Acta Medica Okayama

Volume 25, *Issue* 1 1971 *Article* 4

FEBRUARY 1971

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Hajime Ogura* Takuzo Oda†

*Okayama University,

†Okayama University,

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Hajime Ogura and Takuzo Oda

Abstract

The incorporation of tritiated thymidine into DNA molecules was studied by electr.:m microscopic autoradiography. To make autoradiogram in electron microscopic level, DNA was extracted from rat ascites hepatoma (AH 130) cells after in vitro incubation with tritiated thymidine. Extracted DNA samples were rotary shadowed with platinum palladium and covered with emulsion. Silver grains demonstrated on autoradiogram indicated tritiated thymidine to have incorporated into DNA molecules themselves. The incorporation was further confirmed by liquid scintillation counting of TeA soluble and insoluble fractions after DNase or RNase treatment of the DNA preparations.

*PMID: 4334468 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **25**, 37—41 (1971)

STUDIES ON TRITIATED THYMIDINE INCORPORATION INTO DNA MOLECULES BY ELECTRON MICROSCOPIC AUTORADIOGRAPHY

Hajime OGURA and Takuzo ODA

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

Received for publication, November 12, 1970

CAIRNS studied the mechanism of DNA replication in *E. coli* chromosomes by light microscopic autoradiography using tritiated thymidine (1, 2) and proposed a theory on the replication of circular DNA molecules. Parallel relationship between autoradiographic grain numbers and radioactivity counts was shown by HUNT *et al.* (3), but there are some reports about the contamination of tritiated thymidine (4, 5) or incorporation of tritium from thymidine (6) into substances other than DNA molecules themselves. If the radioactive count of DNA preparations is due to tritiated thymidine incorporated into DNA molecules themselves and not due to contamination, autoradiography of extracted DNA in the electron microscopic level will be useful for the study on the mechanism of DNA replication. With this point in mind we have carried out the present study and demonstrated the incorporation of radioactive thymidine into DNA molecules by the electron microscopic autoradiography of DNA extracted from rat ascites hepatoma (AH 130) cells after *in vitro* incubation with tritiated thymidine.

MATERIALS AND METHODS

The following materials served for the present study: rat ascites hepatoma (AH 130) cells; tritiated thymidine obtained from the Radiochemical Center, England (specific activity 5C/mM); autoradiographic emulsion (SAKURA NR-M2 and NR-H2) of Konishiroku, Japan; DNase and RNase (Worthington); and cytochrome *c* (Boehringer).

Labeling of DNA: Ten ml of ascites containing logarithmically growing AH 130 cells were taken out from the peritoneal cavity of a Donryu rat 6 days after intraperitoneal transplantation and transferred in a shaking flask without delay. One mC of tritiated thymidine was added immediately and incubated for 2 hr at 37°C. Control AH 130 cells were also incubated without labeling.

Isolation of DNA: DNA was extracted according to MARMUR's method (7) and purified by RNase treatment.

Autoradiographic preparations: For the light microscopic autoradiography, labeled AH 130 cells and the unlabeled control were smeared on small pieces of cover-slips and fixed with methanol. Each smeared cover-slip was set in each test tube with a hole at the bottom. Emulsion (Sakura NR-M2) was coated by dipping the test tube into the emulsion diluted 2-fold at 45°C. They were exposed for several weeks at 4°C before being developed in a light-tight box.

Development was carried out for 4 min. at 20°C in Conidol-X (Konishiroku) followed by 1 min water wash and fixed for 3 min in Kodax fixer. Developed cover-slips were dipped in 1 % glycerin and stained with 5 % Giemsa solution for 25 min. Mounting the stained cover-slips on slides, they were examined.

For electron microscopic autoradiography, copper grids were dipped into a solution of 4 % collodion and dried immediately on filter paper, thereby encasing the grids in a coat of collodion but leaving the pores open (8, 9). Samples of labeled DNA and unlabeled-control were dissolved in saline sodium citrate (SSC). Final concentration of DNA was 3—6 μg/ml. The samples were spread according to the protein monolayer technique of KLEINSCHMIDT *et al.* (10).

