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CHARACTERIZATION OF CELL-CELL INTERACTIONS IN NORMAL AND t^{w32} HOMOZYGOUS PREIMPLANTATION MOUSE EMBRYOS

BY

PHYLLIS M. JOHNSON

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Biology, South Dakota
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1976

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CHARACTERIZATION OF CELL-CELL INTERACTIONS

IN NORMAL AND t^{w32} HOMOZYGOUS

PREIMPLANTATION MOUSE EMBRYOS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Date

Head, Biology Department Date

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INTRODUCTION

Following fertilization, the preimplantation mammalian embryo undergoes a series of cleavage divisions and develops into a hollow sphere-like blastocyst complete with two distinct populations of cells, trophoblast and inner cell mass (ICM). Prior to the 8-cell stage physical cellular activity is limited to cleavage. At the 8-cell stage, however, blastomeres acquire the ability to form new junctions and increase their overall cell-cell contact with neighboring blastomes (compaction). Cells of 8-cell embryos also exhibit motility and a potential for active cell migration. A comprehensive characterization of cellular interactions in the 8-cell embryo will aid in the understanding of normal and abnormal preimplantation mammalian development.

In addition to investigating cellular interactions during normal mammalian morphogenesis, information pertinent to these interactions may also be derived from observations on abnormal developmental patterns of certain genetically-induced lethal systems. In the house mouse (Mus musculus), information on cell relationships can be obtained from studying lethal T-locus (Brachyury) mutants. The T-locus, located in linkage group IX of autosome 17, was first investigated by Dobrovolskaia-Zavadskaia, 1927 at the Institut Curie. Embryos with the T/T genotype are lethal at 10-11 days gestation (Chesley, 1932), T/+ embryos are viable with offspring possessing short tails (Brachyury), and homozygote recessives having lethal t alleles

(t^n/t^n) undergo death in utero. Interestingly, heterozygotes having a T/t^n genotype possess no tails. Thus by breeding T/t^n heterozygotes, one can maintain a balanced lethal line that produces only viable heterozygotes (T/t^n), since both homozygous dominant (T/T) and homozygous recessive (t^n/t^n) embryos die in utero.

The T-locus may represent an extremely important genetic region specifying the overall morphogenetic control of mouse development. Bennett, 1964 reports that in mice, embryos carrying the T allele (T/+, short tail; T/t^n, tailless; and T/T, lethal at 10-11 days gestation), "T affects the chordamesoderm, the primary inductive system of the embryo....The primary effects (of T) seem to be on the cellular construction of the notochord and on its surface properties, for example stickiness." Since t-lethal alleles (t^{12}, t^{w32}, t^0 or t^6, t^{w5}, and t^9) seem to affect processes of major morphogenetic significance, the T-locus appears to control certain fundamental switch points of mouse development. Silagi, 1962, page 64 concludes, "...the entire region (T-locus plus t-alleles) is responsible for centers of organization in the early embryo which produce substances needed to trigger the chain of inductions which make up development."

The t^{12} and t^{w32} are considered either to be the same (Bennett and Dunn, 1964) or different but closely associated alleles; Hillman and Hillman, 1975, p. 694 state "...the primary affect of these two alleles (t^{12} and t^{w32}) result in the same or in a closely associated developmental aberration". Embryos homozygous for t^{12} and/or t^{w32}

undergo developmental arrest during the morula-to-blastocyst transformation, a critical period of primary embryonic differentiation. Such arresting embryos should therefore provide a presumably abnormal system for analysis of cellular relationships.

Immediate objectives of the following studies were to characterize and compare normal versus lethal mutant (t^{w32}/t^{w32}) cellular interactions during preimplantation mouse development. Principal experimental methods used included embryo culture and microscopy. Control and mutant embryos were analyzed in vitro to identify chronological events and phenotypic characteristics of t^{w32} homozygotes. Cell-cell associations of uncompacted and compacted 8-cell control (ICR) embryos were analyzed by quantitative electron microscopy. Lastly, light microscopic analyses were performed on control and mutant (t^{w32}/t^{w32}) morulae.

This thesis is organized according to the following plan. The literature review contains information on many aspects of previous research done on the t^{w32}/t^{w32} or t^{12}/t^{12} embryo. Following that, reports of experiments done by this investigator are presented. Each report will contain a literature review, methods, results, and discussion pertaining to that experiment. A discussion of all experimental results will attempt to compare present findings with those of previous investigators. Finally, conclusions will be drawn regarding changes that occur in cell-cell relationships during preimplantation mouse embryogenesis and their overall morphogenetic significance.

LITERATURE REVIEW

Morphologic and In Vitro Studies

The t^{12} allele is the earliest acting (ontogenetically) known lethal in mammals. In the homozygous condition (t^{12}/t^{12}) embryos undergo developmental arrest during the morula-to-blastocyst transformation. Smith, 1956 suggested the failure is due to a lack of organization in the cells rather than an inadequate number of cells. In support, Lewis and Wright, 1935 reported fixed and stained blastocysts with only 30 cells. The t^{12}/t^{12} mutants appear to possess both rounded inner cell mass (ICM) cells and flattened trophoblast cells on the surface (Smith, 1956). Lewis and Wright, 1935 state that at the time of blastocoel formation although trophoblast cells appear flattened on the surface they project deeply into the morula. As blastocoelic fluid increases so does the flattening. Smith, 1956 then suggested a failure of blastocoel fluid formation or a failure of the trophoblast layer to retain the fluid as causes of the lethality. This retention failure could be due to gaps in the trophoblast layer, cell loss, lack of differentiation, or lack of adhesion between these cells. Calarco and Brown, 1968 found that t^{12}/t^{12} mutant cells are less mobile than normal cells and appear to be "more rounded and less closely applied to one another than do cells of normal embryos."

Nucleolar aberrations may also occur in t^{12} homozygotes; mutant nucleoli do not assume elongate shapes as they do in controls (Smith, 1956). In experimental crosses ($\male +/t^{12}$ x $\female +/t^{12}$) nucleoli either

undergo elongation within control blastomeres, or they remain rounded well up to late morula stages in arresting (presumably mutant) embryos (Hillman et al., 1970).

Dense agranular intranuclear bodies with fibrils radiating from them are found in t^{12} homozygotes but not in controls (Hillman et al., 1970; Hillman and Hillman, 1975). These fibrillogranular bodies can be identified as early as 2-cell stages in t^{12}/t^{12} embryos but are not observed in t^{w32}/t^{w32} embryos.

At the time of blastocyst formation normal embryos possess ribosomes mainly in clusters (polysomes) or in association with endoplasmic reticulum; free or single ribosomes are rarely found. Calarco and Brown, 1968 observed that mutant embryos possess both polysomes and granular endoplasmic reticulum plus greater than control numbers of single ribosomes. These single ribosomes were most often found in close association with crystalloid-like material but lacking the characteristic arrangement of crystalloids. Such bizarre ribosome-crystalloid arrangements were suggested by Calarco and Brown, 1968 to represent another phenotypic expression of the lethal t^{12} allele.

Another characteristic of t^{12} and t^{w32} homozygotes is the presence of excessive levels of cytoplasmic and intranuclear lipids. Excess cytoplasmic and nuclear lipids are observed in all cleavage stages but become more numerous as the embryos become older, approaching the time of lethality (Hillman and Hillman, 1975).

