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Expression of the *Tcp*-1 locus of the mouse during early embryogenesis

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SUMMARY

The synthesis of proteins by preimplantation mouse embryos was studied by two-dimensional (2D) gel electrophoresis of proteins labelled with [^{35}S]methionine. The product of the *Tcp*-1 locus of the mouse, p63/6.9, is synthesized by morula and blastocyst stage embryos, but is not detectable in 2-cell embryos. These results indicate that maternal expression (oocyte-specific) of this gene is not occurring and that this gene may be developmentally regulated. Blastocysts which are allowed to implant *in vitro* have rates of protein synthesis that permit fluorographs of single embryos to be performed. These embryos can be unambiguously identified for their *t*-complex genotype. Quantitation of the amounts of the Tcp-1a and Tcp-1b allelic products in morula embryos suggested delayed expression of the paternal allele.

INTRODUCTION

An understanding of the nature of a process is frequently advanced by the analysis of mutations affecting that process. The *t*-complex of the mouse provides numerous mutations which have pronounced effects on spermiogenesis and embryonic development (for review, see Bennett, 1975; Erickson, Hammerberg & Sánchez 1980). Genes within this region causing developmental arrest are typically recessive lethals that belong to numerous genetic complementation groups (Bennett, 1975; Guénet, Condamine, Gaillard & Jacob, 1980; Nizetic, Figueroa & Klein, 1984). Each group demonstrates a distinct time and pattern of developmental aberrations. Many of these lethals appear to disorganize or completely inhibit the formation of a single embryonic cell type. For example, t^{w18} homozygous embryos fail to generate mesoderm cells (Spiegelman & Bennett, 1974); while t^0 homozygous embryos exhibit reduced mass and aberrant morphology of ectodermal cells (Gluecksohn-Schoenheimer, 1940). These observations have led to the suggestion that *t* embryonic lethals are mutations of genes controlling differentiation steps during embryogenesis (Bennett, 1975). However, several *t* lethals acting during preimplantation and early postimplantation stages

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(t^{12}, t^{w5} and t^{0}) have been proposed to be generalized cell lethals based on the inability of their cells to undergo prolonged growth *in vitro* (Wudl, Sherman & Hillman, 1977). More recently, it has been shown that an embryo–carcinoma cell line was t^{w5} homozygous (Magnuson, Epstein, Silver & Martin, 1982) suggesting that the organizational hypothesis of developmental arrest still applies to early acting *t* lethals.

Experimental approaches in the study of *t* embryology have largely relied on the *in vitro* culture of preimplantation embryos. These attempts have been hampered by the small quantity of embryos obtainable and the inability to unambiguously deduce the *t* genotype of the embryos. The discovery of a protein (Silver, Artzt & Bennett, 1979), p63/6.9, that is synthesized by both somatic and germ cells and coded for by a gene, *Tcp*-1, within the *t*-complex provides a molecular probe for examining both embryonic lethality in *t* embryos and normal development. This gene encodes two known allelic variants: p63/6.9b, expressed by all wild-type 17th chomosomes and p63/6.9a, expressed by all complete *t* haplotypes examined to date. That these allelic electromorphs do not arise via post-transcriptional or post-translational modification events has been demonstrated using cell-free translation (Danska & Silver, 1980). Although the *Tcp*-1^{a} allele is an inviolate component of the *t* complex, a biological function for this gene or its protein has yet to be found. It does not cause embryonic lethality since t^{w82} homozygous animals are viable and *Tcp*-1^{a} homozygous. It is a major newly synthetic protein of adult testicular cells and a minor protein in other tissues. However, its expression in embryos has not been reported, although it has been shown to be expressed by a pluripotent stem cell line derived from a t^{w5}/t^{w5} embryo (Magnuson, Epstein, Silver & Martin, 1982) and by the F9 teratocarcinoma cell line (Silver & White, 1982).

