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Towards understanding paternal extragenic contributions to early amphibian pattern specification: the axolotl *ts–1* gene as a model system

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SUMMARY

As a model system for understanding the role sperm extragenic components might play in early embryogenesis the genetics and phenotype of the *ts–1* axolotl (*Ambystoma mexicanum*) mutant gene are reviewed. That mutant gene displays parental effects. It exhibits both maternal (egg-mediated) as well as paternal (sperm-mediated) phenotypic effects. A variety of possible modes of action of the *ts–1* gene are reviewed. Comparisons of various precedents to the *ts–1* genetic data are made. In addition, novel models which account for the *ts–1* phenotypic data are presented.

INTRODUCTION

Since the eggs of so many species including various marine invertebrates (Morgan, 1927), several insects (Morgan, 1927), a few lizards (Maslin, 1967), and some birds (Olsen, 1960), are capable of undergoing parthenogenesis (development of an embryo from an unfertilized egg) the sperm's contribution to early embryogenesis is generally regarded to be dispensable. True parthenogenesis, in which the egg is activated by electrical shock or pricking to initiate gynogenetic (haploid) development is, however, not observed in any of the common laboratory amphibia. For example, it has not been observed in anurans such as *Xenopus* and *Rana*, or in urodeles such as *Ambystoma* (axolotl) or *Pleurodeles*. Gynogenesis in those species requires, as a step in the activation procedure, the introduction of either inactivated (e.g. irradiated) sperm, blood cells, or cell extracts into the uncleaved egg (Fraser, 1971). Various investigations have dealt with the identification of the active factors in cell extracts which exhibit the capacity to promote cleavage in artificially activated eggs. Attention has focused primarily on components of the centrosome. It appears that amphibian eggs lack a fully functional centrosome. They may, however, contain either a partial centriole (Ramirez & Huff, 1967) or perhaps even a complete but inactive centriole (Manes & Barbieri,

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1977). Not surprisingly, microinjection of centriole preparations into *Xenopus* eggs promotes spindle formation and fragmentary cleavage (Maller *et al.* 1976).

Emerging from those studies is the idea that the sperm's contribution to early amphibian embryogenesis is primarily a structural one. Evidence for an informational role associated with early pattern specification is lacking. Any such informational role could be expected *a priori* to be subtle, and therefore difficult to detect. A genetic approach would probably be, in principle, the most productive way to uncover an informational function. Yet there exist no practical opportunities for employing selective screening methods for paternal mutants in the common laboratory amphibia. Furthermore, although mutagenesis is possible (Armstrong & Ortiz, 1978), it is cumbersome. A protracted generation time (approx. 1 year or longer) further complicates potential genetic analyses.

Nevertheless, several axolotl mutant genes which affect early pattern specification have been recognized during the course of an intensive inbreeding program at the Indiana University Axolotl Colony (reviewed by Malacinski, 1978). Most of the 'early-effect' mutants were originally recognized as maternal effects. Eggs spawned by females homozygous for the mutant gene arrest during early postfertilization stages, regardless of the genotype of the activating sperm. Included are the genes *o* (ova deficient), *cl* (cleavage arrest), *f* (fluid imbalance), *v* (blastula arrest), and *ts–1* (temperature sensitive; gastrula arrest).

This latter mutant (*ts–1*), although initially described as a maternal effect (Briggs & Briggs, 1984), has recently been discovered also to display a dramatic paternal effect. Recent genetic analyses and cytological studies indicate furthermore that a non-genetic component produced during both oogenesis and spermatogenesis apparently is involved in gastrular morphogenesis. In many regards the *ts–1* mutant phenotype is both unusual and unique. No similar mutant gene has yet been recognized in other developmental genetic systems. This gene provides, therefore, a rare opportunity to analyse the possibility that the amphibian sperm contributes informational as well as structural components to the developing embryo.

This report reviews the *ts–1* mutant phenotype. Most of the details which have so far emerged concern the maternal effect displayed by eggs spawned by *ts–1/ts–1* females. Much of that information will be considered here. The paternal effect will also be described. This latter aspect (paternal effect) provides evidence that is consistent with the notion that an extragenic component of axolotl sperm which is normally introduced to the egg at fertilization functions during early postfertilization embryogenesis and may be required for the completion of gastrulation.

REVIEW OF *ts–1* MUTANT PHENOTYPE

Brief description of ts–1 *developmental genetics:*

The *ts–1* gene displays a *parental* effect: the phenotype includes both a maternal effect as well as a paternal effect. One or the other of those effects is conditional (temperature sensitive), hence the designation *ts–1*. The maternal effect is

Table 1. ts − 1 *maternal effect and paternal rescue*

Cross	Genotypes		Embryonic survival	
	female	male	25°C	10°C
a	*ts − 1/ts − 1*	*+/+*	−	+
b	*ts − 1/ts − 1*	*+/ts − 1*	−	+
c	*ts − 1/ts − 1*	*ts − 1/ts − 1*	−	−
d	*+/+*	*+/+*	+	+
e	*+/+*	*+/ts − 1*	+	+
f	*+/+*	*ts − 1/ts − 1*	−	−

exhibited as a developmental arrest at gastrulation of eggs shed by a female which is homozygous for the *ts−1* gene and raised at 25 °C (Briggs & Briggs, 1984). Introduction of the wild-type allele at fertilization fails to prevent that 25 °C developmental arrest. Development at 10 °C is, however, normal if eggs shed by a homozygous recessive female are fertilized by sperm from either a wild-type or a heterozygous male (Table 1). Thus, it is possible to speculate that a paternal effect is being observed, namely the conditional (temperature-sensitive) rescue of eggs shed by *ts−1* females.

