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The Use of Iodinated RNA for Gene Localization

(molecular hybridization/chromosomes/iodine-125)

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ABSTRACT **Carrier-free iodination of 5S RNA with ^{125}I yields a probe suitable for use in cytological RNA–DNA hybridization studies. The genes coding for 5S ribosomal RNA in *Drosophila* could be localized by autoradiography after a 2-day exposure, whereas a 2-month exposure is needed when the best available [^{3}H]RNA is used. The procedure introduces one covalently bound ^{125}I atom per 100 nucleotides, resulting in a specific activity of over 10^{8} dpm/μg of RNA. The method may be readily applied to other problems involving molecular hybridization techniques.**

Molecular hybridization of radioactive RNA or DNA to chromosomes offers the opportunity for localizing genes or specific DNA sequences by autoradiography. One needs to isolate in high purity a specific gene product that is either labeled or can be used as a template to transcribe a labeled probe; the probe must have specific activity high enough to yield a detectable number of disintegrations at the site where it hybridizes; there must be a sufficient number of complementary nucleotides in one region of the chromosome to bind detectable amounts of the probe. The higher the specific activity of the probe, the smaller is the template that may be detected.

Molecular hybridization *in situ* has been successfully applied to diploid cells that contain repeated large DNA sequences (1–5) or highly amplified specific genes during specific times of cell development (6). Localization of smaller sequences that are not highly redundant has been successful only in giant chromosome preparation of some dipterans (7, 8); identification of hybridized regions required months of autoradiographic exposure to obtain convincing numbers of silver grains.

All successful gene localizations have been obtained with ^{3}H-labeled RNA or DNA. The main requirement for direct chemical labeling of isolated RNA is that sufficient label be introduced into the RNA without loss of its ability to hybridize specifically with its template. The labeling isotope should have a reasonably short half-life since the maximal specific activity of an isotope is inversely related to its half-life. We have chosen to work with ^{125}I, which has a 60-day half-life, 1/70th that of ^{3}H. Commerford (9) has developed mild conditions for iodinating nucleic acids. He showed that the iodinated nucleic-acid product contained iodine almost exclusively at the C5 position on cytosine, and that DNA containing 10% substitution of iodine in cytosine preserved its capacity for hybridization. The specific activity of ^{125}I is about 2200 Ci/mmol. If we assume an average cytidine content of 25%, iodination of 10% of all cytidines would yield DNA with a specific activity of 55 Ci/mmol of nucleotide. Preparation of nucleic acid of comparable specific activity labeled with ^{3}H would require introduction of two tritiums into each nucleotide throughout the molecule.

^{125}I decays by electron capture. The resultant Auger electrons have varying energies and produce autoradiographs somewhat inferior to ^{3}H in resolution, but with greater efficiency. Ada *et al.* (10) have estimated that one silver grain is produced for each five ^{125}I disintegrations.

In this paper, we report the successful use of Commerford's reaction in labeling 5S RNA with carrier-free ^{125}I. The product of the reaction hybridizes specifically with the DNA at the 56F region of *Drosophila melanogaster* salivary chromosomes, which codes for 5S RNA (7). Furthermore, the hybridization efficiency was comparable to 5S [^{3}H] RNA labeled *in vivo* with [^{3}H]uridine. The highest specific activity of the [^{125}I]RNA obtained thus far is about 50- to 100-times higher than that achieved *in vivo* with ^{3}H and is equivalent to the complementary RNA (10^{8} dpm/μg) synthesized *in vitro* by *Escherichia coli* polymerase (1).