In this paper, we provide evidence that the *Tcp*-1 locus is expressed in morula, blastocyst and implantation-stage embryos, but not by 2-cell-stage embryos. Thus, this gene and its product may now provide an important biochemical and genetical marker in two-dimensional gel analyses of *t* mutations affecting development.

MATERIALS AND METHODS

Animals

Mice were maintained under a 14 h light, 10 h dark cycle with pine bedding and pelleted diet and water always available. This photoperiod results in a mean time of female ovulation of midnight to 1 am. Gestational periods indicated as days *postcoitum* are already corrected for the actual time of fertilization. CD-1 male mice were purchased from Charles River or random bred from their stock. The t^{0} haplotype were originally from Dr. S. Waelsch but have been maintained in our mouse colony for more than 15 generations. The Rb(16.17)7Bnr stain was obtained from Dr. C. J. Epstein. This stock is homozygous for a Robertsonian translocation between chromosomes 16 and 17. Throughout this paper this chromosome is designated Rb7.

Materials

Acrylamide and agarose were obtained from Bio-Rad Laboratories. L-[^{35}S]methionine (sp. act. 900–1200 Ci mmol^{-1}; Radiochemical Centre, Amersham, U.K.) was used for all protein-labelling studies. Specially pure sodium lauryl sulphate was obtained from BDH (Poole, U.K.)

and ultrapure urea was from Schwartz-Mann (Orangeburg, N.Y.). Trypsin, Dulbecco's phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were from GIBCO (New York, N.Y.). Ampholines were from LKB (Bromma, Sweden). All other reagents were obtained from the Sigma Chemical Co. (St. Louis, Missouri).

Embryo labelling

Embryos were flushed from oviducts or uteri with PBS and immediately transferred to DMEM medium without unlabelled methionine but containing 1–4 mCi ml^{-1} [^{35}S]methionine and 1% FCS. After 4–6 h of incubation, the embryos were washed and placed in 25 μl of lysis buffer (SDS sample buffer). Culture of blastocysts was done in DMEM (complete amino acids) with 10% FCS until they artificially implanted onto the culture dish surface.

Two-dimensional polyacrylamide gel electrophoresis

Isoelectric focusing followed by polyacrylamide gel electrophoresis was performed according to the basic methodology of O'Farrell (1975) with the modifications described by Silver *et al.*