Although Calarco and Brown, 1968 found no variance in mitochondria of normal and mutant embryos, Hillman and Hillman, 1975 observed two

kinds of mitochondrial variants in the 8-cell, early morula, and late morula t^{w32}/t^{w32} embryos. Normal mitochondria are of two types: (1) round or ovoid with arc-shaped cristae and electron-dense matrix and (2) they are elongate with parallel cristae with matrices of lesser density. In contrast t^{w32} homozygotes contain only the round mitochondria electron-dense matrices (Hillman and Hillman, 1975). Mitochondria with crystalline inclusions, although not found in all embryos judged to be t^{w32} mutants, are also considered to be part of the lethal mutant phenotype (Hillman and Hillman, 1975).

When arrested embryos are examined, individual blastomeres are in different stages of degeneration ranging from healthy cells still undergoing mitosis to severely pycnotic cells. This phenomenon indicates an asynchronous cell death (Hillman and Hillman, 1975). The precise time of embryo death (the phenolethal period) appears to be a function of the number of viable cells in the embryo. In addition to asymmetric cell death, t^{w32} homozygotes also possess binucleate cells (Hillman and Hillman, 1975); these are not found in 2-cell and 4-cell embryos but become increasingly frequent as embryos age.

Thus, a number of t^{12}- and t^{w32}-induced developmental abnormalities have been identified by use of the electron microscope. Together, these characteristics describe the lethal t^{12} or t^{w32} syndrome. To summarize, such symptoms or characteristics of the t^{12} and/or t^{w32} lethal syndrome include: (1) abnormal cell-cell junctions, (2) nucleolar aberrations, (3) excess nuclear and cytoplasmic lipid inclusions, (4) nucleolar fibrillogranular bodies, (5) abnormal ribosome-crystalloid arrangements,

(6) abnormal mitochondrial variants, (7) binucleate cells, and (8) asynchronous cell death. In addition to ultrastructural analyses, behavioral observations of developing mouse embryos *in vitro* also provide data on characteristics of lethal mutants.

Following extensive *in vitro* observations on t^{12}/t^{12} and control embryos, Mintz, 1964a reports that mutants possess greater cytoplasmic granularity than controls. Mintz, 1964b further suggests that the changes which normally occur during cleavage (and absent in t^{12} homozygotes) are regulated by a "developmental clock". Two morphogenetically significant changes controlled by the "developmental clock" include increased adhesiveness between cells and increased cell motility during cleavage.

Following scanning electron microscopy of mouse embryos Calarco and Epstein, 1973 report a progressive series of changes affecting cell adhesiveness including interdigitating microvilli, circular specializations seen on smoother surfaces, and cell spreading which begins at the 8-cell stage. At the 4-cell and 8-cell stages there is some indication that microvilli begin to function in the adhesion of blastomeres (Calarco and Brown, 1969). Interdigitating microvilli serve in adhering blastomeres at the morula stage (Hillman et al., 1970; Calarco and Epstein, 1973; Hillman and Hillman, 1975; Ducibella and Anderson, 1975; and Ducibella et al., 1975).

Prior to blastocoelic fluid elaboration, specialized junctional complexes are formed in normal embryos. Calarco and Brown, 1968 found that although t^{12}/t^{12} cells appeared to be less closely joined, the

specialized complexes were in fact present between most peripheral cells (i.e., presumptive trophoblast cells). In contrast, however, Hillman et al., 1970 reported that degenerating peripheral cells neither form junctional complexes nor possess the interdigitating microvilli.

Microvilli and interdigitating microvilli of cleavage stage embryos form specialized cell associations defined as focal tight junctions (Ducibella et al., 1975). Such junctions are observed at 8-cell stages when cell flattening or spreading (Calarco and Epstein, 1973) occurs and precede zonulae occludentes of the morula stages (Ducibella et al., 1975). Cell flattening appears to begin at initial points of cell contact and propagate peripherally. This "zippering" action allows the cells to maximize their areas of cell-cell contact and become compacted. In compacted embryos individual blastomeres can no longer be distinguished at the light microscope level.

An interesting study by Spiegelman and Bennett, 1974 correlates ultrastructure with cell surface properties in another T-locus lethal mutant, the t^9 homozygote. The t^9/t^9 lethality occurs at the primitive streak stage (8-10 days in utero); apparently mutant primitive streak cells are not able to carry on normal migration. Bennett, 1975 states, "...it appears that in the mesoderm of mutant (t^9/t^9) embryos, abnormalities of cell shape, cellular interactions, and cell movement are related to the lack of surface adhesiveness and microfilament cytostructure."

Physiological Studies

In addition to ultrastructural analyses and *in vitro* observations, physiological investigations of developing mouse embryos also provide data on the generation of T-locus lethal syndromes. One characteristic phenotypic expression of t^{12} and t^{w32} homozygotes is failure of mutant nucleoli to undergo normal elongation during blastocyst formation. This nucleolar abnormality has led to investigations of RNA metabolism in t^{12} homozygotes. Neither Smith, 1956 using Azure B staining, nor Mintz (1964a,c), using ^{3}H-uridine, could demonstrate differences in total RNA content or in rates of RNA synthesis between mutants and controls up to the late morula stage; during blastocyst formation however, they found lowered total RNA content and lowered RNA synthetic rates in mutants as compared to controls. Calarco and Brown, 1968 found no decrease in cytoplasmic basophilia (i.e., Azure B staining) in t^{12}/t^{12} late morulae when compared to control morulae. Testing the hypothesis that t^{12}/t^{12} morulae are deficient in nucleolar RNA synthesis Hillman, 1972 labeled cleavage stage embryos of $+/t^{12}$ *inter se* matings with ^{3}H-5-uridine and measured their incorporation by electron microscope autoradiography. She was unable to show significant differences in either labeling index or label distribution between groups until late morula stages, when mutants had reached an advanced stage of degeneration. Furthermore, Hillman et al., 1970 suggest that the abnormally shaped round nucleoli present only in t^{12}/t^{12} late morulae and early blastocyst embryos "...are a result of degenerative changes within cells which originally contained normal elongated nucleoli." Hillman

et al., 1970 thus conclude that differences in ribosomal RNA content reported by Calarco and Brown, 1968 and Smith, 1956 are probably related to the length of time the mutants had been developmentally arrested. Hillman, 1972 thus concludes that the primary effect of the t^{12} allele is not related to ribosomal RNA metabolism.

Another phenotypic expression of the t^{12}/t^{12} lethality is the abnormal presence of nuclear lipid inclusions and excessive cytoplasmic lipids (Hillman et al., 1970; Hillman and Hillman, 1975). Both abnormalities are evident as early as the 2-cell stage in t^{12}/t^{12}, t^{w32}/t^{w32}, and t^{12}/t^{w32} embryos (Hillman, 1975).

Nadijcka and Hillman, 1975 failed to determine whether increased mutant cytoplasmic lipid results from excessive lipogenesis, lack of utilization of lipids synthesized at normal rates, or both. However, their high resolution (electron microscope) autoradiographic studies did show that cleavage stage mutants (t^{12}/t^{12} and t^{w32}/t^{w32}) and controls: (1) incorporate [^{14}C] pyruvate with most of the excessive lipid labelled, (2) incorporate phospholipid precursors, [^{3}H] ethanolamine and [^{3}H] choline chloride, with <u>no</u> labelling of either control or mutant lipid droplets, (3) do <u>not</u> incorporate [^{3}H] mevalonic acid, a cholesterol precursor, and (4) incorporate the neutral lipid precursor, [^{3}H] palmitic acid with the majority of lipid droplets densely labelled. Apparently, cleavage stage t^{12} and t^{w32} mutants can actively synthesize excessive lipids which are labelled by [^{3}H] palmitic acid, a neutral lipid precursor. Blastomeres found in

normal and mutant cleavage stage embryos are not synthesizing these lipid types or they are impermeable to these lipid precursors.

