

Cycling cytoplasmic factors that promote mitosis in the cultured 2-cell mouse embryo

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Summary

A cytoplasmic component(s), previously shown to rescue the 'blocked' 2-cell mouse embryo *in vitro*, has been demonstrated to peak in activity during the transition between G₂ and M phase and decline thereafter. The possible significance of this component(s) in the regulation of cleavage of the cultured mouse embryo is discussed.

Key words: cytoplasmic factors, mouse embryo, *in vitro*, mitosis, block.

Introduction

In mice, the development of blastocysts from 1-cell embryos *in vitro* is restricted to certain inbred strains and their F₁ hybrids (Whittingham, 1974; Muggleton-Harris *et al.* 1982; Pratt, 1987). Most random-bred strains generate embryos which, if removed from the reproductive tract at any time prior to the early 2-cell stage and cultured under conventional culture conditions, arrest in G₂ phase of the second cell cycle for reasons which are unclear. Although an *in vitro* phenomena this '2-cell' block can provide useful information as the arrest in cleavage operates at a particular stage in the cycle, is strain dependent and can be obviated by injection of 'non-blocking' cytoplasm (Muggleton-Harris *et al.* 1982). It is known that the '2-cell block' is dependent upon the genotype of the female producing the eggs (Goddard & Pratt, 1983; Pratt, 1987), and that during this period the embryos are peculiarly sensitive to fluctuations in environmental conditions and exhibit stringent energy substrate requirements (Whittingham, 1974).

The activity of cytoplasmic components is known to fluctuate during the cell cycle and specific factors associated with different phases have been identified in a variety of species and systems. A metaphase factor accumulates throughout G₂ phase reaching its highest level at the onset of mitosis (Johnson & Rao, 1971). Maturation- or mitosis-promoting factor,

MPF, has been identified in somatic cells and cleaving embryos of sea urchin, clam and *Xenopus* (reviewed Ford, 1985). MPF activity peaks immediately preceding mitosis and declines thereafter (Gerhart *et al.* 1984; Newport & Kirschner, 1984). When micro-injected *in vivo* it catalyses progress through mitosis (or meiosis) to the next interphase (Miake-Lye & Kirschner, 1985). We have examined whether there is a cell-cycle-related component to the injected cytoplasm that rescues embryos that would otherwise block. Our results demonstrate that the effect of the cytoplasmic factor(s) peak during the G₂ stage of the cell cycle. Although the reasons for the *in vitro* arrest are unclear, the manner in which the arrest can be obviated provides some basic information on the delicate balance and/or requirements of the cultured embryos during early cleavage stages.

Materials and methods

Initial studies were undertaken at Worcester Polytechnic Institute, Worcester, MA, USA and the project was completed at MRC Experimental Embryology and Teratology Unit, Medical Research Council Laboratories, Carshalton, Surrey, UK. A 'blocking' and 'non-blocking' strain were used in each case. The 'blocking' strains used were random-bred CD-1 (USA; obtained from Charles River Breeding Labs, Wilmington, MA, USA) and MF-1 (UK; obtained from Harlan Olac, UK, Ltd, Bicester, Oxon, UK) and the

'non-blocking' embryos were obtained from laboratory bred F₁ (C57BL/6LAC×CBA/CaLAC) hybrid female mice (breeding colony obtained from either Charles River and bred at WPI or obtained from Harlan Olac Ltd and bred at EETU Carshalton). CD-1 males were obtained from Charles River and MF-1 males from Harlan Olan Ltd. Superovulation and embryo collection and culture were carried out as described previously (Muggleton-Harris *et al.* 1982; Pratt, 1987).

