Temporal and spatial patterns of the synthesis of tissue-specific polypeptides in the preimplantation mouse embryo

By A. H. HANDYSIDE and M. H. JOHNSON

From the Department of Anatomy, University of Cambridge

SUMMARY

Mouse embryos at different morphological stages in the transition from morula to blastocyst were analysed for the qualitative pattern of their polypeptide synthesis. The first appearance of the individual species of polypeptides specific to trophectoderm or ICM occurred progressively over this developmental period. Populations of inside cells recovered from morulae and early blastocysts synthesized those polypeptides previously shown to be characteristic of ICMs at 3½ days, but not those characteristic of trophectoderm.

INTRODUCTION

The first divergent differentiation amongst the cells of the preimplantation mouse embryo occurs with the formation of the trophectoderm and the inner cell mass (ICM). These two tissues taken from the 3½-day mouse blastocyst differ in their morphology and behaviour (Gardner & Rossant, 1976), their macromolecular synthetic activity (Van Blerkom, Barton & Johnson, 1976) and in their developmental capacity and fate (Gardner & Johnson, 1975; Rossant, 1977; Johnson, Handyside & Braude, 1977; Handyside, 1978). Whilst the restriction in developmental potential evidently occurs only shortly before full expansion of the blastocyst (Johnson et al., 1977; Handyside, 1978), several distinctive differentiative properties may be observed at earlier stages. For example, the labelling index and radiation sensitivity of cells in the morula evidently depend on the position of cells in relation to the external environment (Snow, 1973; Graham, 1973). Since it has also been proposed that cell position determines cell fate (Tarkowski & Wroblewska, 1967), the differentiation of cells by position may causally relate to their subsequent commitment.

The detection of a system of intrinsic markers of differentiation in the embryo would greatly facilitate study of the time course of the differentiative process. We have previously described the synthesis of tissue-specific polypeptides in the component tissues of the 3½-day blastocyst (Van Blerkom et al., 1976). We
report here on the timing of the first appearance of these polypeptides in the synthetic profile of the embryo, and on the spatial distribution of the cells producing the polypeptides.

**MATERIALS AND METHODS**

1. **Embryos**

Inbred mice carrying the αe gene (AgCam) and CFLP (outbred, Anglia Laboratory Animals) mice were used. Embryos were recovered from superovulated mice, mated 2–3½ days previously. Embryos of equivalent developmental stage were pooled. A sample of between five and ten compacted morula-stage embryos were disaggregated by pronase or Ca²⁺-free medium to obtain cell counts. The remainder of the morulae and all the blastocysts or cleavage-stage embryos were placed in 0.1 ml of protein-free medium 16 (Whittingham, 1971) containing 10 μl of [³⁵S]methionine (400–500 Ci/m mole, Radiochemical centre, Amersham) to a final methionine concentration of between 0.5 and 1 μC/ml as described previously (Van Blerkom et al., 1976). After 4 h, the morphology of the embryos was recorded as being (i) at compaction stage, (ii) morulae with no obvious fluid accumulation, (iii) morulae with cells accumulating fluid, (iv) small blastocoelic cavity present, and (v) full blastocoelic cavity present (see Handyside, 1978).

Some late morulae, early cavitating blastocysts and fully expanded blastocysts were immunosurgically treated in order to recover clusters of inside cells (ICs) or inner cell masses (ICMs) (Handyside & Barton, 1977; Handyside, 1978). The ICs and ICMs recovered in this way were incubated in [³⁵S]methionine as described above.

2. **Electrophoretic separation of polypeptides**

Groups of 15–30 embryos, ICs or ICMs were placed in lysis buffer containing 9.5 M urea, 2 % (w/v) NP40 detergent, 2 % ampholines (1.6 % pH range 5–7, and 0.4 % pH range 3.5–10, LKB) and 5 % mercaptoethanol. The samples were frozen and thawed three times to maximize disruption of cells with release of proteins into solution. One μl of each sample was mixed with 0.2 ml of a 50:50 mixture of 10 % TCA and 50 % ethanol, plus 15 μl of BSA stock solution (1 mg/ml) as carrier. After a minimum of 4 h at 4 °C, the precipitate was recovered, washed and counted. The remainder of the sample was applied to a cylindrical 4.0 % acrylamide gel (120 mm long; 6 mm diameter), containing urea, detergent and ampholines as above. The gel was placed to interconnect two tanks containing 0.01 M-NaOH and 0.01 M-H₃PO₄. All gels had been prerun in this system, after application of lysis buffer devoid of sample, for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. The lysis buffer was removed before addition of sample. The sample was then run for a total of 6000 volt hours, the last 1 h of which was always at 800 V (O'Farrell, 1976; Van Blerkom et al., 1976). The pH gradient established across the gel was found
Patterns of polypeptide synthesis in mouse embryos