Excessive lipid levels together with mitochondrial variants previously discussed can occur with or as the result of abnormal aerobic (ATP) metabolism. Both t^{12}/t^{12} and t^{w32}/t^{w32} mutants exhibit nonphysiological ATP metabolism; excessive ATP levels are present up to one developmental stage prior to arrest (8-cell in t^{w32} and early morula in t^{12}), drop until mutants undergo developmental arrest, then returns to control levels (Ginsberg and Hillman, 1975).

A possible consequence of increased aerobic metabolism, other than excess lipid production, is an increase in capacitation and fertilization of t^n-bearing spermotozoa. Hillman, 1975 states, "...increased (ATP) metabolism could result in either increased motility, viability, capacitation and/or ability to fertilize and could cause the t^n-bearing spermatozoa to be meiotically driven." Meiotic drive, resulting from alterations in meiosis, spermiogenesis, or fertilizing capacity, can result in one gene being transmitted at a greater than 50/50 rate over its corresponding allele. Regarding T-locus mice, heterozygous males (T/t^n or $+/t^n$) transmit lethal t^n-alleles at greater than even (1:1) ratios, sometimes as high as 90-99% transmission (Dunn and Bennett, 1964). There is no evidence of abnormal meiosis or that more t^n-bearing spermatozoa are produced. In order to relate both t^n-induced phenomena (i.e., embryo lethalities and meiotic drive) Hillman, 1975 postulates, "...the t^n allele elicits

a metabolic error which results in nonphysiological levels of aerobic metabolism." However, lethal t-alleles may alter normal differentiation patterns of cell surface macromolecules on embryo and sperm surfaces and thereby induce both embryo lethalities and meiotic drive (Bennett, 1975). At present then, there are two major hypotheses to explain primary t^{12}, t^{w32}, and other t^{n} genetic defects; these include abnormal aerobic metabolism (Hillman, 1975) and abnormal surface macromolecular differentiation (Bennett, 1975).

Another approach to the investigation of t^{12}, t^{w32}, and other lethal t-alleles has been the testing of drugs which copy or mimic phenotypic characteristics of the lethal alleles in question. Such drugs are termed "phenocopies." Because of the hypothesis that t^{12} and t^{w32} lethalities are associated with defective RNA synthesis and abnormal nucleolar shape changes, it is possible that mutant metabolism may be "poisoned" by production of one or more antimetabolites (Smith, 1956). Attempts have been made to mimic or phenocopy the effects of the mutant lethality by subjecting normal embryos to chemicals known to have predictable effects. Two of these chemicals include Actinomycin D and Cytochalasim B.

Effects on preimplantation mouse development of actinomycin D, which blocks DNA-dependent RNA synthesis (transcription) of all types, have been investigated (Thomson and Biggers, 1966; Mintz, 1964a; Silagi, 1963; Tasca and Hillman, 1970; Wilson and Stern, 1975; and others). Although dose-response, mRNA longevity, and other data are

interesting in their own right Silagi, 1963 concludes that actinomycin D-induced defects "...are different from those occurring in the t^{12} homozygotes and do not help explain the mode of action of this gene." In short, actinomycin D does not act as a phenocopy of the t^{12} allele.

The hypothesis that t^{w32}/t^{w32} embryos are blocked at the time of morula-to-blastocyst transformation because of abnormalities related to the cell surface (such as motility and the adhesion-recognition response) has led to experiments with cytochalasin B (CB). One primary mode of action of CB seems to be interference of 50Å microfilament function; morphogenetic processes arrested by CB include cell motility, gastrulation, neurulation, salavary gland development, axon elongation, certain secretory processes, and others (Spooner, 1974). Granholm and Brenner, 1976 and other investigators have demonstrated that CB inhibits blastocyst differentiation in mouse embryos perhaps through the disruption of 40-60Å cortical microfilaments essential for motility to occur. Such microfilaments were not found in electron microscope studies of CB-treated embryos conducted by Perry and Snow, 1975. Granholm and Brenner, 1976 also found that CB acts as a partial phenocopy of the lethal t^{12} allele.

In summary, physiological studies on T-locus lethal mutants reveal few clear cut data on t^{12}- or t^{w32}-induced primary phenotypic defects. Nonetheless, lethal mutants do exhibit phenotypic expressions which can be detected as: (1) abnormal metabolic patterns, (2) irregular organelle morphogenesis, and (3) abnormal cell surface

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properties due to faulty surface macromolecule differentiation, absence of functional 50Å cytoplasmic microtubules in blastomere cortices, or both.

MATERIALS

Embryos analyzed in the compaction study were derived from HA/ICR mice, a Swiss-Webster strain obtained from Sasco, Inc. of Omaha, Nebraska. The strain originated in the colony of A. R. Schmidt of Madison, Wisconsin. Original breeding pairs of Ttf/t^{w32}+ (T tufted/t^{w32} wild) mice were obtained from Dr. Dorthea Bennett of Cornell Medical College in May, 1972. To obtain +/t^{w32} embryos, T/t^{w32} males were crossed to Balb/CJ females carrying a +/+ genotype at the T-locus. Balb/CJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

All mice were maintained in a controlled 70° F environment having a 16 hour light/8 hour dark cycle with feed and water available ad libitum. A complete record was kept on litter size, tail lengths, and sex of offspring from Balb/c x Balb/c (+/+ x +/+) and Balb/c x Brachyury (+/+ x T/t^{w32}) mice.

According to Chesley and Dunn, 1936 Brachyury mice (+/T) have tail lengths of from one-eight to seven-eights the length of their normal tailed (+/t^{n}) littermates; their measurements were made at birth. In our experience however, handling of newly born mice to determine sex and tail lengths seemed to increase the incidence of neonate killing by postpartum females. Therefore sexing and tail length determinations were conducted at weaning or 21 days postpartum.

In order to conduct in vitro analyses and light microscopic studies on t^{w32} homozygous lethal embryos the following two crosses were

necessary. In experimental crosses two normal tailed mice of the genotype $+/t^{w32}$ were mated ($♂+/t^{w32}$ x $♀+/t^{w32}$) to produce two viable genotypes, +/+ and $+/t^{w32}$ and the lethal mutant, t^{w32}/t^{w32}. Control crosses consisted of normal tailed males mated to short and kinky tailed females ($♂+/t^{w32}$ x ♀+/T) to produce four viable genotypes, +/+, $+/t^{w32}$, +/T, and T/t^{w32}. Kinky, a tail mutation eight crossover units away from the T-locus in linkage group IX, acts as a marker gene for the Brachyury (+/T) phenotype. Since lethal t-alleles prevent regular recombination in the area of T for approximately ten crossover units (Dunn and Bennett, 1964), the presence of a kinky tail indicates a +/T (short tail) genotype. Thus females with kinky tails were used for control crosses. Tails possessing kinks had lengths of 4.4 to 6.0 cm. Tail lengths without kinks ranged from 6.1 to 8.0 cm. Only females in the upper end of the range were used and were assumed to have the $+/t^{w32}$ genotype. Males assumed by means of tail lengths to be $+/t^{w32}$ were test mated with +/T females. The presence of any tailless offspring (T/t^{w32}) proved that the male genotype was indeed $+/t^{w32}$. Once proven, these $+/t^{w32}$ males were used in both control and experimental crosses.