MATERIALS AND METHODS

Preparation of Drosophila RNA. About 370 g of third instar larvae of *Drosophila melanogaster* homozygous for *sepia* (*se*) were frozen, mixed with dry ice, pounded into a powder, and homogenized directly in a mixture containing one volume of saline buffer (0.1 M NaCl, in 50 mM Tris·HCl at pH 7.4) and two volumes of 80% phenol. After extensive extraction with phenol, the aqueous phase was precipitated with 2.5 volumes of ethanol at −20° overnight. The precipitate was washed with ethanol and ether and air-dried, giving about 2 g of crude RNA. This RNA was put on a DEAE–cellulose column in saline buffer [0.3 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5)], washed extensively with the same buffer, and eluted with 1.0 M NaCl. The eluate was diluted to 0.3 M NaCl, loaded on a new DEAE–cellulose column, and eluted by a salt gradient (0.3–1.0 M NaCl). One tube was selected for this study, containing RNA that eluted at 0.53 M NaCl, the approximate peak of 5S RNA. This RNA, about 3 mg, contained 5S RNA, tRNA, and traces of 9 and 10S RNA, as determined by acrylamide gel electrophoresis. 1 mg of this fraction was digested with Pronase [37°, 14 hr (pH 7)], put on DEAE–cellulose, and recovered in 1.0 M NaCl as above. The RNA was then dialyzed against water,

Abbreviation: SSC, 0.15 M NaCl–0.015 M Na citrate.

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lyophilized, and dissolved in pH 5 buffer (0.1 M Na acetate–40 mM acetic acid), at a final concentration of 1 mg/ml.

Iodination and Purification of 5S RNA. The concentration of reagents used and the method of assembling the reaction mixture differ from those recommended by Commerford only insofar as dictated by the need for working with carrier-free ^{125}I and radiation safety precautions. Commerford recommends an iodide concentration of 25 μM, and since the ^{125}I must be used carrier-free, this requires performing the reaction in microliter volumes. Also, since the ^{125}I as received in sodium hydroxide solution may have autooxidized, we have introduced a treatment with sulfurous acid to reconvert all oxidized forms to iodide. Unreacted sulfurous acid is consumed by the excess of thallic ions in the reaction mixture.

The labeled RNA was originally purified on Sephadex columns as described by Commerford, and some of our cytological data were obtained with material so purified. However, we now prefer to purify [^{125}I]RNA on micro-hydroxyapatite columns, which is simpler and more dependable, and is presented below.

All operations, except for final dialysis, were done in a well-vented hood behind a transparent safety shield in a radiation chemistry laboratory. Contamination of the hands was unavoidable unless two pairs of disposable surgical gloves were used and discarded after short usage.

Reagents: ^{125}I as NaI (New England Nuclear Corp.), 200 mCi/ml in 0.1 N NaOH; 0.3 mM Na_2SO_3 in 0.3 N H_2SO_4; thallic perchlorate (Alfa Chemicals, Burlington, Mass.), 4 mM in 0.2 M Na acetate and 1.0 M acetic acid; 5S RNA, 1 mg/ml, as described above; 0.1 M Na_2SO_3; phosphate buffers at pH 6.8 prepared by mixing equimolar amounts of NaH_2HPO_4 and Na_2HPO_4, 0.05–0.4 M; 4 × SSC (SSC = 0.15 M NaCl–0.015 M Na citrate); hydroxyapatite, in 0.01 phosphate (a gift of Dr. C. A. Thomas, Jr. of Harvard University).

Assembly of Reaction Mixture:

(*1*). 5 μl of ^{125}I solution were delivered from a micropipette onto a plastic surface, mixed with 2 μl of 0.3 mM Na_2SO_3, taken up in a 20-μl capillary tube, and allowed to stand for 15–30 min. Sterilized Drummond micropipettes (Biolab, Inc., Derry, N.H.) were used to dispense all reagents.

(*2*). The above mixture was expelled into a drop containing 4 μl of thallic perchlorate solution and 4 μl of 5S RNA solution. The mixture was taken up in the capillary tube, heat-sealed, and placed in a test tube within a 60° water bath and heated for 20 min.

