

## DIRECT VISUALIZATION OF LABELED NUCLEOTIDES IN DNA USING ELECTRON MICROSCOPY AND AUTORADIOGRAPHY

G. H. WEBER, U. HEINE, M. COTTLER-FOX, and G. BEAUDREAU. From the Virus Studies Section, Viral Oncology, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Maryland 20014 and the Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

### INTRODUCTION

Recent advances in the visualization of nucleic acids by electron microscopy allow a correlation between ultrastructure and genetic content. Partial denaturation of phage DNA in formaldehyde (7, 8) gives a denaturation map of the nucleic acid, demonstrating adenine- and thymine-rich regions. Likewise, hybridization experiments and electron microscope examination of DNA from wild-type and deletion mutant phage (3) result in a map of the physical position of the deletions.

Autoradiography of nucleic acids, however, has been limited almost exclusively to the light microscope. Experimental evidence at this level has demonstrated the replication of bacterial (2,

11) and mammalian (5, 6) cell DNA at forklike or bidirectional growing points.

The present paper describes a technique in which the ultrastructural analysis of purified nucleic acids can be coupled to autoradiographic procedures. Radioactively labeled DNA, isolated from L cells, was spread for electron microscopy using a modification of the Kleinschmidt and Zahn technique (9). Autoradiographic procedures that followed allowed for both direct visualization of the DNA through the developed emulsion and detection of areas of active isotope incorporation by the presence of developed grains. An attempt in this direction has been made previously concerning the visualization of recombinant DNA molecules of bacteriophage T1 (1).

## MATERIALS AND METHODS

### *Tissue Culture*

Cultures of L cells were grown in T-60 tissue culture flasks (Falcon Plastics, Oxnard, Calif.) using growth medium consisting of 80% minimal essential medium (MEM), 10% tryptose phosphate broth, and 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and incubated in a humidified, 5% CO<sub>2</sub> atmosphere at 37°C. 24 h after subculturing, the cell medium was decanted and replaced with growth medium containing radioactive label ([<sup>3</sup>H]thymidine [42 Ci/mmol] at 14 μCi/ml; [<sup>3</sup>H]deoxyadenosine [8.8 Ci/mmol] at 4.8 μCi/ml; [<sup>3</sup>H]deoxycytidine [21.2 Ci/mmol] at 4.8 μCi/ml; and [<sup>3</sup>H]deoxyguanosine [5.0 Ci/mmol] at 4.8 μCi/ml) (Amersham/Searle Corp., Arlington Heights, Ill.). 48 h after addition of the label the cells were washed with phosphate buffered saline and removed with 0.25% Viokase (Grand Island Biological Co.). The cell suspension was centrifuged for 10 min in a clinical centrifuge, the medium decanted, and the cell pellet (approximately 0.1 ml) frozen at -70°C until used.

### *Extraction of Cellular DNA*

DNA was extracted using the procedures of Renger and Wolstenholme (12). Thawed L cells were resuspended in 10 vol of 0.15 M NaCl, 0.01 M EDTA (NE buffer), sodium dodecyl sulfate (SDS) was added to give a final concentration of 1%, and the cell mixture was incubated at 37°C for 30 min. Sodium chloride was added to give a 5 M final concentration and the mixture was iced for 30 min before being clarified by centrifugation (10,000 g for 10 min). The supernate was gently mixed four times with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol) for 5 min. The aqueous phase was diluted with an equal volume of 0.01 M Tris (pH 8.3) and RNase (previously heated to 90°C for 5 min at pH 5.0) was added to give 400 μg/ml. The solution was incubated at 37°C for 30 min and dialyzed for 2 h against NE buffer. The DNA was precipitated by adding 0.55 vol of isopropyl alcohol and storing at -20°C overnight. After centrifugation at 24,000 g for 15 min the DNA pellet was resuspended in 0.01 M Tris, pH 8.0; 0.15 M NaCl; 0.001 M EDTA (TNE buffer) and a sample was taken for radioactive and optical density determination.

### *Preparation for Electron Microscopy*

DNA was spread using modifications of the protein monolayer technique of Kleinschmidt and Zahn (9) and Westmoreland et al. (13). Radioactive L-cell

DNA (23 μg/ml; specific activity  $2.3 \times 10^5$  cpm/μg) was mixed with an equal volume of formamide followed by the addition of 0.1 vol of 0.1% cytochrome *c*. The mixture was then applied to the surface of a water-filled, Teflon-coated trough (20 × 13 × 0.4 cm) with a 0.025-ml microdiluter (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.). The DNA-protein film was picked up on Formvar- and carbon-coated nickel grids, washed in ethanol for 45 s, and allowed to air dry. The grids were rotary shadowed with platinum-carbon at an angle of 8° and afterwards covered with a thin carbon film.

