The effect of the nucleocytoplasmic ratio on protein synthesis and expression of a stage-specific antigen in early cleaving mouse embryos

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Summary

The nucleocytoplasmic ratio of fertilized mouse eggs was manipulated by removing or injecting cytoplasm by micropipette, and bisection of denuded eggs to obtain both pronuclei in one half of the eggs cytoplasm. The experimental eggs were capable of cleavage to the morula stage and, in some instances, developed to the blastocyst stage similar to unmanipulated eggs. The removal of large quantities of cytoplasm by micropipette and injecting them into a recipient egg did not provide sufficient numbers of viable eggs, whereas transfer of smaller quantities (about a quarter of the cytoplasm) was less deleterious, at least for recipient eggs. However, the alteration of the nucleocytoplasmic ratio by this method was not of the correct magnitude for the purpose of this experiment. Therefore, bisection was the preferred method whereby the nucleocytoplasmic ratio was doubled. This resulted in both pronuclei residing in one half of the egg's cytoplasm. Half eggs with one pronucleus (haploid) but retaining a nucleocytoplasmic ratio similar to unmanipulated control eggs served as additional controls for the bisection experiments. Protein synthesis was analysed by two-dimensional gel electrophoresis, showing that the 2-cell- and 4-cell-stage bisected embryos with double and normal nucleocytoplasmic ratio expressed equivalent protein synthesis patterns as control embryos of the same stage. Likewise, the stage-specific surface antigen SSEA-1 did not appear before the 6- to 8-cell stage. Also in cytoplasm transfer experiments, there was no indication that altering the nucleocytoplasmic ratio in either direction changed the timing of stage-specific gene expression. These results support the idea that stage-specific gene activity during early mouse cleavage might proceed in parallel to DNA replication cycles and is independent of the nucleocytoplasmic ratio.

Key words: cleaving mouse embryos, nucleocytoplasmic ratio, bisection, cytoplasmic transfer, protein synthesis, SSEA-1.

Introduction

In many non-mammalian species, cleavage divisions are very fast, consisting mainly of the S- and M-phase only. During this period the egg's cytoplasm is distributed to the resulting blastomeres until the nucleocytoplasmic ratio of somatic cells is reached. However, in early mouse development, the cell cycle time following the first cleavage division is about 10 h (Bowman & McLaren, 1970), and G_1- and G_2-phases of varying lengths are observed (see Smith & Johnson, 1986 for review). Although transcription and translation start early in mouse development and

increase considerably during cleavage (see Magnuson & Epstein, 1981 for review), there is no cell growth (Abramczuk & Sawicki, 1974; Lehtonen, 1980) and the protein content of embryos does not increase before the late blastocyst stage (Brinster, 1967; Schiffner & Spielmann, 1976). Therefore, the nucleocytoplasmic ratio gradually increases with every DNA replication step, reaching the somatic value at approximately the 120-cell stage (Surani, cited from Smith & Johnson, 1986).

In *Xenopus laevis* embryos it was shown that the achievement of a critical nucleocytoplasmic ratio or DNA/cytoplasmic ratio was required for the event of

midblastula transition and the activation of the embryonic genome (Newport & Kirschner, 1982*a,b*). The direct influence of the nucleocytoplasmic ratio on stage-specific gene expression during early mouse development has not been clearly analysed due to several experimental factors. The classical constriction experiments (Spemann, 1928) applied by Newport & Kirschner (1982*a,b*) to the *Xenopus* egg, cannot be utilized due to the small size of the mouse egg. Gross alterations in the DNA/cytoplasmic ratio by the injection of DNA would require the injection of a large volume of liquid and might result in early developmental arrest (Wirak, Chalifour, Wassarman, Muller, Hassell & de Pamphilis, 1985).

Therefore, we decided to alter the nucleocytoplasmic ratio by two methods. In one experiment we removed cytoplasm by micropipette from one egg and injected it to another, an operation which had been successful in overcoming the '*in vitro* two-cell block' in eggs of certain mouse strains (Muggleton-Harris, Whittingham & Wilson, 1982). The advantage of this technique is that only the amount of cytoplasm is altered without quantitatively changing the membrane. The second method we used was bisection of fertilized eggs (Tarkowski, 1977; Barton & Surani, 1983) leaving both pronuclei in one half of the egg or forming haploid eggs. This technique requires removal of cytoplasm as well as membrane components to achieve the required nucleocytoplasmic ratio.

The results presented in this paper compare protein synthesis and stage-specific surface antigen expression in experimental eggs that have had their nucleocytoplasmic ratio manipulated with control eggs at similar stages of development.

Materials and methods

Collection and culture of eggs

For the protein synthesis experiments, fertilized eggs were obtained from F_1 hybrid females (C57BL/6×CBA/H). They were superovulated with 7·5 i.u. of pregnant mare serum gonadotropin (PMSG: Organon) and 5 i.u. human chorionic gonadotropin (HCG: Organon) given 45 to 48 h apart, injecting the HCG usually at 14.00 h. The females were mated with F_1 males (plug date = day 1 of gestation). For the immunofluorescence experiments C57BL/6-JHAN females were naturally mated with males of the same strain.