(*3*). A disposable liquid chromatography column, 0.7 × 4 cm (Biorad Laboratories), was loaded with hydroxyapatite to a height of 1 cm, and was washed with 0.05 M phosphate buffer. A few drops of 0.1 M Na_2SO_3 were added to the buffer in the column just before the reaction mixture was loaded on the column. After loading, the column was allowed to run dry, then washed several times with 0.05 M phosphate. All eluates were collected in 15-ml glass tubes, changed manually. The wash was followed by 0.2 M phosphate, and the first milliliter, containing the [^{125}I]RNA product, was used in further steps. The column was then washed with more 0.2 M phosphate followed by 0.4 M phosphate, without great regard to the total volume of buffer used in each step. Generally, the entire column product was collected in 6–9 tubes. The tube containing the desired [^{125}I]RNA fraction was made 10 mM with respect to Na_2SO_3, and all other tubes were treated with excess amounts of sulfite to prevent iodine volatilization. The radioactivity contained in these tubes and that remaining in the column itself were determined in a manual gamma-ray counter (Tracerlab) using shielding (a brass trunnion cup for a 15-ml centrifuge tube) to attenuate the activity to measurable counts. The total radioactivity in the 0.2 M phosphate fraction was used to calculate the % of iodine incorporated into RNA.

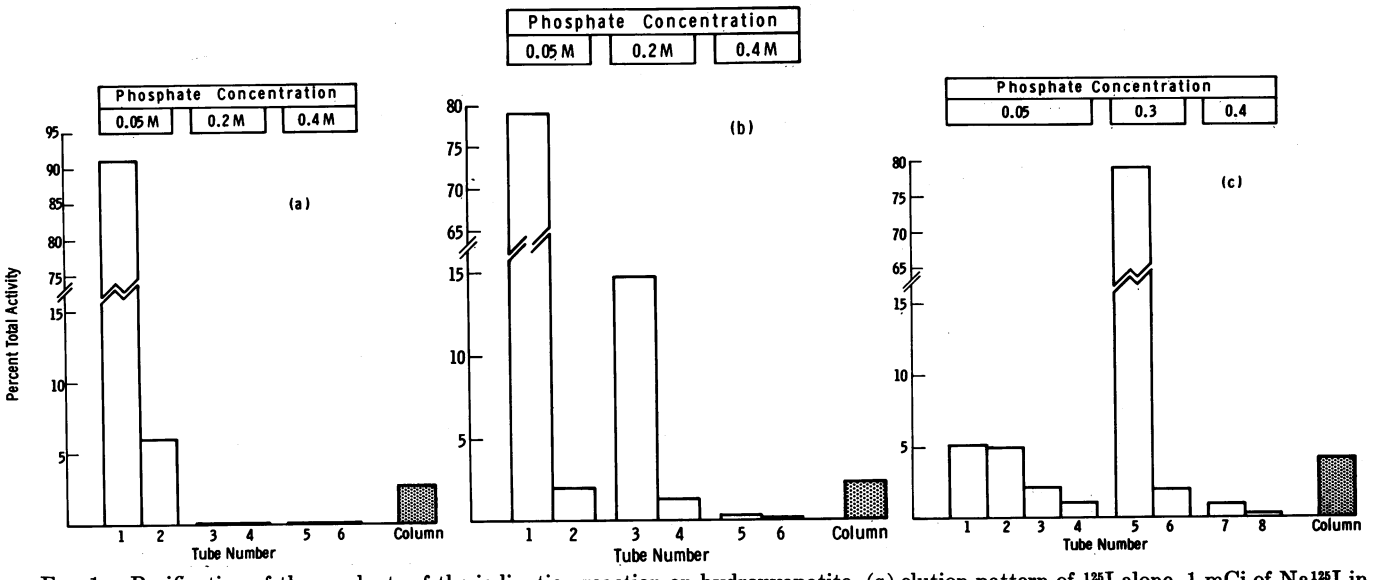

FIG. 1. Purification of the products of the iodination reaction on hydroxyapatite. (*a*) elution pattern of ^{125}I alone. 1 mCi of Na^{125}I in 50 mM phosphate was loaded on hydroxyapatite (0.4 cm bed, 1 cm high). Conditions were the same as for [^{125}I]RNA purification. The volumes in each tube were *not* equal. After elution the column was wrapped in parafilm and its radioactivity was determined in the gamma counter. (*b*) Elution of [^{125}I]RNA after first heating step. Tubes *3* and *5* contained 1 ml of eluate; other tubes contained products of exhaustive washing of the column. (*c*) Elution of [^{125}I]RNA after second heating step. Tubes *5* and *7* contained 1 ml of eluate; all others contained greater volumes of eluate.