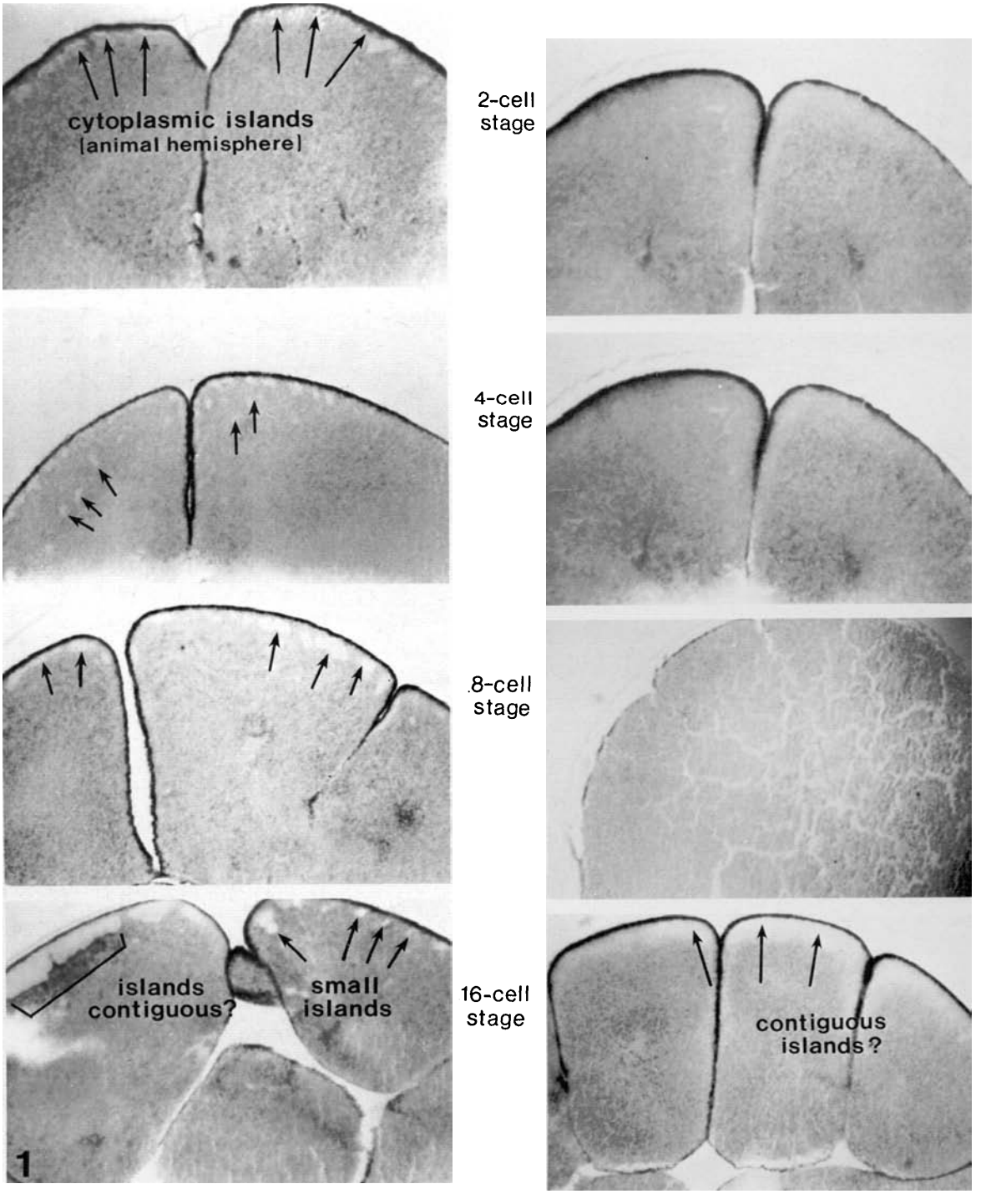
Survival at 10 °C among the progeny of crosses a and b is identical (approx. 90 %). Progeny testing has identified both *+/ts−1* and *ts−1/ts−1* genotypes among the progeny of cross b. Sperm bearing the mutant allele (*ts−1*) can therefore participate in development. Hence, a non-genetic (extragenic) sperm contribution can be considered as rescuing *ts−1* eggs (at 10 °C). Another interpretation is, however, possible and it will be discussed later.