Collection and culture of embryos

The mice were superovulated using 5 i.u. PMS (pregnant mare's serum gonadotrophin, Folligon, Intervet, UK) followed 47–49 h later by 5 i.u. hCG (human chorionic gonadotrophin, Chorulon, Intervet, UK). Cytoplasmic transfer between asynchronous embryos was achieved by creating an 'early' and 'late' group in each batch of mice. The early group was injected with hCG at approx. 15.00 h (this was found to be the earliest injection time at which a high proportion of viable embryos was produced) and the late group was injected at approx. 23.00 h. The females were then placed with CD-1 males (USA) or MF-1 or F₁ males (UK) and detection of a vaginal plug the following morning indicated successful mating. Cumulus masses containing eggs were released from the oviducts at 20 h after the hCG injection and follicle cells were removed by treatment with hyaluronidase. The fertilized eggs (identified by the presence of a second polar body and two pronuclei) were washed through several drops of medium 2+4 mg ml⁻¹ BSA (M2+BSA) and cultured in drops of medium 16+4 mg ml⁻¹ BSA (M16+BSA) under oil at 37°C in 5% CO₂ in air (see Muggleton-Harris *et al.* 1982; and Pratt, 1987, for details of media composition).

Populations of 2-cell embryos of known age were obtained by observing late 1-cell embryos (from 28 h post-hCG onwards) every hour and grouping together all those that had cleaved within the preceding hour. The position of the cells within the second cell cycle was assigned by measuring the time elapsed since cleavage (G₁ phase 0–1 h, S phase approx. 1–6 h, G₂ phase approx. 6–18 h and M phase approx. 18–20 h post-cleavage; Bolton *et al.* 1984). Whenever possible, embryos in the middle of each phase were selected. Amongst G₁ phase embryos only those that had not reformed their nuclear membranes were used.

Cytoplasmic injections

Cytoplasmic injections were carried out on a microscope stage at a temperature of 30–37°C using glass micropipettes (approx. 4 µm diameter at the tip) attached to Agla microsyringes and a DeFonbrune micromanipulator. Penetration of eggs and withdrawal of cytoplasm is aided by exposing embryos to cytochalasin D (CCD) for short periods (not exceeding 45 min, Muggleton-Harris *et al.* 1982). Embryos were therefore incubated for 15 min at 37°C in 5% CO₂ in air in M16+BSA containing 0.5 µg ml⁻¹ CCD in order to maintain a stable pH (between 7.2 and 7.4) in air, embryos were then transferred to drops (50–100 µl) of M2+BSA containing 0.5 µg ml⁻¹ CCD under oil in a plastic culture dish. Most manipulations involved using two to three donor embryos, approximately ten recipient embryos and approximately ten other embryos that had undergone all the

handling procedures but were not injected and hence served as 'uninjected controls'. Donor cytoplasm (approximately 8 pl) was injected into only one blastomere of each recipient. After microinjection, embryos were washed through drops of M16+BSA to remove CCD and then set up in M16+BSA culture drops. Another set of embryos that had not been subjected to any of the handling procedures served as 'non-manipulated controls'. Embryos were scored daily for their developmental progress and any in which blastomeres had lysed within the first 24 h were eliminated from analysis. Those embryos that were 'rescued' by injection developed at the same rate as normal unmanipulated controls and the results are expressed as the percentage of embryos developing to the late morula or blastocyst stage. The normality of the blastocysts that developed was assessed by their ability to expand and hatch from their zonae pellucidae and in some cases by their capacity to attach to the culture dish and develop into conventional trophoblastic outgrowths with an overlying inner cell mass (ICM) when cultured in Eagles Minimal Essential medium plus Hanks's salts (HMEM) supplemented with 20% fetal bovine serum (FBS, Imperial Labs, UK).

Vital staining of embryos with Rhodamine 123

Mitochondria of live embryos were stained (Johnson *et al.* 1980) by incubating them (complete with their zonae pellucidae) in 10 µg ml⁻¹ rhodamine 123 in M2 (without BSA) for 15 min at 37°C. The embryos were then washed through several drops of M2+BSA and viewed on an epifluorescence microscope using appropriate filters.

Results

The experiments were conducted in two different laboratories using mice from different sources (see Materials and methods) but the results were qualitatively similar.