The protein used was cytochrome *c* in final concentration of 0.01 %. 0.3 ml of the final mixture (3—6 μg/ml of DNA in 0.01 % cytochrome *c*, M ammonium acetate, 0.5 % formalin) was gently dropped onto a slanting clean glass slide from which it flowed onto the surface of 0.15 M ammonium acetate solution containing 0.5 % formalin in a glass dish. DNA-cytochrome *c* mixed film was taken up on collodion coated, carbon stabilized grids and dehydrated with 95% ethanol. Rotary shadowing was done on it with platinum palladium. Some samples were stained with uranyl solution (11) after transfer on the DNA-cytochrome *c* mixed film to the grids. The stain solution was prepared by 50-fold dilution with acetone of a stock solution of 5×10^{-3} M uranyl acetate in methanol. Both of these grids were coated with a thin film of emulsion (Sakura NR-H2) by covering a fine wire loop (9) over the grids supported on wood pegs. The covered samples were exposed for several weeks at 4°C before being developed in a light-tight box.

Development procedures were the same as in light microscopic autoradiography. After water wash, the grids were dried and examined in the electron microscope Hitachi HU-11C. With some samples, gelatin was removed by dipping in 0.05N NaOH solution for 20 min. (8).

Counting of radioactivity with liquid scintillation counter: Half ml of DNA solution was treated with DNase (80 μg/ml) in 5 mM $MgCl_2$ for 1 hr at 0°C or RNase (100 μg/ml) for 1 hr at 37°C followed by precipitation with 4.5 ml of 10 % ice cold trichloric acid (TCA), in each case. TCA insoluble residue after centrifugation was washed twice with cold TCA and dissolved in 5 ml of SSC. Half ml of each sample of TCA soluble and insoluble (dissolved in SSC) fractions was put in 6 ml of ethylene glycol monomethyl ether and 10 ml of PPO-POPOP-toluene scintillator mixture (12). Radioactivity counts were taken by Packard TRI-CARB liquid scintillation spectrometer Model 3365.

RESULTS

Numerous localized silver grains were visible on nuclei by the light microscopic autoradiography (Fig. 1), while no visible grains in control samples. By electron microscopic autoradiography, silver grains were visible on DNA fibers in the rotary shadowed samples (Figs. 2, 3). There were a few background grains in some visual fields. Background grains were also visible at the rate of 1 grain per visual field at a magnification of $\times$ 5000 in the control samples, but no silver grain was visible directly on DNA fibers. The removal of gelatin with 0.05N NaOH solution made images rather vague. In uranyl acetate stained samples, silver grains were observed but DNA fibers were not visible. Removal of gelatin or post staining with uranyl acetate solution could not reveal DNA fiber. Radioactivity counts of labeled DNA were 3239cpm/μgDNA (Efficiency 21%). The radioactivity was found mostly in TCA soluble fraction in the DNase treated sample, and it remained in TCA insoluble fraction in RNase treated sample (Table 1).

TABLE 1. RADIOACTIVITY COUNTS IN TCA SOLUBLE AND INSOLUBLE FRACTIONS OF THE DNA PREPARATIONS AFTER TREATMENT WITH DNASE OR RNASE.

DNase Treated		RNase Treated	
TCA Soluble	TCA Insoluble	TCA Soluble	TCA Insoluble
28324	123	78	25641

After treatment with DNase or RNase, DNA preparations were precipitated by cold TCA. TCA insoluble precipitate was dissolved in SSC. Radioactivity was determined in a liquid scintillation counter using ethylene glycol monomethyl ether and PPO-POPOP-toluene scintillation fluid.

DISCUSSION

Both by light microscopic and electron microscopic autoradiography, comparable results were obtaind. DNase and RNase treatments showed that tritiated thymidine was incorporated into DNA molecules themselves. Though samples were shadowed with platinum palladium, silver grains were specifically distributed on DNA fibers in electron microscopic autoradiograms. Some background grains may be due to contamination of free radioactive nucleotides and/or may be caused by unknown physicochemical factors. More adequate technique and suitable emulsion are needed to reduce background grains. Removal of gelatin was not necessary with the rotary shadowed samples. Recently ZADYLAK *et al.* (13) reported the results of autoradiograms of the DNA stained with uranyl acetate

using similar methods as ours, but we could not obtain clear autoradiogram by the uranyl acetate staining. In any event these autoradiographic techniques will contribute to the study of the mechanism on DNA replication.

SUMMARY

The incorporation of tritiated thymidine into DNA molecules was studied by electron microscopic autoradiography. To make autoradiogram in electron microscopic level, DNA was extracted from rat ascites hepatoma (AH 130) cells after *in vitro* incubation with tritiated thymidine. Extracted DNA samples were rotary shadowed with platinum palladium and covered with emulsion. Silver grains demonstrated on autoradiogram indicated tritiated thymidine to have incorporated into DNA molecules themselves. The incorporation was further confirmed by liquid scintillation counting of TCA soluble and insoluble fractions after DNase or RNase treatment of the DNA preparations.