A total of 83 $♂T/t^{w32}$ x ♀+/+ crosses were made with an average of 6.8 young per litter. At 21 days of age 561 Brachyury (+/T) and $+/t^{w32}$ mice were weaned. The number of females numbered 314 (56%), while males numbered 247 (44%).

A total of 225 Balb/CJ x Balb/CJ crosses were made with an average of 9.2 young per litter. Since most males were culled at birth only 114

were weaned. At 21 days 833 females were weaned. Balb/CJ tail lengths ranged from 5.8 to 8.0 cm with 68% of all tail length measurements between 6.6 and 7.3 cm. Tail lengths in Brachyury stock (+/T) ranged from 4.4 to 8.0 cm, but there was no predominant tail length. This latter observation appears to be an indication of the range of +/T mice tail lengths of from one-eight to seven-eights normal tail length (Chesley and Dunn, 1936).

INTERCELLULAR GAP DIFFERENCES IN UNCOMPACTED AND COMPACTED EIGHT-CELL ICR MOUSE EMBRYOS AS REVEALED BY QUANTITATIVE ELECTRON MICROSCOPY[1]

Phyllis M. Johnson[2] and Nels H. Granholm[3]

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ABSTRACT:

During the compaction process individual blastomeres take on new properties which foreshadow significant morphogenetic events including morula-to-blastocyst transformation and segregation of ICM and trophoblast cell populations. It has previously been reported that compaction results in an overall maximization of blastomere contact. The objectives of the present study were to: (1) quantitatively characterize gap differences in cell-to-cell contacts in uncompacted and compacted embryos, (2) identify junctional arrangements which arise during compaction, and (3) discover changes in morphology and distribution of cytoplasmic organelles during compaction.

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2. Botany-Biology Department, South Dakota State University.

3. Electron Microscope Laboratory, South Dakota State University, Brookings, South Dakota, 57006.

Thin sections acquired two fifths and four fifths (40 and 80 μm respectively) of the way through 100 μm diameter 8-cell uncompacted and compacted embryos (3 of each group) were analyzed. Selection of sections on grids and of junctions within sections for analysis at 10,000 and 100,000 diameters involved procedures to insure randomization. The final analysis of individual junctions on prints was accomplished using a randomized quantitative model.

Results show: (1) compaction is accompanied by increased cell-cell contact and decreased volume of intercellular space between blastomeres, (2) blastomeres of compacted embryos possess greater ($P<0.01$, chi-square) overall adhesive cell contacts than blastomeres of uncompacted embryos, (3) 37.3% of the total junctions measured in both uncompacted and compacted 8-cell embryos were found to be within the 0 to 100Å gap class, and (4) compacting embryos display microvilli, microvillus-cell body junctions, interdigitating microvillus-microvillus junctions, and tight junctions.

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INTRODUCTION

A major morphogenetic event in early mouse development takes place at the 8-cell stage. Within a very few hours following the third cleavage division, plasma membranes of individual blastomeres become more closely applied to each other maximizing their areas of cell-cell contact. Both the rapidity of this compaction process and the extent of involvement of all blastomeres suggest some rather fundamental and highly coordinated changes in the physiology and behavior of 8-cell mouse blastomeres.

Lewis and Wright, 1935 suggested that compactness and uncompactness of 8-cell embryos may be due to changes in surface tension, gelation,

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adhesiveness, cleavage plane orientation, and/or combinations of these factors. Interestingly they also found variations in the extent of compactness in older embryos; some morulae and early blastocysts possessed partially rounded blastomeres at their peripheries. Recognizing compaction as a qualitatively different phenomenon from earlier blastomere interactions Mulnard, 1967 used the term "globulization" to convey the meaning that significant changes in blastomere shape and extent of cell-cell contact were occurring at the 8-cell stage. Based on time-lapse cinemicrographic observations of asynchronously fused cleavage stage embryos Mulnard, 1971 p. 271 stated that the globular stage "...is a transitory period of high adhesiveness...which is characterized by the fact that the blastomeres take the widest mutual contact possible with the result that they become tightly pressed against each other..."

Although Calarco and Brown, 1969 and Ducibella et al., 1975 have qualitatively characterized the kinds of junctions which arise during compaction, we do not know the extent or frequency distributions of the various kinds of cell-cell junctions before and after compaction. Such data would be of considerable interest in the determination of: (1) total blastomere surface area in contact with neighboring blastomeres, (2) quantitative junctional differences between internal (presumably ICM anlagen) and peripheral (presumably trophoblast anlagen) cells, (3) total blastomere surface in adhesive contact (250Å or less intercellular distance) with neighbors, (4) extent of microvillus type junctions, and (5) other junctional phenomena.

This study was undertaken to provide quantitative junctional data on the compaction process. Objectives were to quantitatively characterize cell-cell contacts in uncompacted and compacted 8-cell mouse embryos, identify the generation of new junctional arrangements during compaction, and relate quantitative junctional information to physiologic, morphologic, and behavioral aspects of preimplantation mouse morphogenesis.

MATERIALS AND METHODS

The origin, maintenance, recovery, and culture of ICR mice and mouse embryos have previously been comprehensively outlined (Granholm and Brenner, 1976). In this experiment embryos were flushed (BMOC-3, GIBCO) at 60-62 hpc (hours post coitum) and placed in four groups, i.e. 4-cell, uncompacted 8-cell, semicompacted 8-cell, and compacted 8-cell. The scoring criteria, essentially those of Ducibella and Anderson, 1975, are based on the clarity and individual integrity of blastomere membranes. Blastomere outlines of uncompacted 8-cell embryos are clear and distinct. Embryos were judged to be compacted when all eight blastomeres were observed to possess appositional boundries or diastemic zones which obscured the symmetrically-round individual blastomere outlines. A semicompacted group consisted of embryos in which one or more of the eight blastomeres had not formed appositional boundaries with adjacent blastomere(s). Semicompacted embryos were not used in this study.

Compacted and uncompacted embryos were prepared for electron microscopy (Enders, 1971). Thick sections of 1-2 μm were obtained from

the first 40 μm of the approximately 100 μm diameter embryos, and viewed at 200x on a Nikon phase microscope for verification of cell number and state of compaction. Gray to silver thin sections were cut with a diamond knife and collected on uncoated 150 mesh copper grids. Sections were stained with uranyl acetate (Watson, 1958) followed by lead citrate (Sato, 1958).

Sections were viewed on an Hitachi HU-12 electron microscope and photographs were taken at plate magnifications of 4,000x and 20,000x. Instrument magnifications were calculated at each session by taking pictures of a standard calibrated magnification grid. For final analysis, micrographs were prepared at print magnificants of 2.5x and 5x to yield final magnifications of 10,000x and 100,000x respectively.

