RNA synthesis in isolated nuclei of lactating mammary cells in presence of unmodified and
mercury-labeled CTP

Ranjan Ganguly and M.R. Banerjee

Tumor Biology Laboratory, School of Life Sciences, University of Nebraska, 201 Lyman Hall,
Lincoln, NE 68588, USA

Received 17 July 1978

ABSTRACT

Isolated nuclei of lactating mouse mammary gland were capable of supporting
DNA-dependent RNA synthesis in vitro in presence of unmodified and mercurated
CTP (Hg-CTP) at high ionic condition at 25°C. In presence of unmodified CTP,
\([3H]UMP\) incorporation into RNA increased linearly up to 180 min. The kinetic
pattern of the reaction and the rate of RNA synthesis were essentially similar
when CTP was replaced by Hg-CTP. Both in unmodified and Hg-CTP containing
reactions, 70-80% of RNA synthesis was inhibited by α-amanitin. Presence of
poly(A) in a small portion of the in vitro synthesized messenger-like RNA was
detectable by oligo(dT) cellulose chromatography. Both poly(A)⁺ and poly(A)⁻
RNAs sedimented with a clear peak around 15S region in a formamide-sucrose
denaturing gradient. The Hg-RNA after separation from endogenous nuclear RNA
by SH-agarose affinity column chromatography also sedimented around 15S region
in a formamide-sucrose gradient. The Hg-RNA synthesized in the isolated mammary
cell nuclei in vitro should now permit monitoring hormonal regulation of
specific gene (casein) transcription in the mammary cells by molecular
hybridization of the Hg-RNA with cDNA to casein mRNA.

INTRODUCTION

The synergistic actions of prolactin and a glucocorticoid (hydrocortisone
or corticosterone) are required for lactogenesis, including casein synthesis,
and in recent years elucidation of the mechanisms involved in this multiple
hormone regulated specific gene expression in the mammary cells has been of
considerable interest 1-8. Studies in our laboratory have demonstrated that an
enriched glucocorticoid environment in the post-partum mothers is conducive to
the maintenance of larger ribosomal aggregates, active in casein synthesis in
the lactating mammary gland 9. We have also reported that the glucocorticoid in
presence of prolactin is required for the induction as well as maintenance of
the casein mRNA itself 5-7. The same series of studies further revealed that
cellular concentration of the casein mRNA is also modulated by the intensity of
suckling 6, a stimulus known to regulate the pituitary prolactin release by
indirect activation of the hypothalamo-hypophyseal control 8. These observations
thus raise the question concerning the discrete role of the glucocorticoid and
the polypeptide hormones on the transcriptional regulation of the mRNA for the milk-protein. Elucidation of the above question requires a cell-free RNA synthesis system, which permits reliable monitoring of transcription of the specific mRNA for the milk protein.

Widnell and Tata\(^9\) first reported that isolated nuclei of liver cells in a short term cell-free RNA synthesis system can support a predominant synthesis of DNA-like RNA at high ionic conditions. Recently, a few reports have indicated that faithful RNA synthesis in isolated nuclei at high ionic conditions can be sustained for an extended period of time\(^{10-12}\). Moreover, reports also indicate that isolated nuclei and chromatin can support synthesis of mercurated RNA (Hg-RNA) \textit{in vitro} in presence of mercury-labeled nucleotide (Hg-CTP or Hg-UTP) in the reaction mixture\(^{13-17}\). The technical feasibility of separation of the Hg-RNA synthesized \textit{in vitro} from pre-existing excess nuclear RNA by SH-agarose affinity column chromatography\(^18\) appears to provide the tool for monitoring specific gene transcription \textit{in vitro}\(^{13-15,17}\). In this report we present the results of studies on a predominantly RNA-polymerase II directed extended RNA synthesis system, derived from isolated nuclei of lactating mammary cells and also the synthesis of Hg-RNA in a similar \textit{in vitro} model.