For nucleocytoplasmic ratio alteration by bisection fertilized eggs were collected around noon of day 1, freed from the cumulus cells by treatment with hyaluronidase (Sigma, 150 i.u. ml^{-1}) and washed several times through Hepes-buffered mouse embryo culture medium M2 (Fulton & Whittingham, 1978). Zonae pellucidae were removed by treatment with pronase (Calbiochem, 5 mg ml^{-1}) in M2 with polyvinylpyrrolidone but without bovine serum albumin. The eggs were washed again several times in M2 and transferred into M16 (Whittingham, 1971) for recovery

from the pronase treatment for at least 2 h. Culture was performed in plastic dishes (Falcon or Sterilin) in droplets of medium 16 under paraffin oil (BDH or Fisher Scientific) at 37°C in an atmosphere of 5% CO_2 in air or an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Cytoplasmic micromanipulation

The method for cytoplasmic removal and injection followed that described in a previous publication (Muggleton-Harris *et al.* 1982). Batches of ten eggs were placed in M2 plus cytochalasin D at 0·5 μg ml^{-1} for 15 min at 37°C prior to the manipulation. The experimental eggs were manipulated approximately 23–26 h post HCG. The experimental and control eggs were placed in small drops of M2 plus cytochalasin in a 60 mm plastic Petri dish with a glass coverslip insert. The drops were overlaid with paraffin oil. A Leitz Diavert inverted-phase microscope, and De Fonbrune micromanipulators with Agla syringes were used for all micromanipulations. Control eggs for the manipulative procedure were processed through M2 plus cytochalasin but were not manipulated. After manipulation the eggs were washed and transferred to M16 under oil for further development.

Attempts were made to remove and inject half of the egg cytoplasm but the survival rate of the operated eggs was extremely low. Therefore, approximately one quarter of the donor egg's cytoplasm was removed and injected into the recipient egg. The amount of cytoplasm was not calculated in a precise fashion but the same pipette size allowed an approximation of the amount injected. Such eggs from which cytoplasm had been removed (increased nucleocytoplasmic ratio; Fig. 1, lane C) and eggs that had received cytoplasm (reduced nucleocytoplasmic ratio; Fig. 1, lane D) were capable of cleaving and developing to the blastocyst stage. However, there was an unexpected degree of developmental failure, especially in eggs from which cytoplasm had been removed. Thus the method of bisection was used for all subsequent experiments.

Bisection of eggs

Bisection of fertilized eggs was performed in the afternoon of day 1. The methods of Tarkowski (1977) and Barton & Surani (1983) were modified in the following ways. Operations were performed in M2 over 1% Agarose (dissolved in PBS-A and incubated with M2) at about 5°C or at room temperature. Hand-pulled flame-polished micropipettes were used for deformation of the eggs into a cylindrical shape (Fig. 2A). Then the eggs were bisected by hand with a fine-drawn glass needle under a Wild dissecting microscope. The plane of dissection was chosen in such a way that either one pronucleus ended up in each half of the egg or one part of the egg contained both pronuclei and an enucleate cytoplasmic fragment was left (Fig. 1). Control eggs without a zona pellucida were kept with experimental eggs over agar for a period equivalent to the manipulation time. After the operation the bisected eggs were transferred to M16 and cultured individually in drops of M16 for up to 3 days. The eggs developed to the 2-cell stage by day 2, the 4-cell stage (or 4- to 8-cell stage) by day 3 and compacted at day 4 (Fig. 1). The majority of eggs containing one pronucleus retain a haploid genome during further

development (Tarkowski & Rossant, 1976; Tarkowski, 1977). Though these haploid half embryos are not normal, their nucleocytoplasmic ratio is comparable to that of untreated cleaving controls.

Radioactive incorporation and electrophoresis

For radioactive labelling, embryos were taken on the afternoon of day 2 and 3 and were transferred to M16 containing [^{35}S]methionine (New England Nuclear) at a final concentration of $0.5–1.0 \, mCi \, ml^{-1}$ (specific activity $\sim 1000 \, Ci \, mmol^{-1}$) for a period of $4–4\frac{1}{2}$ h. During the necessary handling procedure the zona-less embryos tended to aggregate and disaggregate. Some of the 4-cell embryos (controls as well as bisected embryos) started the next cleavage during the period of incorporation. Processing of

embryos for biochemical analysis was done as described previously (Petzoldt & Hoppe, 1980).

Two-dimensional gel electrophoresis was performed according to O'Farrell (1975). For fluorography (Laskey & Mills, 1975) gels were treated with Amplify (Radiochemical Centre, Amersham) and the Kodak X-omat AR film was used for exposure. Two to fifteen embryos were applied per gel.

