APPEARANCE OF 7 - METHYLGUANOSINE - 5' - PHOSPHATE IN THE RNA OF MOUSE 1-CELL EMBRYOS THREE HOURS AFTER FERTILIZATION

R. J. Young

Reproductive Biology Unit, Department of Obstetrics and Gynecology
Cornell University Medical Center
1300 York Avenue, New York, New York 10021

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Summary: [^{3}H]-guanosine-labelled RNA isolated from 1-cell mouse embryos 1-3 hours after fertilization was digested with a mixture of RNase A, and ribonucleases T_1 and T_2 followed by nucleotide pyrophosphatase. Radioactive 7-methylguanosine-5'-phosphate and Gp were present in the digest with a high ratio of the former to the latter. This result suggests that the mouse ovum contains a store of mRNA which is made available for translation immediately after fertilization by the addition of 7-methylguanosine to the 5'-terminus. The presence of the Gp in the digest shows that guanosine is incorporated into internal positions in the RNA, and may mean that RNA is synthesized for a short period after fertilization.

It is unclear at present if transcription of the embryonic genome occurs throughout pre-implantation development of the mammalian embryo. Inhibitors of RNA synthesis can prevent cleavage of rabbit embryos at the 2-cell stage of development, and mouse embryos even earlier, at the 1-cell stage (1, 2). These observations indicate that synthesis of new RNA is required at all stages of mammalian embryogenesis, in contrast to echinoderms where early embryonic development is dependent on maternal mRNA (3, 4). However, attempts to demonstrate RNA synthesis throughout the cleavage stages of mammalian embryogenesis have not been successful suggesting that RNA synthesis does not occur at all stages of embryonic development. Thus, although rabbit and mouse 2-cell embryos incorporate [^{3}H]-uridine *in vitro* into heterogenous RNA (5, 6), the mouse 1-cell embryo incorporates very little [^{3}H]-uridine

Abbreviations: 7-methylguanosine, m^7G; 7-methylguanosine-5'-phosphate, pm^7G; human chorionic gonadotrophin, HCG.

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into TCA insoluble material, and efforts to characterize the [^{3}H]-uridine-labelled material as RNA have been unsuccessful (6-11). On the other hand [^{3}H]-adenosine and [^{32}P]-phosphate are readily incorporated *in vitro* into TCA insoluble material by the mouse 1-cell embryo; here also [^{32}P]-phosphate-labelled RNA has not been detected in labelled embryos (12). The mouse 1-cell embryo also incorporates [^{3}H]-guanosine into TCA insoluble material, but does so at a lower level than [^{3}H]-uridine and with different kinetics (10). Whereas, [^{3}H]-uridine is incorporated at a constant low level from the time of fertilization until the onset of pronuclear DNA synthesis, [^{3}H]-guanosine incorporation drops from its relatively high level immediately after fertilization to a much lower level 3-4 hours later and remains at this very low level until pronuclear DNA synthesis. This communication reports studies on the utilization of [^{3}H]-guanosine by the mouse 1-cell embryo during the period 1-3 hours after fertilization.

MATERIALS AND METHODS

Collection and culture of mouse ova: Virgin random-bred Swiss female mice 7-12 weeks old were superovulated by intraperitoneal injection with pregnant mare's serum and HCG 46-48 hr later. Immediately after the second injection each female was placed with a male (Balb CJ X C57 BL/6J) and checked for the presence of a vaginal plug next morning. Mated females were sacrificed 16 hr post HCG and the ova collected as described (10). Washed ova were incubated at 37° with gentle agitation 17-19 hr post HCG in Whitten's medium containing 8 -[^{3}H]-guanosine at 500 μCi/ml and 1% dimethyl sulfoxide as previously described (10). Approximately 50% - 60% of the ova were pronuclear after the incubation period.

