LABELING OF RNA OF ISOLATED NUCLEOLI WITH UTP-^{14}C*

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The development of methods for the isolation of nucleoli (1-3) from mammalian liver and tumor cells has provided an opportunity for the study of the biosynthetic reactions that occur in these nuclear organelles. Evidence that the rate of turnover of RNA in the nucleolus is very high has been obtained by autoradiographic studies (4) and more recently by experiments on uptake of the isotope of labeled orotic acid into RNA of nucleoli of livers of control and thioacetamide-treated rats (5,6). Maggio et al (3) have also studied the uptake of ^{32}P into nucleoli of guinea pig livers. In the present experiments, conditions and cofactor requirements for incorporation of the isotope of UTP-2-^{14}C into nucleolar RNA were determined. Along with the inhibitory effects of Actinomycin D and DNase, these experiments provide supporting evidence that the biosynthesis of RNA in the nucleolus is DNA-primed. Inasmuch as the product is largely GC-type RNA (3,7), the data suggested that isolated DNA segments rich in GC are derepressed for biosynthesis of nucleolar components.

MATERIALS AND METHODS

Preparation of Nucleoli: Nucleoli of rat liver were isolated as previously described (1) except for a modification in the

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centrifugation procedure in which 20 ml of sonicate was layered over an equal amount of 0.88 M sucrose in a 50 ml lusteroid centrifuge tube and centrifuged at 2000 x g for 20 min. in an International Refrigerated Centrifuge Model PR-2 at 4° C. The precipitated nucleoli were washed once with 0.25 M sucrose and suspended in 0.025 M Tris buffer at pH 8.2; each reaction vessel contained nucleoli from 5 gm of rat liver.

Reaction Mixture: The reaction mixture for control experiments contained the following in a final volume of 2.0 ml: 20 μg pyruvic kinase (PK), 10 μmoles phosphoenol pyruvate (PEP), 2 μmoles ATP, 0.25 μmoles each of GTP and CTP, 5 μmoles $MgCl_2$, 50 μmoles Tris-HCl at pH 8.2, 0.2 μC UTP-2-^{14}C (11.3 mc/m M, Schwarz Bioresearch, Inc.) and an aliquot of nucleoli.

The reaction mixtures were shaken for 15 min. at 37° C and then were rapidly chilled in an ice bath. 0.1 ml of a solution containing 1 mg/ml UTP-^{12}C was added to each tube followed by 2 ml of 20% trichloroacetic acid (TCA). The precipitate was washed three times with cold 5% TCA, washed twice successively with 90% ethanol and absolute ethanol. The RNA was hydrolyzed in 0.3 N KOH at 37° C for 16 hours. The hydrolysate was acidified with perchloric acid (PCA) to precipitate DNA and then was neutralized with 0.5 N KOH. One aliquot was used for a determination of radioactivity in a gas flow counter with a Micromil® window, and another was used for determination of nucleic acids. RNA was determined by Drury's modification of the orcinol method (8) and DNA was determined by a Burton's modification of the diphenylamine method (9).

DNase in a concentration of 100 μg/ml in 0.25 M sucrose, 7.5 mM with respect to $MgCl_2$ and 0.05 M with respect to Tris (pH 7.4) was incubated with nucleolar preparations for periods of 5 to 30 mins. at 25° C. The nucleoli were sedimented by

centrifugation at 2000 x g for 10 mins., washed once with 0.25 M sucrose and then were incubated in a standard reaction mixture.

RESULTS

Cofactor requirements: Table 1 shows that optimal incorporation of the isotope of UTP-2-^{14}C into RNA required Mg^{++}, and all four nucleotide triphosphates (10). Omission of one nucleotide suppressed the uptake of UTP-2-^{14}C into RNA but the omission of GTP and CTP produced a marked decrease in RNA labeling. In the presence of small amounts of ATP, omission of energy generating systems only decreased the activity slightly. However, complete omission of ATP and the ATP generating system inhibited the incorporation markedly. Omission of ATP suppressed the activity only in part when an ATP generating system was present. Since Mg^{++} was found to be essential to this system (Table 1) concentrations of 2.5 and 5 mM $MgCl_2$ were compared at various pH for their effects on labeling of RNA. Since the higher concentration of $MgCl_2$ was not stimulatory, a concentration of 2.5 mM was routinely used. Manganese ion apparently stimulated the incorporation of isotope into RNA at low concentrations, but was inhibitory at higher concentrations. The optimal pH for incorporation of the isotope of UTP-2-^{14}C into RNA was 8.2 (Fig. 1).

