MOLECULAR HYBRIDIZATION OF RADIOACTIVE DNA TO THE DNA OF CYTOLOGICAL PREPARATIONS

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Abstract.—A method is presented for detecting the cellular location of specific DNA fractions. The technique involves the hybridization of a radioactive test DNA in solution to the stationary DNA of a cytological preparation. Sites of DNA binding are then detected by autoradiography. Experiments with DNA of the toad Xenopus are described.

The technique of DNA-DNA hybridization has been applied to a variety of genetic problems since its introduction by Schildkraut, Marmur, and Doty.\(^1\) Hybridization of purified DNA has been used to investigate homologies between the DNA of phage and the DNA of the host bacterium,\(^2\) to study genetic relationships among higher organisms,\(^3\)\(^,\)\(^4\) and to examine the relation of particular DNA fractions to the rest of the genome.\(^5\) Reannealing kinetics have been used as a measure of genome complexity.\(^6\)\(^,\)\(^7\) Recently, substitutions and deletions in phage \(\lambda\) DNA have been mapped from electron micrographs of hybrid molecules.\(^8\)

We have now developed a technique which permits the localization of DNA-DNA hybrids in cytological preparations. In this method the DNA of the cytological preparation is denatured \textit{in situ} and then hybridized with a radioactive test DNA in solution. The binding of the test DNA to the cellular DNA on the slide is detected autoradiographically.

Materials and Methods.—(A) Preparation of radioactive test DNA: The radioactive DNA used in our hybridizations was extracted from tissue cultures\(^9\) of the mouse and the toad \textit{Xenopus} grown for several days in a medium containing 5 \(\mu\)c/ml of thymidine-\(H^3\) (specific activity 11.3 C/mM). The cells were lysed in a sodium doxycyl sarcosinate-proprona solution (0.5% Sarcosyl Geigy, 50 \(\mu\)g/ml self-digested pronase, 0.1 \(M\) EDTA, 0.05 \(M\) Tris, pH 8.4) and incubated at 37°C for 2 hr. Residual protein was removed by extraction with water-saturated phenol. The aqueous solution was brought to 0.1 \(M\) NaCl and the nucleic acids were precipitated with 2 volumes of 95% ethanol. The precipitate was washed with 70% ethanol, redissolved in 0.1X SSC, and treated with both pancreatic ribonuclease and ribonuclease \(T_1\). In the final step of purification, \textit{Xenopus} DNA was centrifuged to equilibrium in a CsCl density gradient. Mouse satellite DNA was separated on a CsSO\(_4\) gradient.\(^10\) The specific activity was determined by spotting known amounts of the DNA on nitrocellulose filters and counting in toluene-PPO-POP in a scintillation counter. The \textit{Xenopus} DNA had a specific activity of 130,000 cpm/\(\mu\)g. The specific activity of the mouse DNA was 200,000 cpm/\(\mu\)g.

The purified radioactive DNA was dissolved in 2X SSC at 1–2 \(\mu\)g/ml. It was sheared by sonication with the special microtip of a Branson Sonifier (model W140D) for four 1-min intervals interspersed with cooling periods. The sonication tube was immersed in ice water throughout the procedure. Although no molecular-weight determinations were made on these preparations, comparable treatments had earlier produced DNA of about 250,000 daltons. The DNA was denatured in a boiling water bath for 5 min, then chilled, and used immediately for hybridization.

(B) Hybridization procedure: (1) Small pieces of tissue were fixed briefly in ethanol-acetic acid (3:1) and then transferred on a needle to a drop of 45% acetic acid on a micro-
scope slide. The slides had previously been dipped in a solution containing 0.1% gelatin and 0.01% chrome alum, and then stored dry until use.

(2) The tissue was minced in the 45% acetic acid and the larger pieces were removed before the remaining cells were squashed under a cover slip. Slides were frozen on a flat block of dry ice for several minutes. The cover slips were then flipped off with a razor blade and the slides were placed in 95% ethanol. After this step they were air dried.