Isolation of labelled material: After incubation, fertilized ova were washed 4-6 times with medium containing 0.1 mg/ml guanosine and transferred to 0.1 M Tris - HCl, pH 7.2, 1% SDS containing 20 μg yeast tRNA. The suspension of embryos was repeatedly frozen and thawed, the lysate adjusted to pH 5 with 20% sodium acetate, pH 5, and the labelled product precipitated with 2 volumes of ethanol at - 20°. The precipitate was collected by centrifugation at 4°, redissolved in Tris - HCl, pH 8, and reprecipitated with ethanol in the presence of GTP and GDP. When the soluble fraction was required, SDS was omitted from the lysing buffer.

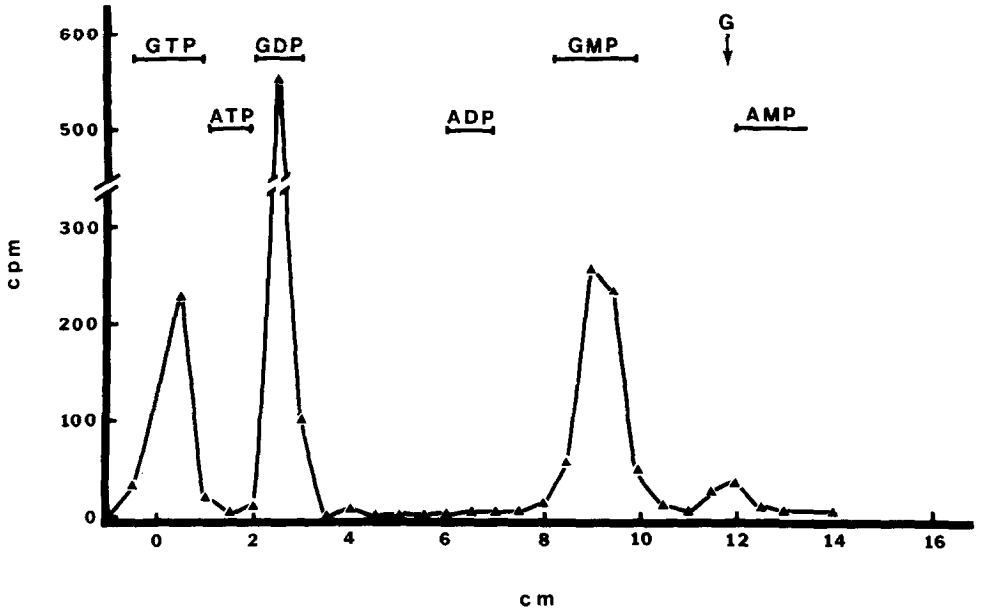

Figure 1. Thin layer chromatography of [^{3}H]-guanosine nucleotides present in mouse 1-cell embryos. The soluble fraction from [^{3}H]-guanosine-labelled mouse 1-cell embryos was chromatographed together with markers on a PEI-cellulose sheet in solvent B. Radioactive spots were located by cutting the chromatogram into 0.5 cm strips and eluting the strips with a solution of 0.3 M LiCl in 0.05 M acetic acid and 7 M urea before scintillation counting.

Enzyme digestion: The labelled precipitate was incubated with self-digested pronase (nuclease free, Calbiochem), 100 µg/ml in Tris - HCl, pH 7.6 for 2 hr at 37°, and the digest extracted with phenol - chloroform as described by Perry et al. (13). Phenol was removed from the aqueous phase with ether and the aqueous layer incubated for 8 hr at 37° with the ribonucleases T_1 (25 units) and T_2 (20 units), ribonuclease A (10 µg) and 2.5 mM EDTA in 50 mM sodium acetate, pH 5. The pH of the digest was brought to 7.5, $MgCl_2$ added to 10 mM, and incubated for a further 45 min with 50 µg GTP and nucleotide pyrophosphatase (0.05 units).

Chromatography: Thin layer chromatography was performed on PEI-cellulose sheets (Baker). Solvents were: A, 0.5 M phosphate, pH 3.4; B, step formate (14); C, 1 M LiCl; D, 0.15 M sodium borate - 0.5 M boric acid. After chromatography the positions of marker nucleotides were determined with a U.V. lamp, the radioactivity eluted with 0.05 M acetic acid - 7 M urea-0.3 M LiCl and measured by scintillation counting in a toluene based scintillation mixture containing BBS3 (Beckman) emulsifier.