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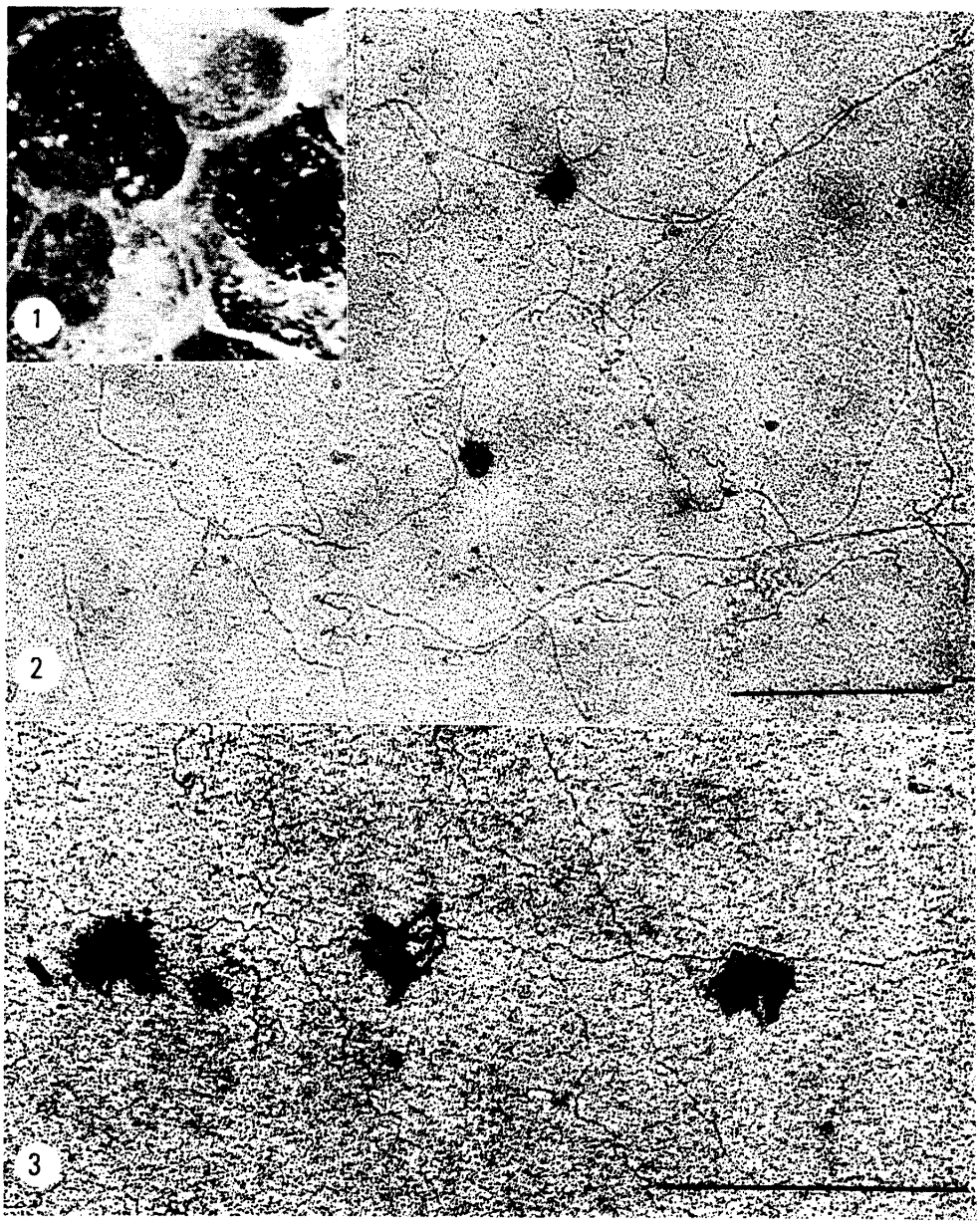

Fig. 1 Light microscopic autoradiography of AH 130 cells. Silver grains on nuclei show incorporated tritiated thymidine.

Fig. 2 Electron microscopic autoradiography of tritiated thymidine incorporated into DNA fibers, rotary shadowed with platinum palladium. Marker represents 1 μ.

Fig. 3 Electron microscopic autoradiography of tritiated thymidine incorporated into DNA fiber, rotary shadowed with platinum palladium. Marker represents 1 μ.