Immunofluorescence microscopy

Half embryos and controls were analysed for the appearance of the stage-specific antigen SSEA-1 in the afternoon of day 3 and 4, using the monoclonal antibody a-SSEA-1 (Solter & Knowles, 1978; kindly provided by Dr D. Solter, Philadelphia). All procedures were performed as described elsewhere (Petzoldt, 1986) but using the M2 medium. The
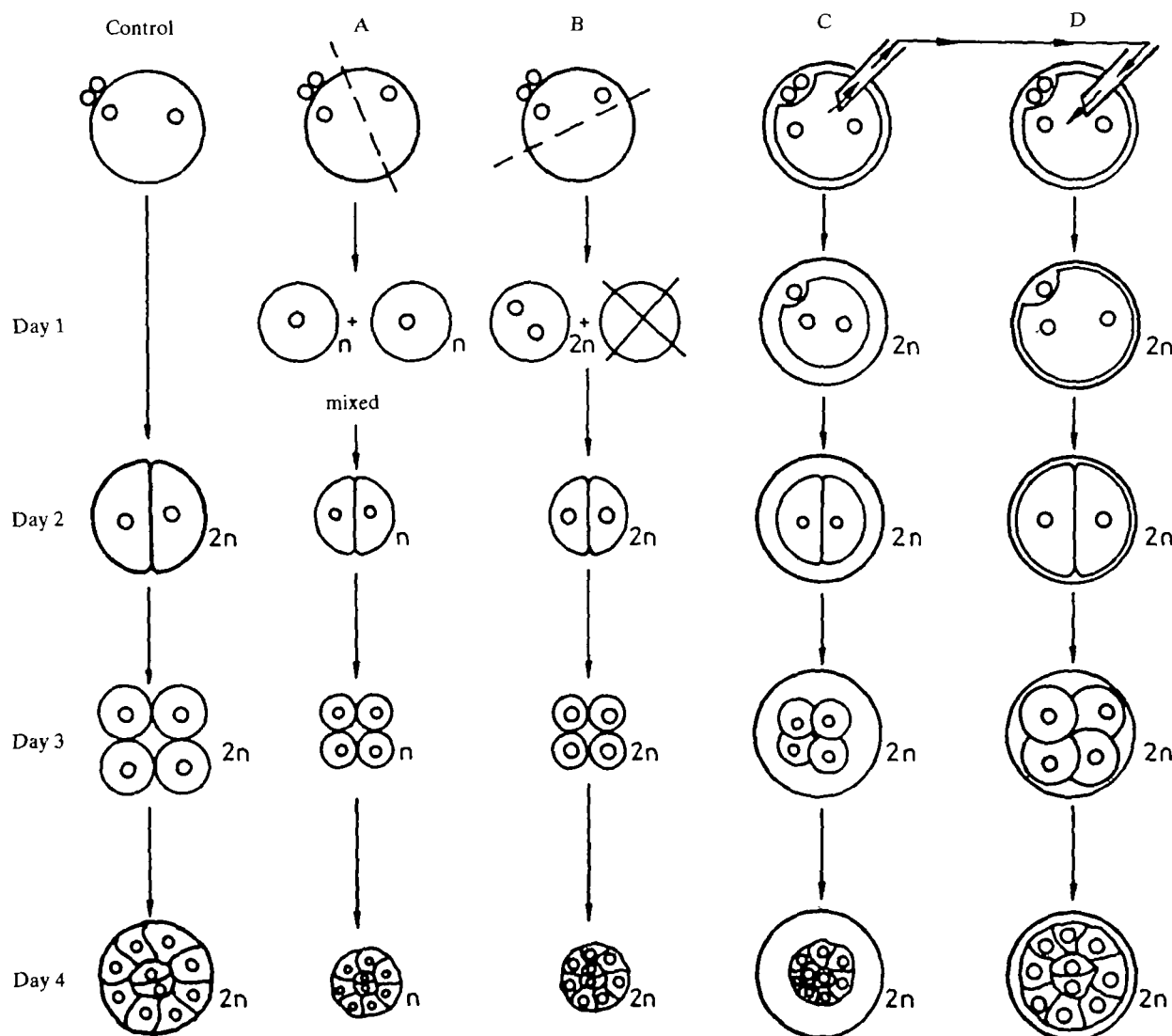

Fig. 1. Methods to increase or decrease the nucleocytoplasmic ratio in fertilized eggs. Line A, bisection of eggs to maintain a normal nucleocytoplasmic ratio; half eggs with one pronucleus only, haploid development. Line B, bisection of eggs to achieve a double nucleocytoplasmic ratio; half eggs with two pronuclei, diploid development. Line C, removal of cytoplasm to achieve an increased nucleocytoplasmic ratio; eggs with two pronuclei, diploid development. Line D, injection of cytoplasm into eggs to achieve a decreased nucleocytoplasmic ratio; eggs with two pronuclei, diploid development. Control eggs were zona-denuded and underwent the same culture condition as bisected eggs.
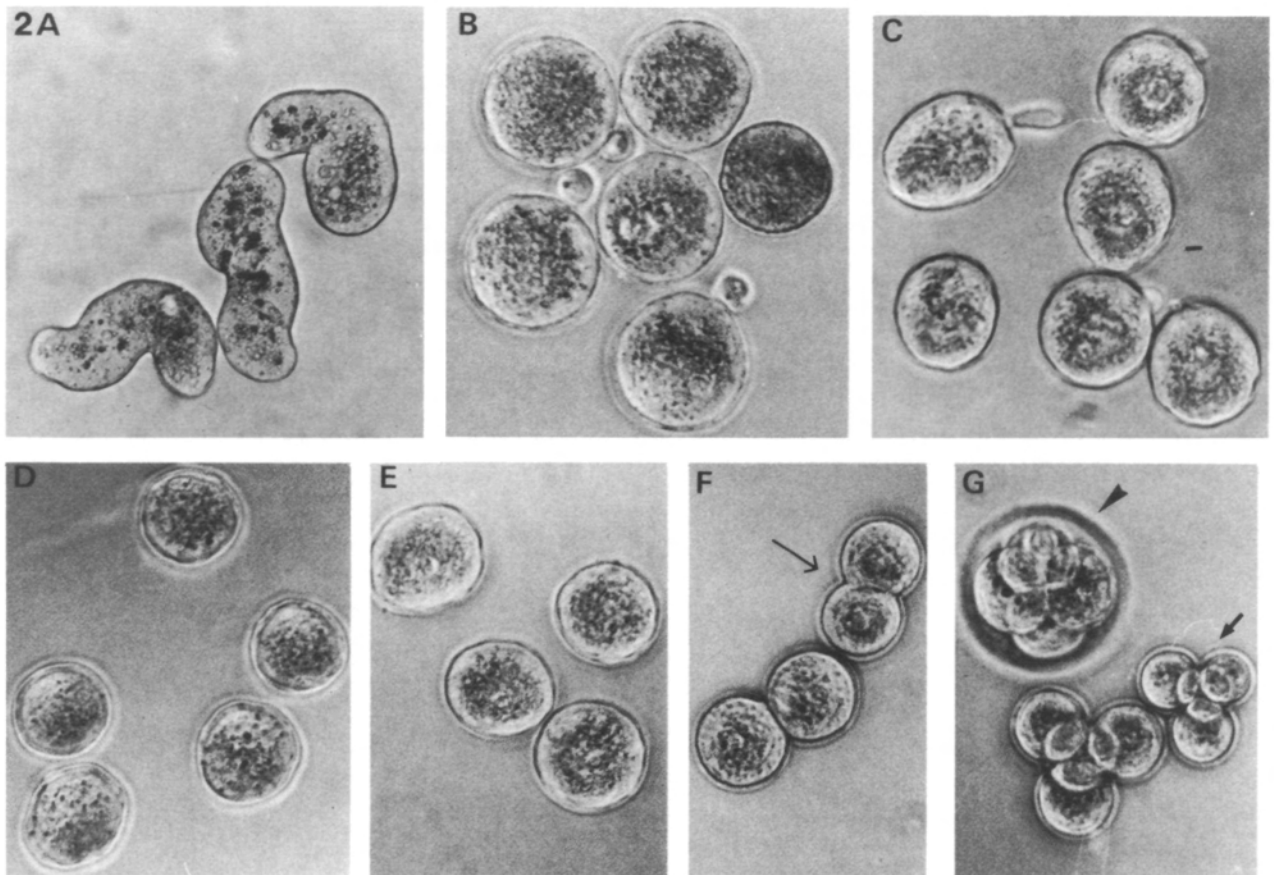
Fig. 2. Bisected fertilized eggs and subsequent development: (A) deformed fertilized eggs; (B) denuded fertilized control eggs; (C) half eggs with two pronuclei; (D) anucleate half eggs; (E) half eggs with one pronucleus; (F) denuded 2-cell control embryo and 2-cell embryo derived from a half egg containing two pronuclei (arrow); (G) 4-cell control embryo with (arrowhead) and without zona pellucida, and 4-cell embryo derived from a half egg containing two pronuclei (arrow). Magnification: (A) ×210, (B–G) ×230.