The majority of embryos from all strains underwent first cleavage during the time interval 28–33 h after the hCG injection although embryos developed from F₁ eggs tended to be the earliest to divide. The '2-cell block' is not an 'all or none' phenomenon and a small but variable proportion of embryos from blocking strains may develop at a normal rate beyond the 2-cell stage (Whittingham, 1974). In the present study, 4% of fertilized CD-1 eggs and 0.4% of fertilized MF-1 eggs developed into blastocysts (Table 1).

The ultimate aim of these experiments was to establish whether the 'rescuing' capacity of F₁ donor cytoplasm fluctuated during progress through the cell cycle. Our initial studies, however, were designed to determine the optimal stage in the cell cycle of blocking embryos for the receipt of rescuing cytoplasm and to confirm that the manipulations involved in injecting cytoplasm were not deleterious. These results are discussed first.

Using CD-1 embryos, we found that the greatest

Table 1. Percentage of embryos developing to the late morula or early blastocyst stage following injection of cytoplasm at the 2-cell stage*

		Blocking			Non-blocking	
(1) Non-manipulated		CD-1	4(134)		F ₁	76(45)
		MF-1	0.4(270)		F ₁	68(176)
(2) Uninjected		MF-1(G ₂)	0(27)		F ₁	45(11)
					F ₁ (G ₁)	67(52)
					F ₁ (G ₂)	63(32)
(3) Injected		CD-1 recipients†			F ₁ recipients	
		G ₂ E§	G ₂ L	S	G ₂ E§	G ₂ L
(A) Donor F ₁	G ₁	50(20)	—	—	—	—
	S	0(26)	—	40(5)	—	—
	G ₂ E§	52(46)	—	—	86(7)	—
	G ₂ L	—	25(28)	—	—	54(13)
(B) Donor CD-1	G ₂ E	13(16)	—	—	—	—
	G ₂ L	—	0(24)	—	—	—
		MF-1 recipients‡			F ₁ recipients	
		G ₁	S	G ₂	G ₁	G ₂
(C) Donor F ₁	G ₁	42(12)	0(8)	22(23)	72(25)	73(11)
	S	—	—	0(13)	—	—
	G ₂	—	38(8)	33(57)	—	42(43)
(D) Donor MF-1	G ₂	—	—	3(32)	—	78(27)

* Those embryos that degenerated within the first 24 h were excluded from analysis. The results are expressed as the percentage of remaining embryos that developed to the late morula or early blastocyst stage. The number of remaining embryos is in parenthesis.

† Data pooled from two experiments conducted at WPI, USA.

‡ Data pooled from four experiments conducted at EETU Labs, Carshalton.

§ G₂E = early G₂ phase, i.e. 6–10 h post-cleavage.

|| G₂L = late G₂ phase, i.e. 10–14 h post-cleavage.

degree of blastocyst development (52 %) was attained when the recipient embryos were in the early G₂ phase (i.e. 6–10 h post-cleavage) (Table 1). In all of these embryos, the midbody (i.e. the remnant of the cleavage furrow which provides cytoplasmic continuity between the two blastomeres) was still present, since injection of a single blastomere was sufficient to rescue the entire embryo. When treated with CCD in late G₂ (approx. 14 h post-cleavage) all types of embryo exhibited the dramatic reorganization of cytoplasm and membrane reported previously (mouse, Muggleton-Harris *et al.* 1982; *Xenopus*, Elinson, 1983). Cytoplasm from all types of embryo became viscous and difficult to withdraw and the surface membrane acquired a rubbery consistency making it hard to penetrate. Although it is possible to carry out successful transfers of cytoplasm at this late stage, embryo survival is poor. In view of these changes, recipients in early G₂ were used wherever possible. The blastocysts that developed from all transfers were morphologically normal. In experiments where blastocysts were transferred into explant medium (see Materials and methods) 90 % or more generated normal trophectodermal outgrowths with overlying ICMs.

