TRITIATED ACTINOMYCIN D AS A CYTOCHEMICAL LABEL FOR SMALL AMOUNTS OF DNA

BONNIE SUE EBSTEIN. From the Department of Biology, Yale University, New Haven, Connecticut 06520

INTRODUCTION

Actinomycin D blocks DNA-dependent RNA synthesis by binding to DNA (Reich et al., reference 15). On a cellular level, several radioautographic studies have demonstrated the in vivo binding sites of radioactive actinomycin (de Vitry, reference 19; Fraccaro et al., reference 3; Goldstein et al., reference 8; Ro et al., reference 16; Rothstein et al., reference 17; Sonneborn and Rothstein, reference 18). Brachet and Ficq (2) used actinomycin-^{14}C as a cytochemical label for DNA. The commercial availability of tritiated actinomycin D of high specific activity has raised the possibility of extending the sensitivity of this technique. Such an extension would have particular value at the present time when the question of endogenous DNA within organelles is of great interest.

The present report concerns the amphibian oocyte nucleus. These nuclei contain approximately 1,000 nucleoli which are unattached to the chromosomes. Each of these nucleoli has been shown by two methods to contain a small amount of DNA: (*a*) under certain conditions the nucleoli are in the form of rings which can be fragmented by DNase (Miller, reference 13; Kezer, reference 10) (*b*) a Feulgen-positive granule is sometimes seen in association with each intact nucleolus (Brachet, reference 1; Painter and Taylor, reference 14; Macgregor, reference 12; Gall, personal communication). However, the Feulgen reaction in these particles is usually quite weak, and during some stages of oogenesis staining is undetectable. For this reason, actinomycin D-^{3}H was used as an alternate "stain" for nucleolar DNA.

MATERIALS AND METHODS

Actinomycin D-^{3}H at a specific activity of 3.8–4.2 c/mmole was obtained from Schwarz Bio Research Inc., Orangeburg, N.J.

Oocytes were obtained from newts of the species *Triturus viridescens* or *Triturus cristatus carnifex* for the preparation of chromosomes and nucleoli. Only mature oocytes were used for the preparations demonstrating localization of label in nucleoli. So that the number of mature oocytes in the ovary could be increased, a female was given three injections, over a week's time, of 100 IU of chorionic gonadotropin (Antuitrin S from Parke, Davis & Co., Detroit, Mich.) The oocytes were removed from the anesthetized animal, and the nuclei were isolated in 0.1 M "5:1" (5 parts 0.1 M KCl: 1 part 0.1 M NaCl). The nuclei were then transferred to well slides constructed as described by Gall (6). The well chamber had been previously washed with an aqueous solution of cold actinomycin D (Merck, Sharp & Dohme, West Point, Pa.; 10 μg/ml). This step is essential for reducing the background of the radioautographs to a reasonable level, since actinomycin binds strongly to glass.

A drop of actinomycin D-^{3}H was placed in the well chamber at a concentration of 30 μc/ml (about 10 μg/ml) in 0.1 M 5:1. The isolated nucleus was transferred into the well, and the nuclear membrane was removed with forceps. The chromosomes and nucleoli were allowed to spread in the actinomycin D-^{3}H solution for 1–3 hr at 4°C. After this time, they were fixed to the bottom of the well chamber by exposing the preparation to the vapor of 50% ethanol. After fixation the actinomycin solution was washed out by placing the well slide into 0.05 M phosphate buffer, pH 6.8, for 5 min and then into water for 5 min. The preparation was then air dried.

After the well slides had been separated from the slides bearing the chromosomes and nucleoli, the latter were washed in toluene and acetone and then