to extend from 4.5 to 7.0, as judged by segmenting some gels into degassed distilled water, homogenizing the sample, and reading the pH of the homogenate.

Gels in which radioactive polypeptide samples had been separated by isoelectric focusing were then equilibrated with SDS buffer for 2 h, and embedded on the top of a slab (110 x 150 mm) acrylamide gel in a solution of agar in SDS buffer. The gel comprised a top layer (30 mm) of 5 % acrylamide on 110 mm of gradient acrylamide (7.5-15 %). Polypeptides were separated under a 15 mA constant current for 4 h. The separation of polypeptides according to molecular weight was calibrated by referring to a standard phage preparation as described previously (Van Blerkom & Brockway, 1975; Van Blerkom et al., 1976). The gels were then removed from between the glass plates, fixed in 30 % TCA, washed in 7 % acetic acid for 2 h, taken through three changes of DMSO, equilibrated for 2 h in 20 % PPO in DMSO, washed in water to precipitate the PPO, and then washed in 7 % acetic acid. Gels were dried onto card, and applied to preflashed Kodak RP54 film for fluorographic development at -70 °C (Laskey & Mills, 1975).

3. Analysis of films

Each species of radioactive polypeptide produced a distinct spot of grains on the film as revealed after development and fixation. In order to produce films with an approximately similar total exposure to radioactivity, the total amount of radioactivity applied to each first dimensional gel was calculated from the value obtained from the 1 µl precipitated sample. The exposure time was then adjusted so that the value of days exposure x counts per minute applied was approximately 10^7. Use of the preflashed film effectively converts the relationship between cpm and grain density to a linear function (Laskey & Mills, 1975). Since incorporation of labelled amino acids into protein rises rapidly as the morula transforms into a blastocyst, it was necessary either to apply greater numbers of morulae to the gel to achieve the same total counts applied, or to use longer exposure times for morulae. In practice, a combination of these approaches was used. Whilst this approach should give an approximate equivalence between films derived from different gels, the comparison clearly does not work in some cases and there are several possible reasons for this. First, for long exposure times of over 4 weeks it is necessary to take into account the half-life of the [35S]methionine. Secondly, the isoelectric focusing range used excludes basic polypeptides including histones. Thus, in comparisons between tissues with differing histone synthetic activity, the number of counts incorporated will not be equivalently reflected in the number of counts separated. Of relevance to this paper, early cleaving morulae seem to incorporate proportionately more methionine into excluded polypeptides than do blastocysts, since they consistently produce slightly fainter gels than would be predicted from values of incorporated counts. It is reasonable to suspect that this might
be due to histone synthesis. Thirdly, although experimental conditions are standardized, variation between experiments in the proportionate migration of non-excluded polypeptides into first and second dimensional gels may occur. Therefore, in the experiments described here, the predicted exposure time for the counts incorporated was calculated, the gels were exposed to film for a fraction of this time, a visual comparison of the films was made, and the gels were then re-exposed for the full calculated time, adjusted as a result of subjective inspection of sample film as necessary.

Comparison of films was made by selecting a standard reference film derived from a blastocyst, and comparing all other films to it. Polypeptide spots were classified as clearly detectable, not detectable, trace detectable or not scorable. For each category of embryo or part-embryo a minimum of four good films were examined. Scores for each polypeptide spot on each film are recorded in the tables. Three categories of seven polypeptides each were analysed. The first category (numbers 2, 3, 4, 5, 6, 13 and 16) were polypeptides limited to trophectoderm at 3½ days; the second (numbers 8, 9, 10, 11, 12, 14 and 15) were polypeptides limited to ICM at 3½ days; the third (numbers 17, 18, 19, 20, 21, 22 and 23) were polypeptides common to both ICM and trophectoderm at 3½ days. This numbering system follows that used previously (Johnson et al.) 1977; note that in that paper there were three errors; for T11 and T12 read I11 and I12, and transpose T5 and T6 in the table). The polypeptides selected as representative of each category are not the only polypeptides showing these features.