(*4*). The [^{125}I]RNA eluate was reheated for 30 min at 70°, and repurified on a new hydroxyapatite column as in step *3*.

(*5*). The [^{125}I]RNA was dialyzed against 800 ml of 4 × SSC for 24 hr at 4° with 3–4 changes of dialysate. The radioactivity of the dialysates was monitored. 100 μg of unlabeled yeast RNA can be added to the [^{125}I]RNA before dialysis to reduce loss of RNA on membrane surface.

^{3}H-Labeled 5S RNA. The 5S [^{3}H]RNA was material prepared as described by Steffensen and Wimber (8). It had a specific activity of 4 × 10^{6} dpm/μg.

Cytology. Preparation of *Drosophila* salivary gland chromosomes, denaturation, and autoradiography were done as described (7, 8). Slides were treated before and after hybridization as described by Pardue *et al.* (3). Hybridization was done for 16 hr at 36° in 50% formamide in 2 × SSC with [^{125}I]RNA concentrations of 0.1–0.5 μg/ml. Autoradiographs were exposed from 1–20 days with [^{125}I]RNA and 60 days with [^{3}H]RNA.

RESULTS

Yield and Purification of [^{125}I]RNA. In our early experiments, the reaction products were separated on a 0.9 × 60 cm G50 Sephadex column. The elution profile was similar to that reported by Commerford (9). About 28% of the ^{125}I eluted as radioactivity associated with the RNA in the void volume of the column. The contents of those tubes containing this fraction were pooled, Na_2SO_3 was added to a final concentration of 0.01 M, and the solution was heated at 70° for 30 min. The product of this reaction was put on DEAE–cellulose and washed with 0.3 M sodium chloride, which removed 20–25% of the radioactivity as iodide. The [^{125}I]RNA was then eluted with 1.0 M NaCl and dialyzed against 4 × SSC. About 10% of the radioactivity was lost on dialysis,