Morphogenesis of mutant eggs – gastrula arrest

At 25 °C, eggs shed by a *ts−1* homozygous female uniformly (90 %) arrest at gastrulation, regardless of the genotype of the fertilizing male. Wild-type eggs also consistently (>90 %) arrest when fertilized by sperm from a *ts−1/ts−1* male (Table 1). Until the onset of gastrulation, however, those eggs are morphologically indistinguishable from control embryos (wild-type eggs fertilized with wild-type sperm). Cleavage proceeds normally and the shift from synchronous to asynchronous division (Signoret & Lefrense, 1971) appears to occur on schedule.

During gastrulation, usually at the early-dorsal-lip stage (st. 10), but certainly no later than the mid-yolk-plug stage (st. 11½), morphogenesis comes to a halt. Typically, the animal hemisphere takes on a convoluted appearance and the vegetal hemisphere shows signs of exogastrulation. While normal (control) embryos at 25 °C are proceeding through neurulation, *ts−1* embryos begin cellular cytolysis. Within 1–2 days most of the cells in *ts−1* embryos appear to have degenerated.

As indicated in Table I, eggs shed by homozygous recessive females fertilized with sperm from wild-type or heterozygous male, and raised at 10 °C exhibit an


cytoplasmic islands
(animal hemisphere)
2-cell stage
4-cell stage
8-cell stage
16-cell stage
islands contiguous?
small islands
contiguous islands?
1

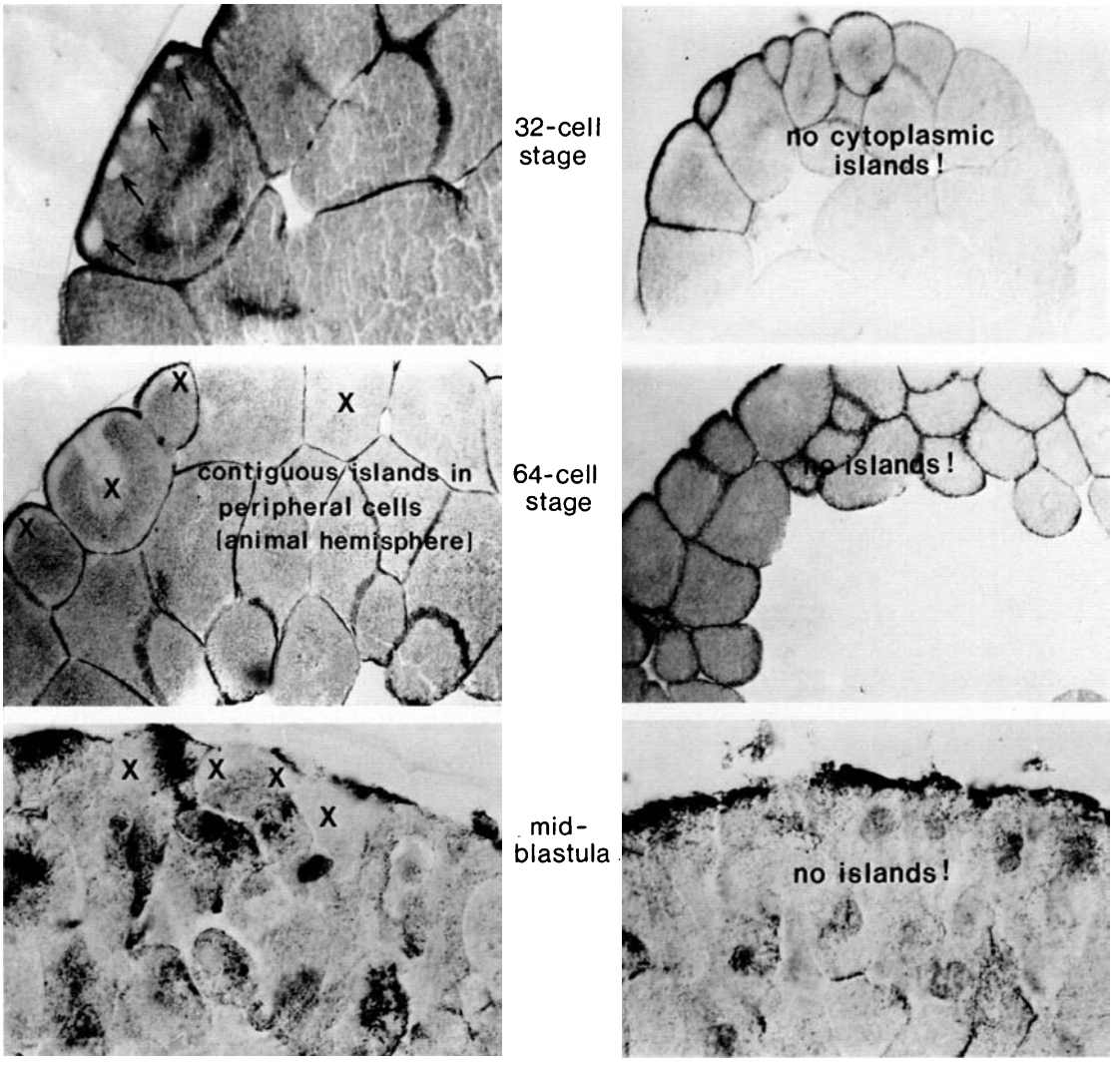


Figure 1. Areas of yolk-free cytoplasm in the animal hemisphere of eggs shed by 25 °C *ts−1/ts−1* female (left column). Those 'clear islands' (arrows) persist throughout early embryogenesis, but are not present in wild-type eggs (right column). Paraffin sections stained with Feulgen–fast green.