The kinetics of incorporation of isotope into RNA in this system were studied under the optimal incubation conditions described above. As shown in Fig. 2, maximum incorporation was found at 30 min. of incubation. At 15 min. it reached almost 80% of the maximum.

Actinomycin D inhibited the incorporation of the isotope of UTP-2-^{14}C into RNA. At concentrations of 0.25 and 0.5 μg/ml, more than 50% inhibition was found (Fig. 3).

TABLE 1

COFACTOR REQUIREMENTS FOR INCORPORATION OF ISOTOPE OF UTP-2-^{14}C INTO NUCLEOLAR RNA

The reaction mixture (complete system) contained the following in final volume of 2.0 ml: 20 µg pyruvic kinase (PK), 10 µmoles phosphoenol pyruvate (PEP), 2 µmoles ATP, 0.25 µmoles each of GTP and CTP, 5 µmoles $MgCl_2$, 50 µmoles Tris-HCl ph 8.2, 0.2 µC UTP 2-^{14}C (11.3 mc/mM, Schwarz Bioresearch, Inc.) and nucleoli obtained from 5 gm of rat liver.

	Specific Activity cpm/mg RNA	µµM incorporated per mg RNA
Complete System	15,000	1630
-ATP	7,800	850
-GTP	4,600	500
-CTP	5,400	590
-GTP-CTP	2,500	270
$-Mg^{++}$	4,200	460
-ATP-PK-PEP	3,600	390
-PEP-PK	11,700	1270
$-Mg^{++}+Mn^{++}$ 2 µmoles	18,800	2040
5 µmoles	6,600	660
10 µmoles	2,700	290
DNase treated	680	70