dilution of α-SSEA-1 was 1:100, that of the second antibody (rabbit anti-mouse IgM/FITC, Nordic) 1:50. Negative controls were carried out using the same protocol but replacing the first antibody by mouse ascites fluid containing IgM (diluted 1:100 in M2, kindly provided by Prof. Dr D. Haustein, Marburg and Dr E. Pfaff, Heidelberg). The embryos were examined for immunofluorescence with a Leitz Orthoplan immunofluorescence photomicroscope. Also during this procedure, denuded embryos tended to aggregate and disaggregate, and some 4-cell embryos started the next cleavage division.

Size determinations

Photographs of the bisected eggs were taken with a Leitz inverted microscope. From the plane of the pictures the volume of the fragments was determined using either the formula for spheres or for ellipsoids.

Results

When the nucleocytoplasmic ratio of fertilized eggs was altered the amount of cytoplasm removed or injected was not quantified exactly. However, by

experimentation it was found that approximately one quarter of the cytoplasmic content could be withdrawn from the donor and injected into the recipient with a reasonable survival and cleavage. At the 2-cell stage, both receiver and donor embryos expressed similar protein synthesis patterns to control embryos (gels not shown). Although the survival of the donors was quite low, the recipients were able to cleave further. They sometimes started compaction at day 3 and synthesized polypeptide sets equivalent to those of normal controls at that time and stage (Fig. 3). Although this method failed to provide sufficient experimental material of the required nucleocytoplasmic ratio, it did provide an additional control for the manual bisection method and supportive data that a change of the nucleocytoplasmic ratio, at the level achieved, does not influence stage-specific gene expression during early cleavage.

In a major experiment, fertilized eggs were manually bisected leaving one pronucleus in each half of the cytoplasm or both in one half. Using this procedure eggs were not dissected into exact halves,
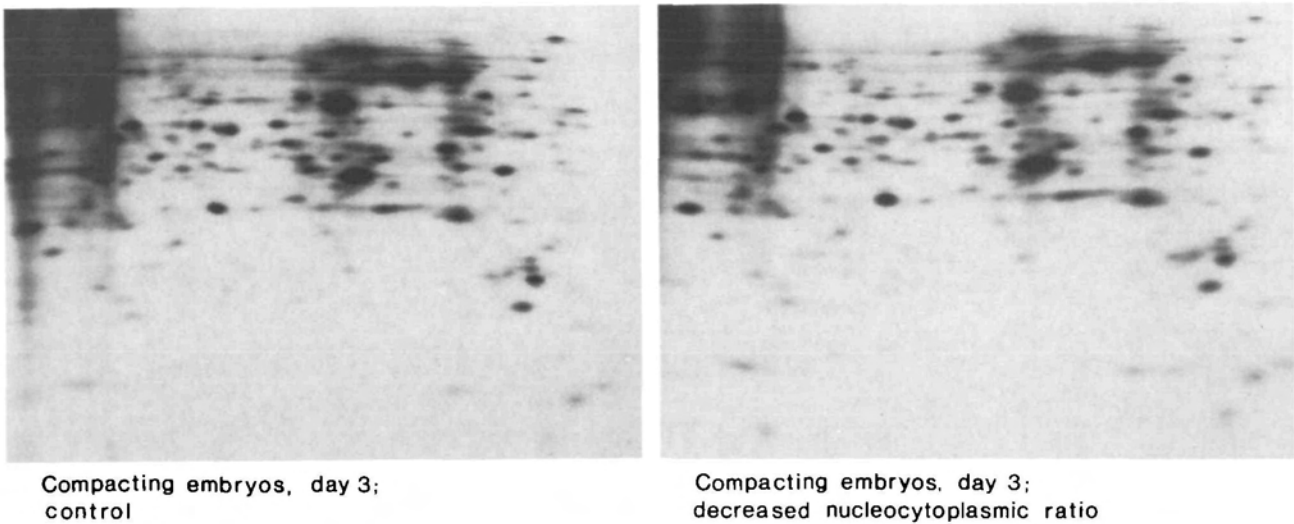
Compacting embryos, day 3;
control

