter initial fragment of replication is contained in 20S-peak is now under investigation.

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