In this study methods of randomization and quantitation were modified from previous procedures (Johnson, 1972). To select a section to be photographed a grid was scanned until a section was found in which the outer embryo profile was contained completely within the grid space. At a magnification of 4,000x the embryo was photographed in order to give a complete composite profile. Then, at 20,000x every cell-cell contact was photographed sequentially along its entire length. Care was taken not to photograph contacts more than once and to be certain every contact was photographed. Prints were made, taped together, and measured as follows. Beginning at one end of a contact, the measurement of the cell-cell gap distance was made at the first point where the plasma membranes were well fixed, sectioned normally, and had an intercellular space of 1000Å or less. Marks were made on

the prints at 1,000Å intervals along the entire length of each contact, until the contact became continually divergent with a gap distance of 1000Å or greater. Gap measurements were made at each of the 1000Å intervals. If membranes at a measuring point were not favorable to measurement that point was skipped. The subsequent measurement was made at the next well defined and measurable 1000Å interval.

Measurements were assigned to one of the following gap classes: 0-50Å, 50-100Å, 100-150Å, and so on at 50Å intervalls to 950-1000Å, and finally >1000Å. Data were obtained using both uncompacted and compacted 8-cell embryos. These data were compared statistically using a X^2 test of the null hypothesis that no differences existed between gap distance frequency distributions of uncompacted and compacted embryos.

Observations were also made on the types of junctions present, morphogenesis of organelles, and other ultrastructural changes occurring within uncompacted and compacted embryos.

RESULTS

Cell-Cell Contacts

Our data show that there is a greater overall intercellular distance between blastomeres in uncompacted versus compacted embryos. Relative frequencies of the various classes of gap distances are shown in Table 1 and graphically illustrated in Fig. 10. Compacted embryos have a larger percentage of their gap distances in the smaller gap distance range (compare cumulative percent of total for 0-250Å junctions), while uncompacted embryos have a greater percentage in the

Table 1. Frequency of intercellular distances between blastomeres of uncompacted and compacted 8-cell ICR mouse embryos.

Gap in Å	UNCOMPACTED			COMPACTED		
	Total number of measurements	Percent of total	Cumulative percent of total[1]	Total number of measurements	Percent of total	Cumulative percent of total[1]
0≤50	78	15.5	15.5	85	8.7	8.7
>50≤100	109	21.7	37.3	275	28.5	37.3
>100≤150	41	8.2	45.4	93	9.5	46.8
>150≤200	35	7.0	52.4	169	17.4	64.2
>200≤250	9	1.8	54.2	68	7.0	71.1
>250≤300	18	3.6	57.8	62	6.4	78.0
>300≤350	5	1.0	58.8	19	2.0	80.0
>350≤400	23	4.6	63.3	48	4.9	85.0
>400≤450	7	1.4	64.7	13	1.3	86.8
>450≤500	10	2.0	66.7	36	3.7	90.5
>500≤550	8	1.6	68.3	3	0.3	90.8
>550≤600	13	2.6	70.9	14	1.4	92.2
>600≤650	5	1.0	71.9	1	0.1	92.3
>650≤700	26	5.2	76.9	9	0.9	93.2
>700≤750	7	1.4	78.3	3	0.3	93.5
>750≤800	24	4.8	83.1	19	2.0	95.5
>800≤850	17	3.4	86.5	1	0.1	95.6
>850≤900	10	2.0	88.4	6	0.6	96.2
>900≤950	3	0.6	89.0	4	0.4	96.6
>950≤1000	39	7.8	96.8	20	2.1	98.7
>1000	16	3.2	100.0	13	1.3	100.0
Total	503			961		
Number of embryos examined			3	3		
Total length of all cell contacts			184.0 µm	378.0 µm		
Total length of all contacts examined			50.3 µm	96.1 µm		
Percent of total length measured			27.3%	25.4%		

[1]The cumulative percent of total at each gap class is the sum of the percent of total for that class and all smaller classes.

large gap distance range (compare cumulative percent of total for 250-1000Å junctions). The most frequent intercellular distance for both compacted and uncompacted embryos was found to be the 50-100Å gap. Cumulative percent column figures at 150-200Å and 200-250Å gap classes reveal clear differences in gap distances between blastomeres of uncompacted versus compacted embryos. In the 0-100Å gap class, cumulative percent values are the same (37.3%) for both groups. Interestingly, in the gap class of 0-50Å, uncompacted embryos possessed nearly twice as many junctions (15.5%) as compacted embryos (8.7%). In fact, in the ten 50Å gap intervals from 0-500Å, percent of total values in compacted embryos always exceed percent of total values in uncompacted embryos except for the 0-50Å gap class. In contrast, just the opposite holds true in the ten 50Å gap classes from 500-1,000Å. These trends are clearly displayed in Fig. 10 showing two major crossings at the 50Å and 500Å points.

The overall chi-square value for all gap class differences (twenty-one, 50Å gap classes from 0-1,000Å and >1,000Å) is 196.3. The P=0.01 value for 20 degrees of freedom is 37.6. In order to statistically test for differences in adhesive contacts (0-250Å), a chi-square determination was conducted using the five 50Å gap classes from 0-250Å. The chi-square value for adhesive contacts is 54.8, and the P=0.01 value with four degrees of freedom is 13.3.

The greater amount of intercellular space present in uncompacted versus compacted embryos is most evident where three blastomeres come together (Figs. 1 and 2). Gap distances of 50Å or less are numerous

in uncompacted (15.5% of total), but they are both short in length and are bounded on each end by areas of larger irregular gap distances (Figs. 3 and 4). Compacted embryos have longer areas of close association extending to the periphery of cell contacts (Figs. 5-9). As compaction begins, microvilli and microvillus-type junctions are formed. Focal tight junctions are apparent at some points of microvillus contact (Fig. 5). Our observations have confirmed the findings of Ducibella et al., 1975 and Ducibella and Anderson, 1975 regarding the generation of qualitatively new kinds of cell-cell contacts during compaction. Areas of close cell-cell contacts become extended during compaction.

Morphogenesis of Organelles During Compaction

Organelles in compacted and uncompacted embryos did not appear to be qualitatively different (Figs. 1 and 2). In both groups: (1) ribosomes were found mostly in clusters, but a few were associated with the differentiating endoplasmic reticulum, (2) granular endoplasmic reticulum were associated with crystalline bodies and mitochondria, and (3) mitochondria were found to contain central vacuoles and assumed both round and elongate profiles. Finally, differences in nuclei and nucleolar morphogenesis between groups were not observed.

DISCUSSION

Numerical data (Table 1 and Fig. 10) showed that compaction of 8-cell ICR mouse embryos was accompanied by adjacent blastomeres flattening against one another with blastomere surfaces coming into

greater overall cell-cell apposition. Frequency distributions of intercellular gap distances between the two groups were different ($P<0.01$, chi-square). Figure 10 and Table 1 reveal that 50-500Å gap distances predominate in compacted (81.8% of total junctions measured) versus uncompacted (51.2% of total junctions measured) embryos. Within the 0-250Å intercellular gap classes, i.e., those judged to exert adhesive influences (Farquhar and Palade, 1963; Ducibella et al., 1975; Trinkaus, 1969 for review), we found a significant difference ($P<0.01$, chi-square) between groups. Compaction therefore is accompanied by an increase in those kinds of junctions considered to be adhesive. Thus our data support the observations of Lewis and Wright, 1935 and Mulnard, 1967, 1971 regarding changes in blastomere adhesiveness as one causal factor in compaction. Interestingly, the cumulative percent total of junctions having intercellular gap distances of 0-100Å within both uncompacted and compacted groups was identical (37.3%), while 15.5% of uncompacted versus 8.7% of compacted embryo junctions possessed 0-50Å gap distances. These latter junctions, focal in nature and observed in uncompacted embryos, probably serve as membrane starting points or organizational foci to promote zippering or increased membrane apposition during compaction.