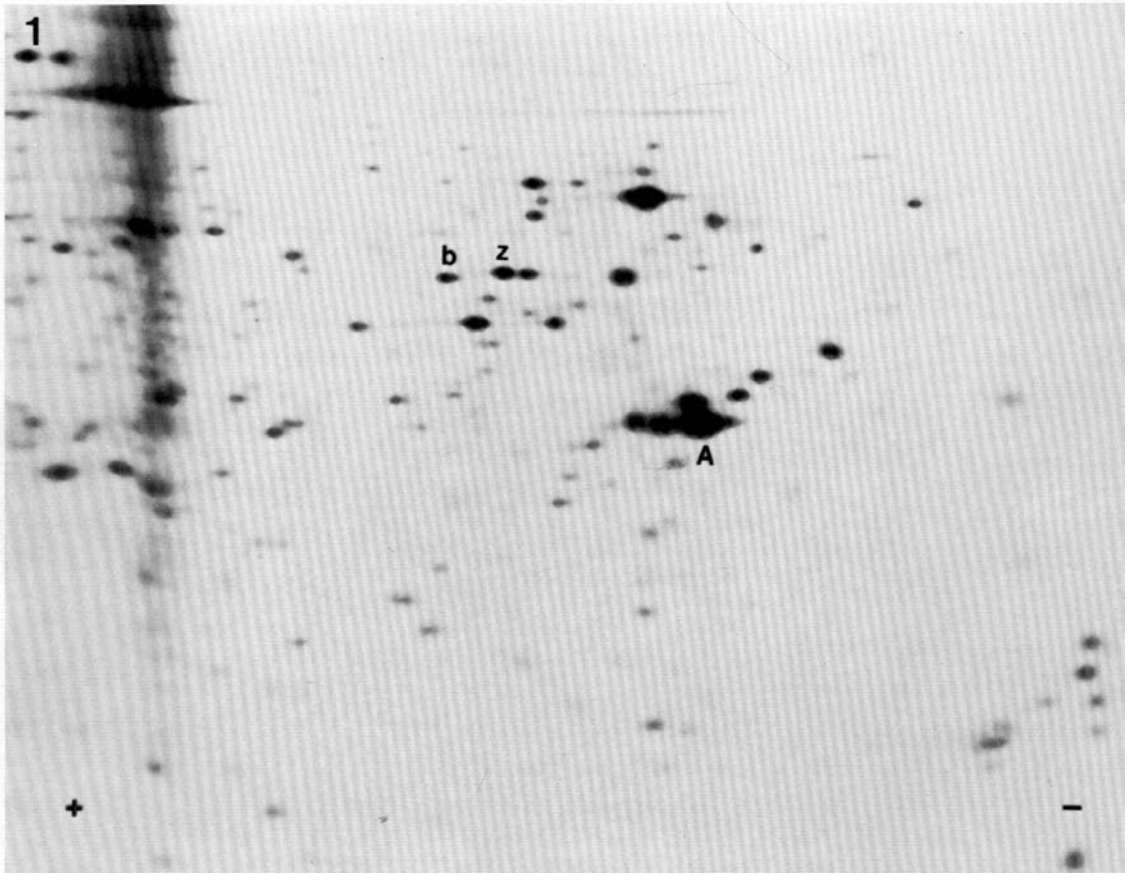
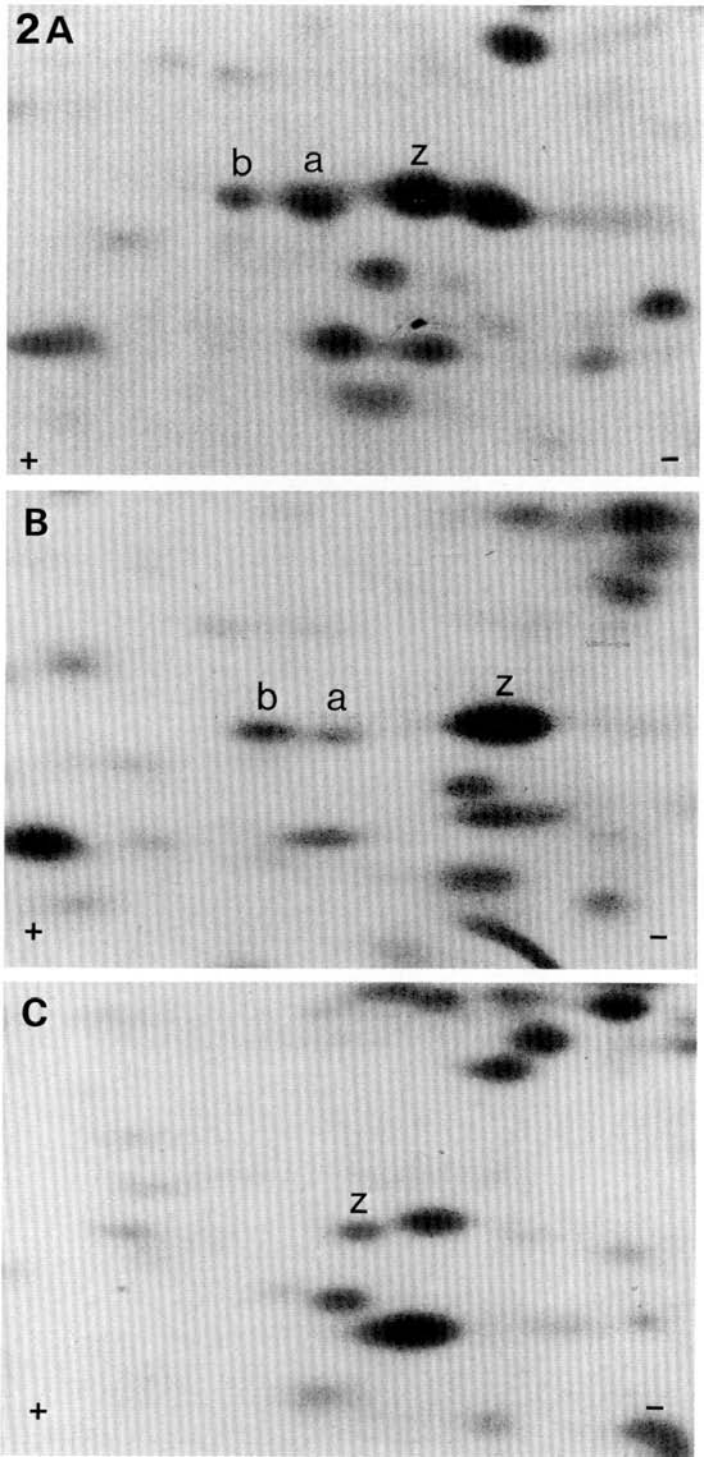