Compacting embryos, day 3;
decreased nucleocytoplasmic ratio

Fig. 3. Fluorographs of two-dimensional gels to compare day-3 compacting embryos with normal and decreased nucleocytoplasmic ratio. Eight embryos were applied per gel. Running directions as in Fig. 4.

especially when both pronuclei ended up in one cytoplasmic fragment. This fragment was significantly larger than the enucleated one ($P < 0.001$), when the volume of bisected eggs was measured using an equivalent sample of eggs from a series of experiments (Fig. 2C,D; Table 1). Although these calculations showed that half eggs with two pronuclei were slightly larger than those halves with one pronucleus (Fig. 1E; Table 1), this difference was not found to be significant ($P > 0.1$). Half eggs with two pronuclei were regarded therefore as real half eggs. The nucleocytoplasmic ratio is double that of the control eggs and half eggs with one pronucleus only, but identical to the ratio in control 2-cell embryos. At the 2-cell stage these eggs have a nucleocytoplasmic ratio equivalent to a 4-cell embryo and at the 4-cell stage a ratio equivalent to that of an 8-cell embryo.

When bisected eggs were kept in culture the majority cleaved, reaching the 2-cell stage the next day and the 4-cell stage the following day, but they were smaller than control embryos (Fig. 2F,G). A considerable number compacted at day 4. There was

Table 1. *Volume of egg fragments after bisection*

	No. of eggs	Volume, arbitrary units (±standard deviation)
Fertilized eggs (control)	16	4007 (±621)
Half eggs with one pronucleus	23	1782 (±394)
Half eggs with two pronuclei	42	1924 (±391)
Egg fragments without pronucleus	40	1193 (±375)

no general delay in development observed following bisection (Tarkowski, 1977; Barton & Surani, 1983). In our experiments we found that operated eggs were delayed in comparison with the controls at the first cleavage division. This delay was also visible at the following cleavage stages and more apparent in haploid than in diploid bisected embryos.

