RNA synthesis at the two-cell stage of mouse development

By JOHN KNOWLAND and CHRIS GRAHAM
From the Department of Zoology and the Sir William Dunn School of Pathology, University of Oxford

SUMMARY
This paper describes a method for quantitative extraction of nucleic acids from cultured pre-implantation mouse embryos in a form suitable for electrophoresis, and its application to the analysis of RNA synthesized during early mouse development. The nucleic acids synthesized by early mouse embryos have been identified by the use of RNase, DNase and mild alkaline hydrolysis. No obvious differences in the kinds of RNA synthesized in the blastocyst, trophoblast or embryonic fibroblast were found. At the two-cell stage of mouse development, which is the earliest that can be successfully labelled with radioactive precursors of RNA, all major RNA classes with similar electrophoretic mobilities to the RNA species of adult cells are synthesized, and it is likely that 28s and 18s RNA are synthesized at this stage. It is suggested that the onset of rRNA synthesis may not be associated with a particular stage of embryonic development.

INTRODUCTION
Study of the synthesis of defined classes of RNA may provide information about gene activity during development. The most suitable classes of RNA for such a study are those that can be easily resolved, and they include ribosomal and soluble RNA. Recent work (Emerson & Humphreys, 1971) suggests that in sea-urchin embryos ribosomal RNA (rRNA) synthesis may start earlier than has been thought previously, and may not be associated with a particular stage of development. The earliest stage at which rRNA synthesis has been definitely found in mouse embryos is the four-cell stage (reviewed by Graham, 1971). Here we describe experiments on the RNA synthesis in the two-cell mouse embryo.

MATERIALS AND METHODS
Supply of embryos
Female mice were from a randomly breeding closed colony of Swiss albino animals (strain PO). Six- to ten-week old virgin females were super-ovulated (Runner & Palm, 1953). Eight i.u. each of PMSG (pregnant mare serum gonadotrophin) and HCG (human chorionic gonadotrophin), (Gestyl and Pregnyl, Organon Laboratories, U.K.), were injected intra-peritoneally 48 h apart.

1 Authors' address: Department of Zoology, South Parks Road, Oxford, U.K.
After the injection of HCG each female was placed with one male of the PO strain.

Mating was revealed by the presence of a copulation plug on the first day of pregnancy, and two-cell embryos were dissected from the oviduct of mated females between 35.5 and 44.0 h post HCG injection. Blastocysts were dissected from the uterus between 93.25 and 93.50 h post HCG injection, at which time they contain on average 46.6 cells (Barlow, Owen & Graham, 1971). In both cases the embryos were stored at 37 °C during collection in equilibrated culture medium after 3 separate rinses in 1 ml of medium to remove contaminating cells.

Culture and labelling

Embryos were cultured in White's medium (Table 1). The water used in this medium was deionized, and then glass distilled 6 times. Fresh medium was made up each week. No antibiotics were present. The medium contained 5% (v/v) foetal calf serum (Flow Laboratories), which has previously been found to promote the uptake of uridine (Woodland & Graham, 1969). The medium was equilibrated with a gas mixture of 90% N₂, 5% CO₂, 5% O₂.

The medium was placed in 0.2 ml drops under liquid paraffin (Boots Pure Drug Co., U.K.; method of Brinster, 1963) and up to 100 embryos were cultured in each drop. The embryos were labelled for 11–16 h in medium containing 100 μCi/ml of [5-³H]uridine (specific activity 33.8 Ci/mM; the Radiochemical Centre, Amersham, U.K.). After incubation, any embryos with more than two cells were discarded. The remaining embryos were then rinsed 3 times in culture medium. The zona pellucida was removed from two-cell embryos with pronase (Mintz, 1967) to reduce the risk of contamination by micro-organisms adhering to it. The denuded embryos were again rinsed 3 times in culture medium before freezing and storage at –70 °C.