Fig. 1. 2-D gel fluorogram of [^{35}S]methionine-labelled, NP-40 soluble proteins of 70 blastocysts derived from +/+ × +/+ matings. 100 000 c.p.m. loaded, 4-day exposure. This autoradiogram was not preflashed. Basic (+) and acidic (−) ends are indicated. Relative molecular masses decrease from top to bottom. The p63/6.9b, z, and actin (A) protein are indicated.


2A
b
a
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C
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(1979). Gels were washed in two changes of dimethyl sulphoxide (DMSO), impregnated with PPO, dried in a gel dryer and exposed to preflashed Kodak X-Omat XR-5 film at −70 °C for the indicated number of days.

Microdensitometry

Measurements of the relative amount of p63/6.9a and p63/6.9b protein in two-dimensional gel fluorographs were performed using a Joyce-Loebl microdensitometer. Absorption peaks were traced in the isoelectric focusing dimension with the beam width adjusted to encompass the diameter of the protein spots. Areas in mm^2 under each absorption peak were then measured with a Leitz Digitizing Analyzer. Except where indicated, the Kodak X-Omat XR-5 film was preflashed according to the method of Laskey & Mills (1975) in order to generate a linear relationship between spot optical density and protein concentration.

RESULTS

The synthesis of p63/6.9 by preimplantation embryos was examined by *in vitro* labelling with [^{35}S]methionine. Two-dimensional fluorographs of 2-cell ($1\frac{1}{2}$ days *postcoitum*), morula ($2\frac{1}{2}$ days *p.c.*) and early blastocyst ($3\frac{1}{2}$ days *p.c.*) stage proteins were performed.

Identification of these proteins was based on a constellation approach; comparing our gels with those previously published by L. M. Silver and co-workers (1979, 1980, 1982), followed by genetical tests for identity. For example, embryos from wild-type CD-1 parents gave the protein pattern seen in Fig. 1. In this case, p63/6.9b is easily detectable; as is the p63/6.9z protein, a nearby reference protein with no known allelic variants. This protein has been found to be expressed by testicular cells for all strains of mice examined to date (Silver & White, 1982). In contrast, blastocysts from the cross t^0/Rb7 × t^0/Rb7 show the presence of both p63/6.9a and p63/6.9b (Fig. 2A). Morula-stage embryos from +/+ × +/t^0 matings also express both p63/6.9a and b (Fig. 2B). However, 2-cell embryos from +/+ × t^0/Rb7 show no detectable amounts of either protein (Fig. 2C). The number of 2-cell embryos labelled (79) and the total radioactive counts run for this gel (68000 c.p.m.) indicate that the p63/6.9 protein is either not synthesized at this stage of development or that the amounts produced are not detectable by these techniques. The number and intensity of other proteins seen in this autoradiogram (entire gel not shown) suggest that the lack of p63/6.9 is not due to an overall depression of protein synthesis.