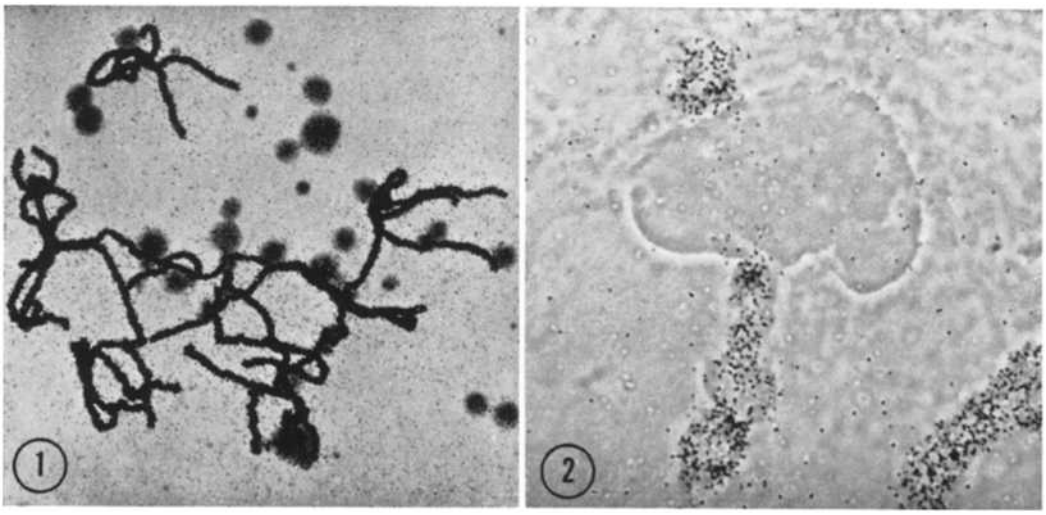

FIGURE 1 Actinomycin D-^{3}H binding to the chromosomes of a mature oocyte of *T. cristatus carnifex*. The black appearance of the chromosomes is due to the heavy concentration of silver grains. The nucleoli have not yet reached the stage of development when they exhibit sharply localized labeling, although the chromosomes have reached the contracted stage typical of mature oocytes. Exposure, 2 wk. × 268.

FIGURE 2 Actinomycin D-^{3}H binding to the fourth chromosome in a salivary gland cell of *Chironomus thummi thummi*. Labeling is largely restricted to regions of high DNA concentration. There are, however, a few grains (mostly out of focus) over the central portion of the nucleolus. Exposure, 1 day. × 800.

air dried. Enzyme treatments were carried out at this point by placing a large drop of RNase (100 μg/ml in 0.01 M phosphate, pH 6.2) or DNase (160 μg/ml in 0.01 M phosphate, 0.003 M $MgCl_2$, pH 6.2) on the preparations. The slides were kept in a moist chamber for 2 hr at 37°C. At the end of this time, the enzyme solutions were washed off in water.

All slides were dipped into subbing solution (0.1% gelatin, 0.01% chrome alum) and air dried. They were then coated with Kodak NTB-2 liquid emulsion and dried. Slides were stored in lightproof boxes at 4°C during the exposure period. Preparations were developed for 3 min in Kodak D19, washed briefly in water, and fixed in Kodak Rapid Acid Fixer for 2½ min. Staining of some of the preparations was carried out with 0.2% fast green at pH 2. The preparations were mounted in Zeiss water-soluble mounting medium or in water.

Squash preparations were made of the salivary glands of *Chironomus thummi thummi* larvae in the last instar. The glands were dissected out, fixed in 45% acetic acid, and squashed with a cover slip on a slide which had been previously washed in aqueous non-radioactive actinomycin D (10 μg/ml). The preparation was then frozen on dry ice, the cover slip was flipped off, and the slide was plunged into 95% ethanol. The slide was then brought down to water and air dried. Enzyme treatments were carried out at this point as described above.

A drop of actinomycin D-^{3}H (1 μg/ml in 0.1 M 5:1) was then placed over each preparation for 1 hr at room temperature. After this time, the slides were washed for 5 min in 0.05 M phosphate buffer, pH 6.8, for 5 min in water and then air dried. The slides were then subbed and coated with emulsion, as described above.

RESULTS

The binding of actinomycin D-^{3}H in both the oocyte and salivary gland preparations was limited to DNA. In the preparations of chromosomes and nucleoli from oocyte nuclei, the chromosomes were always heavily labeled (Fig. 1). The same specificity of labeling can be observed in Fig. 2, which shows a chromosome from a squash of a *Chironomus* salivary gland. Heavy actinomycin D-^{3}H label is found along the length of the chromosome but the isotope has clearly not bound to the attached nucleolus to any significant degree.