The two-dimensional electrophoretic system is essentially a qualitative system of separation. However, quantitative variation in the presence of certain species of polypeptides obviously occurs. Indeed, since the polypeptides being analysed may represent highly conserved, constitutive polypeptides (Johnson and Van Blerkom, unpublished), it is probable that synthesis of most if not all may occur in most cells regardless of phenotype, but that the proportionate synthesis of each will vary with phenotype. Furthermore, in cells or groups of cells spanning a differentiative transition, a quantitative increase in any given species of polypeptide may occur, and the homogeneity of its intensity in different electrophoretic separations of cells at equivalent developmental stages will be determined by the efficiency of staging criteria (see Discussion). Therefore, as long as scrupulous efforts are made to match films for similar intensity (see above) and as long as unclear spots are recorded as such, it is legitimate to expand a semiquantitative detectable/non-detectable scale into three categories of detectable, weakly detectable and non-detectable.

RESULTS

The polypeptide synthetic profile of the 3-day late morula is shown in Fig. 1. Twenty-one polypeptides are indicated as described in Materials and Methods.
Fig. 1. Fluorograph of [³⁵S]methionine-labelled polypeptides synthesized by a 3-day late morula with some fluid accumulating cells and separated by two-dimensional electrophoresis. Horizontal separation is by isoelectric focusing over the pH range indicated. Vertical separation is by SDS gradient acrylamide electrophoresis, giving separation by molecular weight (as indicated by 1, 5 and 10 x 10⁴ M.W.). Polypeptide species 2, 3, 4, 5, 6, 13 and 16 (position indicated by →) are limited to trophoblast at 3½ days as described previously by Van Blerkom et al. (1976); note that spot 6 (overlaid partly by a persistent spot to top right) and spot 13 are barely detectable, and that spot 4 is very weak; polypeptide species, 8, 9, 10, 11, 12, 14 and 15 (position indicated by ➡) are limited to ICM at 3½ days; note that spot 10 is barely detectable and that spot 9 is relatively weak; polypeptides 17, 18, 19, 20, 21, 22 and 23 (position indicated by ➤) are common to both ICM and trophoblast at 3½ days; note that spot 23 is barely detectable.

In Fig. 2, the time that each of these polypeptides is first detected is recorded in relation to the morphological development of the morula through to the blastocyst. It will be apparent (i) that none of the polypeptides are synthesized actively in the early compacting morula, and (ii) that the synthesis of different polypeptides is initiated at different times during the morphological transformation to the blastocyst.

Fig. 3. shows an electrophoretic separation of polypeptides from ICs of an
Fig. 2. Diagram to indicate relative synthesis at different developmental stages of the various tissue-specific polypeptides illustrated in Fig. 1. Each small square represents detectability of a single species of polypeptide on a single gel. The code number of the individual polypeptides are as in Fig. 1. Block T summarizes the detectability of trophectodermal polypeptides, block I summarizes detectability of ICM polypeptides and block C summarizes detectability of polypeptides common to both ICM and trophectoderm. The synthetic profile of polypeptides was analysed for five developmental stages: C = 8- to 12-cell cleavage stage; EM = 12- to 25-cell early morula; LM = 25- to 30-cell late morula with fluid-accumulating cells; ECB = early cavitating blastocyst with cavity forming in culture; FEB = fully expanded blastocyst. Empty squares = polypeptide not detectable; cross-hatched squares = polypeptide weakly detectable; solid squares = polypeptide clearly detectable; starred squares = polypeptide not scorable.

early expanding blastocyst, and Fig. 4 records the polypeptide synthetic profiles of ICs taken from late fluid-accumulating morulae and from early expanding blastocysts. It will be apparent that the ICs do not make all those polypeptides made by the equivalent-stage intact embryo. Rather, only those polypeptides common to both ICM and trophectoderm, and, with the exception of T5, those limited to ICM are synthesized.