**MATERIALS AND METHODS**

**Isolation of Nuclei**

The methods described by Yu\(^9\) was used with some modifications to suit the conditions of the lactating mammary tissue. Lactating (10-11 day) BALB/c mice nursing 6-8 pups were killed by cervical dislocation; mammary glands were excised and stored at -80°C. Frozen mammary tissue was pulverized in presence of liquid nitrogen and then homogenized in 2.5 vol of 2.3 M sucrose containing 3.3 mM CaCl\(_2\). The homogenate was diluted 8 fold with the same solution, filtered through four layers of cheese cloth and centrifuged at 50,000 xg for 60 min at 4°C. The nuclear pellet was suspended in 0.32 M sucrose-30% glycerol and centrifuged again at 700 xg for 10 min at 4°C. The final nuclear pellet was suspended in sucrose-glycerol solution and stored at -80°C. DNA was estimated by diphenylamine reaction\(^20\).

**In Vitro RNA Synthesis**

The standard reaction mixture in 100 μl volume contained 100 mM Tris-HCl (pH 8.1); 10 mM 2-mercaptoethanol; 4 mM MnCl\(_2\); 0.18 M (NH\(_4\))\(_2\)SO\(_4\) (pH 8.1); 0.6 mM each of ATP, GTP, CTP and 0.1 mM [\(^3\)H]UTP (1 μCi/0.01 μmol, New England Nuclear Corp., Boston). The reaction was started by adding the nuclei (10-20
μg DNA) and the incubation was continued for 180 min at 25°C. For the synthesis of Hg-RNA the composition of the reaction mixture was same as described above except that CTP was replaced by Hg-CTP (P-L Biochemicals Inc., Wisconsin), 2-mercaptoethanol was substituted by 20 mM monothioglycerol and [3H]UTP concentration in the reaction mixture was reduced to 0.02 mM. Consistent with an earlier report, thioglycerol was found to be a better sulfhydryl reagent for Hg-RNA synthesis in the mammary nuclear system (data not shown). In reactions with Hg-CTP the nuclei (20 μg DNA) were incubated for 40-60 min at 25°C. RNA synthesis was measured by monitoring [3H]UMP radioactivity in the TCA-precipitable or phenol-chloroform extractable material of the reaction product.

Characterization of the RNA Synthesized In Vitro

The RNA synthesized by the isolated nuclei after 180 min incubation was analyzed for its poly(A) content by oligo(dT) cellulose column chromatography. Synthesis of Hg-RNA was determined by SH-agarose affinity chromatography and the RNA eluting in the bound fraction was also analyzed in a formamide sucrose gradient. The poly(A)− and poly(A)+ RNAs were also characterized by sedimentation analysis in SDS-sucrose and formamide-sucrose gradients. The detailed procedures are given in appropriate figure legends.

RESULTS

RNA Synthesis in the Isolated Nuclei in Presence of Unmodified and Hg-CTP

Figure 1 shows the data on the kinetics of RNA synthesis in the standard reaction mixture containing unmodified CTP and 0.1 mM of [3H]UTP. A burst of RNA synthesis in the isolated nuclei was present during the initial 10 min period and this was followed by a linear increase of [3H]UMP incorporation into acid-precipitable product up to 180 min. The initial burst of activity constituted 1/3 of the total incorporation observed at 180 min and during the linear phase (between 10 and 180 min) the rate of precursor incorporation was 0.024 pmol per μg DNA per min. It is of interest to note that the incorporation rate during the initial 10 min period was 10 times greater than subsequent period of incubation. When the nuclei were incubated in presence of 10 μg/ml α-amanitin, RNA synthesis reached a maximal level at 10 min and then remained unaltered through the rest of the incubation period. At 180 min 77% of RNA synthesis was inhibited by the fungal toxin.