Fig. 3 is a photomicrograph of a lampbrush chromosome preparation made in actinomycin D-^{3}H. The chromosomal labeling is still present after RNase treatment, whereas DNase leaves no detectable amount of actinomycin bound to the chromosomes (Fig. 4). These same results were obtained when enzyme treatments were carried out *before* the chromosomes were exposed to actinomycin D-^{3}H.

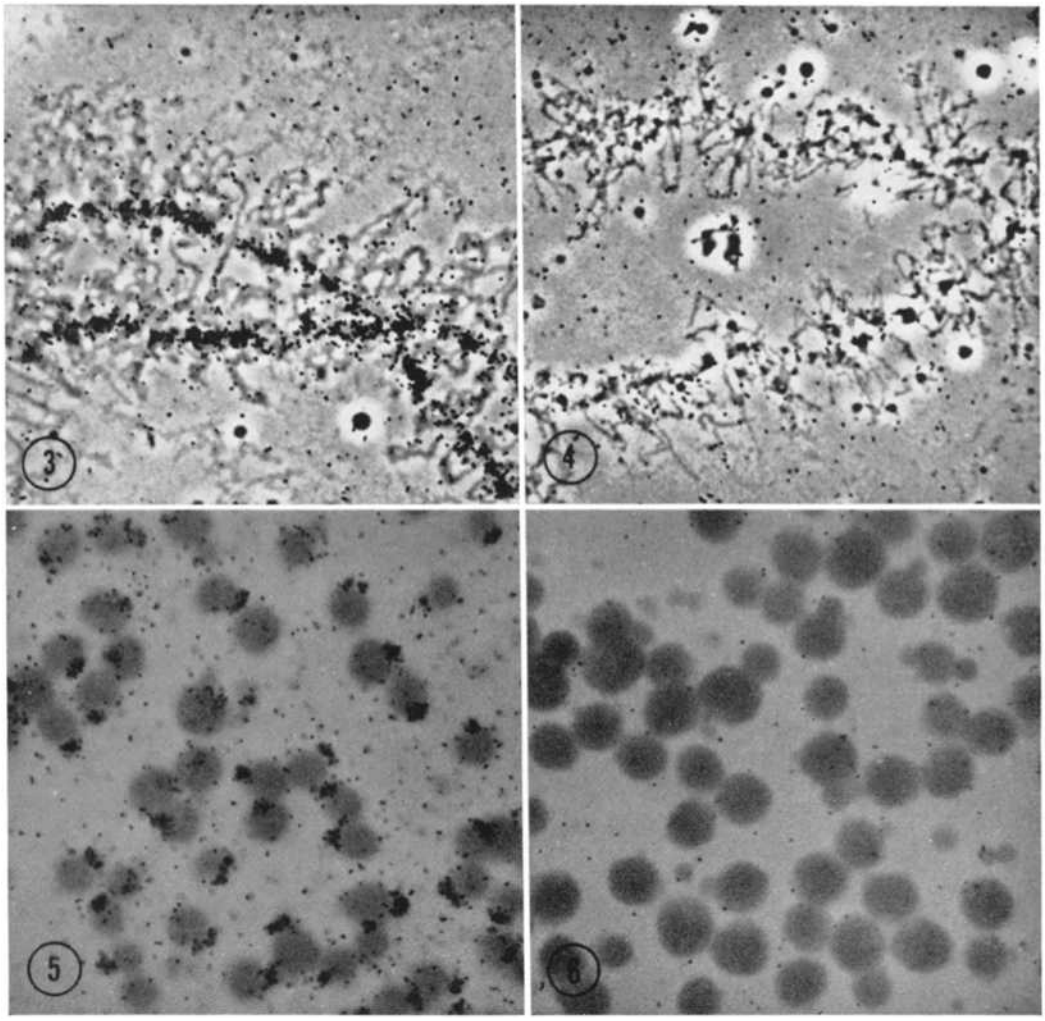

FIGURE 3 Actinomycin D-^{3}H binding to a portion of a lampbrush chromosome from an oocyte of *T. viridescens*. Only the chromomere axis appears labeled above background. Exposure, 4 wk. × 800.