2- and 4-cell embryos were incubated with [^{35}S]methionine and analysed for their protein synthesis by two-dimensional polyacrylamide gel electrophoresis. The periods for radioactive labelling were chosen so that they included a common part of the cell cycle for controls and bisected embryos. Embryos of the 2-cell stage were at the G_2-phase at that time, the majority of 4-cell embryos passed from S- to G_2+M-phase (see Smith & Johnson, 1986 for the variations of cell cycles during first cleavage divisions in mouse embryos). Fig. 4 shows representative gels (each gel was selected from at least five gel runs for every stage analysed) for 2- and 4-cell stages. It is clearly visible that 2-cell control embryos and 2-cell bisected embryos with normal (haploid half eggs) as well as with double (diploid half eggs) nucleocytoplasmic ratio express protein synthesis patterns that are closely related to each other. A set of proteins predominant in 2-cell stages is marked by arrows and a triangle. Equivalently, 4-cell controls and bisected embryos with normal and double nucleocytoplasmic ratio exhibit protein synthesis patterns that are similar to each other but definitely different from those of the 2-cell embryos. Here a variety of polypeptide spots more typical for 4-cell stages is indicated by arrowheads (Fig. 4). There are variations between different gels of the same stage but, evidently, 2-cell embryos with a double nucleocytoplasmic ratio, which is equivalent to that of a 4-cell control embryo,
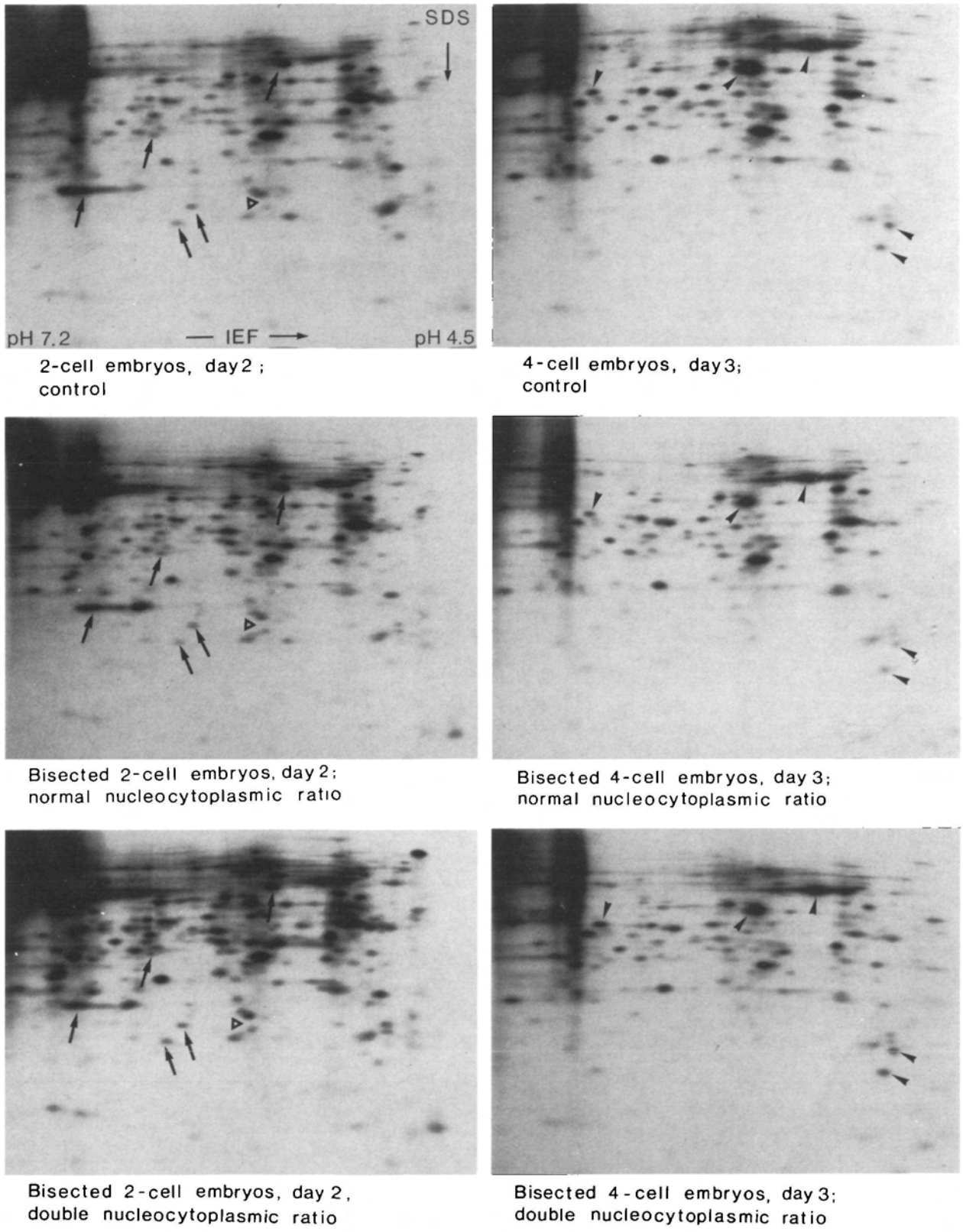
Fig. 4. Fluorographs of two-dimensional gels of cleaving bisected eggs with normal (haploid half eggs) and double (diploid half eggs) nucleocytoplasmic ratio. Arrows, polypeptides quantitatively or qualitatively more predominant in 2-cell embryos than in 4-cell embryos. Triangle, group of polypeptides expressed stronger in 2-cell embryos than in 4-cell embryos. Arrowheads, polypeptides quantitatively or qualitatively more predominant in 4-cell embryos than in 2-cell embryos. Five to fifteen embryos were applied per gel. Running directions (first dimension: IEF, isoelectric focusing; second dimension: SDS, sodium dodecyl sulphate electrophoresis) are indicated on upper left fluorograph.

synthesize proteins typical for the 2-cell and not the 4-cell stage.

In additional experiments embryos of day 3 at the 4-cell or 4- to 8-cell stage and compacted embryos of day 4 were analysed for the expression of the stage-specific antigen SSEA-1. In control embryos and bisected diploid embryos with double nucleocytoplasmic ratio of day 4, 100 % reacted with the monoclonal antibody against SSEA-1, but only 71 % of the bisected haploid embryos with normal nucleocytoplasmic ratio of day 4 were positive (Table 2; Fig. 5). Almost no reaction was found in day-3 embryos (Fig. 5), independent of whether they were at the 4-cell or 4- to 8-cell stage. Only one bisected embryo with normal nucleocytoplasmic ratio showed a slight reaction with the antibody (Table 2). These results also show that the expression of a stage-specific antigen, which normally appears at the 6- to 8-cell stage (Solter & Knowles, 1978), is not accelerated in 4-cell embryos with double nucleocytoplasmic ratio.

Table 2. *Immunofluorescence experiments with anti-SSEA-1* *

	Total no. of embryos (no. of experiments)	No. of positive embryos (% of total no.)
Control embryos, day 3	18 (4)	0 (0)
Bisected embryos with normal nucleocytoplasmic ratio, day 3 (haploid half eggs)	25 (5)	1 (4)
Bisected embryos with double nucleocytoplasmic ratio, day 3 (diploid half eggs)	27 (5)	0 (0)
Control embryos, day 4	17 (3)	17 (100)
Bisected embryos with normal nucleocytoplasmic ratio, day 4 (haploid half eggs)	24 (4)	17 (71)
Bisected embryos with double nucleocytoplasmic ratio, day 4 (diploid half eggs)	15 (4)	15 (100)

* Only experiments with at least one positive control embryo present were considered.

Discussion

Early development is characterized by a variety of morphogenetic processes and the stage-specific activation of the embryonic genome. The time schedule of all these events is regulated by different programmes including cytoplasmic clock mechanisms, counting of DNA replication cycles and the quantitative nucleocytoplasmic (or DNA/cytoplasmic) ratio (see Satoh, 1982, for review). Such different programmes might cooperate within the same embryos each controlling a different developmental event as was shown for cleavage synchrony, midblastula transition and onset of gastrulation in *Xenopus laevis* (reviewed by Kirschner, Newport & Gerhart, 1985).