At the time of compaction, interdigitating microvilli and focal tight junctions arise. Focal tight junctions are usually seen at the point of contact of a microvillus with an opposing cell (Fig. 5). During the morula stage, focal tight junctions precede the formation of zonulae occludentes which are thought to be necessary for maintaining

blastocelic fluid (Ducibella and Anderson, 1975). The increase in microvilli and microvillus-like contacts during compaction may function to enhance the increase adherence between blastomeres (Calarco and Brown, 1969; Calarco and Epstein, 1973) as well as to promote the flattening which begins at initial points of adhesive cell contacts and extends or "zippers" over blastomere surfaces.

Our data do not provide specific information on precise causes of compaction. Although increased cellular apposition occurs, it is not clear which factors initiate, promote, and accomplish compaction. Changes in adhesiveness, motility, deformability, and cortical function of 8-cell blastomeres may be involved. Mintz, 1964 discussed cell movements within mid-cleavage embryos following zona removal by pronase. She observed the presence of lobe-shaped and narrow cellular processes and the displacement of entire blastomeres. She stated (p. 277) "cell movements in the egg appear to be random rather than ordered." When naked mid-cleavage embryos (ICR and C57BL/6 distinguishable on the basis of different cytoplasmic granularities) were fused Mintz, 1964 observed active cell migration during sphere reconstitution. Recently the role of cytoplasmic microfilaments during compaction has been investigated (Ducibella and Anderson, 1975; Granholm and Brenner, 1976); cytochalasin B at 5.0 and 4.0 μg/ml causes compacted 8-cell embryos to become uncompacted. Colchicine (1.0 μg/ml), colcemid (7.5 μg/ml dissolved in DMSO), and lowered temperatures for 1.0 hour had no observable effects on compaction (Ducibella and Anderson, 1975). Time-lapse cinemicrographic information of synchronous and asynchronous

embryo fusion (Mulnard, 1971) indicates that the stage of the cell cycle may also influence incidence and extent of membrane apposition. Also, certain purely physical factors may promote compaction; the compacted state may represent a more stable thermodynamic equilibrium configuration than the uncompacted state. Thus the precise roles of intercellular, intracellular, purely physical factors, and others during compaction are at present unknown.

One way to analyze the compaction process would be to characterize a developmental system that spontaneously fails to undergo the normal compaction response. Two of the Brachyury or T-locus developmental lethals, t^{12}/t^{12} and t^{w32}/t^{w32}, may represent such cases (see Hilman, 1975 for current review). Cultural observations on lethal t^{w32} homozygotes reveal that some mutants may never completely compact (Granholm et al., 1976). Calarco and Brown, 1968 have shown that t^{12} homozygotes do elaborate specialized cell junctions between presumptive trophoblast cells, but no extensive quantitative experiments have been conducted to date to assess: (1) the number of these presumably essential junctions formed in lethal mutants, and (2) their degree of normality. Moreover, Calarco and Brown, 1968, p. 171 report that blastomeres of t^{12} homozygous morulae "appear more rounded and less closely applied to one another than do the cells of normal embryos." The inability of t^{12}/t^{12} blastomeres to maintain close intercellular contact may be related to the cell surface. Mintz, 1964 states that in mixed aggregates of blastomeres from normal and from t^{12}/t^{12} embryos (genetic mosaics), intermixing of the blastomeres occurs to a lesser

degree than controls, suggesting that mutant cells have less motility than control cells.

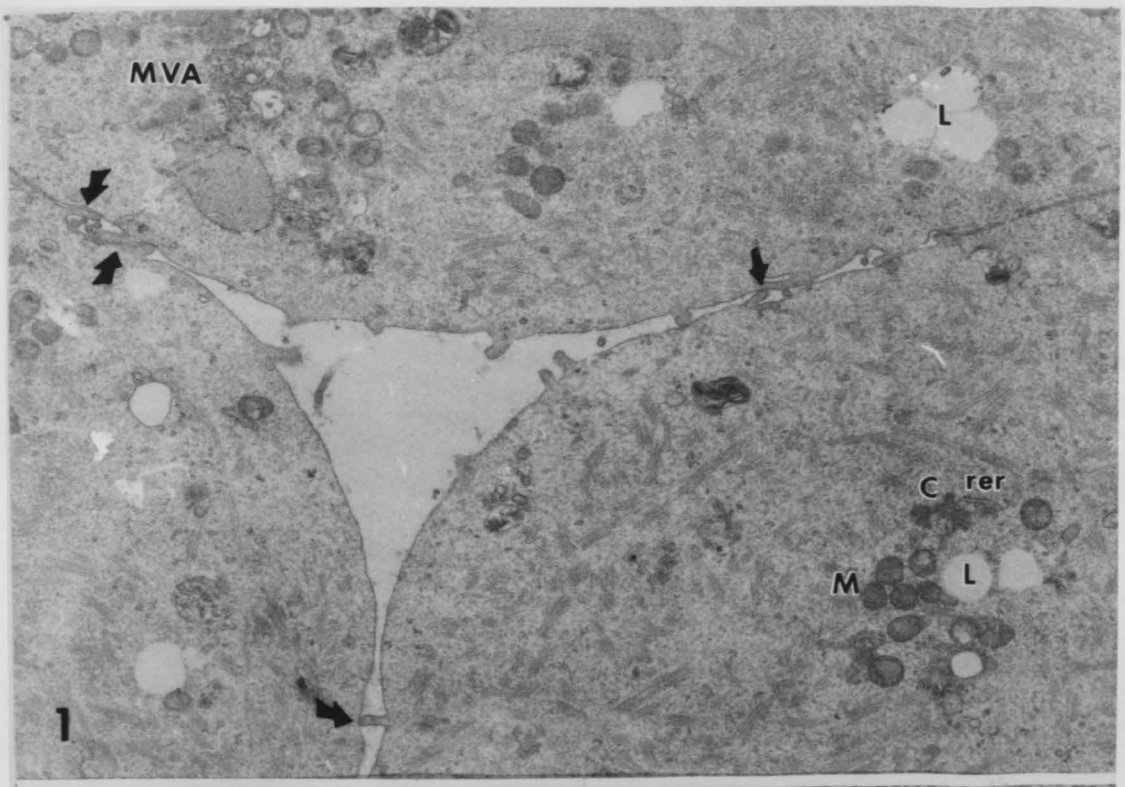
The publications by Spiegelman and Bennett, 1974, with regard to another T-locus lethal (t^9/t^9), deals directly with the concepts of abnormal cellular associations, abnormal cell contacts, failure of mutant primitive streak cells to elaborate normal cytoplasmic extensions (filopodia), and abnormal surface adhesiveness. Bennett, 1975 states the following interesting points with regard to the t^9 homozygote: (1) mutant primitive streak cells appear to be immobilized, (2) mutant cells probably possess "membrane-controlled defects of cell-cell interactions," (3) mutant cells do not elaborate filopodia; the broad and stubby extensions that are produced do not possess cortical microfilaments as seen in controls, (4) cellular junctions between mutant cells are never normal, and (5) "...it appears that in the mesoderm of mutant embryos (t^9/t^9) abnormalities of cell shape, cellular interaction, and cell movement are related to the lack of surface adhesiveness and microfilament cytostructure." Thus certain of the T-locus mutants may enable us to learn specific requirements for normal cell-cell interaction during mouse morphogenesis.