FIGURE 4 DNase treatment of lampbrush chromosomes spread in actinomycin D-^{3}H. The label over the chromomere axes is not above background. The black clumps along the chromosomal axes are chromomeres, not clumps of silver grains. Exposure, 4 wk. × 800.

FIGURE 5 Localized binding of actinomycin D-^{3}H to the nucleoli of a mature oocyte of *T. viridescens*. This binding represents the site of nucleolar DNA. Exposure, 4 wk. × 800.

FIGURE 6 Nucleoli treated with DNase show absence of actinomycin D-^{3}H binding. Exposure, 4 wk. × 800.

Closer examination of Fig. 3 reveals that detectable labeling is found only along the chromomere axes of the lampbrush chromosomes. The label over the lateral loops does not appear to be above background. This situation is in striking contrast to the pattern of labeling found after whole oocytes are incubated in tritiated uridine. In the latter case, the isotope is heavily incorporated into the loops but not into the chromomere axis (Gall and Callan, reference 7).

It can be observed in Fig. 3 that the chromosome loops are in their normally extended form. Loop

retraction was never observed in chromosomes isolated into actinomycin, contrary to the report by Izawa et al. (9) that loops contract within 10 min after the chromosomes are isolated into the actinomycin solution. On the other hand, incubation of *intact* oocytes in actinomycin results in complete retraction of the loops within a few hours.

In preparations made from mature oocytes of either species of *Triturus*, nucleoli show asymmetrically localized labeling (Fig. 5). The localized grains are still observed after RNase treatment but are removed by DNase (Fig. 6). Sometimes a few residual grains are left over the nucleoli, but these are not localized.

DISCUSSION

It has been demonstrated that actinomycin D-^{3}H can be used to label DNA in radioautographic preparations. The binding is limited to DNA, as shown by the absence of chromosomal label in oocyte preparations after DNase treatment. The few residual grains sometimes left after DNase treatment might represent a low level of nonspecific binding. The specificity of the actinomycin binding is further demonstrated by the difference in labeling patterns found along lampbrush chromosomes after actinomycin D-^{3}H binding, on the one hand, and uridine-^{3}H incorporation into RNA, on the other hand. The actinomycin binding parallels the pattern of Feulgen staining, while the uridine incorporation parallels the distribution of RNA (Gall, reference 4).

The labeling pattern of actinomycin D-^{3}H in both oocyte and salivary gland preparations clearly follows the distribution of DNA. The chromosome loops, which also contain DNA, are the apparent exception to this generalization. However, since the loops are thought to be in a highly extended state (Gall, reference 5), one would expect the grain density over them to be low. Consequently, loop labeling might not be detected above background.

The ease with which the localized binding of actinomycin D-^{3}H can be detected in the nucleoli of mature oocytes is in marked contrast to the difficulty of detecting the nucleolar DNA by Feulgen staining. It appears therefore that actinomycin D-^{3}H can be used to label small amounts of DNA cytochemically with even greater sensitivity than Feulgen staining.

The localized binding of actinomycin to *Triturus* oocyte nucleoli confirms the presence of DNA in these nucleoli and also confirms its asymmetrical location, as revealed earlier by Feulgen staining (Brachet, reference 1; Painter and Taylor, reference 14; Macgregor, reference 12; Gall, personal communication). In addition, after incorporating a short pulse of uridine-^{3}H, *Triturus* oocyte nucleoli show asymmetrical labeling (Gall, personal communication; Macgregor, reference 12; Lane, reference 11). Thus the site of localization of DNA within the nucleolus and the site of RNA synthesis appear to be the same. Furthermore, it has been found that actinomycin D inhibits the incorporation of uridine-^{3}H into nucleolar RNA in *Triturus viridescens* oocytes (Izawa et al., reference 9). These experimental results considered together show clearly that the nucleolar DNA in amphibian oocytes is directing the synthesis of RNA in the nucleoli, a conclusion also reached by Macgregor (12).

Studies with actinomycin D-^{3}H for the detection of DNA in oocyte nucleoli are now being carried out on oocytes at all stages of development. In addition, this technique is being applied to cytological preparations for the detection of DNA in other cellular organelles.