For assessment of the type(s) of nuclear RNA polymerase activity in RNA synthesis, nuclei were incubated in the standard reaction mixture containing different concentration of α-amanitin (Figure 2). About 70% of RNA synthesis
FIGURE 1. Kinetics of RNA synthesis in isolated nuclei (20 µg DNA) of lactating mammary cells in presence of unmodified CTP and 0.1 mM [3H]UTP. RNA synthesis was determined by measuring [3H]UMP radioactivity in the TCA-insoluble product on Whatman no. 3 filter disks by liquid scintillation spectrometry. Incorporation at zero time (1.2 pmol/µg DNA) was subtracted from each time point. O——O no α-amanitin; ••• + 10 µg/ml α-amanitin.

was inhibited in presence of 2.5 X 10⁻⁷ M (0.25 µg/ml) of the toxin and the level of inhibition remained essentially unaltered when α-amanitin concentration was raised to 1 X 10⁻⁶ M. Since RNA polymerase III is known to be inhibited by the toxin at a concentration range of 10⁻⁵-10⁻⁴ M22, the present observation suggests that the enzyme sensitive to α-amanitin in the present assay system mostly represents RNA polymerase II.

Figure 3 shows the results on the kinetics of RNA synthesis in presence of unmodified and Hg-CTP at low concentration (0.02 mM) of [3H]UTP. In either condition there was a ten minute initial burst of RNA synthesis and this was followed by a linear increase of precursor uptake during the 60 min period. The rate of [3H]UMP incorporation during 10 to 60 min of incubation in unmodified and Hg-CTP containing reaction mixture was about 0.007 and 0.006 pmol per µg DNA per minute. These results demonstrate that substitution of CTP by Hg-CTP does not cause any significant alteration in RNA synthesis in the isolated nuclei of lactating mammary cells.

Table 1 shows that in the standard assay system containing unmodified CTP, incorporation of the radioactive precursor into an acid insoluble product at
FIGURE 2. α-amanitin sensitivity of the reaction in presence of unmodified CTP. Nuclei (20 μg DNA) of lactating mammary cells were incubated for 180 min in the standard reaction mixture containing different concentration of α-amanitin and RNA synthesis was monitored as described in Figure 1.

180 min was dependent on the availability of template DNA (as indicated by actinomycin-D sensitivity) and also on the presence of all four ribonucleotides. Virtually all (98%) radioactivity was rendered acid soluble after incubation of the reaction product in 0.3 N KOH for 18 hr at 37°C. The U/G ratio of the reaction product was 1.66. In the Hg-CTP supplemented assay system [3H]UMP incorporation was virtually abolished by actinomycin-D and nearly 70% of the reaction was inhibited by α-amanitin (Table 2). The reaction was also dependent on the presence of Hg-CTP and 96% of the acid insoluble radioactivity in the reaction product of Hg-CTP containing assay system was hydrolysable by pancreatic RNase and 0.3 N KOH.

Oligo(dT) Cellulose and SH-agarose Column Chromatographic Analysis of the RNA

The RNA transcribed in vitro in the extended assay in presence of unmodified nucleotides was extractable by the phenol-chloroform method.23 Chromatography of the RNA on oligo(dT) cellulose affinity column showed that
FIGURE 3. Kinetics of RNA synthesis in isolated mammary cell nuclei (20 µg DNA) in presence of unmodified or Hg-CTP and 0.02 mM \([^{3}H]UTP\). The reaction condition was the same as described in Methods. At the end of the incubation the reaction product was extracted by phenol-chloroform method\(^{23}\) in presence of mouse mammary RNA (25 A\(_{260}\) units) as carriers, washed twice with 3 M NaOAc, pH 6.0 and dissolved in distilled water. RNA synthesis was determined by monitoring \([^{3}H]UMP\) radioactivity in the extracted reaction product in toluene-triton X-100 scintillation mixture. Incorporation at zero time (0.01 pmol/µg DNA) was subtracted from each time point. \(\Theta\ldots\Theta\) unmodified CTP; \(\Theta\ldots\Theta\) Hg-CTP.

while a major portion of the radioactive RNA eluted in unbound fractions with high ionic buffer, a small, but measurable amount, also eluted with low ionic condition (water) as bound fraction (Figure 4). Similar affinity chromatography of the RNA transcribed in presence of \(\alpha\)-amanitin showed considerably reduced radioactivity both in the unbound and bound fractions (Figure 4). The results shown in table 3 also revealed that 17% of the \(\alpha\)-amanitin sensitive RNA was bound to the column and this is presumably due to polyadenylation of the RNA transcribed \textit{in vitro}. 
TABLE 1: RNA SYNTHESIS IN ISOLATED MAMMARY CELL NUCLEI UNDER VARIOUS ASSAY CONDITIONS IN PRESENCE OF UNMODIFIED CTP.