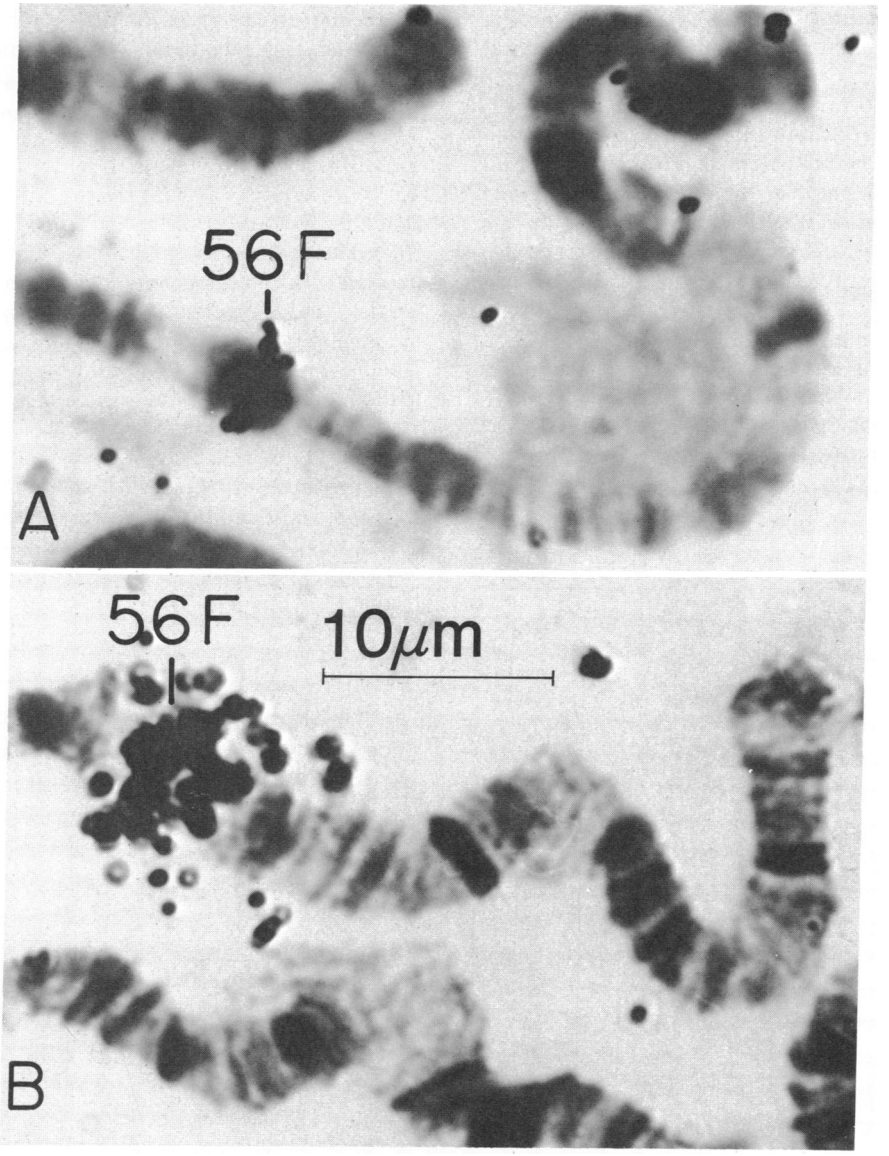

FIG. 2. Autoradiographic comparison of 5S [^{3}H]RNA with 5S [^{125}I]RNA. The RNA was hybridized to salivary gland chromosomes of *Drosophila melanogaster*. Magnification, ×3000. (*A*) [^{3}H]RNA, about 4 × 10^{6} dpm/μg and 60-day exposure. The silver grains are located over the bands at 56F, on chromosome 2R. (*B*) [^{125}I]RNA, about 1.16 × 10^{8} dpm/μg, exposure time 4 days. Radioactivity of the RNA probe due to a 3.3% substitution of cytosine with [^{125}I]iodocytosine.

most of the loss occurring in the first 4 hr of dialysis, suggesting that the activity was associated with molecules much smaller than 5S RNA. Further heating of aliquots obtained from DEAE-cellulose column for 5, 10, 20, or 30 min at 74° did not cause any further loss of iodine from the [125I]RNA. All reheated samples hybridized successfully with *Drosophila* chromosomes *in situ*.

Fig. 1 presents the results of [125I]RNA purification on hydroxyapatite at room temperature, our current practice. The columns were allowed to run dry between steps, obviating the need for a fraction collector and minimizing radiation exposure to personnel. The radioactivity in each eluate and that remaining on the column was measured in a gamma counter through a suitable attenuator. When unreacted 125I was put on this column, at least 97% eluted in the loading buffer (Fig. 1*a*); the rest remained bound to the column and was not eluted at the buffer concentration used to elute the [125I]RNA. Fig. 1*b* presents the separation of [125I]RNA after the first heating step. The first [125I]RNA tube in Fig. 1*b*, containing about 1.0 ml of eluate, was made 0.01 M in sulfite; the tube was heated at 70° for 30 min, diluted to 0.05 phosphate, and loaded onto a second hydroxyapatite column. This second separation (Fig. 1*c*) shows that about 18% of the total radioactivity of the [125I]RNA was heat labile and came off in the loading buffer. Thus, it was probably iodide liberated from an unstable reaction product, as discussed by Commerford (9).