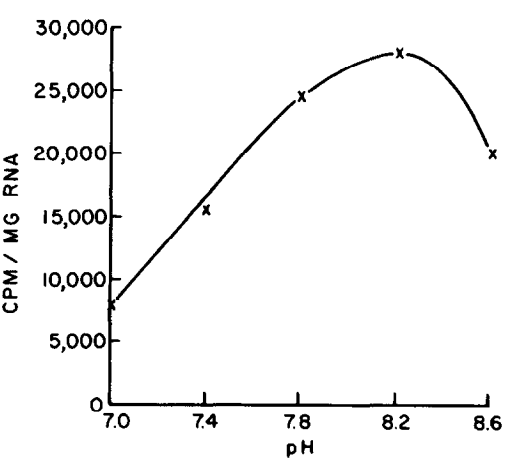

Fig. 1. Effect of pH on labeling of nucleolar RNA with UTP-2-^{14}C

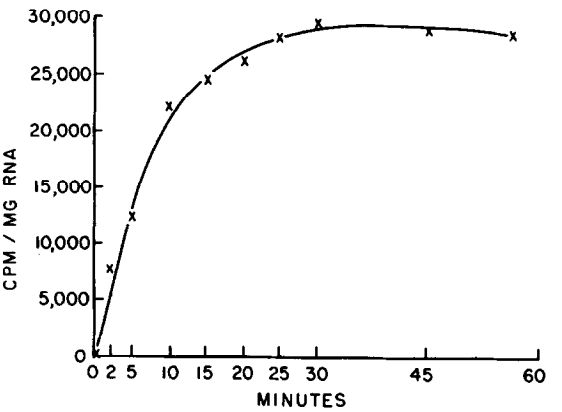

Fig. 2. Kinetics of labeling of nucleolar RNA with UTP-2-^{14}C

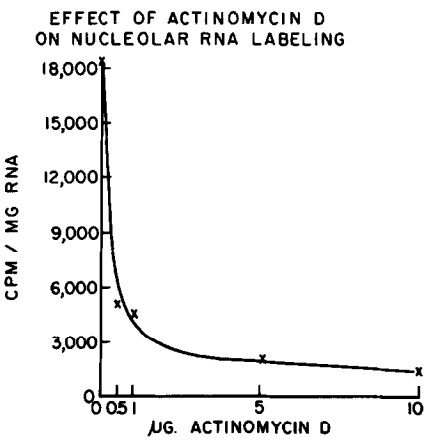

Fig. 3. Actinomycin D was added to the complete system described in Table 1. The incubation time was 15 mins. The abscissa is µg Actinomycin D per 2 ml reaction mixture.

When the nucleoli were pretreated with DNase as described under Materials and Methods more than 95% inhibition of uptake of isotope into RNA was found (Table 1) regardless of whether the nucleoli were incubated with DNase 5 mins. or longer. When nucleoli were treated similarly in the absence of DNase, a reduction in activity of approximately 25% was found.

DISCUSSION

These experiments provide evidence that RNA synthesis in the nucleolus is DNA-dependent. There are three types of DNA associated with the nucleolar preparations, namely, intranucleolar DNA in small chromatin fibers (11), perinucleolar nucleolus-associated chromatin (4) and adventitious contaminants. The latter were excluded as a significant source of primer and enzymes by the careful controls used in these experiments such that only nucleolar preparations of high purity were utilized for in vitro studies. Evidence that the product formed by nucleolar DNA is different from that formed by other DNA of the nucleus has emerged from studies on the base composition of nucleoli that show that nucleolar RNA is largely of the GC-type (3,7) and further that this RNA is synthesized rapidly (12). It has not been possible in these studies to rule out completely the role of RNA-dependent RNA polymerase in synthesis of components of nucleoli. Although the system employed in these studies incorporates labeled RNA precursors into RNA, it should be noted that the overall RNA content of the preparation is decreased by 15-25% during the course of 15 minutes of incubation. The reduction in RNA content may be related to the activity of the nucleoside phosphorylase of Harris (13) or to RNase activity. Regardless of the source of the degradative reactions, it is necessary to develop systems for net synthesis of RNA before satisfactory studies can be made on the products formed in isolated nucleoli.

SUMMARY

Isolated nucleoli were found to incorporate isotope of UTP-^{14}C into RNA under conditions similar to those reported by Weiss (10). The system requires Mg^{++} and all four nucleoside triphosphates.

Mn^{++} at low concentrations could replace Mg^{++}. The optimal pH for the reaction was 8.2. Addition of an energy-generating system enhanced the incorporation which was DNA-dependent as shown by the marked inhibition of incorporation by actinomycin D and DNase I.

REFERENCES

1. Muramatsu, M., Smetana, K. and Busch, H., Cancer Res. 23: 510 (1963).
2. Desjardins, R., Smetana, K., Steele, W. J. and Busch, H., Cancer Res. (in press).
3. Maggio, R., Siekevitz, P. and Palade, G. E., J. Cell Biol. 18: 293 (1963).
4. Busch, H., Byvoet, P. and Smetana, K., Cancer Res. 23: 313 (1963).
5. Adams, H. R. and Busch, H., Biochem. Biophys. Res. Comm. 9: 578 (1962).
6. Adams, H. R. and Busch, H., Cancer Res. 23: 576 (1963).
7. Muramatsu, M., Adams, H. R. and Busch, H., Proc. Am. Assoc. Cancer Res. 4: 46 (1963).
8. Drury, H. F., Arch. Biochem. 19: 455 (1948).
9. Burton, K., Biochem. J. 62: 315 (1956).
10. Weiss, S. B., Proc. Natl. Acad. Sci. 46: 1020 (1960).
11. Smetana, K. and Busch, H., Exp. Cell Res. 31: 198 (1963).
12. Muramatsu, M., Hodnett, J. L. and Busch, H., in preparation.
13. Harris, H., Proc. Roy. Soc., B, 158: 79 (1963).