

The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo

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SUMMARY

The 2-cell stage of mouse embryogenesis is characterized by two phases of α -amanitin-sensitive polypeptide synthetic activity, which appear to mark the first major expression of the embryonic genome, as assessed by examination of *in vitro* translates of mRNA. Using populations of embryos synchronized to the first cleavage division, we have established that DNA replication takes place over the period 1 to 5.5 h after the first cleavage division; the two bursts of putative transcription take place before and immediately after DNA replication, and the translation products are detectable in each case within 3–4 h. In addition, we have shown that suppression of cytokinesis and the second round of DNA replication does not affect synthesis of the α -amanitin-sensitive polypeptides, and that neither DNA replication nor the loss of maternal mRNA that take place during the 2-cell stage are dependent upon synthesis of the α -amanitin-sensitive polypeptides.

INTRODUCTION

Early development in embryos of most, if not all, species appears to take place largely or exclusively under the control of the maternal genome (Denny & Tyler, 1964; Brachet, Ficq & Tencer, 1968; Woodland, Flynn & Wyllie, 1979; Braude, Pelham, Flach & Lobatto, 1979; Rosenthal, Hunt & Ruderman, 1980; Woodland & Ballantine, 1980; Wells, Showman, Klein & Raff, 1981; Van Blerkom, 1981; Pratt, Bolton & Gudgeon, 1983) with the sequential activation and utilization of components synthesized and stored in the oocyte during oogenesis (Davidson, 1976). The duration of this period of exclusive maternal control varies in different species (Davidson, 1976; Johnson & Pratt, 1983). In the mouse embryo, the period of exclusive maternal regulation appears to extend from ovulation to cleavage to the early 2-cell stage (i.e. a period lasting 18 to 22 h; Pratt *et al.* 1983; reviewed by Johnson, 1981). Although limited synthesis of RNA on the DNA template of mouse 1-cell zygotes has been reported (Moore, 1975; Young, Sweeney & Bedford, 1978; Clegg & Pikó, 1982, 1983), this activity is apparently not involved in proximate development. Thus, physical enucleation, or treatment with the transcriptional inhibitor α -amanitin, do not appear to affect the normal sequence of morphological and molecular changes that take place during development to the early 2-cell stage (Braude *et al.* 1979; Petzoldt,

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Hoppe & Illmensee, 1980; Schultz *et al.* 1981; Van Blerkom, 1981; reviewed by Johnson, 1981). Indeed it has been demonstrated that these changes are explicable by sequential activation of subsets of mRNA (Braude *et al.* 1979; Cascio & Wassarman, 1982; Pratt *et al.* 1983) and by post translational modifications to polypeptides (Van Blerkom, 1981; Cascio & Wassarman, 1982; Pratt *et al.* 1983).

Several lines of investigation have indicated that the transition from maternal to embryonic control of development in the mouse embryo takes place during the 2-cell stage. Genetic evidence suggests that by the late 2-cell stage, the embryonic genome is active transcriptionally (Sawicki, Magnuson & Epstein, 1981; reviewed by McLaren, 1979; Magnuson & Epstein, 1981). Biochemical evidence shows that the first major incorporation of RNA precursors into heterogeneous RNA takes place during the 2-cell stage (Woodland & Graham, 1969; Knowland & Graham, 1972; Clegg & Pikó, 1977, 1982; Levey, Stull & Brinster, 1978; Pikó & Clegg, 1982) and coincides with major qualitative changes in the profile of polypeptides synthesized (Van Blerkom & Brockway, 1975; Cullen, Emigholz & Monahan, 1980; Levinson, Goodfellow, Vadeboncoeur & McDevitt, 1978; Howe & Solter, 1979). Furthermore, using both one- and two-dimensional characterization of polypeptide synthetic profiles, we have presented evidence previously that the first detectable polypeptide synthetic events that are sensitive to α -amanitin occur in two phases, at the early and mid 2-cell stages (Flach *et al.* 1982). The first α -amanitin-sensitive event is completed soon after the first cleavage division, and results in the synthesis of a complex of polypeptides of relative molecular mass (M_r) $\sim 67 \times 10^3$ within a few hours; the synthesis of these polypeptides has been confirmed in an independent study in which the polypeptides were identified as heat-shock proteins (Bensaude, Babinet, Morange & Jacob, 1983). The second event occurs later, from the mid 2-cell stage onwards, and results in many changes in the polypeptide synthetic profile of 2-cell embryos. We have suggested that these events might represent bursts of transcriptional activity occurring prior to and immediately after the second round of DNA replication (Luthardt & Donahue, 1975; Sawicki, Abramczuk & Blaton, 1978).

In our previous report, there was considerable heterogeneity of developmental staging among embryos of the same chronological age which arose due to variation in developmental rates up to the 2-cell stage. This heterogeneity made the precise timing of events impossible. Here we present data in which synchronized populations of 2-cell embryos of different ages have been examined for changes with time in their polypeptide synthetic profiles, DNA content, and sensitivity to α -amanitin. This approach has permitted the definition of the precise sequence of putative transcription, translation and DNA replication in the second cell cycle of mouse embryogenesis. In addition, we have investigated the influences of inhibitors of cell division, DNA replication and transcription on events taking place during the 2-cell stage in an attempt to establish whether these three parameters are related causally. We have demonstrated that the two bursts of putative transcriptional activity form a sandwich about DNA replication, and

that each burst is coupled tightly to utilization of the new transcripts. Furthermore, we have shown that neither the second round of DNA replication, nor the presumed activation of the embryonic genome and subsequent translation of embryonic transcripts, are dependent on the first round of cytokinesis. Moreover, activation of α -amanitin-sensitive polypeptide synthesis takes place in the absence of the second round of DNA replication.

MATERIALS AND METHODS

Preparation and culture of embryos

Female HC-CFLP (Hacking & Churchill Ltd) and F₁LAC (C57BL ♀ × CBA/Ca ♂, bred in the laboratory) mice aged 3–5 weeks were superovulated with intraperitoneal injections of 5 i.u. of PMS (Folligon), followed 48 h later with 5 i.u. hCG (Chorulon). For *in vivo* fertilization, the females were caged with HC-CFLP males and a vaginal plug taken as an indication of successful mating. Late 1-cell zygotes were flushed from the oviducts with phosphate-buffered medium 1 + 0.4 % (w/v) bovine serum albumin (PB1 + BSA; Whittingham & Wales, 1969) at 26 h post hCG (hphCG), collected and cultured in drops of medium 16 + 0.4 % (w/v) BSA (M16+BSA; Whittingham, 1971). All incubations were carried out under paraffin oil in Sterilin tissue culture dishes at 37 °C in an atmosphere of 5 % CO₂ in air.

For *in vitro* fertilization, spermatozoa were collected from the vas deferens and epididymidis of male HC-CFLP mice and incubated in 500 μ l drops of Whittingham's medium containing 3 % (w/v) BSA (W + 30; Fraser & Drury, 1975) for 1½ h. Cumulus masses were recovered from the oviducts of F₁LAC female mice at 12½ hphCG and placed in 1 ml drops of W + 30. Insemination was carried out at 13½ hphCG with a suitable volume of sperm suspension to give a final sperm concentration of (1–4) × 10⁶ ml⁻¹. Fertilized eggs were recovered after 4 h incubation and transferred to M16 + BSA for further culture.

Assay of nuclear DNA content by microdensitometry

Embryos were incubated for 15 min in Ca²⁺- and Mg²⁺-free Hank's medium (Gibco) at 37 °C, air dried onto clean microscope slides, fixed in a mixture of ethanol and acetic acid (3:1) for 5 min followed by ethanol, acetic acid and formaldehyde (85:5:10) for 1 h, dried and stored at -12 °C. Samples were stained using Schiff's basic stain for the Feulgen reaction: 5 N HCl for 55 min at 26 °C; double-distilled water for 5 min; freshly prepared and filtered Schiff's stain for 2 h in the dark; freshly prepared sulphurous acid (1 % potassium metabisulphite + 0.1 N HCl in a ratio of 1:1) for three washes of 10 min each; tap water rinse; dehydration in graded alcohols through to xylene. Samples were mounted in Depex and analysed within two weeks of staining using an integrating microdensitometer (Vickers Instruments Ltd) which determines the quantity of DNA

in nuclei by measuring the light absorption at a wavelength of 560 nm. A sample of mouse liver was fixed and stained simultaneously in all experiments to provide reference values for the absorption shown by nuclei containing 2C and 4C amounts of DNA.

RNA extraction and translation in vitro

Total RNA was extracted from 2-cell embryos and translated *in vitro* in the presence of [³⁵S]methionine in a message-dependent rabbit reticulocyte lysate exactly as described by Braude & Pelham (1979), except that the concentration of EDTA in the extraction buffer was reduced from 0.01 M to 0.001 M. 10 μ l samples of translation mixture were added to an equal volume of double-strength lysis buffer (O'Farrell, 1975) in preparation for resolution by two-dimensional SDS polyacrylamide gel electrophoresis.

