¹²⁵I-Labeled DNA·RNA Hybrids in Cytological Preparations

(human placental DNA/chromosomes/metaphase/autoradiography)

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ABSTRACT RNA complementary to bulk human-placental DNA was synthesized *in vitro* both in the presence and absence of ³H-labeled ribonucleotides. The ³H-labeled RNA was used directly for hybridization to the DNA of human metaphase chromosomes, whereas the unlabeled complementary RNA was labeled chemically with ¹²⁵I before hybridization. A comparison of autoradiographs produced by either isotope revealed no qualitative differences in the chromosomal annealing sites of the same population of RNA molecules. Since ¹²⁵I-labeled nucleic acids give similar, if not identical, results as do ³H-labeled nucleic acids in *in situ* hybridization experiments, their use should make possible the localization of genetic elements for which tritium labeling methods are either inadequate or not possible.

Within the past few years considerable progress has been made in determining the chromosomal localization of certain genetic elements by the direct hybridization of a radioactively labeled nucleic acid to the DNA of metaphase chromosomes (1-4). In all such experiments the hybrid products detected by autoradiography contained DNA or RNA molecules labeled to very high specific activity with [³H]nucleotides. Those experiments that involved DNA·RNA hybridization *in situ* were particularly successful in instances where relatively large quantities of a single class of RNA molecules, such as ribosomal RNA (5, 6) or transfer RNA (7), could be labeled *in vivo* with [³H]RNA precursors. In other equally successful experiments it was possible to synthesize, by *in vitro* transcription, ³H-labeled RNA molecules complementary to a defined, repetitious population of DNA molecules and, thus, to determine their positions in the genome by hybridization to chromosomes (8-12). However, further progress in determining the chromosomal localization of additional genetic elements, such as structural genes, may be limited if the methodology is dependent on the use of tritium for labeling hybrid products. Such limitations exist because in most cells only a small percentage of the total population of RNA molecules represent the product of a single structural gene. Hence, the direct *in vivo* labeling of messenger RNA (mRNA) molecules, either by cell culture or whole animal techniques, to high specific activity by incorporation of [³H]nucleotides is not practical. Also, since methods are presently not available for fractionating mixed populations of DNA molecules into single populations representing structural genes, the transcription of a ³H-labeled RNA sequence complementary to a structural gene is not possible.

As a means of overcoming the limitations imposed by the use of ³H-labeled nucleic acids for *in situ* hybridization experi-

ments, the possibility of their replacement by nucleic acids labeled chemically with ¹²⁵I (13) was investigated. The mode of decay of ¹²⁵I includes the emission of an Auger electron (14) similar in path length to the tritium β -particle; hence, it is an ideal radionuclide for use in high-resolution autoradiography. Since radioactive iodine atoms can be incorporated into nucleic acid molecules whose biological synthesis is already complete, the difficulties involved in labeling nucleic acids to high specific activity during molecular synthesis, by either *in vivo* or *in vitro* techniques, are avoided. In a previous report (15) we showed that the introduction of ¹²⁵I into the molecular structure of bulk mammalian RNA resulted in no detectable alterations in either the ability of the RNA to hybridize with DNA on filters or in its secondary structure. In the present report, we show that identical populations of RNA molecules, labeled either with ³H or ¹²⁵I, give qualitatively identical patterns of label distribution when hybridized *in situ* to human metaphase chromosomes.

MATERIALS AND METHODS

Synthesis of ³H-Labeled and Unlabeled Complementary RNA (cRNA). RNA complementary to human placental DNA was synthesized *in vitro* with DNA-dependent RNA polymerase from *Escherichia coli*. The enzyme was isolated from late-log phase *E. coli* B cells (Miles Laboratories) by a combination of the methods of Burgess (16) and Bautz and Dunn (17). In this procedure enzyme fraction 4 of Burgess is further purified on columns of DNA-cellulose (18) and Bio-Gel A 1.5 m. High specific activity [³H]cRNA was prepared as described (19). Unlabeled cRNA was synthesized in a 5.0-ml reaction mixture containing: 0.1 M Tris·HCl (pH 7.8), 50 mM KCl, 12 mM MgCl₂, 4.8 mM 2-mercaptoethanol, 1.6 mM (each) ATP, GTP, CTP, and UTP, 800 μ g of placental DNA, and 300 units of polymerase (16). The mixture was incubated at 37° for 3 hr. DNase I was added to a final concentration of 10 μ g/ml and the mixture was incubated at 23° for 30 min. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and the mixture was extracted with an equal volume of redistilled phenol saturated with 15 mM NaCl-1.5 mM Na citrate (pH 7.0). The RNA in the aqueous phase was concentrated by flash evaporation, applied to a 0.6 $\times$ 75-cm G-25 Sephadex column, and eluted with distilled H₂O.