RESULTS AND DISCUSSION

Since adenosine is readily incorporated into TCA insoluble material (10) the possibility that the low incorporation of [^{3}H]-

guanosine is due to conversion of guanine to adenine and the subsequent incorporation of this base into TCA insoluble products was explored. Thin layer chromatography in solvent B of supernatants from embryo lysates showed that four radioactive components were present (fig. 1). Three of these were identified as GTP, GDP and GMP which were present as internal markers, and the fourth had the same R_f as guanosine which was present as an external marker. A similar result was obtained in solvent A except that guanosine did not separate from GMP. The percentage of the total recovered radioactivity present as GTP, GDP and GMP was 17%, 40% and 38% respectively. This result showed that immediately after fertilization guanine is not converted to adenine and that [^{3}H]-guanosine is incorporated into TCA insoluble products, probably RNA.

Mouse ova contain very little rRNA (15, 16). Because of the difficulty in obtaining large numbers of embryos and the low level of [^{3}H]-guanosine incorporation, no attempt was made to fractionate embryo lysates into polysome fractions or to separate RNA into [poly (A)-] and [poly (A) +] RNA. However, the method used for RNA extraction (13) does release [poly (A) +]RNA, and control experiments in which E. coli or rat liver rRNA was present showed that RNA degradation did not occur during pronase digestion or phenol - chloroform extraction of embryo lysates. Other preliminary experiments demonstrated that small (0.1% of that present in the soluble fraction) amounts of GTP and GDP were present in the aqueous layer after phenol - chloroform extraction and that these were the only soluble contaminants.

A lysate of [^{3}H]-guanosine-labelled embryos was extracted with phenol-chloroform and the extract digested with a mixture of RNase A and ribonucleases T_1 and T_2 followed by nucleotide pyrophosphatase. Analysis of the digest by thin layer chromatography on PEI-

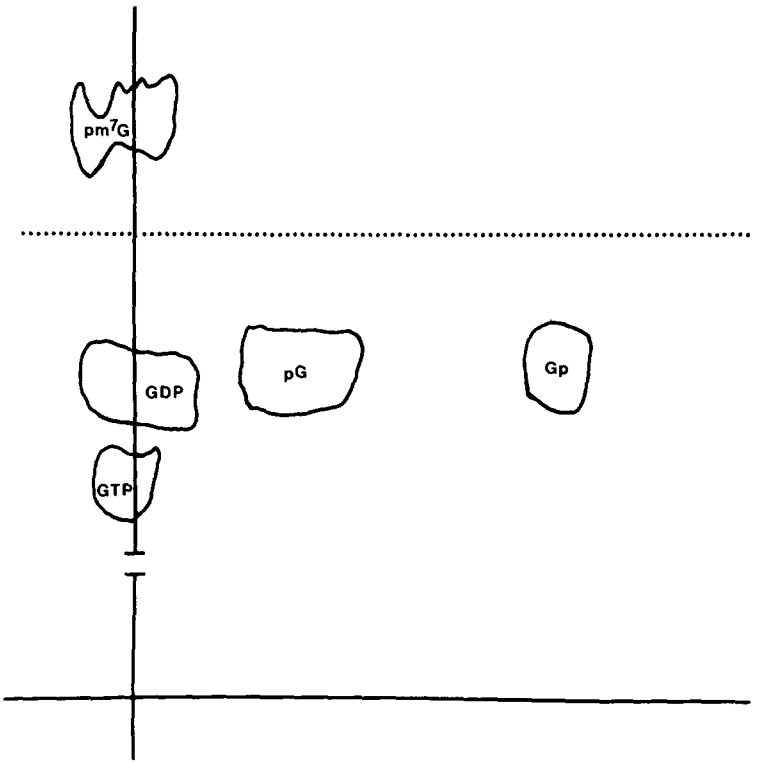

Figure 2. Thin layer chromatography of the enzyme digest of [^{3}H]-guanosine-labelled mouse 1-cell embryos. The digest and markers were chromatographed on a PEI-cellulose sheet in water then dried and the dried sheet developed in solvent C in the same direction (bottom to top). The sheet was cut along the dotted line, and the lower part washed in anhydrous methanol before development in solvent D (left to right).