One- or two-dimensional SDS polyacrylamide gel electrophoresis

Polypeptide synthetic profiles were analysed after one- or two-dimensional SDS polyacrylamide gel electrophoresis. Although a more detailed analysis is possible after two-dimensional electrophoretic separation, in experiments where multiple samples were to be compared, this approach was not practical. The two-dimensional electrophoretic mobility of the 67×10^3 complex of polypeptides has already been characterized in two independent studies (Flach *et al.* 1982; Bensaude *et al.* 1983), and that of the characteristic late 2-cell polypeptides has been described previously (Flach *et al.* 1982). Therefore, despite the inevitable loss of resolution that results from superimposition of polypeptides, the majority of the analyses were restricted to one-dimensional SDS polyacrylamide gel electrophoresis.

For one-dimensional separation, embryos were incubated for 1.5 or 3 h in 5 μ l [³⁵S]methionine (1000–1400 Ci/mMol, Amersham International Ltd) in 50 μ l of M16 + BSA. Equal numbers of embryos were rinsed three times with PB1 free of BSA and placed in 5 μ l double-strength SDS sample buffer (Laemmli, 1970). Samples were separated according to molecular weights on either linear gradient (7.5–15 %) or uniform (10 %) SDS polyacrylamide gels as described previously (Flach *et al.* 1982). For comparative analysis at $\frac{1}{2}$ h intervals, an equal number of embryos (usually 8–15) were harvested at each time point in any given experiment. Thus, differences in the relative intensities between tracks in any one gel will reflect either genuine incorporation differences at different time points, or random variations between embryos or in recovery losses at the various stages of processing of the embryonic polypeptides. Pilot experiments were carried out, in which the incorporation of label into protein in each sample was measured, and the amount of protein applied was adjusted such that for any one gel, incorporated counts applied were identical for each track. However, this did not produce any appreciable reduction in the minor variations in intensity due to random losses. Furthermore, since the application of labelled protein from

identical numbers of embryos gives additional information, this procedure was used throughout. In some cases, densitometric traces of gels were made, and the proportions of changing polypeptides assessed quantitatively.

For two-dimensional separation, [³⁵S]methionine-labelled polypeptides derived from *in vitro* translation were separated in the first dimension according to isoelectric points in cylindrical 4 % acrylamide gels, and in the second dimension according to molecular weights on uniform (10 %) SDS polyacrylamide gels (Braude & Pelham, 1979). After electrophoresis, gels were processed as described by Bonner & Laskey (1974) and exposed to preflashed Fuji RX X-ray film (Laskey & Mills, 1975) for fluorography at -70°C .

Pulse-chase experiments

The stock solution of [³⁵S]methionine (1000–1400 Ci/mMol) was diluted 1:10 with double-distilled water and lyophilized in aliquots of 5 μl in order to reduce the cytotoxic effects of the labelled precursor (MacQueen, 1979; Van Blerkom, 1981). To each lyophilized aliquot was added 50 μl M16 + BSA supplemented with 0.01 μM unlabelled methionine to further dilute the [³⁵S]methionine. Embryos were incubated in this medium for 1 h and either harvested immediately for one-dimensional SDS polyacrylamide gel electrophoresis as described in the previous section, or washed three times in warm, pre-equilibrated M16 + BSA containing unlabelled methionine, phenylalanine and leucine, each at a concentration of 100 μM , to facilitate rapid efflux of incorporated labelled methionine by exchange diffusion (Holmberg & Johnson, 1979; Kaye *et al.* 1982). Washed embryos were then cultured for a further period in fresh drops of the same medium. At the appropriate time, embryos were harvested for one-dimensional SDS polyacrylamide gel electrophoresis as described above. This procedure has been shown previously to result in detection of post-translational modifications to pre-existing polypeptides (Pratt *et al.* 1983).

Assay for putative transcription

Because of the low permeability of early embryos to radioactive precursors of RNA such as [³H]uridine (Clegg & Pikó, 1977), it is not possible to obtain reliable quantitative data on the earliest synthesis of mRNA. The timing of putative transcriptional activity was, therefore, assessed indirectly, by measuring the sensitivity of embryos to α -amanitin. While changes produced by the action of any drugs must be interpreted with caution due to possible non-specific side effects (discussed at length in Flach *et al.* 1982), α -amanitin does show selective effects on RNA polymerase II, and therefore on mRNA synthesis, for the first few hours of treatment in a variety of eukaryotic systems (Kedinger *et al.* 1970; Lindell *et al.* 1970; Hadjiolov, Davera & Mackedowski, 1974) as well as in the mouse embryo (Levey & Brinster, 1978). However, the possibility cannot be excluded that other, as yet undescribed, side effects of α -amanitin could explain our observations, such as effects on splicing of selected species of hnRNA, or cell

cycle variation in α -amanitin uptake or access to RNA polymerase II. We therefore refer throughout to events that are inhibited by α -amanitin as 'putative transcriptional' events.

RESULTS

1. Collection of timed 2-cell embryos

Variation in the time of division of 1-cell zygotes to yield 2-cell embryos is considerable after *in vivo* fertilization followed by embryo recovery at 26 hphCG and culture in M16 + BSA (Fig. 1). This variation may be reduced by *in vitro* fertilization, especially if the source of the spermatozoa is limited to the cauda epididymidis (Fig. 1). This observation confirms previous reports that there is considerable variation in times of ovulation (in hphCG) and in the interval between coitus and fertilization (Edwards & Gates, 1959; Krzanowska, 1964; Nicol & McLaren, 1974). In order to reduce asynchrony further, 1-cell zygotes (derived either by *in vivo* or *in vitro* fertilization) were cultured, and examined at half-hour intervals. Any 2-cell embryos formed within the previous half hour were picked off and cultured separately. Embryos at this stage of development proved to be particularly sensitive to low temperature or pH variation. All manoeuvres were therefore performed with small numbers of embryos, on a heated stage with pre-equilibrated, warm medium under oil.

In experiments using fertilization *in vitro*, embryonic development was timed in terms of either hours post-insemination (hpi) or hours post pick-off after division to the 2-cell stage (hppo) (which occurs over the period 16–20 hpi). For fertilization *in vivo*, since the times of ovulation, insemination and fertilization

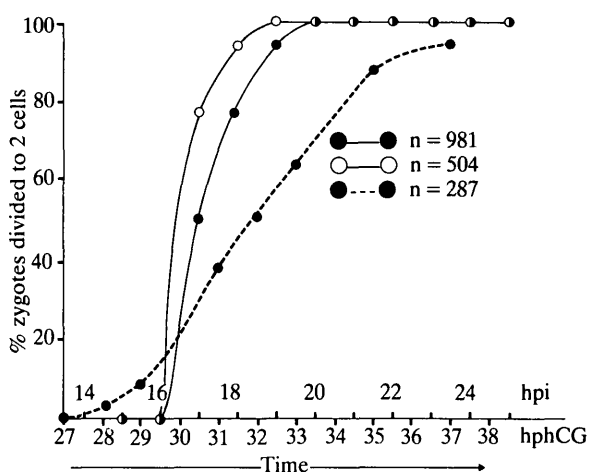


Fig. 1. Timing of first cleavage division of mouse embryos after fertilization *in vivo* (●---●); *in vitro* with sperm from both vas deferens and epididymus (●—●); *in vitro* with sperm from epididymus only (○—○). Time axis is expressed in hours post hCG (hphCG) and hours post insemination (hpi). n = number of embryos scored.

vary considerably, it is not possible to express time in hpi. Development was therefore timed in hours after the injection of hCG (hphCG) (which induces ovulation between 11–13 hphCG), or in hppo after division to the 2-cell stage (which takes place 27–35 hphCG).