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Activity</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>5.38</td>
<td>--</td>
</tr>
<tr>
<td>Omit ATP, GTP, CTP</td>
<td>1.07</td>
<td>80</td>
</tr>
<tr>
<td>+ 10 μg Actinomycin D</td>
<td>1.19</td>
<td>78</td>
</tr>
<tr>
<td>+ 10 μg α-amanitin</td>
<td>1.45</td>
<td>73</td>
</tr>
</tbody>
</table>

a RNA was transcribed for 180 min at 25°C in presence of 20 μg nuclear DNA. [3H]UMP incorporation was determined by monitoring TCA-precipitable radioactivity on Whatman no. 3 filter disks.

b pmol [3H]UMP incorporation/μg DNA.

TABLE 2: RNA SYNTHESIS IN ISOLATED MAMMARY CELL NUCLEI UNDER VARIOUS ASSAY CONDITIONS IN PRESENCE OF Hg-CTP

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Activity</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.31</td>
<td>--</td>
</tr>
<tr>
<td>Omit Hg-CTP</td>
<td>0.03</td>
<td>90</td>
</tr>
<tr>
<td>+ 10 μg Actinomycin D</td>
<td>0.02</td>
<td>94</td>
</tr>
<tr>
<td>+ 10 μg α-amanitin</td>
<td>0.10</td>
<td>68</td>
</tr>
</tbody>
</table>

a RNA was transcribed for 40 min at 25°C in presence of 20 μg DNA. After the incubation the reaction product was extracted as described in legend of Figure 3 and radioactivity was determined in toluene-triton X-100 cocktail.

b pmol [3H]UMP incorporation/μg DNA

In order to determine whether the RNA synthesized in presence of Hg-CTP retains the Hg-ligand of the CTP molecule, the RNA was chromatographed on a SH-agarose column (Figure 5). About 70% of the RNA was bound to the column and eluted with 2-mercaptoethanol. This observation is consistent with recent reports on the level of binding of in vitro synthesized Hg-RNA to SH-agarose or SH-agarose.
FIGURE 4. Acid precipitable radioactivity in the oligo(dT) unbound and bound fractions of RNA transcribed in isolated nuclei. RNA was synthesized in a 10 fold scaled up reaction volume in presence of unmodified CTP as described in Materials and Methods. After 180 min incubation purified mouse mammary total RNA was added to the reaction mixture as carrier and the RNA in the reaction mixture was extracted in presence of unmodified CTP as described in Materials and Methods. The isolated RNA was washed twice with 3 M NaOAc (pH 6.0) and dissolved in high salt buffer containing 0.5 M KCl-0.01 M Tris-HCl pH 7.6. The RNA was then heated at 65°C for 2 min, cooled rapidly and applied to an oligo(dT) cellulose column (1 x 4 cm) equilibrated with the same buffer at room temperature. The column was then washed with 7 bed vol. of high salt buffer and 1 ml fractions were collected. Bound RNA was eluted with water. Acid precipitable radioactivity on Whatman no. 3 filter disks was measured by liquid scintillation spectrometry according to the standard method. Elution position of the unbound (♦) and bound (●) RNA was determined by $A_{254}$ of the carrier RNA monitored by a UA-2 continuously recording UV analyzer (ISCO). ♦—♦ without α-amanitin; ●—● with 10 μg/ml α-amanitin.

The results also show that the RNA synthesized in presence of unmodified CTP failed to bind to the column and was eluted in the unbound fraction.