altogether different fate. They develop normally through gastrulation, organogenesis and larval stages right up to sexual maturity. Indeed, this conditional (temperature-sensitive) survival provides a useful way to obtain both heterozygous (Table 1 – crosses a and b) and homozygous (Table 1 – cross b) breeding stock.

Eggs from a wild-type female fertilized with sperm from a *ts−1/ts−1* male arrest at both temperatures (Table 1 – cross f). Gastrular arrest in those embryos resembles, in general terms, the aberrant morphogenesis just described for crosses a–c (Table 1). That failure of wild-type eggs to develop beyond the late blastula or

early gastrula stage is open to two alternative interpretations. According to the first interpretation, the product of the normal allele of *ts−1* is required for gastrula morphogenesis. When that gene product is lacking, as in cross f, morphogenesis ceases at the mid–late blastula stage. According to the second, sperm from a homozygous recessive male contain an altered gene product which is destructive, and leads to gastrular arrest.

Gastrular arrest represents, therefore, the primary gross morphological effect of the mutant gene. Clearly, the product of the normal allele of the male's *ts−1* gene is either directly or indirectly involved in one or another event which is eventually associated with gastrular pattern specification.

At intermediate temperatures (eg. 18 °C) the mutant phenotype associated with crosses a and b is expressed in a variable fashion. Many embryos exogastrulate, and others display a swollen prospective gut. Some actually develop as far as the tadpole stage, but fewer than 20 % survive beyond larval stages.

Temperature shift data

A preliminary series of 'shifts up' and 'shifts down' in temperature have been carried out with embryos from cross a (Briggs & Briggs, 1984). The data reveal that it is necessary for eggs from a *ts−1/ts−1* ♀ × +/+ ♂ cross to be raised at 10 °C only during the period between the 9th and 12th cleavage for survival beyond the completion of gastrulation to be realized. That period coincides with the stage during which the synchronous cell division program becomes asynchronous (Signoret & Lefrense, 1971). It is not clear, however, whether at higher temperatures (eg. 25 °C) the *ts−1* gene product is inactivated, or whether an event which is normally stabilized by that gene product is altered. That is, it is unknown whether 25 °C acts directly or indirectly on the *ts−1* gene product which is involved in gastrular morphogenesis. Further experiments are called for, including temperature pulse analyses and the isolation from sperm of the active component.

Cytological examination of ts−1 *embryos*

Histological analysis of eggs shed by *ts−1/ts−1* females indicates that by the first cleavage division cytoplasmic abnormalities are detectable. Localized in the animal hemisphere are a series of clear 'islands' or 'patches' of yolk-free cytoplasm. Similar 'clear islands' are not present in control eggs (wild-type eggs fertilized with wild-type sperm) (Fig. 1). Those islands persist throughout early embryogenesis. They remain more or less constant in size and continue to be localized in the animal hemisphere (Fig. 1). Examination with the transmission electron microscope reveals, most notably, the absense of yolk platelets and other larger inclusions (Fig. 2). Those islands are present only in eggs raised at 25 °C. It is not known whether they disappear when eggs are shifted down to 10 °C.

Those 'clear islands' represent somewhat of a paradox. Although they definitely represent the first detectable phenotypic effect of the *ts−1* gene, their presence does not condemn an embryo to certain gastrular arrest. Those same 25 °C eggs which

display islands can be rescued if shifted down to 10 °C prior to the 9th cleavage. Nevertheless, their study will be pursued since they may represent a valuable probe for the analysis of the cytoplasmic organization. It is known for example that the amphibian egg cytoplasm is organized into a series of density compartments (Neff, Wakahara, Jurand & Malacinski 1984). The *ts−1* gene may eventually be useful for understanding the structural basis of those density compartments.

Cytological examination of wild-type eggs fertilized with ts−1 *sperm:*

Although wild-type eggs fertilized by *ts−1* sperm (Table 1 – cross f) share a common fate with 25 °C *ts−1* eggs (crosses a–c), gastrular arrest, they lack the cytoplasmic islands illustrated in Fig. 1. Careful examination of eggs from cross f failed to reveal the 'clear islands' described above (Fig. 3). That observation reinforces the notion that 'clear islands' are not a prerequisite for gastrular arrest. Rather, the 'clear islands' most likely represent a side effect of the action of the mutant allele of the *ts−1* gene during the construction of the egg in oogenesis.