2. Timing of changes in polypeptide synthesis

At timed intervals after division to the 2-cell stage, groups of *in vitro* fertilized embryos were placed in [³⁵S]methionine for 1.5 h, harvested and the labelled polypeptides synthesized by each group of embryos were resolved by one-dimensional SDS gel electrophoresis (Figs 2 & 3). The synthesis of the relative

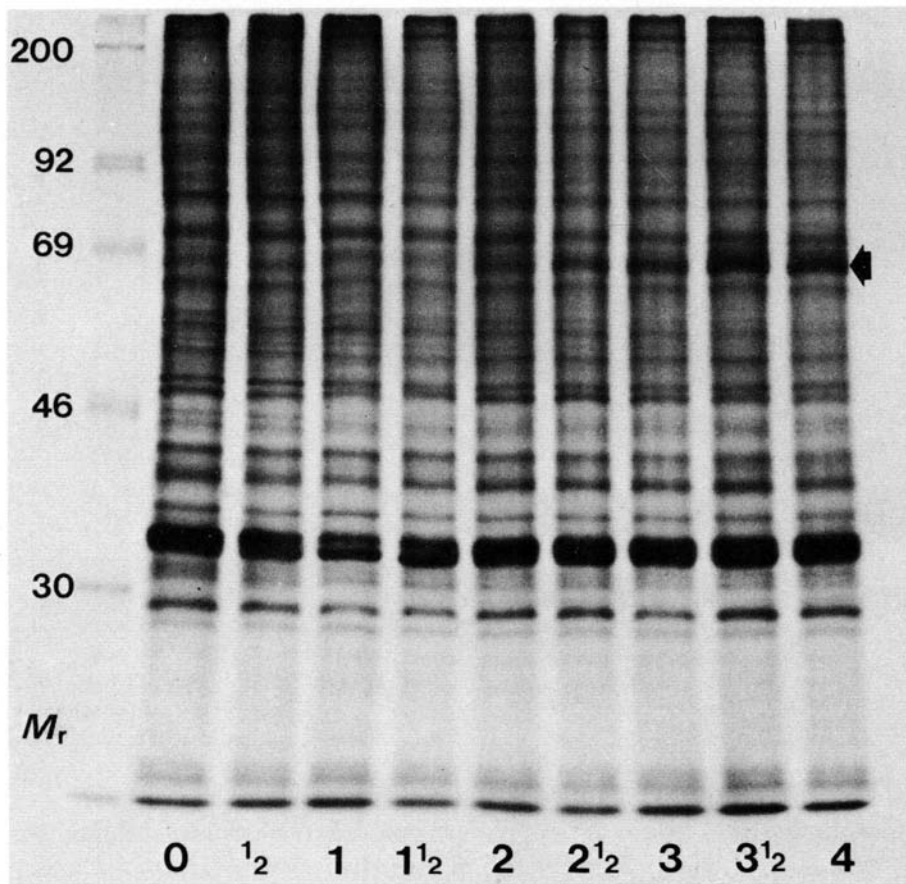


Fig. 2. One-dimensional SDS PAGE separation of radiolabelled polypeptides: M_r relative molecular mass markers: 200, 92, 69, 46 and 30 ($\times 10^3$); embryos cultured in M16 + BSA and labelled: (0) 0–1½ hppo; (½) ½–2 hppo; (1) 1–2½ hppo; (1½) 1½–3 hppo; (2) 2–3½ hppo; (2½) 2½–4 hppo; (3) 3–4½ hppo; (3½) 3½–5 hppo; (4) 4–5½ hppo (hppo – hours after 2-cells picked off having divided from 1-cell within previous ½ h). Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides. Densitometric traces of this region show an increase in synthesis of 67×10^3 polypeptides from 0.46% to 3.2% of total polypeptide synthesis between tracks (½) and (4).

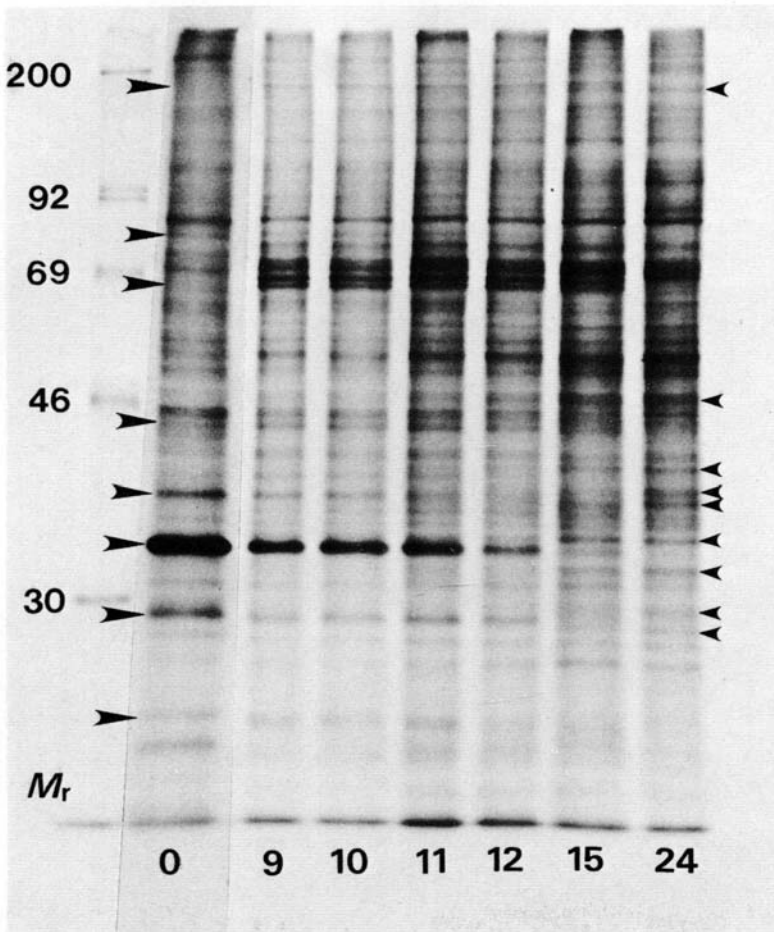


Fig. 3. One-dimensional SDS PAGE separation of radiolabelled polypeptides: M_r relative molecular mass markers as for Fig. 2; embryos cultured in M16 + BSA and labelled (0) 0–3 hppo; (9) 9–10½ hppo; (10) 10–11½ hppo; (11) 11–12½ hppo; (12) 12–13½ hppo; (15) 15–16½ hppo; (24) 24–27 hppo. Details as for Fig. 2. Large arrowheads indicate positions of representative early 2-cell polypeptides whose synthesis ceases during the 2-cell stage; small arrowheads indicate positions of representative late 2-cell polypeptides.

molecular mass 67×10^3 complex of polypeptides characteristic of the early 2-cell stage (Flach *et al.* 1982; Bensaude *et al.* 1983) was first detected in embryos placed in label for 1½ h at 2 hppo (Fig. 2). The earliest evidence of the synthesis of new polypeptides characteristic of the mid to late 2-cell stage was observed in embryos placed in label for 1½ h at 11 hppo (Fig. 3), although the majority of changes only became distinct when embryos were placed in label over the ensuing 1–3 h. Over the same period, a number of polypeptides that were synthesized by the 1-cell and early 2-cell embryo could no longer be detected, suggesting that their synthesis was reduced substantially.

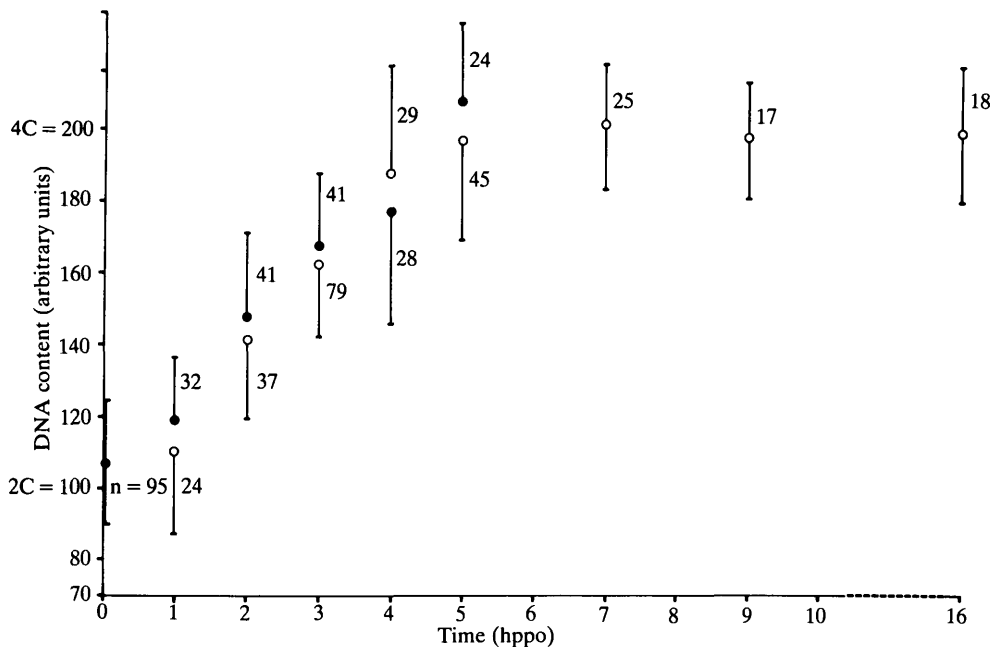


Fig. 4. Time course of DNA replication in nuclei of 2-cell mouse embryos cultured from late 1-cell stage in M16 + BSA (○) or M16 + BSA + 11 $\mu\text{g/ml}$ α -amanitin (●), as judged by microdensitometric analysis at $\lambda = 560 \text{ nm}$ after the Feulgen reaction. Each point represents the mean, and bars show the standard deviation, of several nuclei (number of nuclei per sample indicated beside each point).