**Sedimentation Analysis of the RNA**

Oligo(dT) cellulose unbound and bound RNA fractions, synthesized in pres-
TABLE 3: Oligo(dT) Cellulose Chromatography of the RNA Synthesized in Presence or Absence of α-Amanitin

<table>
<thead>
<tr>
<th>RNA</th>
<th>[3H]UMP cpm</th>
<th>Total</th>
<th>Jnbound</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td></td>
<td>16,805</td>
<td>14,252 (84.8%)</td>
<td>2,553 (15.2%)</td>
</tr>
<tr>
<td>In presence of α-amanitin sensitive</td>
<td></td>
<td>3,050</td>
<td>2,835 (93.0%)</td>
<td>215 (7.0%)</td>
</tr>
<tr>
<td>α-amanitin sensitive</td>
<td></td>
<td>13,755</td>
<td>11,417 (83.0%)</td>
<td>2,338 (17.0%)</td>
</tr>
</tbody>
</table>

RNA was transcribed in presence of unmodified CTP with or without 10 μg/ml α-amanitin for 180 min at 25°C. The composition of the reaction mixture was the same as described in methods except that 3 μCi/0.01 μmol [3H]UTP was used. Product extracted by phenol-chloroform method was fractionated on cellulose column and acid precipitable radioactivity in each fraction was counted as described in Figure 4 with 30 cpm background subtraction setting in the counter. Numbers in parenthesis indicate % of total radioactivity (bound and unbound).

ence of nonHg-CTP, were further characterized by sedimentation analysis in sucrose density gradients (Figure 6). After centrifugation in a SDS-sucrose gradient a major portion of both the unbound and bound fractions of RNA sedimented slower than 28S (Figure 6A). It was estimated that 47% of the labeled RNA in the unbound fractions sedimented slower than 18S, 39% between 18S-28S and 14% faster than 28S. The radioactive RNA in the bound fraction sedimented in the order of 39%, 46% and 15%, in the respective zones indicated above, suggesting a slightly faster sedimentation of the RNA in the bound fraction. In formamide-sucrose gradient (Figure 6B) the unbound RNAs under denaturing condition resolved into a clear peak around 15S region and no labeled RNA sedimented at or faster than 28S region. The peak of bound RNA resolved at a slightly faster sedimentation zone. No radioactivity was present on top of the gradient indicating a virtual absence of degradation of the labeled RNA during sucrose gradient analysis.

The RNA synthesized in presence of Hg-CTP was also analyzed before and after SH-agarose chromatography. In SDS-sucrose gradient the unfractonated RNA resolved into clear peak around 15S region, and aliquotes of the same RNA sample sedimented a little faster in a formamide-sucrose gradient (Figure 7). Similar characterization of the SH-agarose bound Hg-RNA also showed a peak of radioactivity around 15S region, in a formamide-sucrose gradient (Figure 8).
FIGURE 5. SH-agarose chromatography of the RNA synthesized in presence of unmodified and Hg-CTP. The reaction condition was the same as described in Figure 3 except that the reaction volume was increased 10 fold and incubation was done for 40 min. RNA, synthesized in vitro, was extracted similarly as described in Figure 4 and dissolved in a buffer containing 50 mM NaCl-5 mM Tris-HCl, pH 7.5. The samples were heated at 65°C for 2 min, cooled rapidly and loaded on a 1 ml SH-agarose column (Agthiol™, P-L Biochemicals, Wisconsin) which was equilibrated at room temperature with 15 vol of TN buffer (100 mM NaCl-10 mM Tris-HCl pH 7.5). The column was first washed with 15 column vol of TN buffer and then with 10 vol of the same buffer containing 0.2 M 2-mercaptoethanol. The arrow indicates the position where the mercaptan containing buffer was added. Fractions (1 ml) were collected with a flow rate of 30 ml/hr and radioactivity in each fraction was determined as described in Figure 3. The column was always regenerated before use by washing with 10 bed vol of a buffer containing 0.1 M Tris-HCl, pH 8.0-0.3 M NaCl-1 mM EDTA-20 mM DTT and stored at 4°C in the same buffer containing 0.02% sodium azide. ••••• unmodified CTP; O---O Hg-CTP.