Reproducibility of Commerford's Reaction with Carrier-Free 125I. We have performed over 40 iodination reactions, with *Drosophila* 5S RNA and other types of RNA from several species. *Drosophila* 5S RNA was used for developing the optimum methods for the reaction, with respect to conditions of assembly, purification, choice of reagent concentrations, and assay of the hybridizability of the product. 125I that reacted with RNA in the first heating step varied from 1–42%. This variation was no doubt largely due to variations introduced as we developed our present procedure. When several iodinations were done on the same day with fresh commercial 125I, the results were much more reproducible.

Because of the small volume and high radioactivity of the reaction mixture, it was not possible to determine the final pH of the mixture directly. Commerford reported the high pH dependence of the reaction rate, and chose pH 5.0 as optimal. Our original conditions were designed to approximate this pH by including in the thallic perchlorate solution an excess of acetic acid, calculated to neutralize the alkali in the carrier-free iodide and produce a pH near 5. However, we now neutralize the alkali with sulfuric acid before adding the thallic buffer, our reaction mixture is closer to pH 4, and we increased the amount of thallic ion. These changes gave us a higher yield of [125I]RNA, with no loss in hybridizability. We believe that most of the differences in 125I incorporation that were observed in our early experimentation were due to variability in the composition of commercial radioiodide solution. Pretreatment of the radioiodide with acid sulfite, as now routinely done, generally caused a 3-fold increase in yield. Larger amounts of sulfite showed no additional benefit.

Specific Activity and Degree of Iodination of [125I]RNA. The specific activity of the labeled purified product was calculated assuming a complete recovery of the RNA. This figure is obviously low since losses must have occurred. In the product used for the autoradiograph in Fig. 2, the radioactive yield was 21% of 1.0 mCi. Since 4 μg of RNA were iodinated, this calculates to a specific activity of 5.2 Ci/g or 114×10^6 dpm/μg.

The reaction mixture contained about 0.4 nmol of radioiodine and 2.86 nmol of cytosine. If we assume that all of the iodine was the 125I isotope, and if the iodine was incorporated only into cytosine, the observed iodination corresponds to a 3.3% conversion of cytosine to iodocytosine. Equivalently, about one 125I atom was introduced per 100 nucleotides.

Hybridization Studies. Section 56F of the *Drosophila* salivary gland chromosome, which codes for 5S RNA, could be visualized by a 1- or 2-day exposure when one atom of 125I was introduced per 100 nucleotides of RNA. Proportionately longer times were necessary for detecting the 56F region when 5S RNA of lower specific activity was used. Fig. 2*B* shows an autoradiograph of the 56F region after hybridization with [125I]RNA. Fig. 2*A* shows the same region after hybridization with [3H]RNA. Although the 3H label yields a tighter cluster of grains, identification of the region is not materially more difficult with 125I than with 3H.

Several other much more weakly labeled hybridization sites could be seen. These sites were located in the same regions known to hybridize with tritiated tRNA (8). The sample of 5S RNA used in developing our labeling technique contained an appreciable admixture of tRNA species, as determined by disc-gel electrophoresis of the input RNA. After longer exposure (10 days or more), the nucleoli of the salivary gland chromosomes were also labeled, suggesting that 18 or 28S ribosomal RNA or its degradation products were also present. Such nucleolar labeling could be eliminated by addition of unlabeled 18S and 28S RNA to the hybridization mixture (Steffensen, unpublished observation).

All successful *in situ* hybridizations were obtained with [125I]RNA that had been reheated in the presence of a reducing agent. Samples of [125I]RNA dialyzed against 4 × SSC for hybridization directly after the first purification step did not yield any specific labeling of the 5S DNA region even after a 30-day exposure. This could be due to the presence of appreciable amounts of cytosine and uracil hydrates in the RNA, as suggested by Commerford. The reheating of the RNA in the absence of oxidizing agent was therefore necessary to decompose this hydrate and obtain an [125I]RNA that would hybridize to chromosomal DNA.