Table 1. White's medium

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (mm)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>85.54</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>KCl</td>
<td>4.78</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>L(+) Ca-lactate·4H₂O</td>
<td>1.71</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>1.19</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.19</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>Na-pyruvate</td>
<td>0.33</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Na-lactate</td>
<td>20.00</td>
<td>Sigma: 3-68 ml/l. of 65% 1:1 DL mixture</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>11.90</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>D(+) glucose</td>
<td>5.55</td>
<td>BDH, Analar</td>
</tr>
<tr>
<td>Crystalline bovine</td>
<td></td>
<td>Armour</td>
</tr>
<tr>
<td>Plasma albumin</td>
<td>5 g/l.</td>
<td>Pharmaceuticals</td>
</tr>
</tbody>
</table>

This is a modification of the medium described by Whitten & Biggers (1968).
Embryonic fibroblasts were cultured from trypsinized embryos on the 14th day of pregnancy. They were labelled with $[^{32}P]$orthophosphate (10 $\mu$Ci/ml) in reinforced Eagle's medium (Vogt & Dulbecco, 1963) for 24 h and subsequently chased for 6 h in fresh medium.

Trophoblast fragments taken on the 10th day of pregnancy were labelled with $[^{5}\text{-}^{3}H]$uridine (100 $\mu$Ci/ml) for 16 h in reinforced Eagle's medium.

**Extraction of RNA and DNA**

Neither cold (Knowland, 1970) nor hot (Girard, 1967) phenol–sodium dodecyl sulphate (SDS) extraction of nucleic acids from cultured mouse embryos gave high recoveries of acid-insoluble radioactivity or reproducible electrophoretic profiles. The method adopted was designed to achieve thorough deproteinization of nucleic acids. With embryonic *Xenopus* cells it has been consistently successful when cold and hot phenol-SDS extraction have failed (Knowland, 1971). Embryos were homogenized in $0\cdot1$ M Tris-HCl, $0\cdot05$ M NaCl, $0\cdot01$ M disodium EDTA, $0\cdot5\%$ (w/v) SDS (BDH, specially pure), 4 $\mu$g polyvinyl sulphate/ml, pH 7-5. Pronase (Calbiochem, grade B) that had been pre-digested at 1 mg/ml in the same medium for 1 h at 37 °C was added to 200 $\mu$g/ml, and the mixture was incubated for 2 h at 37 °C. The digest was extracted twice with phenol and once with chloroform–octanol (24:1, w/v) at room temperature, made 3 % (w/v) in NaCl and precipitated for 18 h at 4 °C with 2 vol of ethanol. Carrier RNA prepared in the same way from mouse liver was added to the homogenate at the start of the extraction. The precipitate was washed twice with ethanol and stored at −70 °C. This procedure invariably gave at least 95 % recovery of acid-insoluble radioactivity, and the electrophoretic profile of RNA extracted in this way was not altered when it was added to a homogenate and subjected to the entire procedure again.

**Removal of DNA**

Nucleic acid pellets were dissolved in 2 ml of $0\cdot01$ M Tris-HCl, $0\cdot001$ M MgSO$_4$, 2 $\mu$g polyvinyl sulphate/ml, pH 7-5. 40 $\mu$g of RNase-free DNase (Worthington) were added, and the solution incubated at 30 °C. More DNase (20 $\mu$g/ml each time) was added at 10 min intervals, and after the final addition the digest was deproteinized with phenol, and the RNA precipitated with NaCl and ethanol. Three successive additions of DNase were required to digest all the DNA in a nucleic acid extract (Knowland, 1970).

**Electrophoresis**

Nucleic acid samples were analysed by electrophoresis at 5-4 V/cm on 2.7 % acrylamide gels cross-linked with 0.27 % (w/v) ethylene diacrylate (Knowland, 1970). SDS at 0.5 % (w/v) was present during electrophoresis. Gels were scanned at 265 nm in a Joyce, Loebl u.v. scanner and radioactivity was measured in 1 mm slices of the gels (Knowland, 1970).
Fig. 1 Electrophoresis and identity of nucleic acids synthesized in the mouse blastocyst. One hundred blastocysts were labelled with [5-^3H]uridine at 100 μCi/ml for 12 h. The nucleic acid extract was divided into 4 parts before ethanol precipitation. A, Control; the sample was analysed by electrophoresis for 2.5 h. B, Effect of RNase; the sample was dissolved in 2 ml of 0.15 M-NaCl, 0.015 m trisodium citrate and digested with boiled bovine pancreatic RNase at 50 μg/ml for 1 h at 37 °C; 100 μg of pronase that had been pre-digested at 1 mg/ml in 0.15 m-NaCl, 0.015 m trisodium citrate was added and incubation continued for 2 h. The incubate was extracted twice with chloroform-octanol and precipitated with NaCl and ethanol. Carrier RNA was added before electrophoresis to provide an absorbance trace (---). C, Effect of DNase; the sample was treated with three successive doses of DNase as described in Materials and Methods before electrophoresis. Residual degraded DNA is detectable beyond the soluble RNA region. D, Effect of mild alkaline hydrolysis. Following electrophoresis, 1 mm slices of the gel were treated with 0.3 m-KOH at 37 °C for 18 h to hydrolyse both the gel and the RNA. After neutralization with HCl an aliquot was taken from each hydrolysate for measurement of total radioactivity (●—●; corresponding to RNA plus DNA) and trichloroacetic acid-insoluble radioactivity (○—○; corresponding to DNA) was measured in the remainder.
**RESULTS**