3. Timing of DNA replication

In order to establish the time course of DNA replication after the first cleavage division, synchronized groups of 2-cell embryos derived by fertilization *in vitro* were fixed at hourly intervals after pick-off and stained with Schiff's basic stain for the Feulgen reaction. The DNA content of individual nuclei was analysed by microdensitometry, mouse liver cells providing 2C and 4C reference values. Since standard values vary between experiments, the results of separate experiments were normalized with respect to standard values of 2C = 100, 4C = 200. The time course of DNA replication in data pooled from three experiments is shown in Fig. 4 (open circles). DNA replication commences in embryos fixed at 1 hppo, and is completed in embryos fixed at 5 hppo.

4. Timing of α -amanitin sensitivity of polypeptide synthesis

2-cell embryos derived from fertilization *in vitro* were taken at various times post pick-off (hppo), placed in α -amanitin and cultured further for either 8 h or 27 h. For the last three hours of these culture periods, the embryos were incubated in [^{35}S]methionine. Control embryos, and embryos that were inseminated and cultured in the continuous presence of α -amanitin, were also incubated in [^{35}S]methionine over the same 3 h labelling periods. In the case of

embryos cultured for 27 h either with or without α -amanitin, the incidence of division to 4-cells at the end of the labelling period was recorded. The labelled polypeptides in each group of embryos were resolved by SDS polyacrylamide gel electrophoresis (Figs 5 & 6), and the appearance of the $M_r 67 \times 10^3$ complex of polypeptides recorded for the 8 h groups, and of the late 2-cell polypeptides recorded for the 27 h groups. Embryos placed in α -amanitin before 1 h post pick-off (1 hppo) failed to synthesize the 67×10^3 complex (Fig. 5), whereas embryos placed in α -amanitin at later times did. Embryos placed in α -amanitin prior to 8 hppo failed to synthesize late 2-cell polypeptides and synthesized only traces of

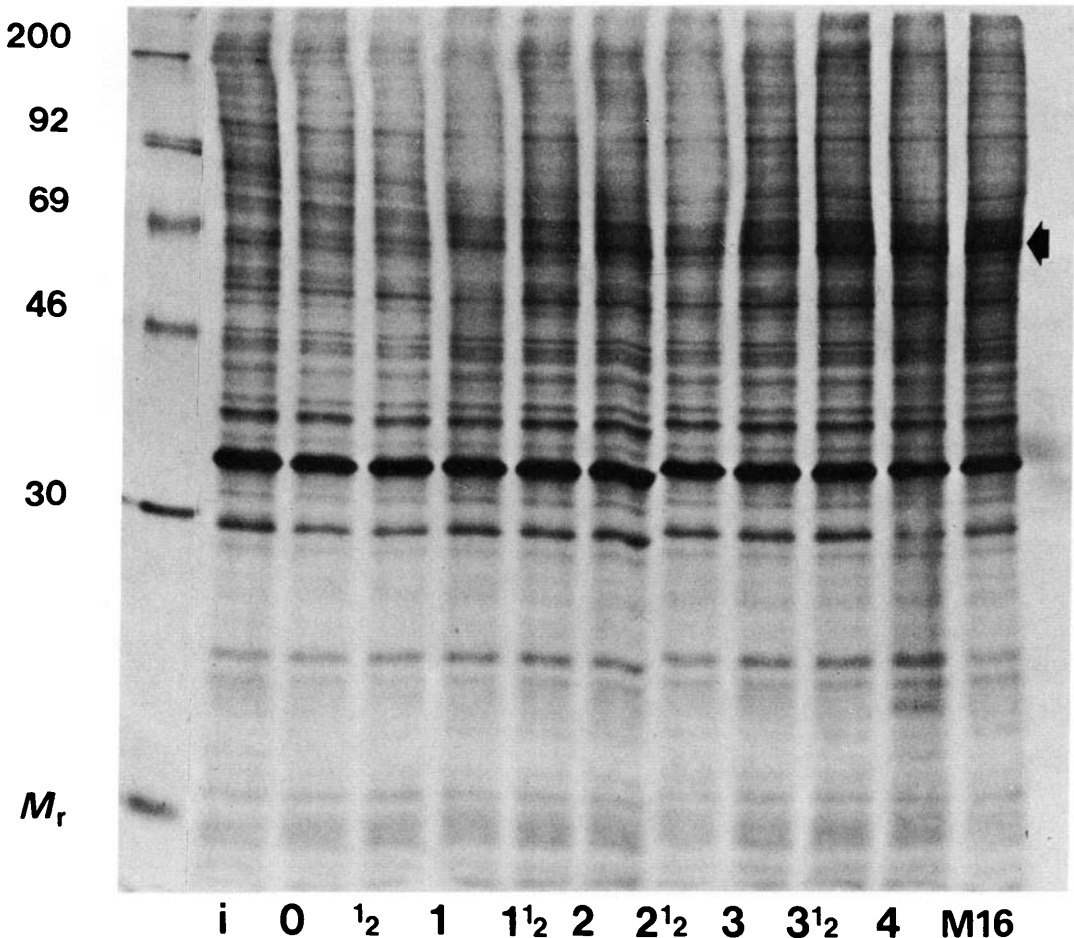


Fig. 5. One-dimensional SDS PAGE separation of radiolabelled polypeptides: (M_r) relative molecular mass markers as for Fig. 2; embryos cultured in M16 + BSA and transferred to M16 + BSA + $11 \mu\text{g/ml}$ α -amanitin at (i) 4 hpi; (0) 0 hppo; ($\frac{1}{2}$) $\frac{1}{2}$ hppo; (1) 1 hppo; ($1\frac{1}{2}$) $1\frac{1}{2}$ hppo; (2) 2 hppo; ($2\frac{1}{2}$) $2\frac{1}{2}$ hppo; (3) 3 hppo; ($3\frac{1}{2}$) $3\frac{1}{2}$ hppo; (4) 4 hppo; (M16) control embryos cultured in M16 + BSA without exposure to α -amanitin. All embryos were labelled 8–11 hppo. Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides. Densitometric traces of this region show an increase in synthesis of 67×10^3 polypeptides from 0.83 % to 5.6 % of total polypeptide synthesis between tracks (i) and (M16).

early 2-cell polypeptides (Fig. 6) and failed to cleave (Fig. 7, lower panel), whereas embryos placed in α -amanitin at 9 hppo or later cleaved (Fig. 7), ceased the synthesis of early 2-cell polypeptides and synthesized late 2-cell polypeptides (Fig. 6). From this we infer that an early α -amanitin-sensitive (putative transcriptional) event has occurred by 0.5–1 hppo, and a later event by 8–9 hppo.

In order to establish whether the $M_r 67 \times 10^3$ complex of polypeptides is synthesized *de novo* or as a result of α -amanitin-sensitive post-translational modification of presynthesized polypeptides after the first cleavage division, a pulse-chase experiment was undertaken. Late 1-cell embryos derived from eggs fertilized *in vivo* were taken at 28 hphCG and labelled for 1 h in diluted,