Embryos that are placed in culture media continue to synthesize p63/6.9 when *in vitro* 'implantation' is allowed to occur. Hatching of the blastocyst from the zona pellucida usually takes place on day 3 of culture, when embryos are flushed as

Fig. 2. Partial 2-D gel fluorographs of NP-40 soluble proteins of blastocyst (A), morula (B) and 2-cell embryo (C) stages. Each fluorograph shows the p63/6.9a, b and z proteins. Basic (+) and acidic (−) ends as indicated. Relative molecular masses decrease from top to bottom. (A) 68 blastocysts from the cross +/t^0 × +/t^0. 20,000 c.p.m. loaded, 56-day exposure. (B) 62 morulae from the cross +/+ × +/t^0. 100,000 c.p.m. loaded, 16-day exposure. (C) 79 2-cell embryos from the cross +/+ × +/t^0. 68000 c.p.m. loaded, 20-day exposure.

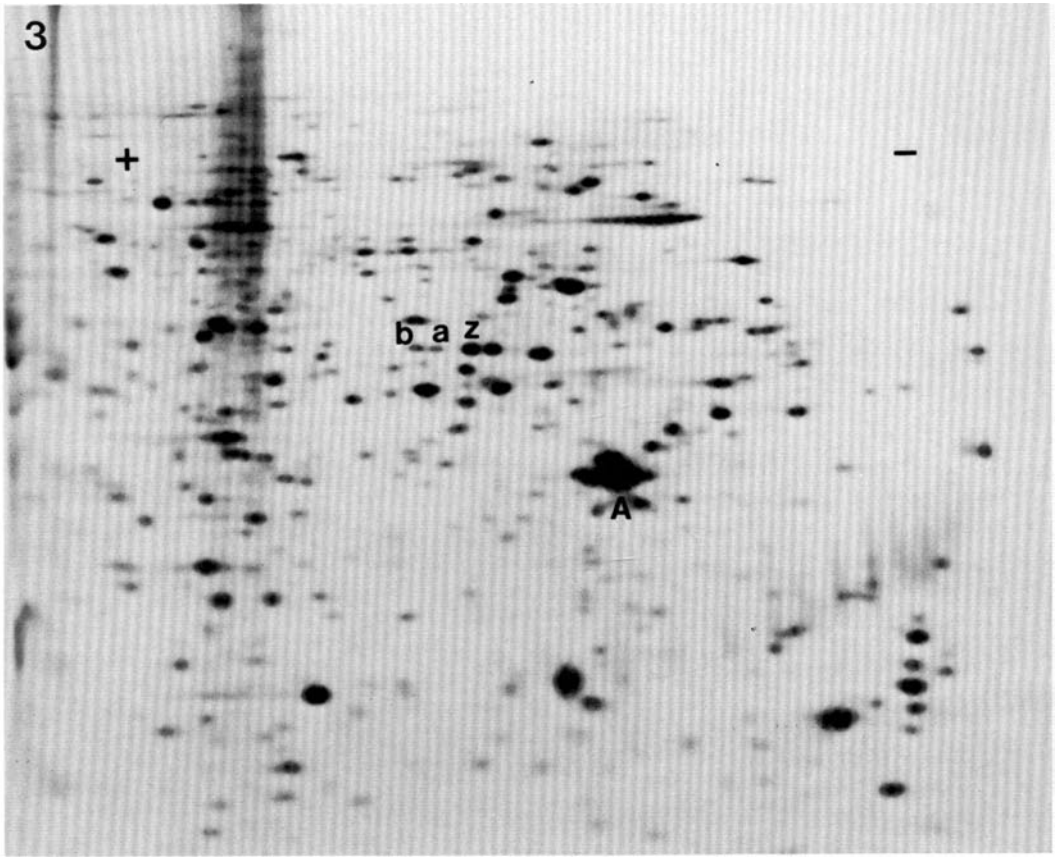

Fig. 3. 2-D gel fluorograph of NP-40 soluble proteins of a single explant stage embryo (day 4 *in vitro*) obtained from a $+/t^0 \times +/t^0$ mating. 99000 c.p.m. loaded, 6-day exposure. Basic end (+) on left, acidic end (−) on right. Relative molecular masses decrease from top to bottom. p63/6.9 proteins are designated a, b, and z. The actin protein is designated A.