DISCUSSION

These results indicate that a change in the polypeptide synthetic profile occurs as the embryo transforms from a cleavage state to a blastocyst. The profile shows a progressive change and presumably is a molecular correlate of, and may indeed underlie, the morphological changes. The progressive nature of this change in profile is shown both by polypeptides eventually limited to one or other of the constituent tissues of the blastocyst, and by polypeptides synthesized by both tissues. Thus, the polypeptides previously described as specific for the component tissues of the blastocyst are evident prior to the overt appearance of two distinct tissues. Some variation between gels of different embryos at equivalent developmental stages is apparent. This variation may arise from slight stage mismatching over a period of relatively rapid changes. It is also possible that an inappropriate time-base was used to stage embryos. For example, cell numbers or absolute time could be used. Cell number assessment would be difficult if embryos were also to be used for studies on electrophoretic
Fig. 3. Fluorograph, details as for Fig. 1, but of polypeptides synthesized by ICs isolated from an early cavitating blastocyst with small blastocoelic cavity. Trophoectodermal polypeptides – note the absence of all these spots except for numbers 5 and 16 where weak traces are detectable. ICM polypeptides – note that all ICM spots are clearly detectable. Common polypeptides – note that all common spots are clearly detectable.

Fig. 4. Diagram, details as for Fig. 2, to indicate relative synthesis by inside cells isolated from embryos of different ages of the various tissue-specific polypeptides illustrated in Fig. 1. Inside cells were isolated from late morulae with fluid accumulating cells (LM) or from early cavitating blastocysts (ECB).
separation of synthesized polypeptides. Embryos of identical age in absolute
time show considerable range in morphology, in total incorporation of counts
and in electrophoretic profile (Johnson et al., 1977). Thus, a morphological
staging seems to be the best criterion to use over this period of rapid develop-
mental change. The important point to emerge from the data is that a clear
developmental trend in polypeptide synthetic activity is evident.

The results of the immunosurgical experiments make it highly probable that
the tissue-specific polypeptides are synthesized in spatially distinct cells over
this developmental period. Those polypeptides eventually to be limited to the
ICM are synthesized by inside cells. Those eventually to be limited to the
trophectoderm, with the exception of T5, are not. Presumably synthesis of T5
by inside cells must cease during blastocyst expansion, but a more precise timing
of this 'switch-off' is required. In the absence of data for isolated populations
of outside cells, it is not possible to say with absolute confidence that there is
a spatial difference in the synthetic activity of the cells of the morula. Such
a conclusion seems to be likely, however, at least for the trophodermal
polypeptides. This conclusion also strongly supports the notion that cell fate
and cell position are related at this stage.

The evidence presented above suggests that inside cells are differentiated
from outside cells at an early developmental stage. Similar conclusions have
been reached from examinations of labelling indices, radiation sensitivity, and
Thus, the differentiative divergence leading to the distinctive tissues of the 3½-day
mouse blastocyst may be initiated with the first appearance of inside cells. In
contrast, the commitment of inside cells to an ICM fate occurs later (Johnson

Polypeptide synthesis and/or post-translational modification occur in the
cytoplasm, and it is not yet clear which of the temporally and spatially defined
changes in polypeptide synthetic profile described above are dependent on
changing genetic instruction and which on reorganization of pre-existing
cytoplasmic information. Transcription of new embryonic genetic information
and its utilization to form protein is occurring in embryos of these stages, as has
been demonstrated by use of antigenic and enzymic markers (Muggleton-Harris
& Johnson, 1976; Wudl & Chapman, 1976). However, the ability to differentiate
between two cell populations in the morula on the basis of polypeptide profiles
does not require any assumption of differential gene expression or inactivation,
since positional-sensitive activation of cytoplasmic message could yield the
same result. We are presently investigating these alternatives.

We wish to thank Debbie Eager, Sheila Barton and Gin Flach for their technical help,
and Dr H. Pratt, Dr P. Braude, Dr J. Rossant and Dr M. Edidin for their helpful discussion.
The work was supported by an M.R.C. grant to M. H. J., a Wellcome grant to A. H. H., and
a grant from the Ford Foundation to Professor C. R. Austin.
Patterns of polypeptide synthesis in mouse embryos

REFERENCES


(Received 27 August 1977, revised 24 October 1977)