Iodination of Unlabeled cRNA. cRNA prepared as described above was labeled (15) with ¹²⁵I, with the following modifications: the concentrations of TiCl₃ and Na ¹²⁵I were raised to 7.5 mM and 5 mCi/ml, respectively, in a total reaction mixture of 200 μ l.

Abbreviation: cRNA, complementary (to DNA) RNA.

***In situ* Hybridization, Autoradiography, Staining, and Photomicroscopy.** Air dried slide preparations of metaphase chromosomes from human lymphocyte cultures were treated with 0.2 N HCl, 0.07 N NaOH, and 50 $\mu\text{g}/\text{ml}$ of RNase A (Worthington) exactly as described (20) before hybridization with either ^3H - or ^{125}I -labeled cRNA in 0.30 M NaCl-30 mM Na citrate at 60° for 12-16 hr (the exact conditions of hybridization are given in the Figures). After hybridization, the slides were treated with 20 $\mu\text{g}/\text{ml}$ of RNase A at 37° for 30 min, immersed in 3 liters of 0.30 M NaCl-30 mM Na citrate, stirred at 4° for 3-4 hr, dehydrated with alcohol, then allowed to air-dry. In some experiments, the slides were treated with a 25 ng/ml solution of trypsin (Worthington) in phosphate-buffered saline, pH 7.0 (Gibco) for 1 min at room temperature after hybridization and RNase treatment. For autoradiography, the slides were dipped in Ilford K-5 radionuclide emulsion (diluted with 3 parts H_2O for each 1 part of emulsion) and air-dried. The slides were exposed at 4° for the times indicated before developing and staining in 5% Giemsa. Photographs were taken with a Zeiss photomicroscope under bright-field optics.

RESULTS

The results of experiments designed to demonstrate differences between hybrid products formed with either ^3H - or ^{125}I -labeled cRNA in *in situ* hybridization experiments are shown in Fig. 1a and b. In Fig. 1a the cRNA was labeled with ^{125}I , while in Fig. 1b the cRNA was labeled with ^3H . The results of this experiment demonstrate that the distribution of grains over chromosomes, whether produced by ^3H - or ^{125}I -labeled hybrid products, is qualitatively the same. The only apparent difference between the two experiments was the observation of a 2- to 3-fold higher level of background grains when autoradiographs of ^{125}I -labeled hybrids *in situ* were compared to those labeled with ^3H . As the autoradiograph shown in Fig. 2 indicates, most of the background grains seen in autoradiographs represented by Fig. 1a could be eliminated by treatment of the hybridized preparations with low concentrations of trypsin. Trypsin treatment did not, however, remove any of the grains localized over chromosomes.

Other experiments were designed to reveal differences, if any, in the behavior of ^3H - and ^{125}I -labeled cRNA in competition experiments. To solutions containing ^3H - or ^{125}I -labeled

FIG. 1. Autoradiographs of ^{125}I - and ^3H -labeled cRNA hybridized *in situ* to human metaphase chromosomes. To air-dried slides prepared for *in situ* hybridization, 34 ng of either (a) ^{125}I -labeled cRNA (1.3×10^6 cpm/ μg RNA) or (b) ^3H -labeled cRNA (1×10^7 cpm/ μg RNA), in 100 μl of 0.30 M NaCl-30 mM Na citrate (pH 7.0) was added. The slides were incubated at 60° for 16 hr, treated at 37° for 30 min with 20 $\mu\text{g}/\text{ml}$ of RNase in the same buffer, washed with stirring in this buffer at 4° for 4 hr, then dehydrated with alcohol before coating with emulsion. Exposure times were (a) 33 days and (b) 21 days. In competition experiments, 6.8 μg of either unlabeled bulk human RNA (c) or unlabeled cRNA (d) were mixed with 34 ng of ^{125}I -labeled cRNA (1.3×10^6 cpm/ μg RNA), in 100 μl of the above buffer, before hybridization. Subsequent steps were the same as a and b. Exposure time (c and d) was 33 days.