cellulose (fig. 2) showed that pm^{7}G, pG and Gp but not GDP and GTP were present. Recent studies have shown that the nucleoside m^{7}G, is a minor constituent of eukaryotic mRNA and is attached via a 5'-5' pyrophosphate linkage to the 5'-terminus of the RNA forming the cap structure m^{7}G(5')ppp(5')X^m (17-21). This structure is resistant to nucleases, and digestion of embryo lysates with a mixture of RNase A, and ribonucleases T_1 and T_2 would result in cleavage of the [^{3}H]-guanosine-labelled RNAs present to give nucleoside-3'-phosphates, and products from the 5'-terminus such as nucleotides of structure pXp, ppXp etc., and the cap structure above.

The small quantity of material available prevented attempts to isolate the 5'-terminal cap, but identification of pm^7G after further digestion of the lysate with nucleotide pyrophosphatase suggests that the cap structure is present in the [^{3}H]-guanosine-labelled mRNA of mouse embryos because nucleotide pyrophosphatase releases pm^7G from the cap but not from other nucleotides present at the 5'-terminus. Unmethylated guanosine has also been found in the 5'-terminal cap structure of insect oocyte mRNA (22), and the presence of pG in the enzymic digest may mean that this structure is also present in mouse embryos. However, a more likely source of pG is from GTP which is present as a contaminant in the mouse embryo lysates, but which is absent after enzymic digestion. It is probable that the unmethylated guanosine 5'-5' pyrophosphate cap structure is not present in mouse embryo mRNA.

The presence of Gp in the digest of embryo lysates shows that [^{3}H]-guanosine is incorporated into embryo RNA in internal positions. This may arise either by 3'-terminal addition to pre-existing RNAs, by replacement of a base within the polynucleotide chain of tRNA by a guanine residue (23-26), or by synthesis of new RNA. It is not known at present which of these alternatives is correct. However, the differences in the kinetics of incorporation of uridine and guanosine by the 1-cell embryo (10), the inability to isolate [^{3}H]-uridine-labelled RNA from the embryo (6, 7) and the absence of RNA polymerase activity in the pronuclei of 1-cell embryos (27) all suggest that new RNA is not made by the 1-cell embryo. Nevertheless it is possible, as suggested by the kinetics of incorporation (10), that a low level of RNA synthesis occurs up to 3 hours after fertilization but then ceases and further transcription of the embryonic genome does not occur until after the first cleavage. Attempts to

detect RNA synthesis in the 1-cell embryo later than 3 hours after fertilization would be unsuccessful.

The ratio of the radioactivity found in embryo lysates as pm^7G to that found as Gp varied between 1.6 and 0.4. If new mRNA is synthesized immediately after fertilization a much lower ratio would be expected since many guanosine residues are incorporated into internal positions but only one is incorporated at the 5'-terminus. Therefore, the high ratio of pm^7G to Gp suggests that immediately after fertilization [^{3}H]-guanosine is attached as m^7G via a 5'-5' pyrophosphate linkage to the 5'-terminus of pre-existing mRNA. Since the cap appears necessary for translation of mRNA (see review (28)), the result implies that the mouse ovum contains a store of inactive mRNA which immediately after fertilization is capped and made available for translation. The pattern of proteins synthesized by the 1-cell embryo is different from that of the unfertilized ovum or the 2-cell embryo (29, 30). While heterogenous RNAs are synthesized by the 2-cell embryo, very little if any, RNA synthetic activity can be detected in the 1-cell embryo or unfertilized ovum (6-11). It appears that in mammals maternal mRNA is utilized for development of the fertilized ovum up to at least the first cleavage, and the store of maternal mRNA is activated after fertilization by addition of a pm^7G residue to the 5'-terminus. This behavior is quite different from that of the echinoderms where maternal mRNAs are capped and their translations after fertilization does not require attachment of a pm^7G residue to the 5'-terminus (31 - 33).