### *Autoradiography and Electron Microscopy*

Grids were prepared for autoradiography according to the method of Lettré and Pawletz (10). Platinum wire loops were immersed in diluted Ilford L-4 emulsion and the film allowed to air dry. The grids were covered with that area of the emulsion showing a gold interference color, attached to glass slides with tape, transferred to light tight boxes, and kept at 4°C for 4-6 wk. Microdol X was used as the developer after first stabilizing the emulsion for 30 min in 70% ethanol vapor. The grids were examined in a Siemens 1A electron microscope operating at 80 kV with a 50-μm objective aperture. The microscope was calibrated with a 54,800 lines per inch grating replica (Ernest Fullam, Inc., Schenectady, N. Y.) and all photographs taken at a magnification of 10,000.

## RESULTS AND DISCUSSION

Tritium-labeled deoxyribonucleic acid, isolated from L cells, is shown in Fig. 1. Prepared as described in Materials and Methods, L-cell DNA has routinely resulted in smoothly contoured strands. The lengths of the molecules, however, were variable, most probably due to shearing during isolation. Exposed grains seen here and in Figs. 2 and 3 indicate the sites of active incorporation of radioactive substrates. These figures were obtained from DNA with a specific activity of  $2.3 \times 10^5$  cpm/μg, which was exposed to emulsion in the dark for 6 wk. In these preparations more than 95% of the exposed grains could be associated with the strands.

As is evident in Fig. 1, not all DNA molecules show exposed grains, possibly a result of the insensitivity of the technique or of DNA molecules that did not replicate in the presence of the labeled substrate. One should be able to obtain a greater amount of label per unit length of DNA with synchronized cells, higher specific activity labeling, and increased exposure time.



FIGURE 1 Tritium-labeled L-cell DNA prepared according to Kleinschmidt and Zahn (9) and Westmoreland et al. (13) and covered with Ilford L4 emulsion (10). Specific activity:  $2.3 \times 10^5$  cpm/ $\mu$ g. Exposure time: 6 wk. Bar, 1  $\mu$ m.  $\times 28,500$ .