blastocysts. Implantation onto the culture dish surface takes place on day 4. At this time, these explants increase the level of protein synthesis so that two-dimensional gels from single embryos can be obtained. Fig. 3 is the protein pattern of a single explant derived from a $+/t^0 \times +/t^0$ mating. This embryo can now be unambiguously genotyped as *Tcp*-1^b/*Tcp*-1^a.

Early blastocysts from t^0/Rb7 × t^0/Rb7 matings appear to synthesize more p63/6.9a than b (Fig. 2A). However, this difference is not likely to be due to altered rates of synthesis and/or degradation but rather to the relative amounts of t^0 genotypes present in the embryo population. The t^0/Rb7 males used in these crosses donate the t^0 allele to 99% of their offspring (mean, 99·2; S.D., ±1·8; $P \ll \cdot001$). This transmission ratio distortion would result in a population of

embryos in which approximately one half are t^0/t^0 and one half are $+/t^0$. Thus, a p63/6.9a to b ratio of 3:1 would be expected if there is one equally transcribed copy on each type of chromosome.

In order to test whether the observed amount of p63/6.9 allelic forms noted in our gels correspond to an effect of *t* complex determined transmission ratio distortion on the genotypes in the embryo pool, we performed microdensitometric tracings of these proteins. The results can be seen in Table 1. In the case of blastocysts, the observed and the expected p63/6.9a to b (a/b) ratios for both the $+/+ \times t^0$/Rb7 and the t^0/Rb7 $\times$ t^0/Rb7 matings are nearly identical (1·02 *vs* 1·00 and 2·98 *vs* 3·00, respectively). It should be noted that t^0/Rb7 females do not exhibit distortion of segregation ratios (mean % *t* offspring = 61; $P = 0{\cdot}1$). It is clear from these values that the ratio of a to b in blastocysts is the result of transmission ratio distortion. Furthermore, mechanisms operating to change equimolar amounts of p63/6.9a and b (e.g. gene dosage, differential synthesis or degradation) may not be in effect.

However, morula-stage embryos do not give a/b ratios which fit this hypothesis. In this case, the expected a/b of 1·00 for the $+/+ \times t^0$/Rb7 cross is twice as much as that observed (0·48). It is probable that the observed ratio is the result of delayed expression of the paternally derived 17th chromosomes.

The ratios of a to b observed (1·53) for pooled explants is considerably lower than expected (3·00) given transmission ratio distortion. However, it has been previously reported (Erickson & Pederson, 1975) that putative t^0 homozygous embryos when implanted *in vitro* lose their inner cell masses, which for normal embryos sit on top of a layer to trophoblast cells. Thus, this embryo pool would contain a reduced amount of *t* specific proteins since the t^0/t^0 embryos are contributing less biomass. Given the above, we would expect the a/b ratio to shift in favour of b, as already observed. Moreover, we would expect that normal explants would contain approximately equal amounts of p63/6.9a and b. This is apparently the case for each of two individual explants that were typed as *Tcp*-1^b/*Tcp*-1^a (a/b = 0·75 and 1·14).

Table 1. *Microdensitometry of* Tcp-*1 gene products (p63/6.9a,b) expressed in embryos*

Stage	Cross or genotype (♀) (♂)	Number	Areas (mm²) a	Areas (mm²) b	a/b	Expected genotype ratio	Expected a/b
Morula	$+/+ \times t^0$/Rb7	62	493·4	1022·9	0·48	100% $+/t^0$	1·0
Blastocyst	$+/ \times t^0$/Rb7	55	1061·9	1043·0	1·02	"	1·0
Blastocyst	t^0/Rb7 $\times$ t^0/Rb7	44	593·1	198·9	2·98	50% t^0/t^0; 50%t^0/Rb7	3·0
Explant	t^0/Rb7 $\times$ t^0/Rb7	92	978·6	638·7	1·53		3·0
Explant	t^0/Rb7	1	302·1	404·1	0·75	—	1·0
Explant	t^0/Rb7	1	624·2	548·3	1·14	—	1·0
Explant	Rb7/Rb7	1	—	243·0	—	—	—
Explant	t^0/t^0	1	99·4	—	—	—	—