DISCUSSION

The results of the present assay system show that the lactating mammary cell nuclei are capable of incorporating [3H]UMP into an acid-insoluble product for extended period of time. The inhibitory effect of actinomycin-D, dependence of the reaction on ribonucleoside triphosphates, including hydrolysis of the acid-insoluble product after RNase and KOH treatment are indicative of a DNA-dependent RNA synthesis. The pronounced sensitivity of the reaction
FIGURE 6. Density gradient analysis of the oligo (dT) cellulose bound and unbound RNA transcribed for 180 min in isolated nuclei. RNA was transcribed in the standard reaction mixture containing [3H]UTP and [3H]ATP as the labeled precursors. The reaction product was extracted and chromatographed on oligo(dT) cellulose column as described in Figure 4. Unbound and bound fractions were pooled separately and precipitated with cold ethanol. For SDS-sucrose (10-40%) gradient analysis, RNA was dissolved in the buffer containing 10 mM Tris-HCl pH 7.5-0.1 M NaCl-0.05% SDS. The RNA was then layered on 12 ml gradients made in the same buffer and centrifuged for 16 hr at 20,000 RPM at 25°C in a SW 41 rotor. BMV 14S, 20S and 25S RNAs were centrifuged as marker in parallel tubes. For formamide (70%)-sucrose (5-22%) gradient analysis RNAs were dissolved in a buffer containing 3 mM Tris-HCl pH 7.4-3 mM Na-EDTA-70% formamide (deionized); heated at 65°C for 5 min, cooled rapidly, and layered on a 11 ml gradient made in the same buffer. The gradients were centrifuged at 36,000 RPM for 24 hr at 25°C in a SW 41 rotor. Fractions (0.3 ml) were collected from the top of the gradients using a gradient fractionator (ISCO) fitted with UA-2 UV analyzer and radioactivity was determined in toluene-triton X-100 liquid scintillation mixture. The absorbance of the marker RNAs was measured at 254 nm for SDS-sucrose and at 280 nm for formamide-sucrose gradient. (A) SDS-sucrose and (B) formamide-sucrose gradients. •••• bound RNA; rainfall unbound RNA.

to α-amanitin and the greater than 1.0 U/G ratio further suggest that the product is messenger-like RNA and the reaction is predominantly catalyzed by DNA dependent RNA polymerase II.

Earlier reports have indicated that a prolonged synthesis of RNA in isolated nuclei requires a higher concentration of the ribonucleotides. The present results show that the kinetic pattern of RNA synthesis in the isolated mammary cell nuclei remains essentially similar both in presence of high and low concentration of [3H]UTP in the reaction mixture. However, the rate of
FIGURE 7. Sedimentation profile of Hg-RNA in (A) SDS-sucrose and (B) formamide-sucrose gradients before fractionation on SH-agarose column. RNA was transcribed as described in Materials and Methods and extracted by the phenol-chloroform method as described\(^6,23\). Mouse mammary ribosomal RNA was used as carrier. The treatment of the RNA, making of gradients and centrifugation were done as described in Figure 6. 0.3 ml fractions were collected and counted in a Beckman LS 350 liquid scintillation system in toluene-triton X-100 liquid scintillation mixture.

RNA synthesis increases in presence of higher \(^{3}H\)UTP concentration. The kinetic pattern and the rate of RNA synthesis remain unaltered when CTP is replaced by Hg-CTP.

Polyadenylation is believed to be a post-transcriptional processing of the 3'-end of the mRNA\(^{24-26}\) and it is known that the poly(A) mRNA binds to oligo(dT) cellulose column at high ionic condition\(^{21}\). Since a detectable amount of RNA transcribed in the mammary cell nuclei binds to oligo(dT) cellulose, it is conceivable that a portion of the RNA has completed the chain elongation step \textit{in vivo}. Preliminary experiments on the incorporation of \(^{32}\)P-GTP into RNA and subsequent release of \(^{32}\)pppGp after KOH hydrolysis used as a measure of RNA chain initiation\(^{27}\) have shown that a detectable amount of \(^{32}\)P-GTP was incorporated into RNA (data not shown), suggesting some RNA chain initiation in the extended mRNA transcriptional system. Similar qualitative evidence of RNA chain initiation was also observed in isolated hen oviduct nuclei during extended period of mRNA synthesis \textit{in vitro}^12.