*Identity of nucleic acids labelled by [5-3H]uridine*

In cultured mouse fibroblasts, radioactivity from [5-3H]uridine enters both DNA and RNA (Adams, 1968). The same was found to be true of mouse embryos (Fig. 1). In blastocysts, the peaks labelled by [5-3H]uridine correspond in order of increasing electrophoretic mobility to DNA, 28 s rRNA, 18 s rRNA and 4 s RNA of adult cells. The pattern of labelling obtained in blastocysts (Fig. 1), in which the main RNA species labelled are ribosomal and soluble
RNA, agrees with those obtained previously using other methods (Ellem & Gwatkin, 1968; Woodland & Graham, 1969; Piko, 1970; Monesi et al. 1970). A very similar pattern was also found in the trophoblast on the 10th day of pregnancy (Fig. 2). We conclude that the methods used for extraction and analysis of RNA provide a sensitive and reliable method for detecting the synthesis of the main RNA classes during early mouse development.

**RNA synthesis in two-cell embryos**

Three batches of 1000 2-cell embryos were labelled for 11–13 h with [5-3H]-uridine at 100 μCi/ml. 32P-labelled RNA extracted in the usual way from day-14 mouse embryo fibroblasts was added to each batch at the start of extraction to provide an accurate internal marker of rRNA. The nucleic acid precipitates were treated 5 times with DNase before analysis. Fig. 3 shows that at the two-cell stage the mouse embryo synthesizes mainly high-molecular-weight heterogeneous RNA and also low-molecular-weight RNA. Small peaks of radioactive RNA run with the marker 28s and 18s RNA, suggesting that rRNA may be synthesized at the two-cell stage. Very similar results were obtained from the other two batches of two-cell embryos.
**DISCUSSION**

*Extraction of nucleic acids*

One aim of this work has been to establish a method for quantitative extraction of RNA from cultured mouse embryos in a pure form suitable for electrophoretic analysis. Another has been to identify the nucleic acids synthesized by early mouse embryos. The extraction method used consistently gives at least 95% recovery of trichloroacetic acid-insoluble radioactivity, and RNA does not seem to be degraded during extraction. Thus, its electrophoretic profile is not affected by a second passage through the procedure, and the high-molecular-weight RNA made at the two-cell stage is not degraded (Fig. 3). Conclusions about RNA synthesis based on the use of radioactive precursors of RNA are valid only if the RNA analysed is undegraded and free from contaminants. The experiments illustrated in Fig. 1 show that radioactivity from [5-3H]uridine enters both DNA and RNA. The electrophoretic method separates DNA from most RNA, but reliable conclusions about high-molecular-weight RNA synthesis require the removal of contaminating DNA by exhaustive treatment with DNase (Fig. 3).

**RNA synthesis at the two-cell stage**

Most of the RNA synthesized at the two-cell stage does not enter the cytoplasm. Autoradiography of two-cell embryos exposed to tritiated uridine for 3-4 h showed that most of the labelled RNA was retained in the nucleus (Mintz, 1964; Hillman & Tasca, 1969). During these labelling times, the nucleoli were lightly labelled (Mintz, 1964), and radioactivity was found in the nucleoplasm and at the periphery of the primary nucleoli, which contain electron-dense granules associated with chromatin filaments (Hillman & Tasca, 1969). There was heavy labelling of the nucleus and slight labelling of the cytoplasm above background when two-cell embryos were exposed to tritiated uridine at 100 μCi/ml for 6 h (Graham, unpublished). There is a similar retention in the nucleus of most of the RNA synthesized during early cleavage in the sea-urchin embryo (Kijima & Wilt, 1969).