Eggs from cross f display pycnotic nuclei and chromosomal abnormalities as early as the 128-cell stage (Fig. 3). This observation contrasts with data to be described below for *ts−1* eggs.

Chromosomal abnormalities of ts−1 *blastulae*

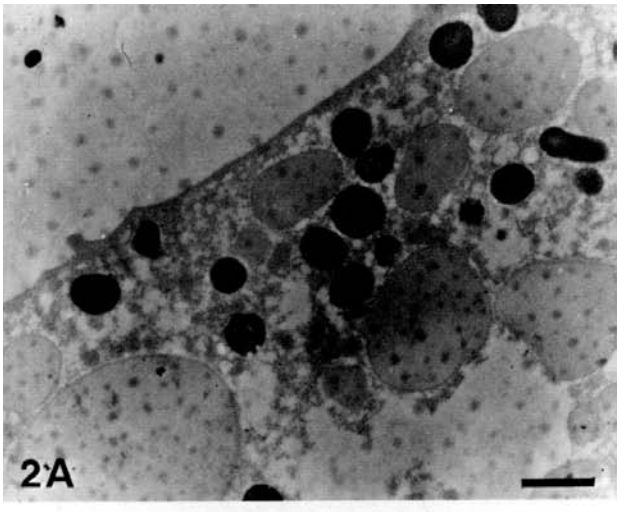
Prior to the arrest of morphogenesis at gastrulation but after the temperature-sensitive period has begun, anaphase or telophase bridges, occasionally together with more severe chromosomal abnormalities (eg. variable chromosome numbers), are common in the 25 °C *ts−1* embryos of cross a (Briggs & Briggs, 1984). Those phenotypic effects probably, however, follow rather than precede the initial events which condemn an embryo to gastrular arrest.

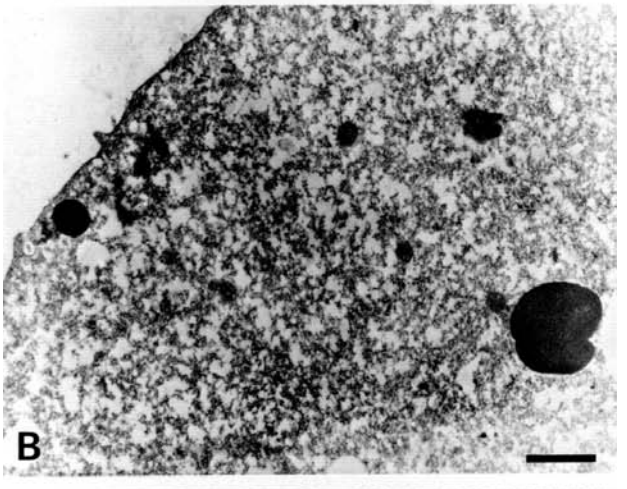
REVIEW OF SPERM CONTRIBUTIONS TO EARLY EMBRYOGENESIS

In order to develop a model for *ts−1* gene action, several precedents for sperm contributions to embryonic survival or early pattern specification will be reviewed, and their relevance to the *ts−1* mutant phenotype discussed.

Wild-type allele contributions to pattern specification

Needless to say, the sperm contributes a haploid genome to the zygote. In most species the presence of that haploid genome is, however, not required for early pattern specification since development to the organogenesis stage proceeds in gynogenetic haploids. The *ts−1* mutant phenotype cannot be explained simply by rescue mediated by the introduction of the wild-type allele at fertilization. Among the progeny of cross b both *+/ts−1* and *ts−1/ts−1* animals were identified. A *ts−1* allele introduced at fertilization is therefore capable of supporting development (at 10°C) all the way to sexual maturity. One explanation for that observation is that an extragenic component of sperm is required by all eggs, including those from


2A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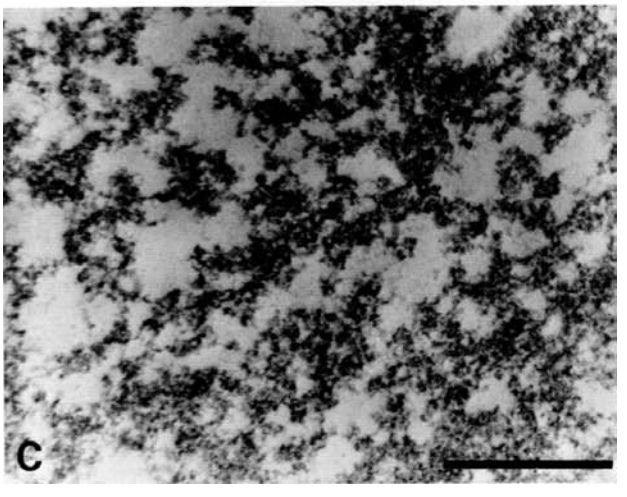
C

+/+ females (cross f), for survival beyond gastrulation. The data from cross c supports that interpretation: Males lacking the wild-type allele of the *ts−1* gene could be producing sperm which are unable to rescue *ts−1* eggs, presumably because those sperm lack the extragenic component.