DISCUSSION

Insertion of one 125I atom per 75 nucleotides yields an RNA of as high a specific activity as is presently obtainable by the use of the *E. coli* polymerase system. For detection by autoradiography, 125I appeared to yield about twice the number of grains that would be obtained from [3H]RNA of equal specific activity.

Fig. 2 provides a direct comparison of the relative merits of RNA labeled with 3H *in vivo* and similar material labeled chemically with 125I. The principal advantages of using 125I are due to the higher specific activity of the labeled probe and the lower cost of producing it. The yield in grains per day of exposure was about 50-times greater for the iodinated than for the tritiated sample of RNA. Since the difference in the specific activities was about 25-fold, and if hybridization

efficiency were the same, then ^{125}I appeared to yield a 2-fold increase in autoradiographic efficiency as well.

Compared to ^{3}H, the apparent autoradiographic resolution of ^{125}I is much poorer. The scatter of electrons from ^{125}I decay has been calculated by Ertl *et al.* (11) and compared to that of ^{3}H. Over 55% of the grains observed in film should be no further from the source of radiation than grains obtained after ^{3}H decay, i.e., within a radius of 0.5 μm of the source. The most energetic electrons, 13%, will result in grains within a 16-μm radius; the remaining energy will be deposited within 3.5 μm of the source of radiation. Thin emulsions will diminish the fraction of grains found in the halo about a point source, and are therefore preferable for use in localizing genes in diploid chromosomes. When an autoradiograph is slightly overexposed, (Fig. 2*B*), a halo of grains is in fact obtained within a radius of about 10 μm about the 56F region, plus a very dense cluster of grains directly over the region itself. In underexposed autoradiographs some cells showed as good a resolution as that obtained from tritium (figure not shown). Under most experimental conditions, a series of slides exposed for different lengths of time will therefore yield information on the approximate and the precise localization of the molecular hybrid from the over- and underdeveloped autoradiographs, respectively.

Since direct chemical labeling can be done on any RNA after isolation, the hybridization properties of any available purified species of RNA can be studied. When tissues or cells must be labeled *in vivo*, the maximum specific activity that may be obtained is limited not only by cost of materials, but also by dilution in precursor pools and by limits to the amounts of radioactive nucleotide that may be given without killing a growing cell population from internal radiation.

Our best yields are still poorer than those of Commerford, who worked with milligram rather than microgram quantities of RNA and with trace amounts of ^{125}I. His trace labeling experiments have been repeated by others (12, 13) and although higher iodination yields were obtained, the specific activities of such [^{125}I]RNA was about 50-times smaller than in our system. We do not know all the factors that depress the iodination rate in carrier-free iodination reactions. In almost every case tested, a fresh bottle of ^{125}I gave better results when first used. Older lots of ^{125}I gave generally poorer results in terms of percent of iodine incorporated into RNA. However, sulfurous acid treatment and careful deproteinization of the RNA always increased the efficiency of the iodination reaction.

Further study of the carrier-free iodination reaction may define conditions under which more of the cytidines can be substituted with ^{125}I. In practice, when difficult to separate (i.e., relatively rare) species of RNA are to be iodinated, the chemical purity of the RNA (its freedom from protein contamination) is most likely to be the limiting factor in the iodination reaction. Recently we obtained much higher specific activities than those reported here when large and easily purified ribosomal RNA molecules were iodinated.

Successful localization of specific genes by RNA-DNA hybridization depends not only on the specific activity of the RNA, but also on the fraction of the chromosomal template available for hybrid formation. In *Drosophila* (7) and in humans (14) cytological hybrids contain about 3–6% as much RNA as there is template DNA. With an order of magnitude improvement in the specific activity of the radioactive RNA and an improvement in the methods used for annealing RNA to cytological preparations, we should be well on our way towards localizing individual genes in diploid chromosomes.

NOTE ADDED IN PROOF

A recent paper by N. H. Scherberg and S. Refetof describes a similar synthesis of iodinated RNA [(1973) *Nature New Biol.* **242,** 142–145]. Their observation on the shelf-life of the labeled RNA is very much in accord with our own experience.

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