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REFERENCES

1. Manes, C. (1973). Dev. Biol. 32, 453-459.
2. Golbus, M.S., Calarco, P.G., and Epstein, C.J. (1973). J. Exp. Zool. 186, 207-216.
3. Gross, P.R. (1968). Ann. Rev. Biochem. 37, 631-660.
4. Brown, D.D., and Dawid, I.B. (1969). Ann. Rev. Genet. 3, 127-154.
5. Manes, C. (1971). J. Exp. Zool. 176, 87-96.
6. Knowland, J., and Graham, C. (1972). J. Embryol. Exp. Morphol. 27, 167-176.
7. Woodland, H.R., and Graham, C.F. (1969). Nature 221, 327-332.
8. Monesi, V., and Salfi, V. (1967). Expt. Cell Res. 46, 632-635.
9. Daentl, D.L., and Epstein, C. J. (1971). Dev. Biol. 24, 428-442.
10. Young, R.J., Sweeney, K., and Bedford, J.M. (1977). Submitted for publication.
11. Graham, C.F. (1973) in Regulation of Mammalian Reproduction (Segal, S., Crozier, R., Corfman, P.A. and Condliffe, P.G., eds.) pp 286-301, C.C. Thomas, Springfield, Illinois.
12. Young, R.J. (1976). Fed. Proc. 35, 1690.
13. Perry, R.P., LaTorre, J., Kelley, D.E., and Greenberg, J.R. (1972). Biochim. Biophys. Acta. 262, 220-226.
14. Randerath, E., and Randerath, K. (1964). J. Chromatog. 16, 126-129.
15. Young, R.J., Stull, G.B., and Brinster, R.L. (1973). J. Cell Biol. 59, 372a.
16. Olds, P.J., Stern, S., and Biggers, J.D. (1973). J. Exp. Zool. 186, 39-46.
17. Rottman, F., Shatkin, A., and Perry, R.P. (1974). Cell 3, 197-199.
18. Adams, J.M., and Cory, S., (1975). Nature 255, 28-33.
19. Wei, C.M., Gershowitz, A., and Moss, B., (1975). Cell 4,379-386.
20. Desrosiers, R., Friderici, K., and Rottman, F. (1974). Proc. Nat. Acad. Sci., U.S.A.
21. Perry, R.P., Kelley, D.E., Friderici, K., and Rottman, (1975). Cell 4, 387-394.
22. Kastern, W.H., and Berry, S.J. (1976). Biochem. Biophys. Res. Comm. 71, 37-44.
23. Hankins, W.D., and Farkas, W.R. (1973). Biochim. Biophys. Acta 213, 77-89.
24. Farkas, W.R. and Singh, R.D. (1973). J. Biol. Chem. 248, 7780-7785.
25. Farkas, W.R., Hankins, W.D., and Singh, R. (1973). Biochim. Biophys. Acta. 294, 94-105.
26. Farkas, W.R., and Charnoff, D. (1976). Nucleic Acid Res., 3, 2521-2529.
27. Moore, G.P.M. (1974). J. Embryol. Exp. Morph. 34, 291-298.
28. Griffin, B. (1976). Nature 263, 188-190.
29. VanBlerkom, J., and Brockway, G.O. (1975). Dev. Biol. 44, 148-157.
30. Cited by McLaren, A., (1976). Ann. Rev. Genet. 10, 361-388.
31. Hickey, E.D., Weber, L.A., Baglioni, C. (1976). Nature 261, 71-73.
32. Surrey, S., and Nemer, M., (1976) Cell 9, 589-595.
33. Faust, M., Milward, S., Duchastel, A., and Fromson, D., (1976). Cell 9, 597-604.