Centriole contributions for stimulating cleavage

As mentioned in the Introduction, it is unclear whether amphibian eggs contain a centriole which has the full potential to become functionally active. It is apparent, however, that sperm fractions which include the centriole can promote cleavage in amphibian eggs (Maller *et al.* 1976). A bonafide contribution of a structural component from the fertilizing sperm to the early embryo is therefore well documented. In the case of the *ts−1* phenotype, more than a centriole is obviously required for temperature-sensitive (10 °C) survival. Eggs which do not survive (eg. 25 °C; cross f) display superficially normal cleavage furrow formation patterns right up to the gastrulation stage.

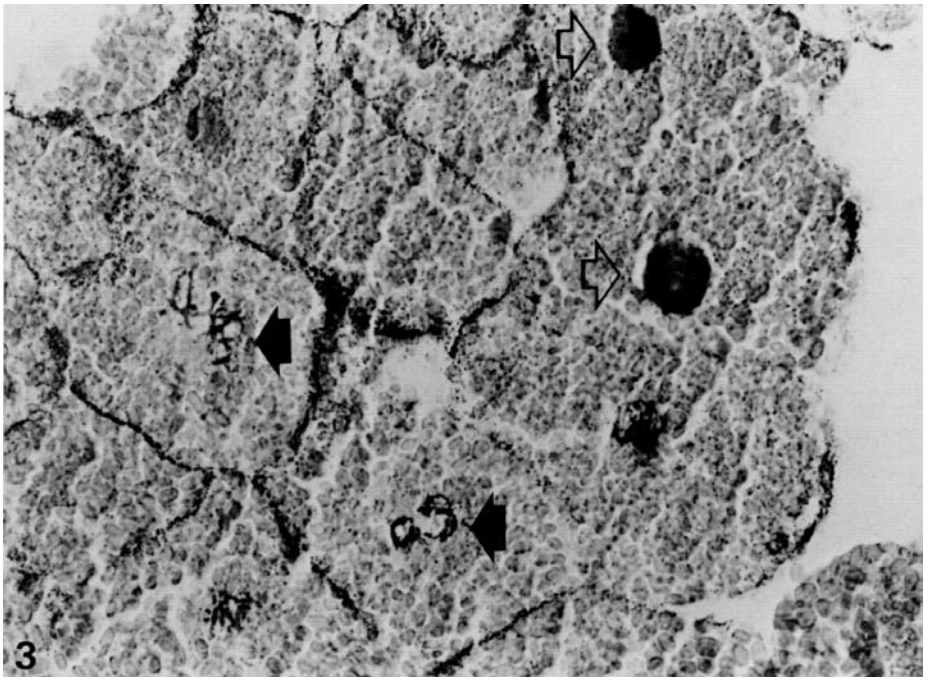

Figure 3. Histological examination of wild type eggs fertilized with sperm from a *ts−1 /ts−1* male (Table 1, cross f). Although 'clear islands' are absent, at the 128-cell-stage pycnotic nuclei (open arrows) as well as chromosomal abnormalities (solid arrows) are visible.

Figure 2. Transmission electron micrographs of (A) animal hemisphere cortical cytoplasm of normal (wild-type) embryo at 2-cell stage (×10 000). Note presence of pigment granules and yolk platelets; (B) similar region of 2-cell egg shed by *ts−1/ts−1* female (cross a), also ×10 000. Note absence of yolk platelets; (C) higher magnification (×50 000) of region shown in (B). Bar for (A) and (B) = 1 μm; bar on (C) = 0·5 μm.

Seminal fluid contributions for increased fertility

Well-documented cases of seminal fluid materials enhancing fertility in insects are available (reviewed by Chen, 1984). In those instances male accessory glands secrete materials into either the seminal fluid or spermatophore. Those secretory products are transmitted to the female during copulation, and either directly or indirectly raise fertility. Among the crosses included in Table 1 fertility (measured by percentage fertile eggs) did not vary significantly among the male genotypes. An influence of the *ts−1* mutant gene on the functional activity of the axolotl spermatophore is consequently ruled out. It should also be mentioned that the role of the spermatophore can be bypassed by artificial insemination methods which employ vas deferens homogenates.

Differentiation states 'imprinted' in the sperm genome persist in embryogenesis

Evidence for the establishment of a stable regulatory mechanism (imprinting) during gametogenesis comes from mammalian nuclear transfer experiments. Specifically, paternal gene expression may be required for the development of extraembryonic tissue (Surani Barton & Norris, 1984; McGrath & Solter, 1984). In the *ts−1* system cross b reveals that both wild-type and *ts−1* sperm are active in promoting development in *ts−1* eggs, since both +/*ts−1* and *ts*/*ts* animals were detected among the progeny. Imprinting is accordingly not a plausible explanation for the extragenic contribution associated with the *ts−1* gene.