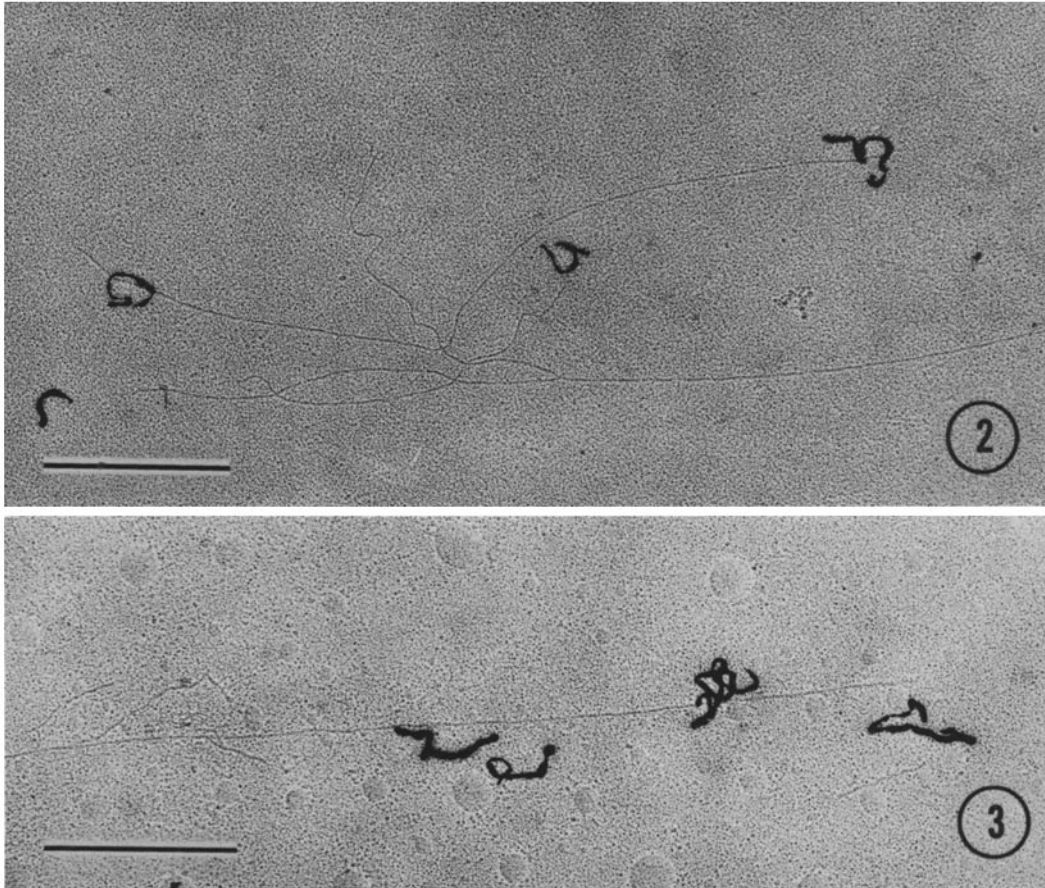


FIGURE 2 Tritium-labeled L-cell DNA prepared as in Fig. 1. The close association of developed grains with the DNA molecules is apparent. No background contamination. Bar, 1  $\mu\text{m}$ .  $\times 24,500$ .

FIGURE 3 L-cell DNA prepared as in Fig. 1. A single DNA molecule is labeled with four grains at different positions along the molecule. Bar, 1  $\mu\text{m}$ .  $\times 24,500$ .

DNA preparations of sixfold lower activity ( $3.7 \times 10^4$  cpm/ $\mu\text{g}$ ) were also examined. Here, as above, virtually all of the developed grains were associated with DNA strands. Although a quantitative analysis was not performed, the decreased number of DNA-associated grains approximated the decrease in specific activity.

Fig. 3 represents the general appearance of DNA molecules showing more than one grain. The presence of the radioactivity at multiple points along a strand seems to indicate the completion of a round of replication with incorporation of radioactive molecules at different points along the molecule. Consequently, with the technique presented here, an ultrastructural and autoradiographic analysis of the replicating

molecules is now possible if suitable pulse label conditions are chosen.

These procedures will allow the electron microscope analysis of purified nucleic acids where it would be desirable to distinguish between radioactive and nonradioactive molecules or portions of molecules. Among the uses of this direct ultrastructural autoradiographic technique might be the examination of hybridized molecules, the sensitivity of labeled and/or substituted nucleic acids to various agents such as X rays or UV light, as well as observations on nucleic acid replication.

We are very grateful to Dr. R. Gerald Suskind for

helpful discussions throughout this work. We also appreciate deeply the excellent technical assistance of Mr. Benjamin Elliott, Jr. and Mr. Douglas Jones. Received for publication 30 October 1973, and in revised form 13 December 1973.

#### REFERENCES

1. BRESLER, S. E., L. P. DADIVANJAN, and M. I. MOSEVITSKY. 1970. Electron microscopic autoradiography of recombinant DNA molecules of bacteriophage T1. *Biochim. Biophys. Acta.* **224**:249-252.
2. CAIRNS, J. 1963. The chromosome of *Escherichia coli*. *Cold Spring Harbor Symp. Quant. Biol.* **33**: 43-45.
3. DAVIS, R. W., and N. DAVIDSON. 1968. Electron-microscopic visualization of deletion mutations. *Proc. Natl. Acad. Sci. U. S. A.* **60**:243-250.
4. FAREED, G. C., C. F. GARON, and N. P. SALZMAN. 1972. Origin and direction of simian virus 40 deoxyribonucleic acid replication. *J. Virol.* **10**:484-491.
5. HAND, R., and I. TAMM. 1973. DNA replication: direction and rate of chain growth in mammalian cells. *J. Cell Biol.* **58**:410-418.
6. HUBERMAN, J. A., and A. TSAI. 1973. Direction of DNA replication in mammalian cells. *J. Mol. Biol.* **75**:5-12.
7. INMAN, R. B. 1966. A denaturation map of the  $\lambda$  phage DNA molecule determined by electron microscopy. *J. Mol. Biol.* **18**:464-476.
8. INMAN, R. B. 1967. Denaturation maps of the left and right sides of the lambda DNA molecule determined by electron microscopy. *J. Mol. Biol.* **28**:103-116.
9. KLEINSCHMIDT, A. A., and R. K. ZAHN. 1959. Deoxyribonucleic acid molecules in protein-mixed films. *Z. Naturforsch. Teil B.* **146**: 770-779.
10. LETTRÉ, H., and N. PAWELETZ. 1966. Probleme der elektronenmikroskopischen Autoradiographie. *Naturwissenschaften.* **53**:268-271.
11. PRESCOTT, D. M., and P. L. KUEMPEL. 1972. Bidirectional replication of the chromosome in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2842-2845.
12. RENGER, H. C., and D. R. WOLSTENHOLME. 1970. Kinetoplast deoxyribonucleic acid of the hemoflagellate. *Trypanosoma lewisi*. *J. Cell Biol.* **47**:689-702.
13. WESTMORELAND, B. C., W. SZYBALSKI, and H. RIS. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. *Science (Wash. D. C.)* **163**:1343-1348.