(3) Dry slides were dipped in 0.5% agar at 60°C and then drained vertically at room temperature until the agar had gelled but not dried. At this point, the DNA in the cytological preparation was denatured by placing the slides in 0.07 N NaOH for 5 min at room temperature. The slides were then washed in 2 changes of 70% ethanol and 2 changes of 95% ethanol before air drying.

(4) About 200 µl of radioactive test DNA was placed over the preparation on the slide and covered with a glass cover slip. Each slide was then placed in a moist chamber made of a 9-cm plastic Petri dish containing filter paper and 10 ml of 2X SSC. The slides were supported on 2 rubber grommets. The hybridization was carried out at 66°C for 12 to 15 hr.

(5) After the hybridization, the cover slips were removed by dipping the slides into 2X SSC. The preparations were then washed through 5 changes of 2X SSC at 60°C to remove any non-specifically bound DNA. A final 2X SSC wash was done at room temperature. The slides were then rinsed in 70 and 95% ethanol and air dried.

(6) Hybridized slides were dipped into Kodak NTB-2 emulsion which had been diluted 1:1 with distilled water. After drying, they were stored in light-tight boxes at 4°C.

(7) Slides were developed for 2 min in Kodak D-19, rinsed in 2% acetic acid, and fixed for 3 min in Kodak Fixer. They were then rinsed in several changes of distilled water and stained for about 10 min in Giemsa stain. The slides were finally rinsed in distilled water, air dried, and mounted under a coverslip.

Results and Discussion: We have recently described a technique for the hybridization of radioactive RNA to its complementary DNA in cytological preparations. In developing that method we used oocytes of the toad, Xenopus laevis, as a test system and studied the binding of ribosomal RNA (rRNA) to the DNA which codes for it (rDNA). The Xenopus oocyte was chosen for the experiments because during oogenesis this cell undergoes a specific replication or amplification of its rDNA. In the late pachytene nucleus, the amplified rDNA constitutes about 70 per cent of the total DNA and forms a cytologically distinct cap partially covering the chromosomes. In our RNA-DNA hybridization experiments, rRNA hybridized specifically with the cap DNA but not with the chromosomes.

For our initial experiments with DNA-DNA hybridization we once again made use of the difference between chromosomal and cap DNA in the Xenopus oocyte. In this case, however, we formed specific hybrids with the chromosomes and not with the cap. This we did by preparing a radioactive test DNA from which we removed the rDNA. As shown earlier by Wallace and Birnstiel, the rDNA is more dense in CsCl than the bulk of Xenopus DNA. Our test DNA was centrifuged to equilibrium in a preparative CsCl gradient, and all fractions more dense than the main band of DNA were discarded. In this way we obtained a DNA that contained essentially all of the Xenopus genome except for the ribosomal cistrons.

When incubated with cytological preparations of Xenopus ovaries, the fractionated test DNA bound to the chromosomes of pachytene oocytes, but not to the cap of rDNA (Figs. 1 and 3). The absence of binding in the cap region dem-
onstrates the specificity of the reaction. Hybridization was also detected in the interphase follicle nuclei (Fig. 4) and in metaphase chromosome groups (Fig. 2). These results indicate that DNA from several stages of the cell cycle can be denatured and successfully hybridized in situ. At present we know very little about the nucleotide sequences involved in the binding. Under the annealing conditions used, only that DNA which is present in the genome in highly multiple copies should exhibit hybridization. The labeling pattern in the metaphase chromosomes shows that this repetitive DNA is widely distributed in the complement of Xenopus. More exact localization of these DNA sequences will require longer autoradiographic exposures.

The DNA-DNA experiments gave essentially the converse of the picture seen in our earlier work in which the cells were hybridized with rRNA (Fig. 5). Ribosomal RNA bound to the cap of rDNA in pachytene oocytes, but showed little or no binding elsewhere. The absence of rRNA hybridization in the follicle nuclei presumably reflected the comparatively small amount of rDNA present in the normal diploid genome. Detection of the ribosomal cistrons in follicle nuclei would require a longer period of autoradiographic exposure than that used in our experiments.

It is important to establish that the binding seen in our DNA-DNA experiments represents true hybrid formation. Four considerations are pertinent to this question.

First, the localization of binding in Xenopus oocytes is specific. As already mentioned the cap of rDNA in pachytene oocytes showed no binding of test DNA from which the rDNA sequences had been removed.