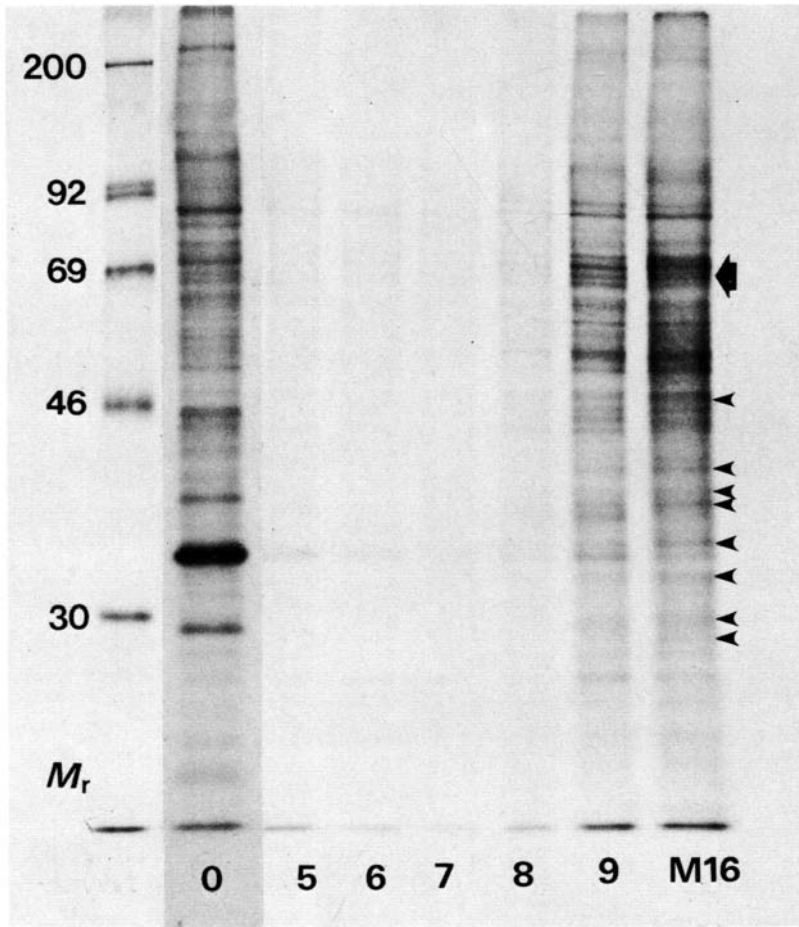


Fig. 6. One-dimensional SDS PAGE separation of radiolabelled polypeptides: (M_r) relative molecular mass markers as for Fig. 2; embryos cultured in M16 + BSA and (0) labelled 0–3 hppo; labelled 24–27 hppo after transfer to M16 + BSA + 11 $\mu\text{g/ml}$ α -amanitin at (5) 5 hppo; (6) 6 hppo; (7) 7 hppo; (8) 8 hppo; (9) 9 hppo; (M16) control embryos cultured in M16 + BSA and labelled 24–27 hppo without exposure to α -amanitin. Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides; arrowheads indicate positions of representative late 2-cell polypeptides.

lyophilized [³⁵S]methionine in M16 + BSA containing 0.01 μM unlabelled methionine. After the labelling period, one group of embryos was harvested immediately for SDS polyacrylamide gel electrophoresis, and the remaining embryos were washed and incubated for a further 8 h (to 36 hphCG) in M16 + BSA supplemented with unlabelled methionine, leucine and phenylalanine. Under these conditions, all the embryos cleaved to 2-cells, and the prolonged presence of [³⁵S]methionine produced no evidence of cytotoxicity. A final group of control mid 2-cell embryos were labelled for 1 h at 36 hphCG in diluted, lyophilized [³⁵S]methionine in M16 + BSA containing 0.01 μM unlabelled methionine. The labelled polypeptides synthesized by each group of embryos were

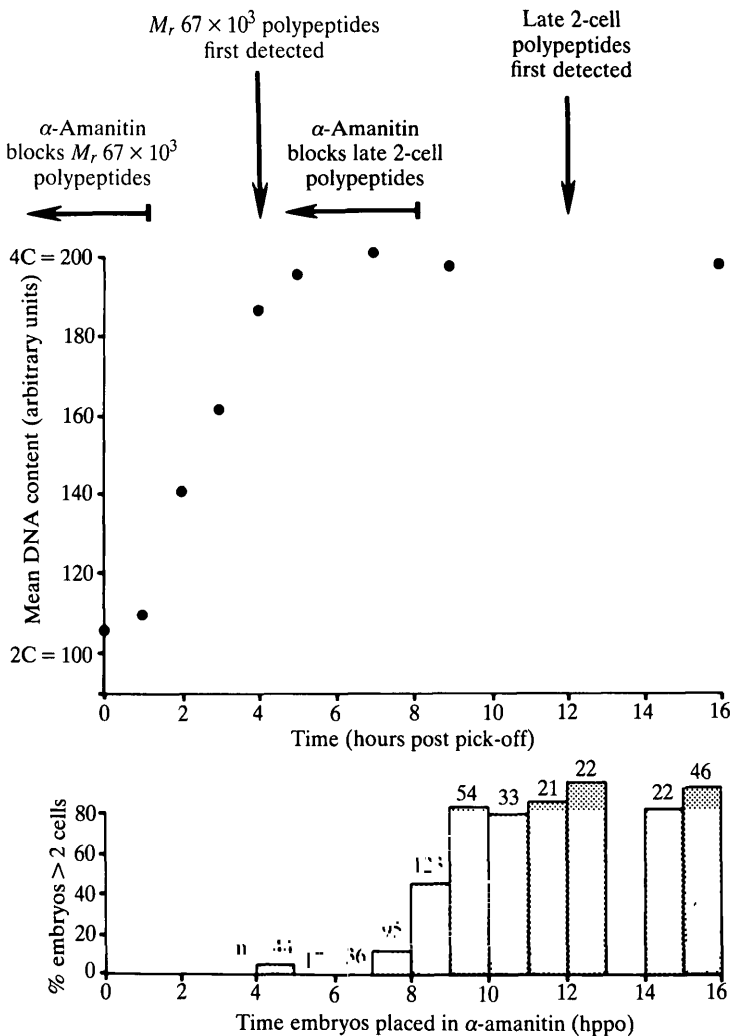


Fig. 7. Summary diagram illustrating temporal relationship between cell division (first cleavage division = time 0; second cleavage division = histogram: lower panel), DNA replication (solid dots); α -amanitin-sensitivity, and translational activity.

resolved by one dimensional SDS polyacrylamide gel electrophoresis (Fig. 8). Embryos that were labelled with a pulse of [^{35}S]methionine at the late 1-cell stage (28–29 hphCG) did not synthesize the 67×10^3 complex of polypeptides, whether they were harvested immediately or whether the label was 'chased' for a further 8 h (tracks b and d, Fig. 8), whereas embryos that were labelled at the mid 2-cell stage (36–37 hphCG) did synthesize these polypeptides (track c, Fig. 8). This result indicates that the $M_r 67 \times 10^3$ complex of polypeptides is not the product of post-translational modification to polypeptides synthesized at the late 1-cell stage.

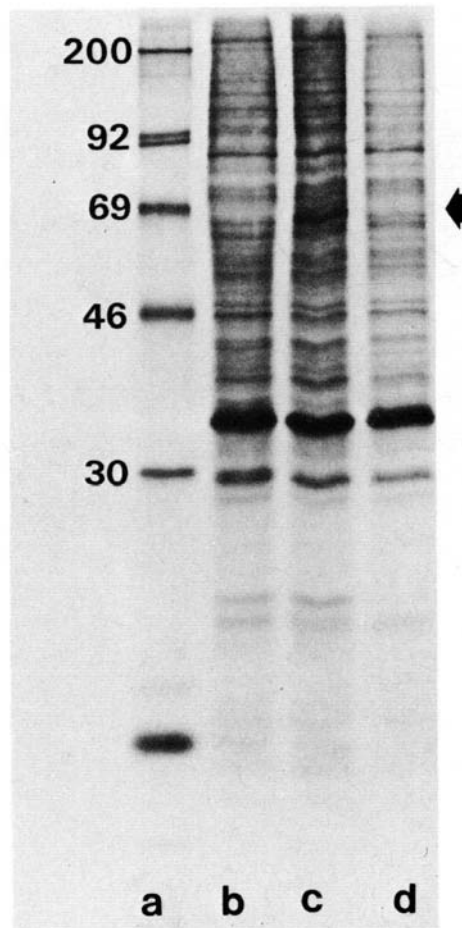


Fig. 8. One-dimensional SDS PAGE separation of radiolabelled polypeptides: (a) relative molecular mass markers as for Fig. 2; embryos labelled in M16 + BSA + $0.01 \mu\text{M}$ unlabelled methionine containing diluted, lyophilized stock [^{35}S]methionine: (b) late 1-cell embryos placed into label at 28 hphCG and harvested at 29 hphCG; (c) mid 2-cell embryos placed into label at 36 hphCG and harvested at 37 hphCG; (d) mid 2-cell embryos placed into label at the late 1-cell stage, washed at 29 hphCG and cultured in M16 + BSA containing $100 \mu\text{M}$ unlabelled methionine, phenylalanine and leucine, and harvested at 37 hphCG. Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides.