In recent years, the action of different timing mechanisms has been analysed also in mouse embryos. The existence of cytoplasmic clocks was verified as being responsible for certain morphogenetic events, e.g. in enucleated eggs cortical activity was observed at the time nucleate eggs cleave (Waksmundska, Krysiak, Karasiewicz, Czołowska & Tarkowski, 1984). For compaction and blastocyst formation conflicting data are reported. Experiments of Smith & McLaren (1977) led to the conclusion that cavitation of blastocysts is controlled by the counting of replication cycles. By using aphidicolin, an inhibitor for DNA polymerase-α, other investigators demonstrated that compaction as well as blastocyst formation occur at the correct time, even when one or two preceding DNA replication steps are blocked (Alexandre, 1982; Dean & Rossant, 1984). Embryos should have reached at least the 4-cell stage before exposure to the drug (Smith & Johnson, 1985). These results show that these morphogenetic changes are independent of a defined number of DNA replications but triggered by an unknown event occurring sometime during cleavage but not evident at the 1-cell stage. Recent results from Prather & First (1986) postulate that such an internal programme for the process of cavitation can be overcome by cell–cell interaction. Chimaeras formed from a single 8-cell blastomere and a 2-cell embryo cavitate at a time intermediate between both programmes. Such a programme can be manipulated even further. Treatment of 2-cell embryos with wheat germ agglutinin induced premature compaction- and cavitation-like events, thus inducating that proteins and glycoproteins necessary for these processes might be present already at that stage (Johnson, 1986). Actually, this idea is supported by experiments to inhibit protein synthesis at the 2- to 4-cell stage. In such embryos an accelerated expression of some features occurred, which are typical for compaction (Kidder & McLachlin, 1985; Levy, Johnson, Goodall & Maro, 1986). None of these results really clarify which mechanisms
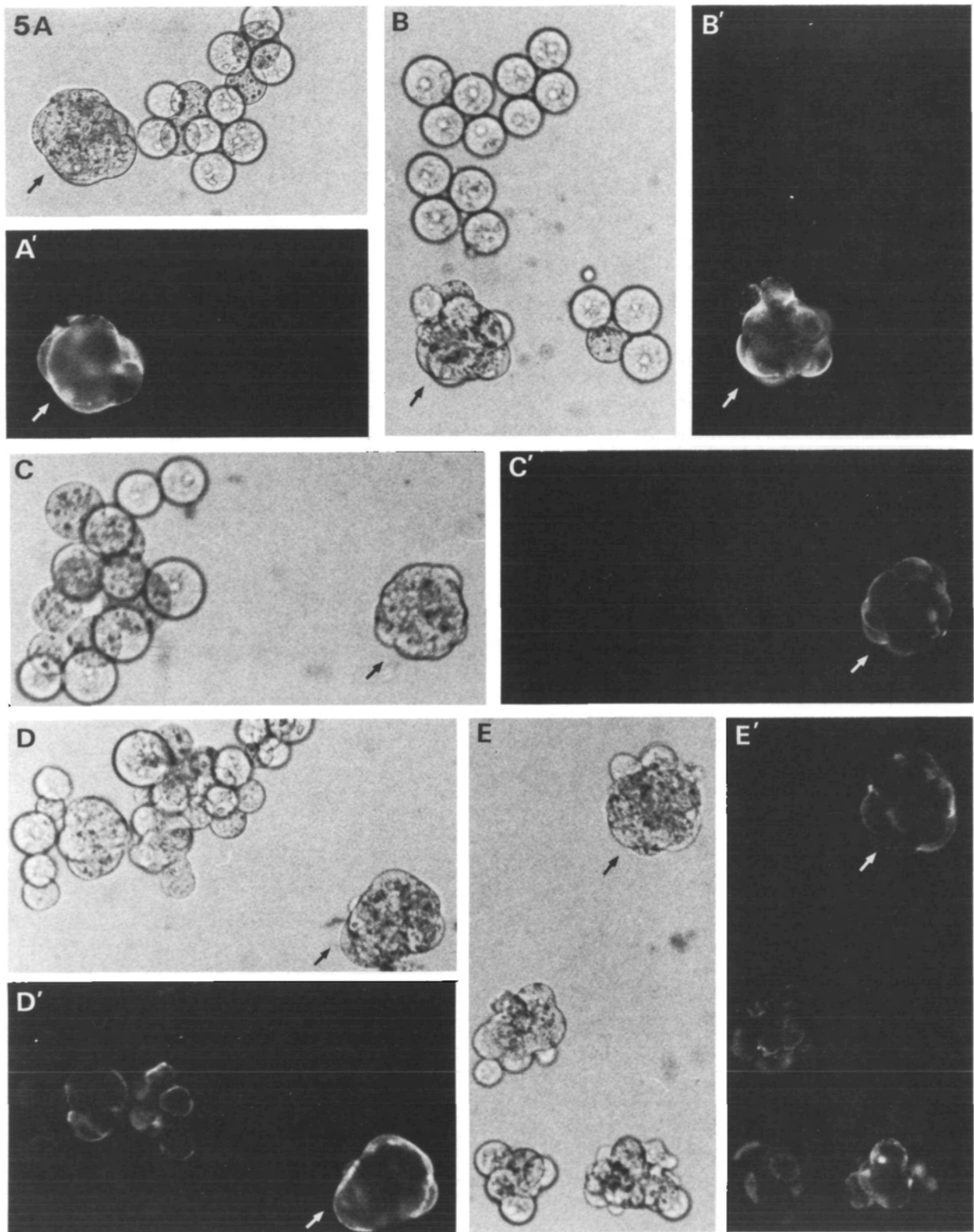
Fig. 5. Immunofluorescence microscopy of cleaving bisected eggs with normal (haploid half eggs) and double (diploid half eggs) nucleocytoplasmic ratio, using a monoclonal antibody against the stage-specific embryonic antigen SSEA-1. A–E normal light; A′–E′ fluorescence. Arrows mark control morulae (day 4), which were added to each sample of the experimental embryos. (A,A′) bisected 4- to 8-cell embryos (day 3) with normal nucleocytoplasmic ratio; (B,B′) bisected 4- to 8-cell embryos (day 3) with double nucleocytoplasmic ratio; (C,C′) 4- to 8-cell control embryos (day 3); (D,D′) bisected compacting embryos (day 4) with normal nucleocytoplasmic ratio; (E,E′) bisected compacting embryos (day 4) with double nucleocytoplasmic ratio. Magnification: ×220.