Second, there was no binding on slides previously treated with DNase (0.3 mg/ml in 0.001 M MgCl₂, 0.01 M Tris, pH 7.4, 37°C for 4 hr).

Third, slides which had not been treated with alkali showed no label during the first few weeks of autoradiographic exposure. After longer exposures such slides did show some grains over the DNA, exhibiting the same spatial localization seen in the alkali-treated material. These observations suggest that some denaturation of the DNA occurs during the fixation and squashing of the material. In our earlier RNA-DNA experiments we had observed a similar low but specific hybridization on control slides.

Fourth, the hybridization reaction is able to discriminate between different types of DNA. To demonstrate this point we have used mouse satellite DNA, separated from the rest of the mouse DNA by CsSO₄ density centrifugation. It has been shown that the mouse satellite DNA will not cross-hybridize with DNA extracted from other organisms, even those closely related to the mouse. We have found similar specificity in our cytological preparations. Radioactive mouse satellite DNA bound to interphase nuclei and metaphase chromosomes from mouse tissue culture cells, but showed no hybridization with Xenopus cells.

One of the major technical problems in the hybridization technique is that of denaturing the cellular DNA without destroying the morphology. In our experiments, we immobilize the DNA in the cytological preparation with a thin layer of agar, and then denature with alkali. The test DNA must also be denatured, but this is easily accomplished in solution. In addition, we shear the
FIG. 1-4.—Autoradiographs of nuclei from the ovary of the toad Xenopus after cytological hybridization with radioactive Xenopus DNA which lacked the ribosomal cistrons. The DNA of these squash preparations was denatured in situ. The slide was then incubated with a solution of radioactive test DNA from which the rDNA had been removed by separation on a CsCl density gradient. The specific activity of the test DNA was 130,000 cpm/µg. The slides were stained with Giemsa.

Fig. 1 & 3.—Late pachytene oocyte nuclei showing binding of the radioactive test DNA to the chromosome region but not to the cap of amplified rDNA. Exposure 16 days. ×1300.

Fig. 2.—Metaphase chromosomes showing a wide distribution of hybridization with the test DNA. Exposure 12 days. ×1700.

Fig. 4.—Interphase follicle nucleus showing binding of the radioactive DNA. Exposure 12 days. ×1300.

Fig. 5.—Autoradiograph of nuclei of a pachytene oocyte and a follicle cell from Xenopus after cytological hybridization with radioactive Xenopus ribosomal RNA. The ribosomal RNA has hybridized specifically with the cap of amplified rDNA in the oocyte. The conditions of slide preparation, denaturation, and annealing are the same as for Figs. 1–4. The slide was treated with ribonuclease after hybridization. The specific activity of the RNA was 500,000 cpm/µg. The slide was stained with Giemsa. Exposure 1 day. ×1200.
test DNA to facilitate diffusion to sites of potential binding. We also used agar immobilization and alkali denaturation in our earlier experiments with RNA-DNA hybridization.

Two recent reports indicate that heat denaturation is successful in RNA-DNA cytological hybridization. John, Birnstiel and Jones obtained hybridization of rRNA with the amplified rDNA of *Xenopus* oocytes after heat denaturation of squashes. Also using heat denaturation, Buongiorno-Nardelli and Amaldi have successfully hybridized rRNA to the nucleoli in conventional histological sections. Their experiments were performed with tissue from the Chinese hamster.

The technique described here for detecting the spatial localization of specific DNA fractions should be applicable to many cases where the test DNA can be heavily labeled with tritium and isolated in pure form. We are currently using DNA-DNA hybridization to investigate the chromosomal distribution of various repetitive fractions of DNA and now have evidence that the mouse satellite DNA is localized in the centromere region of the chromosomes. The details of these experiments will be published elsewhere.

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This work will be submitted by one of us (M. L. P.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Yale University.

Abbreviations: SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; toluene-PPO-POPPOP, 4 gm 2,5 diphenyloxazole and 50 mg 1,4 bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter toluene; EDTA, ethylenediamine tetraacetate; Tris, Tris(hydroxymethyl)aminomethane.

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