Finally, in order to demonstrate that the mRNA populations of 2-cell embryos do indeed change between the very early and the late 2-cell stages, and that this change is blocked in the presence of α -amanitin, mRNA was extracted from newly formed 2-cell and late 2-cell embryos (18 hpi and 38 hpi respectively) after fertilization *in vitro* and culture in M16 + BSA with or without α -amanitin. Following translation *in vitro* in a rabbit reticulocyte cell-free system in the presence of [35 S]methionine, the labelled polypeptides synthesized were resolved by one- and two-dimensional SDS polyacrylamide gel electrophoresis. The

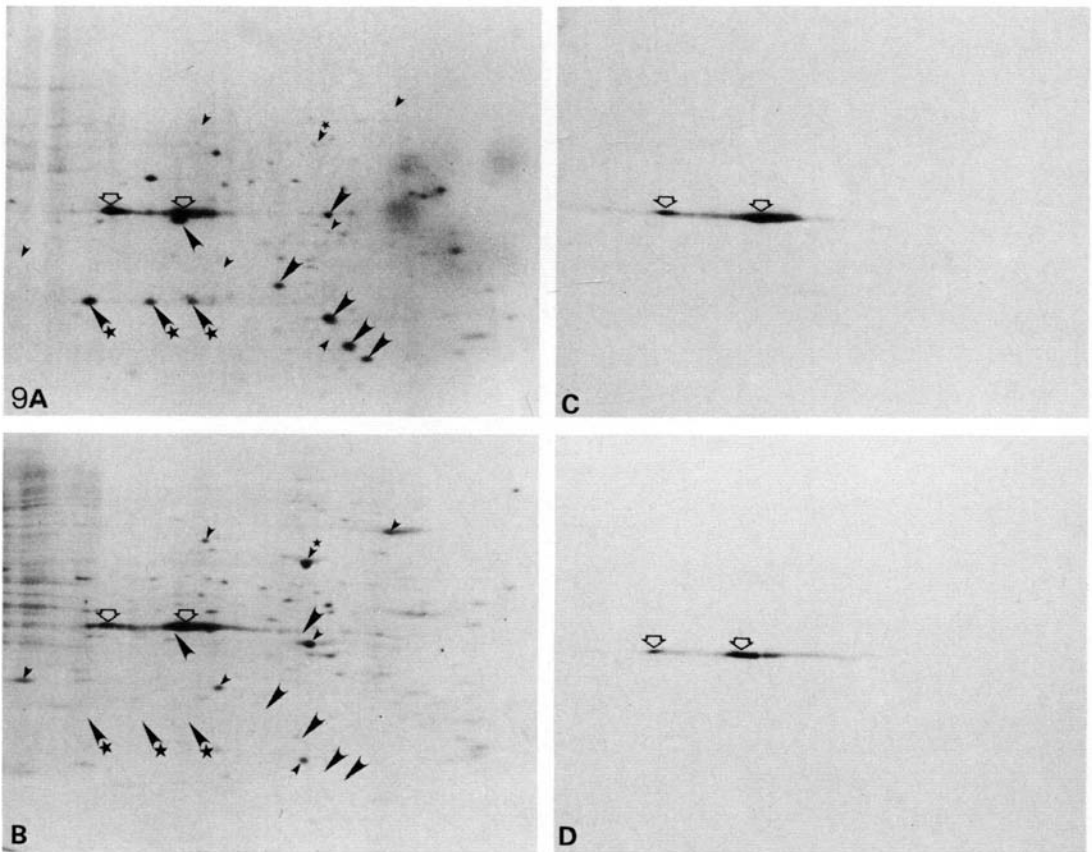


Fig. 9. Two-dimensional SDS PAGE separation of [35 S]methionine-labelled polypeptides translated *in vitro*: (A) on mRNA extracted from control newly formed 2-cell embryos at 18 hpi; (B) on mRNA extracted from control late 2-cell embryos at 38 hpi; (C) on mRNA extracted from late 2-cell embryos at 38 hpi after incubation in α -amanitin from 18 hpi; (D) by rabbit reticulocyte cell-free system independently of exogenous mRNA. Open arrows indicate mRNA-independent incorporation of [35 S]methionine (Braude & Pelham, 1979); larger arrowheads indicate representative early 2-cell polypeptides (asterisks indicate those whose synthesis is discussed in Pratt *et al.* 1983); small arrowheads indicate representative late 2-cell polypeptides (asterisk indicates putative $M, 67 \times 10^3$ polypeptide; see Flach *et al.* 1982). Isoelectric focussing is from left (pH 7.0) to right (pH 4.5).

results of the latter separation are shown in Fig. 9. The patterns of polypeptides translated *in vitro* from mRNA extracted from very early 2-cell and late 2-cell embryos are completely different (panels a and b, Fig. 9), and furthermore, no detectable polypeptides at all are translated *in vitro* from mRNA extracted from late 2-cell embryos that had been incubated in the presence of α -amanitin (panel c, Fig. 9). This result shows that the transformation in polypeptide synthetic profiles that occurs between the early and late 2-cell stages is accompanied by major changes in the populations of translatable mRNA, and that the gain of new transcripts, but not the loss of old transcripts, is blocked by α -amanitin.

5. Investigation of the causal relationships between cytokinesis, DNA replication and putative transcription

We first examined the effect of inhibition of the first round of cytokinesis on subsequent DNA replication and transcription. Late 1-cell embryos (from *in*

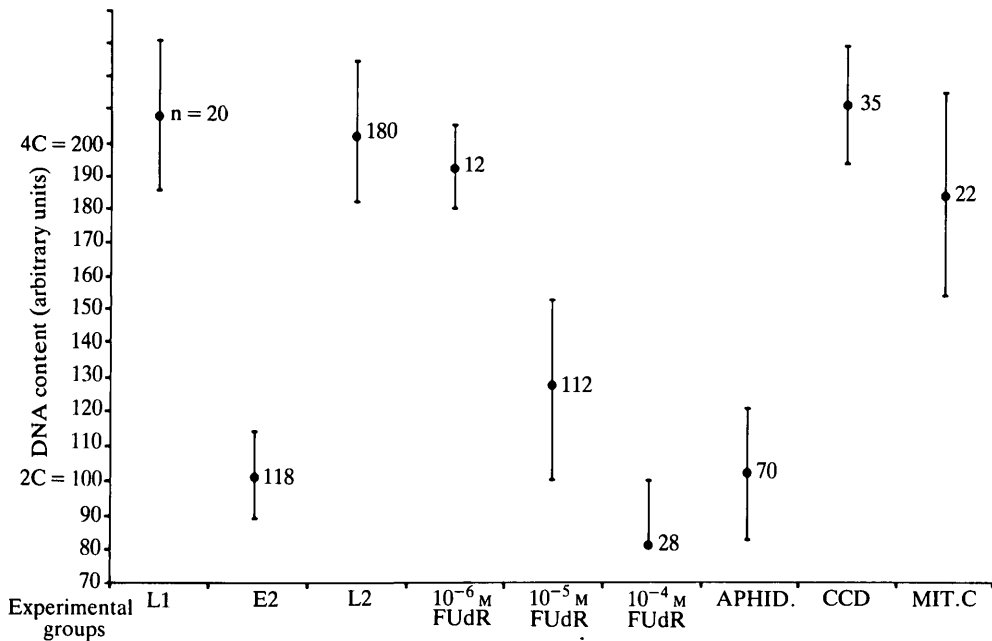


Fig. 10. DNA content of nuclei of embryos, determined by microdensitometric analysis after various culture conditions. Each point represents the mean, and the bars the standard deviation, of several nuclei (number of nuclei per sample marked by each point). L1 = late 1-cell embryos in M16 + BSA; E2 = early 2-cell embryos in M16 + BSA; L2 = late 2-cell embryos in M16 + BSA; 10⁻⁶, 10⁻⁵, 10⁻⁴M-FUdR = late 2-cell embryos cultured from the late 1-cell stage in M16 + BSA + the indicated concentration of 5-fluorodeoxyuridine; APHID. = late 2-cell embryos cultured from late 1-cell stage in M16 + BSA + 2 μ g/ml aphidicolin; CCD = embryos cultured from late 1-cell stage in M16 + BSA + 0.5 μ g/ml cytochalasin D until control embryos were at late 2-cell stage; MITC = late 2-cell embryos cultured from late 1-cell stage in M16 + BSA + 25 μ g/ml mitomycin C.

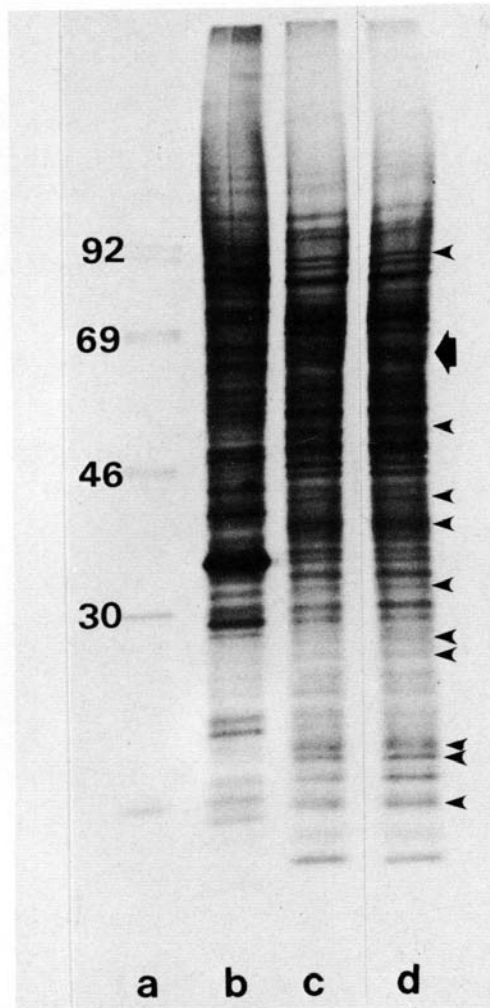


Fig. 11. One-dimensional gradient SDS PAGE separation of radiolabelled polypeptides: (a) relative molecular mass markers as for Fig. 2; (b) control late 1-cell embryos labelled 28–31 hphCG; (c) embryos cultured in M16 + BSA + 0.5 µg/ml CCD from 28 hphCG and labelled 48–51 hphCG; (d) control late 2-cell embryos cultured in M16 + BSA and labelled 48–51 hphCG. Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides; arrowheads indicate positions of representative late 2-cell polypeptides.