Sucrose density gradient analysis of the oligo(dT) cellulose unbound and bound RNA, synthesized by the isolated nuclei further indicates that the structural integrity of the transcripts is sustained in the assay system. In
FIGURE 8. Formamide-sucrose density gradient sedimentation analysis of the SH-agarose bound RNA (Hg-RNA). RNA, synthesized in presence of Hg-CTP, was extracted and fractionated on SH-agarose column as described in Figure 5. The bound fractions were pooled and Hg-RNA was precipitated with ethanol in presence of mouse mammary ribosomal RNA as carrier. The treatment of the RNA, making of the gradient and centrifugation procedure were the same as described in Figure 6. Wheat germ tRNA marker was centrifuged in a parallel gradient. Fractions (0.3 ml) were collected from the top of the gradient and radioactivity was determined as described in Figure 7.

SDS-sucrose gradient RNAs sedimenting faster than 28S is presumably due to RNA-RNA aggregation, because these faster sedimenting RNAs are not present when the RNA is analyzed under denaturing condition in presence of 70% formamide. A slightly faster sedimentation of the oligo(dT) cellulose bound RNA, more visible in the formamide-sucrose gradient, is possibly due to the poly(A) content of the RNA. The virtual absence of radioactivity on the top, as well as in the lower molecular weight zones of the gradients further indicates that the RNA is not degraded while synthesized or during the isolation procedure. In contrast, in assay systems derived from isolated nuclei of rat pituitary11, and hen oviduct12, addition of rat liver RNase inhibitor in the reaction mixture is essential to sustain extended RNA synthesis and/or to prevent degradation of the transcripts.
The characteristics of RNA synthesis in the isolated nuclei of the mammary cells in presence of Hg-CTP is similar to that observed in presence of unmodified nucleotides. Moreover, the pronounced α-amanitin sensitivity of the reaction clearly demonstrates a RNA polymerase II directed Hg-RNA synthesis in vitro. A recent report indicates that synthesis of Hg-RNA in isolated nuclei of mouse L-cell yields relatively smaller, 4S-10S, molecular species of RNA. In contrast the present results of formamide-sucrose gradient analysis demonstrate that the sizes of the RNA synthesized by the lactating mammary cell nuclei both in presence of nonmodified and Hg-CTP are virtually similar, sedimenting mostly as 15S, and this corresponds to the molecular weight of the mouse casein mRNA. The present results thus provide the first demonstration of abundant synthesis of large molecular size Hg-RNAs in a cell-free RNA synthesis system, derived from isolated nuclei of lactating mouse mammary cells.

Recent reports on the separation of in vitro synthesized Hg-RNA from endogenous nuclear RNA by SH-agarose (or -sepharose) chromatography and subsequent molecular hybridization of the Hg-RNA with cDNA to a specific mRNA have improved the feasibility of measuring specific gene transcription in vitro. The results described in the present report demonstrate development of an in vitro RNA synthesis system in which isolated mammary cell nuclei also synthesize Hg-RNA which is mostly messenger-like. The Hg-RNA synthesized in this system includes RNA species of relatively large size, some of which correspond to the molecular weight of mouse casein mRNA. Casein mRNA sequences in the Hg-RNA synthesized in isolated nuclei have been detected by molecular hybridization with cDNA to casein mRNA. These results will be reported elsewhere.

ACKNOWLEDGEMENTS

We thank Professor Myron K. Brakke for his critical reading of the manuscript and for the BMV RNAs used as markers. The authors also appreciate the secretarial assistance of Arvilla Kirchhoff. This work was supported by the Department of Health, Education, and Welfare grant CA-11058 from the National Cancer Institute.

REFERENCES