This study has provided quantitative information on the frequency and distribution of intercellular junctions during the compaction process in 8-cell ICR mouse embryos. As a result of compaction, qualitatively new kinds of junctions arise, and the overall linear extent of preexisting contacts increases in a manner perhaps analogous to zippering. Our data support the concept of increased blastomere

adhesiveness during compaction; 71.1% of compacted versus 54.2% of uncompacted embryo cell contacts were observed within the 0-250Å gap category-intercellular distances considered to exert adhesive forces between cells. Because of the dramatic and highly coordinated events, the relevance of compaction to subsequent morphogenetic phenomena such as cavitation of morulae and differentiation of ICM and trophoblast cells may be extraordinary. Quantitative data presented in this paper should aid both in characterizing the compaction process and in its overall morphogenetic significance.

Fig. 1. Typical uncompacted 8-cell embryo with characteristically large intercellular space where three blastomeres meet. Note the presence of numerous microvilli and microvillus junctions (arrows). Ribosomal endoplasmic reticulum (rer) is seen in close association with crystal aggregates (C), mitochondria (M), and lipid droplets (L). A multivesiculate aggregate (MVA) is also present. X12,000.

Fig. 2. Typical compacted 8-cell embryo with characteristically small intercellular space where three blastomeres meet. Microvilli and microvillus junctions are not as frequent as in uncompacted. Note presence of crystals (C), mitochondria (M), multivesiculate aggregate (MVA) and lipid droplets (L). X12,000.


MVA
L
C
rer
M
L
1

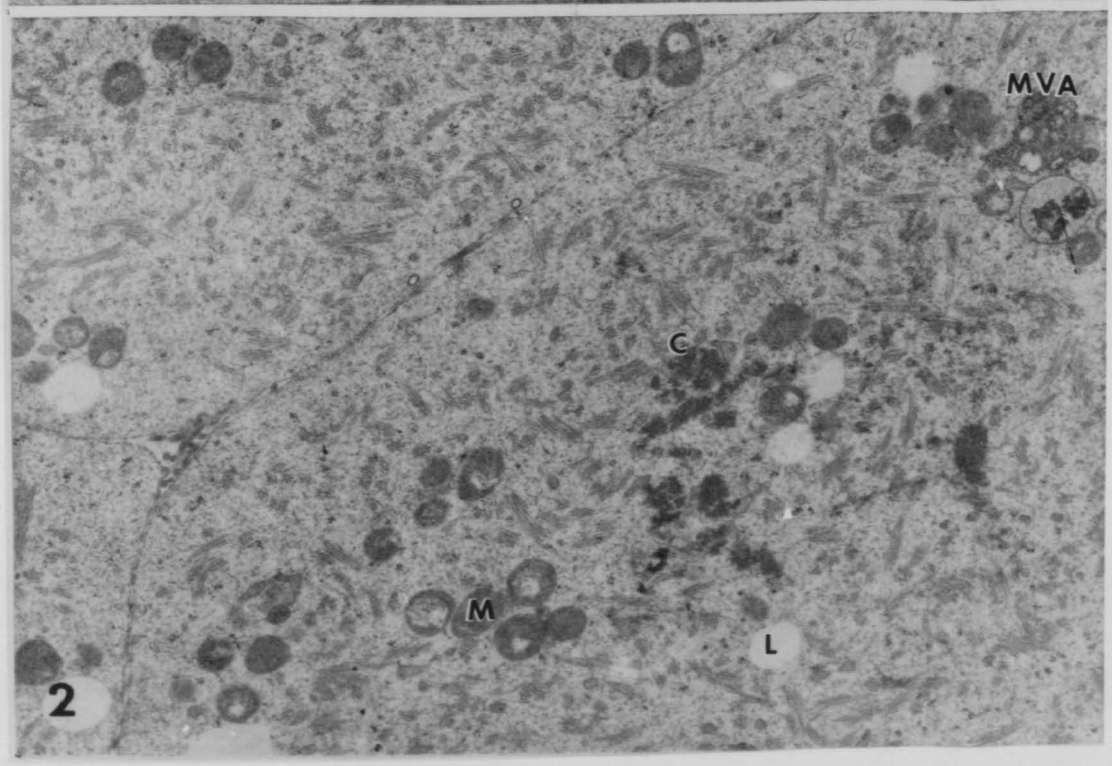

MVA
C
M
L
2

Fig. 3 and 4. Complete uncompacted cell-to-cell contact running from internal triangular space (left margin Fig. 3) toward external zona pellucida (right margin Fig. 4). Figure 3 and 4 are over lapped at *. Note presence of microvilli at open ends of junction. Total length of junction measuring 1000Å or less is 9.0 μm. Of that length 5.7 μm (63%) possesses gap distances of ≤250Å (between arrows). X18,000.

Fig. 5-9. Complete compacted junction running from external zona pellucida (left margin Fig. 5) toward internal triangular space (right margin Fig. 9). Total junctional length measuring 1000Å or less is 13.8 μm, of which 11.0 μm (81%) possesses gap distances of ≤250Å. Note concentration of microvilli at open ends of contact and specialized cell contacts (arrows). X40,000.