Sperm surface proteins retained through early embryogenesis

Several sea urchin sperm polypeptides persist in the embryo up to the late gastrula stage. The membrane fusions occurring between egg and sperm during fertilization generate a 'patch' of sperm-derived proteins on the surface of the embryo (Gundersen & Shapiro, 1984). The role of the 'patch' remains, however, to be resolved. True parthenogenesis is possible in sea urchin eggs, so an obligatory role of 'patch' sperm proteins in pattern specification can be ruled out. At present, there is no reason to expect *ts−1* gene products to resemble components of the sea urchin sperm patch. Indeed, assuming that the cross b data indicate that a sperm extragenic component rescues (at 10 °C) *ts−1* eggs, that component would have to be produced prior to the completion of meiosis. However, acquisition of sperm surface proteins probably occurs during spermatid differentiation, a postmeiotic event.

Conclusion regarding precedents for the ts−1 *gene*

The above examples of paternal contributions indicate that among different species sperm components play various roles. Unfortunately in no instance has evidence accumulated which would serve as a model for the *ts−1* mutant phenotype. As a result it is necessary to formulate a novel model to account for the *ts−1* data.

TWO ALTERNATIVE MODELS WHICH EXPLAIN THE *ts−1* DATA

The genetic data included in Table 1 are open to two alternative interpretations. One interpretation would state that the product of the normal allele of the *ts−1* gene is required for embryonic survival beyond gastrulation. The alternative explanation views the *ts−1* mutant gene product as destructive. Its presence would contribute to embryonic death. Those very different notions will be briefly described below.

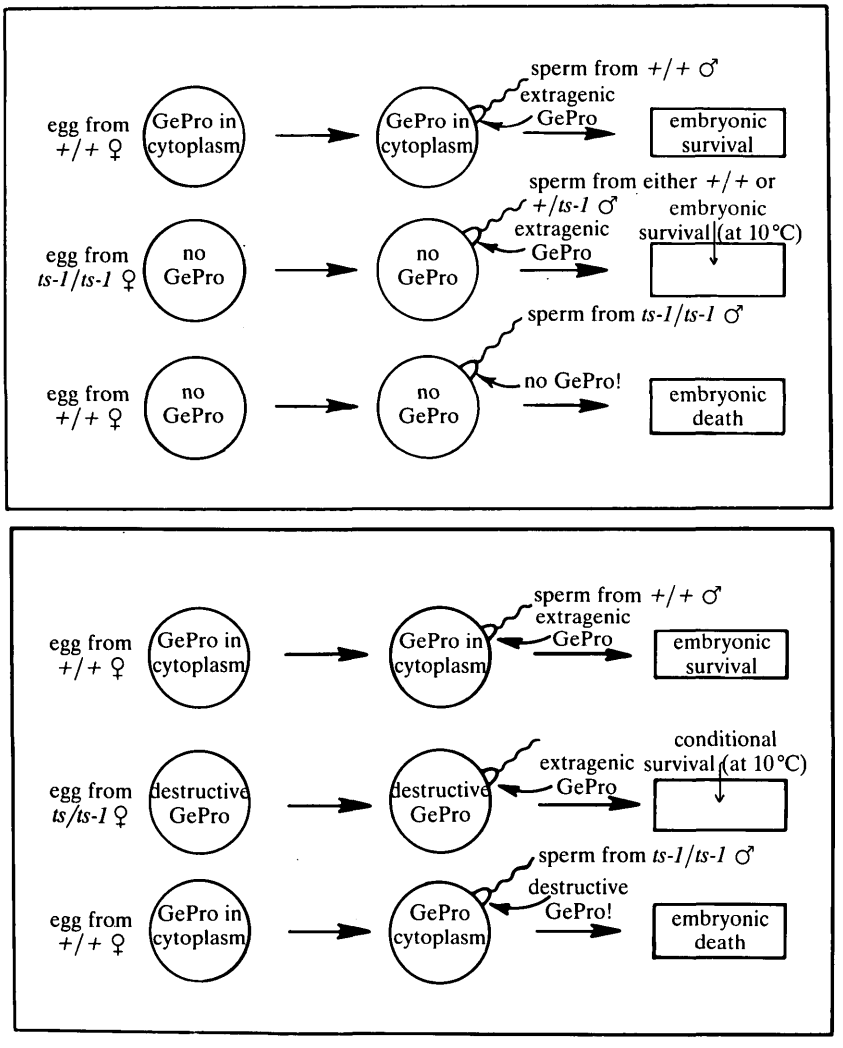

Figure 4. Two alternative models for which explain *ts−1* genetic data. Top: *ts−1* gene product ('GePro') plays a constructive role in postfertilization embryogenesis. Bottom: Sperm from *ts−1/ts−1* male contributes a lethal mutant gene product ('destructive GePro') to egg.

ts−1 *gene product is normally required for embryogenic survival*

The *ts−1* gene product is produced during both oogenesis and spermatogenesis. Wild-type eggs contain *ts−1* gene product, but for embryonic survival both the sperm's contribution of that extragenic component as well as the egg's supply is required. In the absence of the sperm's contribution, eggs spawned by a wild-type female arrest (cross f). Eggs spawned by a homozygous recessive female lack the *ts−1* gene product, but can be rescued (at 10 °C) by the sperm's contribution of the *ts−1* gene product (crosses a and b). This model is illustrated in Fig. 4 (top). The *ts−1* gene is viewed as displaying an unconditional maternal effect ('clear islands' in fertile eggs; developmental arrest at gastrulation – crosses a–c) and a conditional (temperature-sensitive) paternal effect (rescue of the maternal effect at 10 °C – crosses a and b).