As a control for any deleterious effects of manipulation we used F₁ embryos. Nonmanipulated F₁

controls and uninjected F₁ controls, which had been subjected to all the handling procedures except cytoplasmic transfer, were included in every experiment. The ability of F₁ embryos to develop beyond the late morula/early blastocyst stage was reduced by the handling procedures ('uninjected controls') in the first set of experiments but not the second. When F₁ embryos were subjected to synchronous or asynchronous transfers of homologous (F₁) or heterologous cytoplasm their viability was affected to the variable extent inherent in this type of study (range 42–73 %) but was never reduced below 50 % of the normal control values.

Our systematic investigation of cytoplasmic 'rescuing factors' during the F₁ cell cycle used embryos derived from both MF-1 and CD-1 eggs as recipients and demonstrated the existence of rescuing factors in G₁- and G₂- but not S-phase cytoplasm of the 2-cell stage (Table 1). Preliminary experiments also indicate the existence of this factor(s) in cytoplasm of the late 1-cell G₂ stage (23–28 h post-hCG). The rescuing factor(s) appears to be effective when transferred to blocking recipients at any stage in the cell cycle, although the response of S-phase cells seems to vary with the stage of the injected material (Table 1). Throughout these experiments successful 'rescue' gave rates of development comparable to, or slightly

less than, F₁ embryos subjected to homologous injections but always substantially higher than nonmanipulated or uninjected 'blocking' controls.

The 'rescue' was not due to the process of injection itself since the majority of G₂-phase 'blocking' embryos injected with homologous cytoplasm did not develop significantly beyond the 2-cell stage (Table 1). The only exception to this was one small group of CD-1 embryos from the initial series which showed a slight improvement compared with non-manipulated controls (13% versus 4%) but their viability was still substantially less than CD-1 embryos subjected to heterologous injections (52%). Furthermore, the 'blocking' activity is not transferable since G₂-phase F₁ embryos show comparable development to uninjected controls when injected with G₂-phase cytoplasm from the MF-1 'blocking' strain (Table 1).

The distribution of mitochondria in 'blocking' and

'non-blocking' embryos was compared after staining the embryos with the vital dye rhodamine 123. Although some variation between embryos was observed, it was clear that F₁ embryos exhibit a more homogeneous distribution of mitochondria than either CD-1 or MF-1 embryos throughout the cycle and that aggregation of mitochondria in 'blocking' embryos increases substantially towards the end of G₂ phase (Fig. 1) as previously observed (Goddard & Pratt, 1983). Experiments in which F₁ cytoplasm was transferred into MF-1 embryos show that abolition of G₂ arrest in 'rescued' embryos is associated with a change in the organization of mitochondria from an aggregated to a more homogeneous distribution (Muggleton-Harris & Brown, 1988).