vivo fertilization; 28 hphCG) were placed in cytochalasin D (CCD) and cultured until control embryos had developed to the late 2-cell stage (51 hphCG), when the effect of the drug on cleavage was determined. Groups of embryos were either fixed and stained for microdensitometric analysis of DNA content, or placed in [³⁵S]methionine for 3 h, harvested, and the labelled polypeptides synthesized resolved by SDS polyacrylamide gel electrophoresis. Late 1-cell embryos placed in CCD failed to divide to 2-cells, but microdensitometric

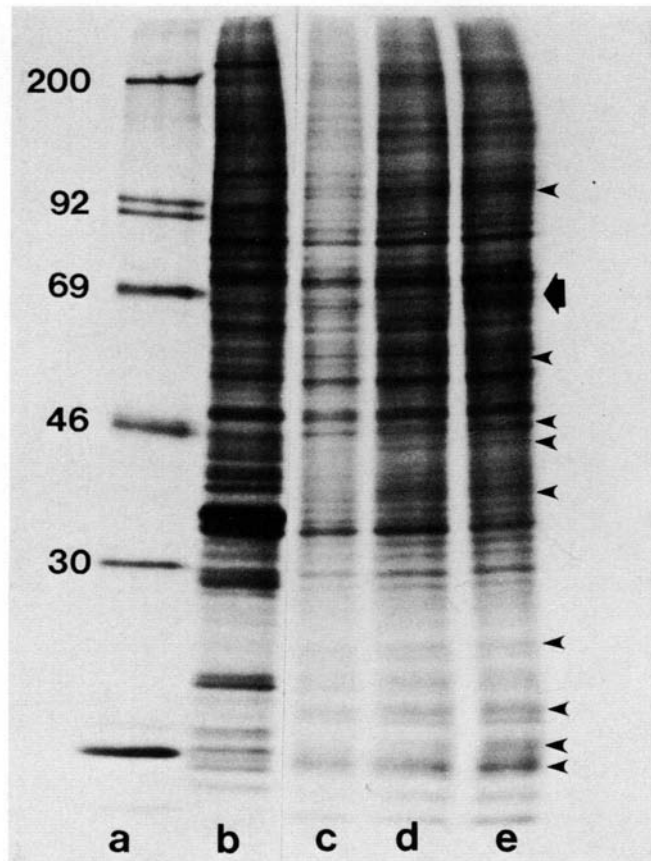


Fig. 12. One-dimensional gradient SDS PAGE separation of radiolabelled polypeptides: (a) relative molecular mass markers as for Fig. 2; (b) control early 2-cell embryos cultured in M16 + BSA and labelled 30–33 hphCG. Late 2-cell embryos labelled 51–54 hphCG after (c) culture in M16 + BSA + 2 $\mu\text{g}/\text{ml}$ aphidicolin from the late 1-cell stage (30–33 hphCG); (d) culture in M16 + BSA + 2 $\mu\text{g}/\text{ml}$ aphidicolin from the mid 2-cell stage (44 hphCG); (e) culture in M16 + BSA without exposure to aphidicolin. Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides; arrowheads indicate positions of representative late 2-cell polypeptides.

analysis revealed that the blocked 1-cell embryos were binucleate, and each nucleus had completed the second round of DNA replication (Fig. 10). Furthermore, analysis of one-dimensional gels indicates that CCD-treated embryos were synthesizing late 2-cell polypeptides (Fig. 11). From these results, we conclude that the first cleavage division is not a prerequisite for DNA replication, nor for the synthesis of α -amanitin-sensitive polypeptides.

We next attempted to establish whether the early burst of putative transcriptional activity is required for the round of DNA replication that follows it. Late 1-cell embryos (from *in vitro* fertilization; 18 hpi) were placed in α -amanitin and cultured until various times after cleavage to 2-cells. At hourly intervals after

pick-off, groups of embryos were fixed, stained and analysed by microdensitometry. Despite inhibition of synthesis of the $M_r 67 \times 10^3$ complex of polypeptides, the time course of DNA replication in α -amanitin-treated embryos was the same as in controls (Fig. 4; closed circles).

Finally, we examined the effect of inhibition of DNA replication on the synthesis of late 2-cell polypeptides. Three inhibitors of DNA replication were used: mitomycin C, aphidicolin and 5-fluorodeoxyuridine (FUdR; at three concentrations). None of the drugs produced any evidence of cytotoxicity at the doses and incubation periods used. Each drug was used with embryos derived

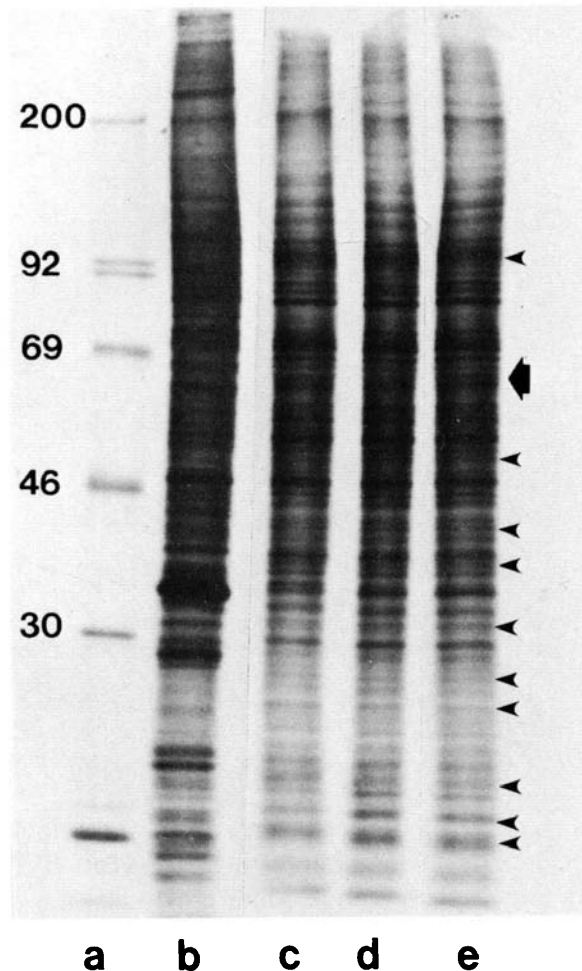


Fig. 13. One-dimensional gradient SDS PAGE separation of radiolabelled polypeptides: (a) relative molecular mass markers as for Fig. 2; (b) control late 1-cell/early 2-cell embryos cultured in M16 + BSA and labelled 30–33 hphCG; late 2-cell embryos labelled 51–54 hphCG after culture in (c) FUdR from the early 2-cell stage (30 hphCG); (d) M16 + BSA; (e) FUdR from the mid 2-cell stage (44 hphCG). Arrow indicates position of $M_r 67 \times 10^3$ complex of polypeptides; arrowheads indicate positions of representative late 2-cell polypeptides.

both from *in vitro* and from *in vivo* fertilization and, as the results were identical for each condition, they are pooled. The drugs were applied either to late 1-cell embryos (17–18 hpi; 30–33 hphCG), which then divided to yield 2-cell embryos, or to very early 2-cell embryos of the same ages in hpi or hphCG. Additionally, each drug was applied to mid 2-cell embryos after the period of DNA replication was completed, as judged directly in control embryos by microdensitometry (27 hpi or 44 hphCG). Embryos were analysed at the late 2-cell stage (38–41 hpi; 51–54 hphCG) for evidence of inhibition of DNA replication (Fig. 10) and for patterns of polypeptide synthesis (Figs 12 & 13).

Mitomycin C did not suppress DNA replication (Fig. 10). If added to late 1-cell or early 2-cell embryos, mitomycin reduced total incorporation of methionine into late 2-cell polypeptides and prevented the appearance of most of the new polypeptides, with only traces of early 2-cell polypeptides remaining (gels not shown). Addition of mitomycin to mid 2-cell embryos did not affect the synthesis of the late 2-cell polypeptides. Thus, mitomycin seems to interfere with the putative activation of transcription at the mid 2-cell stage but by a mechanism that does not depend upon inhibition of DNA replication.