The autoradiographic evidence suggests that there is a little rRNA synthesis at the two-cell stage of mouse development. The low nucleolar labelling observed at this stage in mice may not be general in the Class Mammalia; the nucleoli of the Syrian hamster can be heavily labelled at the two-cell stage (Ukatoji, 1969).

The RNA labelled during a 6 h incubation at the two-cell stage of mouse development has previously been characterized by sucrose-gradient centrifugation (Woodland & Graham, 1969). This study demonstrated the synthesis of low-molecular-weight RNA and also of high-molecular-weight RNA in regions of the gradient which contained marker 28 s and 18 s. However, the high-molecular-weight RNA was not resolved into discrete peaks of radioactivity.
We have used polyacrylamide gel electrophoresis, which has greater resolving power than sucrose-gradient centrifugation, and in this work we extended the labelling period to increase the possibility of detecting rRNA synthesis in two-cell embryos. We have shown that in addition to heterogeneous high-molecular-weight RNA and low-molecular-weight RNA, the gels contain radioactivity in the rRNA region. Three samples, each containing 1000 two-cell embryos, were analysed, and these peaks were observed in every case.

The presence of these peaks does not prove that rRNA is synthesized at the two-cell stage, but it seems likely that this is the case. Certainly at the two-cell stage of mouse development all the major classes of RNA with electrophoretic mobilities similar to those of adult cells are synthesized. In the rabbit, labelled RNA is apparently not found in the rRNA region of a gel until the 200- to 400-cell stage (Manes, 1969).

RNA synthesis in the trophoblast

The pattern of incorporation of radioactivity into RNA in the trophoblast is not significantly different from that in the blastocyst or in embryonic fibroblasts. The biochemical differences between these tissues are therefore not clearly reflected in their pattern of RNA synthesis.

RNA synthesis of the one-cell stage

As in previous work (Mintz, 1964), we have found it difficult to label RNA at the one-cell stage of development. This difficulty is largely due to the failure of orthophosphate, bicarbonate or uridine to penetrate the cell membrane at this time (Woodland & Graham, 1969). However, when 500 one-cell eggs were used, we did find incorporation into trichloroacetic acid-precipitable material. This material could not be resolved by gel electrophoresis and so we can say nothing about the classes of RNA synthesized at this stage.

Due to the scarcity of material, we have been unable to measure the specific activity of UTP at the one- or two-cell stages. We are therefore unable to compare the rate of rRNA synthesis at these stages with the rate observed at later stages.

CONCLUSION

In the mouse it seems likely that there is no stage of development at which rRNA and soluble RNA are not synthesized although it is difficult to label these classes of RNA at very early stages of development. The same is probably true of the sea-urchin embryo (Emerson & Humphreys, 1971). In both these embryos it may be incorrect to think of the ribosomal genes becoming active at a particular stage of development after fertilization.
Le présent article décrit une méthode pour l'extraction quantitative des acides nucléiques d'embryons de souris en culture (au stade de la pré-implantation) sous une forme adéquate pour l'électrophorèse et l'application de cette méthode à l'analyse des RNA synthétisés pendant le développement precoce chez la souris. Les acides nucléiques synthétisés par les embryons de souris (à des stades précoces) ont été identifiés en utilisant la RNase, la DNase et l'hydrolyse alcaline ménagée. On n'a trouvé aucune différence marquante entre les types de RNA synthétisés dans le blastocyste, le trophoblaste et le fibroblaste embryonnaire. Au stade 2 cellules du développement de la souris, qui est le stade le plus précoce où on peut affectuer un marquage au moyen de précursors de RNA radioactifs, toutes les classes principales de RNA à mobilités électrophorétiques semblables à celles des RNA de cellules adultes sont synthétisées et il est vraisemblable que les rRNA 28s et 18s sont déjà synthétisés à ce stade. On propose que le déclenchement de la synthèse de rRNA pourrait ne pas être associé à un stade particulier du développement embryonnaire.

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REFERENCES


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