I would like to thank Dr. Joseph Gall for his invaluable assistance during the course of this work and for a critical reading of this manuscript. I would also like to thank Mr. Roger Pedersen for providing the *Chironomus* larvae.

This investigation was supported by funds from a training grant from the National Institute of Child Health and Development (National Institutes of Health, grant HD-32-04) and from a research grant from the National Institute of General Medical Sciences (GM 12427) to Dr. Joseph Gall.

Received for publication 19 June 1967.

REFERENCES

1. BRACHET, J. 1940. La localisation de l'acide thymonucléique pendant l'oogénèse et la maturation chez les Amphibiens. *Arch. Biol.* **51**: 151.
2. BRACHET, J., and A. FICQ. 1964. Détection cytochimique, au moyen d'actinomycine radioactive, de l'acide désoxyribonucléique (DNA) cytoplasmique des oeufs de Batraciens. *Compt. Rend.* **258**:6258.
3. FRACCARO, M., A. MANNINI, L. TIEPOLO, and A. Albertini. 1966. Incorporation of tritium-labelled actinomycin in a human cell line. *Exptl. Cell Res.* **43**:136.
4. GALL, J. G. 1954. Lampbrush chromosomes from

oocyte nuclei of the newt. *J. Morphol.* **94**:283.

5. GALL, J. G. 1956. On the submicroscopic structure of chromosomes. *Brookhaven Symp. Biol.* **8**:17.
6. GALL, J. G. 1966. *In* Methods in Cell Physiology. David M. Prescott, editor. Academic Press Inc., New York. **2**:37.
7. GALL, J. G., and H. G. CALLAN. 1962. H^3-uridine incorporation in lampbrush chromosomes. *Proc. Natl. Acad. Sci. U.S.* **48**:562.
8. GOLDSTEIN, M. N., K. HAMM, and E. AMROD. 1966. Incorporation of tritiated actinomycin D into drug-sensitive and drug-resistant HeLa cells. *Science.* **151**:1555.
9. IZAWA, M., V. G. ALLFREY, and A. E. MIRSKY. 1963. The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc. Natl. Acad. Sci. U.S.* **49**:544.
10. KEZER, J. 1965. Observations *cited by* Peacock, W. J. 1965. Chromosome replication. *Natl. Cancer Inst. Monograph.* **18**:101.
11. LANE, N. J. 1967. Spheroidal and ring nucleoli in amphibian oocytes. Patterns of uridine incorporation and fine structural features. *J. Cell Biol.* **35**:421.
12. MACGREGOR, H. C. 1967. Pattern of incorporation of H^3-uridine into RNA of amphibian oocyte nucleoli. *J. Cell Sci.* **2**:145.
13. Miller, O. L., Jr. 1964. Extrachromosomal nucleolar DNA in amphibian oocytes. *J. Cell Biol.* **23**:60A. (Abstr.)
14. PAINTER, T. S., and A. N. TAYLOR. 1942. Nucleic acid storage in the toad's egg. *Proc. Natl. Acad. Sci. U.S.* **28**:311.
15. REICH, E., R. M. FRANKLIN, A. J. SHATKIN, and E. L. TATUM. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. *Science.* **134**:556.
16. RO, T. S., K. S. NARAYAN, and H. BUSCH. 1966. Effect of actinomycin D on base composition and nearest neighbor frequency of nucleolar RNA of the Walker tumor and liver. *Cancer Res.* **26**:780.
17. ROTHSTEIN, H., J. FORTIN, and M. L. YOUNGERMAN. 1966. Synthesis of macromolecules in epithelial cells of the cultured amphibian lens. *Exptl. Cell Res.* **44**:303.
18. SONNEBORN, D., and H. ROTHSTEIN. 1966. H^3-actinomycin D: uptake by amphibian lens epithelial cells. *J. Cell Biol.* **31**:163A. (Abstr.)
19. DEVITRY, F. 1964. Etude autoradiographique de l'incorporation d'actinomycine C^{14} chez *Acetabularia mediterranea. Compt. Rend.* **258**:4829.