Aphidicolin suppresses DNA replication (Fig. 10), but is almost without effect on the transition from an early to late 2-cell polypeptide pattern (Fig. 12). Maternally-coded early 2-cell polypeptides disappear and embryo-coded late 2-cell polypeptides appear. One or two minor differences are evident, and total incorporation seems to be reduced slightly, but the major changes in polypeptide synthetic pattern appear to be unaffected by the block to DNA replication.

5-fluorodeoxyuridine showed a dose-dependent effect on DNA replication (Fig. 10), but was without effect on the change in polypeptide synthetic profile at any dose (Fig. 13).

DISCUSSION

The use of highly synchronized populations of 2-cell embryos has permitted a more precise description of the events taking place during the 2-cell stage of mouse development than was possible previously (Flach *et al.* 1982). The temporal relationships established between the first cleavage division, the round of DNA replication that follows it, putative transcriptional activity (as assessed by α -amanitin-sensitivity) and the translation of the presumptive embryonic transcripts during the second cell cycle are summarized in Fig. 7. By 1 h after pick-off (1–1.5 h after cleavage) an α -amanitin-sensitive event has occurred, that results in the detectable synthesis of the M_r 67×10^3 complex of polypeptides within 3 h (3.5–4.0 h after cleavage). DNA replication commences 1 h after pick-off and is completed by 5 h after pick-off (extends between 1.0 and 5.5 h after cleavage). A second burst of α -amanitin-sensitive activity has occurred between 8 and 9 h after pick-off and results in new protein synthesis by 12.5 h after pick-off (12.5–13 h after cleavage). These results provide support for our earlier

suggestion that the two bursts of transcriptional activity in the 2-cell embryo might form a sandwich about DNA replication, and that each burst is coupled tightly to utilization of the transcripts (Flach *et al.* 1982). They also indicate the presence of a very short G₁ phase in the second cell cycle.

Our evidence on the detailed timing of putative transcriptional activity at the 2-cell stage rests on the use of α -amanitin. The assumption that α -amanitin sensitivity does indeed reflect transcriptional activity is reasonable on three grounds: (i) the sharpness of the temporal changes in response to the drug at both 1–2 hppo and 8–9 hppo suggests that non-specific toxic actions of the drug are unlikely to explain its effects; (ii) the sensitivity of the early (M_r 67×10^3) complex of polypeptides to α -amanitin is unlikely to be due to some post-translational modification that is sensitive to the drug, as shown by the pulse-chase experiment; (iii) the major change in mRNA populations that occurs between the newly formed 2-cell and late 2-cell stages includes not only mRNAs coding for polypeptides in the M_r 67×10^3 region, but also for many other new polypeptides. Moreover, the appearance of the mRNA species coding for all these new polypeptides is sensitive to α -amanitin. Taken together, and with the cautions outlined earlier in mind, these results suggest that our equation of α -amanitin-sensitivity with transcriptional activity is not unreasonable.

The establishment of a precise temporal sequence of molecular events over what appears to be the earliest period of large-scale gene expression in the mouse embryo, provides us with a basis for the study of the mechanism(s) by which gene activation might be controlled. We therefore attempted to establish whether there are any causal relationships between cytokinesis, DNA replication and gene expression. When the first cleavage division was inhibited by cytochalasin D, both the replication and putative transcription of DNA were unaffected. This result might be expected in light of previous demonstrations that the first to fifth rounds of cytokinesis are expressions of the developmental programme rather than prerequisites for that expression (Surani, Barton & Burling, 1980; Pratt, Chakraborty & Surani, 1981; Petzoldt, Burki, Illmensee & Illmensee, 1983). Similarly inhibition of the early burst of putative transcription by α -amanitin did not prevent DNA replication, which also might be expected, since the first evidence of translational effects resulting from it was not detected until after replication had commenced. In addition, α -amanitin did not affect the decline in synthesis of 1-cell and early 2-cell polypeptides observed at the mid to late 2-cell stage. This result confirms our previous observation (Flach *et al.* 1982) and extends it by showing that by the mid to late 2-cell stage the mRNA species which code for these early proteins, and which are presumably maternal in origin, are not only inactive *in situ*, but are not translatable *in vitro*, whether or not the embryos are cultured in the presence of α -amanitin. Thus, we can conclude that the whole of the first cell cycle, cleavage to the 2-cell stage, the second round of DNA replication and the loss of maternal mRNA are all regulated at a post-transcriptional level, independently of the expression of the embryonic genome.

Which, if any, of the elements of this maternally-regulated programme influence activation of the embryonic genome? We examined the influence of the second round of DNA replication on this event and found it to have surprisingly few immediate effects on development. A number of studies have suggested that the reprogramming of chromatin assumed to occur before new genes are expressed requires a round(s) of DNA replication, or that a critical number of cyclic nuclear events are required for the activation of transcription. Of the three inhibitors of replication examined, mitomycin C proved to be unsatisfactory. The time course of action of mitomycin C, and its critical active concentration, are rather variable, and the drug tends to produce delayed, drawn out and erratic effects on DNA which occur at the same time as side effects on RNA synthesis (Shatkin, Reich, Franklin & Tatum, 1962). Despite the application of high doses of the drug, DNA replication was affected only marginally and embryonic chromatin became disorganized only after extended exposure (unpublished data). In contrast, mitomycin suppressed totally the presumed *de novo* transcription from embryonic genes, although once activated the mRNAs produced were not obviously affected by the mitomycin. Therefore, the results with this drug were not illuminating.

In contrast, both FUdR (at 10^{-4} M) and aphidicolin reduced DNA replication to insignificant levels. FUdR is a thymidine analogue which inhibits DNA synthesis by rendering thymidine incorporation rate limiting (Cozzarelli, 1977). The relatively high concentration required for effective inhibition probably reflects the large endogenous nucleotide pool in embryos and the slow rate of uptake (Epstein & Daentl, 1971; Quinn & Wales, 1973; Clegg & Pikó, 1977). Aphidicolin inhibits DNA polymerase α which is responsible for most DNA replication (Ikegami *et al.* 1978).

Despite the inhibition of DNA replication produced by either drug, expression of the genes coding for the late 2-cell polypeptides was barely affected, the synthetic patterns being almost indistinguishable from control late 2-cell embryos in which replication had occurred. A few minor, variable differences were observed, and total incorporation seemed slightly less in aphidicolin-treated embryos compared with controls. It is possible that although both drugs inhibited DNA replication, a small but critical portion of the DNA required for expression of the late 2-cell genes might have escaped inhibition. However, there is no evidence to support a selective resistance of potentially active DNA sequences to replication inhibitors of very different types, and indeed even partial suppression of DNA replication in other systems has been associated with a failure of genomic reprogramming (Weintraub, 1975). It is not therefore possible to conclude from these data that the second round of DNA replication in the mouse embryo is required for the putative major gene activation that follows it.

Should this gene expression indeed prove to be independent of the preceding round of DNA replication, constraints are placed on the types of model that can explain gene activation. Such models fall into two general categories within the

broader concept of 'quantal cell cycles' (Dienstman & Holtzer, 1975). The first kind of quantal cycle is seen as a permissive process and the concept invokes specific interactions between the DNA or chromatin and cytoplasmic regulator substances which, after a critical number of nuclear cyclic events, allow transcription to proceed (Davidson & Britten, 1971). For example, the segregation and consequent dilution of cytoplasmic components during cleavage, with consecutive cycles of DNA replication causing an exponential increase in the relative nuclear DNA content, may permit eventual expression of the genome when the ratio of DNA:regulator substance(s) reaches a critical level. The development of acetylcholinesterase activity in ascidian embryos (Sato & Ikegami, 1971*a,b*) and the midblastula transition in *Xenopus* embryos (Newport & Kirschner, 1982) may be controlled in this way. Our results suggest that the mid 2-cell gene activation of the mouse embryo cannot be regulated in this manner.

The second kind of scheme for quantal cell cycles envisages a causal role for the cell cycle, whereby a specific, programmed change in DNA or chromatin is a requirement for gene expression. Transcriptionally active chromatin may be characterized by a number of features, and it has been suggested that these modifications allow gene expression by affecting the tightness of the packing of the chromatin, and thereby presumably the access of RNA polymerase. Several models have been proposed by which stable changes in chromatin might be produced, many of which require a round of DNA replication for their implementation. For example, chromatin modifications allowing gene expression that involve changes in methylation patterns (Razin & Riggs, 1980), nucleosomal organization (Weintraub, 1979; Allfrey, 1980) or histone structure or type (Borun, 1975) appear to require DNA replication in order to operate. Clearly, if such modifications are involved in the proposed mid 2-cell gene activation of the mouse embryo then either they must occur in the absence of DNA replication, or modification of one DNA strand at the first round of replication must be adequate for subsequent altered expression. We are currently investigating these alternatives.

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