DISCUSSION

We have presented evidence that new synthesis of the p63/6.9 proteins does not begin until the morula stage. Thereafter, these proteins are easily detectable in blastocysts and cultured embryos. Our observation that 2-cell embryos do not express either form of the p63/6.9 proteins suggests that synthesis of these products from stored maternal RNA or from newly transcribed sequences is not occurring at this stage.

One- and two-dimensional gel electrophoresis studies of mouse preimplantation embryos have been reported by a variety of investigators. Zygote and early 2-cell embryos demonstrate an almost identical pattern of protein synthesis as compared to oocytes (Van Blerkom & Brockway, 1975; Levinson, Goodfellow, Vadenboncoeur & McDevitt, 1978). The first example of an embryo-derived protein may be the 68kD, α-amanatin-sensitive protein of early 2-cell embryos (Flach *et al.* 1982). These same authors report that a major change from maternal to embryonic control of protein synthesis does not occur until the late 2-cell stage (29–32 h *p.c.*), a stage that roughly corresponds to the 2-cell embryos used in this study (36 h *p.c.*). More recently, two heat-shock proteins (HSP68 and HSP70) have been proposed as the first major products of zygotic gene activity in the mouse, occurring at the 2-cell stage (Bensaude, Balsinet, Morange & Jacobs, 1983). As many as 36 stage-specific polypeptides (SSPs) have been detected for stages extending from ova to early blastocyst (Levinson *et al.* 1978). Such developmental profiles have also been performed for cytoplasmic *versus* nuclear proteins (Howe & Solter, 1979). Tissue-specific spots have been described for microsurgically derived inner cell masses (ICM) and trophoblast vesicles (Van Blerkom, Barton & Johnson, 1976). Although it is apparent that oocyte-specific proteins disappear by the 8-cell stage, there is some controversy over when most SSPs appear. The most dramatic changes in the number of SSPs synthesized occur during the transition from zygote to 8-cell stage (Howe & Solter, 1979), or during the transition from morula to blastocyst (Cullen, Emigholz & Monahan, 1980).

Gene products expressed early in preimplantation development have usually not shown a difference in maternal and paternal allelic expression; e.g., β_2-microglobulin at the 2-cell stage (Sawicki, Magnuson & Epstein, 1981); β-glucuronidase at the 8-cell stage (Wudl & Chapman, 1976). However, delayed expression of the paternal allele of X-linked phosphoglycerate kinase, corrected for the lower expression in extraembryonic membranes, has been recorded (Krietsch *et al.* 1982). We have previously reported an effect of the chromosonal 17 mutation, *T*, causing delay of the appearance of F9 antigen when transmitted by the male (Erickson & Lewis, 1980). Thus, our finding of probable delayed paternal expression of Tcp-1 may be related to shared properties of a region of chromosome 17 and the X-chromosome in regards to imprinting (Erickson, 1985).

There have been few 2-D gel analyses of embryos segregating lethal genotypes. Some examples we are aware of were provided in the Introduction. More recently, quantitatively altered proteins have been detected by 2-D gel analyses of embryos

containing various trisomies (Klose & Putz, 1983). This relative paucity of information coming from 2-D gel studies during embryogenesis underscores the need for usable genetical markers. Our observation that the *Tcp*-1 locus product can be detected in morula, blastocysts and cultured embryos provides an opportunity to do genetic mapping of chromosome 17 linked proteins. This is especially feasible since two-dimensional gel fluorographs of single peri-implantation embryos can now be performed.

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