Discussion

These results demonstrate that the capacity of cyto-

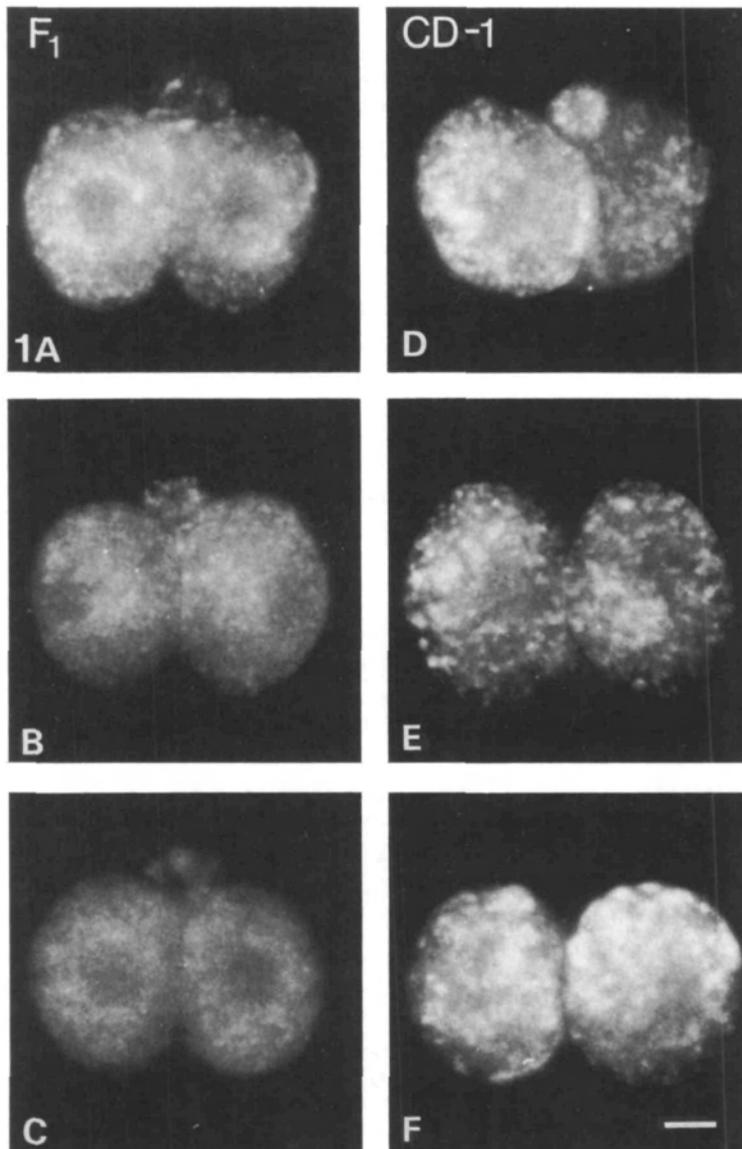


Fig. 1. Patterns of mitochondrial distribution during the second cell cycle in 'non-blocking' (F₁: A,B,C) and 'blocking' embryos (CD-1: D,E,F) as assessed by staining with Rhodamine 123 (details in Materials and methods). (A,D) Early 2-cells (approx 3 h post-cleavage); (B,E) mid 2-cells (approx 10 h post-cleavage) and (C,F) late 2-cells (approx 18 h post-cleavage). Bar, 10 μm.

plasm to initiate mitotic events and 'rescue' embryos that would otherwise arrest in G₂ phase of the 2-cell stage fluctuates during the cell cycle. In the second cell cycle, this activity is negligible during S phase and apparently present during both G₁ and G₂ phases. The G₂-phase activity is apparently associated with progress into mitosis whereas the activity in G₁ (this short phase occupies little more than the first hour of the second cell cycle) may be a remnant of the activity present during the previous mitosis. Indeed preliminary experiments suggest that the activity is also present at the late 1-cell stage.

Some cyclic phenomena have already been described during cleavage of the mouse embryo. For example, early cleavage cycles show an obligatory requirement for protein synthesis for entry into, but not exit from, mitosis (Howlett, 1986b), as well as cycles of post-translational modification of pre-existing polypeptides (Howlett, 1986a), reorganization of membrane and cytoplasm (Muggleton-Harris *et al.* 1982; this paper) and autonomous changes in the cortex which precede each division (Waksmundzka *et al.* 1984).

An injection of the factors present in G₂-phase cytoplasm administered early (0–1 h post-cleavage) or late (6–14 h post-cleavage) in the cycle is sufficient to promote passage through the second cell cycle and presumably also to reinitiate endogenous cycling since subsequent cell cycles are apparently normal (Table 1). Theoretically, this factor could be either an intermediary metabolite (Abramzuk *et al.* 1977; Loutradis *et al.* 1987) or a macromolecule of some kind. However, since such a small volume of cytoplasm (approx. 8 pl, about 4% of the host cell volume) is sufficient to overcome the block we consider it unlikely that the effects we see are mediated *solely* through direct modulation of metabolite concentrations. If this were the case the disparities in metabolite concentration between blocking and nonblocking strains would have to be very large indeed for such a minute volume to elevate the host concentration to an effective level. It seems far more likely that an amplification process of some kind is operating. If the initiating component is a metabolite then it could exert its effect by activating an enzyme cascade or other macromolecular assembly, including untranslated mRNA, in the host cytoplasm. The fact that ensuing cell cycles are normal once the block is obviated demonstrates that there is no qualitative defect in the embryonic genome of the arrested cultured embryo.

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