Mutant ts−1 *gene product is destructive*

The *ts−1* gene product is likewise produced during both oogenesis and spermatogenesis. Eggs constructed in *ts−1/ts−1* females are defective (eg. contain 'clear islands'), and will support embryogenesis only at the permissive temperature (10 °C – crosses a and b). Sperm which developed in *ts−1/ts−1* males contains a mutant gene product which is lethal. In each of the two crosses (c and f) in which sperm contribute lethal (mutant) gene product, to the embryo, developmental arrest ensues (Fig. 4, bottom).

In contrast to the former model, in this model the maternal effect is considered to be conditional (temperature sensitive – crosses a and b). The paternal effect is viewed as being both lethal and unconditional.

Conclusion regarding the two models

Each of the models is entirely consistent with the data included in Table 1. Microinjection tests employing either egg cytoplasmic fractions or sperm homogenates will perhaps be useful in distinguishing between the 'constructive' *versus* 'destructive' contributions postulated above.

CONCLUDING REMARKS

The role that extragenic sperm components play in early postfertilization pattern specification probably varies somewhat from species to species. That role is no doubt subtle, for artificial parthenogenesis is possible in many species. Since amphibian embryos display a requirement for a sperm contribution, they perhaps represent one of the potentially more useful systems for understanding the function of sperm components. Developmental genetics provides, in principle, an approach to gaining insight into subtle or elusive phenomena such as the role sperm extragenic contributions might play in pattern specification. The *ts−1* mutant gene presents several unique phenotypic traits: (a) eggs spawned by *ts−1/ts−1* females display

cytoplasmic abnormalities; (b) spermatocytes as well as oocytes are affected by the *ts−1* gene; (c) the mutant allele contributes, either directly or indirectly, to gastrular morphogenesis; and (d) the phenotype is conditional (temperature sensitive).

Paternal effect mutations are extremely rare. Only a few other examples (eg. *C. elegans* – Wood *et al.* 1980) are known, and they have not yet been extensively exploited. With the inherent advantages of the amphibian embryo for microsurgical manipulation (eg. microinjection) and molecular biology, a detailed investigation of the *ts−1* mutant phenotype is warranted.

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The authors would like to dedicate this paper to the memory of Prof. Robert Briggs (deceased March 4, 1983) and Ms. Francoise Briggs (deceased April 10, 1985). R. B. provided the authors with an introduction to experimental embryology as well as many pleasant personal memories during a 20 year association. F. B. collaborated with the authors during the early stages of the project. She generated enthusiasm for the project in a kind, warm and friendly way. Both left memories which will be cherished by all who had the good fortune to benefit from their association.

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DISCUSSION

Speaker: G. Malacinski

Question from I. Dawid (NIH, Bethesda):
How strongly do you know that the mutated genes in the male and female are the same gene?

Answer:
The data in Table 1 represents a total of perhaps 30 crosses and we have cross-indexed them in all possible ways, followed the segregation in all possible ways, made predictions and done the matings. From this, they *appear* to be the same gene.

Question from Mae Wan Ho (Open University):
It seems to me the data indicates that although it is the same 'GePro', it is modified differently in male and female. It is possible that both modifications are necessary to development and that would explain your data. One way of testing would be to see whether the temperature-sensitive period is the same for male product as for female product. I wonder whether you have tested this?

Answer:
This is a very good point. Of course, another way of looking at it is that the same gene product just has different effects in egg *vs.* sperm, but you are proposing that a temperature-shift experiment on cross a and cross b might sort that out. We have not done it and are very eager to do so.

Question from J. Slack (ICRF, London):
One variable you have not mentioned is the fact that the axolotl is polyspermic. The fertilized egg will contain one sperm which contributed genes and other sperms which contributed non-genetic material. Presumably for some of the quantitative effects, you could count the number of sperm pits and correlate that with the degree of rescue. Have you any comments on this?

Answer:
That is a very good point. We have not done a systematic analysis – we'll do that one day. All I can say is that we don't see any trend in the data which would suggest that polyspermy provides an influence.

Question from D. Smith (Purdue):
I am not sure why you have to have an extragenic sperm product. Why not something made during oogenesis which is used up prior to the time of nuclear activation and then the sperm – wild type or whatever – carries the gene which replaces it?

Answer:

You don't have simple wild-type rescue. If you want the action of the gene to replace what has already been used up, you would expect that the introduction of the wild-type gene would provide the rescue, and we don't get that. In cross b there are both +/ts and ts/ts progeny.