are responsible for the timing of compaction. Different signals might cooperate in unknown combinations (Levy *et al.* 1986).

Stage-specific gene activity in early embryos is completely independent of cleavage with regard to transcription, translation, surface antigen expression and the activation of the paternal genome (Surani, Barton & Burling, 1980; Pratt, Chakraborty & Surani, 1981; Petzoldt, Bürki, Illmensee & Illmensee, 1983; Petzoldt, 1986). Also post-translational modifications of maternal mRNA-derived proteins, which are characteristic for meiosis and mitosis, proceed in cleavage-arrested eggs after cytochalasin D treatment but cease when embryos are arrested in metaphase (Howlett, 1986*a*).

The influence of a cytoplasmic clock on stage-specific gene activity cannot be excluded. Bolton, Oades & Johnson (1984) showed by short-term treatment of late 1-cell eggs or early 2-cell embryos with aphidicolin, that α-amanitin-sensitive late 2-cell proteins were synthesized without an S-phase occurring. When treating early fertilized eggs with aphidicolin, only a few 2-cell-specific proteins showed up the next day (Petzoldt, 1984*a*; Howlett, 1986*b*). When such eggs were incubated with the drug even for 2 or 3 days they did not progress further in stage-specific protein synthesis or in surface antigen expression (Petzoldt, 1984*a,b*). A toxic side effect of aphidicolin cannot be excluded, however, when used over such a long period. Therefore, the action of a cytoplasmic clock to regulate stage-specific gene expression during cleavage cannot be ruled out completely.

We have tried to differentiate between the timing mechanisms by either counting the cycles of DNA replication or measuring the nucleocytoplasmic ratio in cells during cleavage. For this purpose, fertilized eggs were either bisected or the amount of cytoplasm was manipulated using micropipettes. Both techniques disturb the cellular architecture of the egg and its quantitative (and qualitative?) amounts of organelles and cytoplasmic substances. In the case of removal and addition of cytoplasm the disturbance is restricted to cytoplasmic organelles and/or substances. However, the bisection method also removes amounts of the cell membrane. All these components are of major importance for pronuclear movements and regular development after fertilization (see e.g. Schatten, Simerly & Schatten, 1985; van Blerkom & Bell, 1986). Nevertheless, orderly cleavage is neither grossly influenced after bisection (see also Tarkowski & Rossant, 1976; Tarkowski, 1977; Barton & Surani, 1983), nor is it after cytoplasmic transfer experiments, which served as additional controls.

During successive cleavage, such embryos were analysed for stage-specific protein synthesis and surface antigen expression. Protein synthesis patterns

changed in bisected eggs in a manner equivalent to controls. These changes include shifts of proteins translated from maternal mRNA as well as new embryonic gene products, as shown in previous publications (Petzoldt, Illmensee, Bürki, Hoppe & Illmensee, 1981; Petzoldt *et al.* 1983). Even though α-amanitin sensitivity of newly appearing polypeptides was not tested here, it is obvious by comparison of our protein synthesis patterns with those in the literature that these newly appearing spots are α-amanitin sensitive (see Flach, Johnson, Braude, Taylor & Bolton, 1982). The expression of the stage-specific embryonic antigen SSEA-1 was not accelerated and occurred at day 4, when bisected and control embryos compacted.

It was quite clear from our results that doubling of the nucleocytoplasmic ratio does not induce the embryo to express proteins and surface antigens one cleavage step earlier than embryos with normal nucleocytoplasmic ratio. Furthermore, decreasing the ratio by injecting cytoplasm into fertilized eggs did not visibly slow down stage-specific protein synthesis. This is in agreement with previous results, where the nucleocytoplasmic ratio was approximately halved by removing one pronucleus from fertilized eggs and restoring the normal ratio by diploidizing the egg with cytochalasin B (Petzoldt *et al.* 1981). Protein synthesis followed the number of DNA cycles of replication and not the nucleocytoplasmic ratio. On the basis of the results presented here, we favour the idea that the consecutive events of stage-specific gene activity during early mouse development are closely related to the progress of DNA replication as experiments with cytochalasins have indicated previously (Surani *et al.* 1980; Pratt *et al.* 1981; Petzoldt *et al.* 1983; Petzoldt, 1986). The molecular basis for this mechanism is not known. Satoh (1982) has proposed that fertilized eggs contain certain fully or uniformly methylated DNA sequences. During cleavage defined consecutive demethylation takes place at specific sites of the DNA, allowing the embryo the capability to count the replication steps and activate the genome in a stage-specific manner. However, Sanford, Rossant & Chapman (1985) have shown that the DNA in mouse oocytes and preimplantation embryos is undermethylated and *de novo* methylation is not found until implantation. Future work in this area will help elucidate the role of methylation in early mammalian development.

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