FIG. 2. Autoradiograph of trypsin-treated, ^{125}I -labeled cRNA hybridized *in situ* to human metaphase chromosomes. Experimental conditions and the ^{125}I -labeled cRNA preparation used were the same as in Fig. 1. After incubation at 60° for 16 hr and treatment with RNase A, the slides were treated at room temperature for 1 min with 25 ng/ml of trypsin in phosphate-buffered saline (pH 7.0). The slides were then dehydrated with alcohol before washing in stirred 0.30 M NaCl–30 mM Na citrate (pH 7.0) at 4° for 4 hr. Subsequent steps were the same as in Fig. 1.

cRNA, either homologous or heterologous species of RNA molecules were added, in a 200-fold excess by weight, before hybridization. The results of adding homologous competitor to ^{125}I -labeled cRNA are shown in Fig. 1d and Table 1. The competition between homologous species of RNA molecules labeled with either ^3H or ^{125}I , was at least 90%. However, when bulk human RNA was added to either ^{125}I -labeled cRNA (Fig. 1c and Table 1) or ^3H -labeled cRNA (Table 1), the competition was reduced to about 70%. When *E. coli* ribosomal RNA was added to cRNA labeled with either isotope, no competition was observed (Table 1). The most likely explanation for the ability of bulk human RNA to compete reasonably well with cRNA is that both RNA species contain some populations of molecules whose base sequences are either very similar or identical. By the same reasoning, the absence of competition between *E. coli* ribosomal RNA and cRNA would be expected on the basis of the two species of molecules containing completely different base sequences.

DISCUSSION

Throughout this work careful attention was given to the removal of free ^{125}I from preparations of ^{125}I -labeled nucleic acids used for *in situ* hybridization experiments. Indeed, several early experiments clearly confirmed that free ^{125}I , present as a contaminant in preparations of ^{125}I -labeled nucleic acids, would give spurious results due to the high binding affinity of iodine and protein. However, the application of procedures (15) designed to eliminate both free ^{125}I and ^{125}I loosely bound to RNA as hydrates (13) results in ^{125}I -labeled RNA preparations entirely satisfactory for these purposes.

Background levels were consistently 2- to 3-fold higher in autoradiographs of hybrids labeled with ^{125}I than in those labeled with ^3H . The source of this background is likely due to the slow, but continuous, release under annealing conditions (60° for 16 hr) of ^{125}I not covalently bound to RNA (13).

TABLE 1. Comparative evaluation of ^{125}I and ^3H -labeled cRNA hybridized to human metaphase chromosomes in the presence and absence of competitor RNA molecules

Experiment	Average number of grains per metaphase cell*	
	Over chromosomes	Not over chromosomes (background)
^{125}I -labeled cRNA only	303	51
^3H -labeled cRNA only	337	17
^{125}I -labeled cRNA + <i>E. coli</i> rRNA (1:200)	334	53
^3H -labeled cRNA + <i>E. coli</i> rRNA (1:200)	387	19
^{125}I -labeled cRNA + bulk human RNA (1:200)	100	16
^3H -labeled cRNA + bulk human RNA (1:200)	122	6
^{125}I -labeled cRNA + unlabeled cRNA (1:200)	28	5
^3H -labeled cRNA + unlabeled cRNA (1:200)	34	2

* In each experiment, 10 metaphases were examined, and an average of the total number of grains observed was computed.

Consequently, free ^{125}I , generated by annealing conditions, would be available for binding to protein. This possibility is supported by the observation that treatment of the ^{125}I -labeled slide preparations with trypsin reduces the background to a level comparable to that observed in ^3H -labeled slide preparations (compare Fig. 1a and Fig. 2). Since no loss of grains localized over chromosomes occurred when slide preparations were treated with trypsin, its use was adopted as a routine measure in subsequent experiments.

The ^3H -labeled cRNA used in these experiments was about 10-fold higher in specific activity than was the ^{125}I -labeled cRNA. Although the maximum specific activity attainable for nucleic acids labeled with ^{125}I has not been determined, *in situ* hybrids were readily detected when ^{125}I -labeled cRNA was used at a specific activity of 1.3×10^6 cpm/ μg of RNA. For this reason, attempts to increase the specific activity of ^{125}I -labeled cRNA to a level equal to or greater than the ^3H -labeled cRNA were not pursued.

The results of the competition experiments present evidence that the type of interaction between the ^3H - or ^{125}I -labeled cRNA represents sequence-specific annealing to the DNA of chromosomes. Although it was not the intent of these experiments to determine the localization of bulk DNA transcripts, the specificity observed with ^{125}I -labeled cRNA is in close agreement with previous results with ^3H -labeled cRNA (21).

The use of ^{125}I -labeled nucleic acids in *in situ* hybridization experiments should broaden the range of genetic loci that might be mapped at the cytological level. Indeed, it seems theoretically possible to localize the position of any structural gene for which the corresponding mRNA can be isolated.

While this work was in progress, an abstract (22) appeared that demonstrated the use of ^{125}I -labeled nucleic acids to localize the annealing site of 5S RNA in *Drosophila* chromosomes.

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