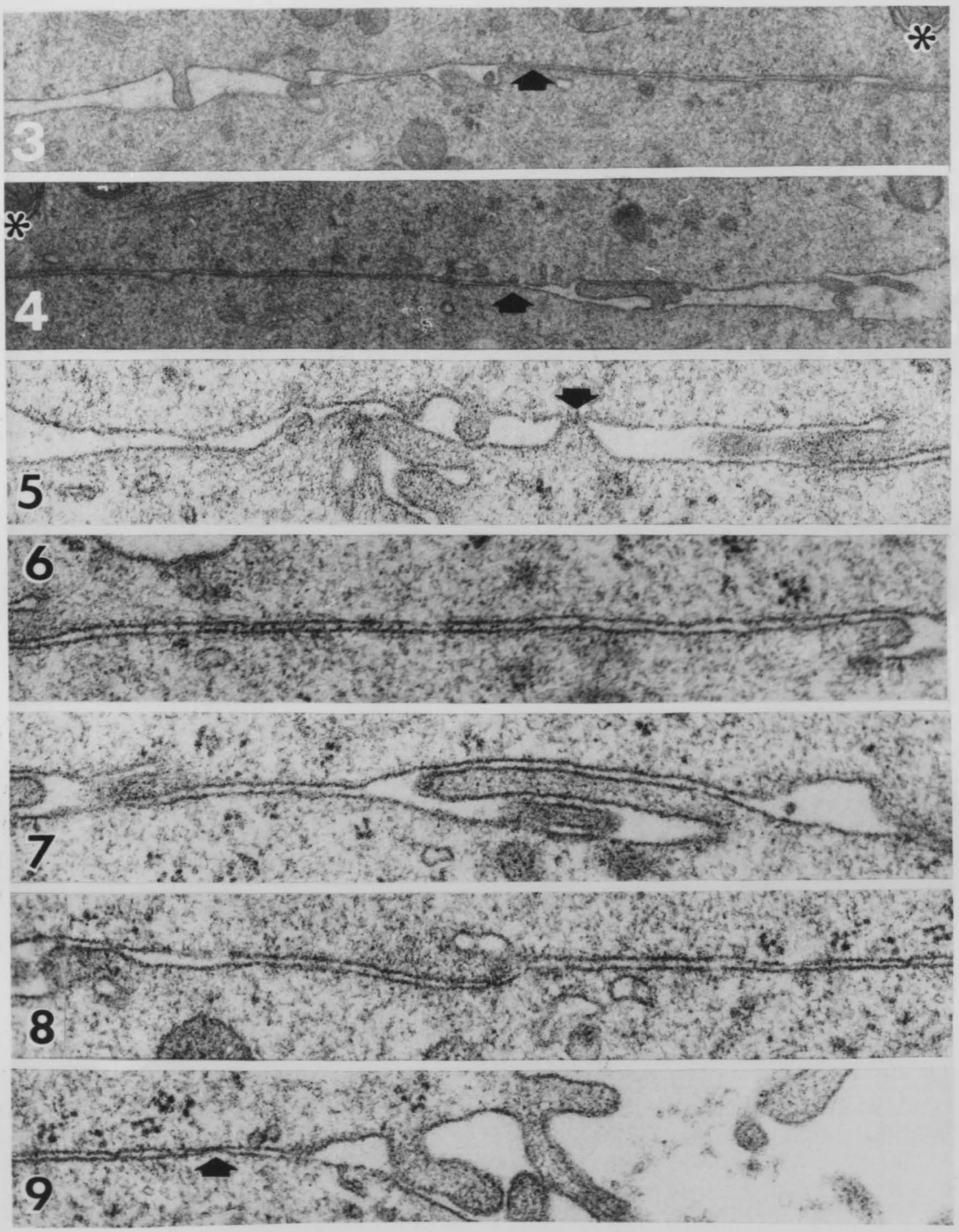

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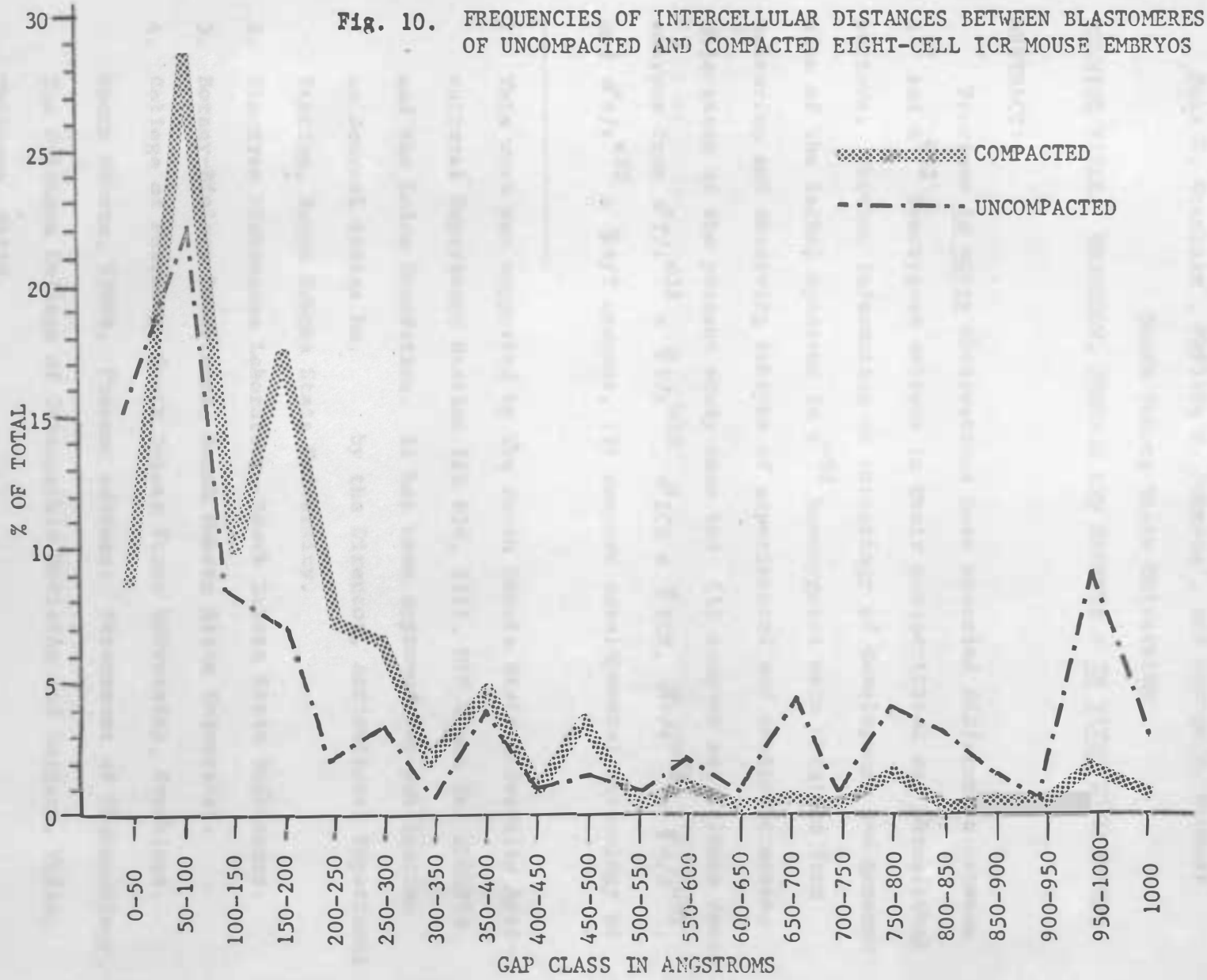
Fig. 10. FREQUENCIES OF INTERCELLULAR DISTANCES BETWEEN BLASTOMERES OF UNCOMPACTED AND COMPACTED EIGHT-CELL ICR MOUSE EMBRYOS



IN VITRO DEVELOPMENT OF t^{w32} HOMOZYGOUS LETHAL EMBRYOS[1]

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RUNNING TITLE: GRANHOLM, JOHNSON AND BRENNER - IN VITRO DEVELOPMENT

ABSTRACT:

Previous in vitro observations have revealed differences between t^{12} and t^{w32} homozygous embryos in their phenocritical and phenolethal periods. Further information on chronology of development and generation of the lethal syndrome in t^{w32} homozygotes were obtained from culturing and observing embryos of experimental and control crosses. Objectives of the present study were to: (1) recover and culture mouse embryos from ♂T/t^{w32} x ♀T/t^{w32}, ♂ICR x ♀ICR, ♂+/t^{w32} x ♀+/t^{w32}, and ♂+/t^{w32} x ♀+/T crosses, (2) compare developmental chronology of

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experimentals and controls, (3) determine percentage of developmentally arrested morulae (t^{w32}/t^{w32}), (4) describe the genesis of the t^{w32}/t^{w32} lethality and (5) discover new information pertaining to t^{w32} gene expression.

Embryos were recovered from experimental and control crosses and cultured from 54-100+ hpc. Observations were made at regular intervals from 54-94 hpc. Embryo development was recorded and photographs were taken at 50 and 100X.

Results on the generation of t^{w32}/t^{w32} lethality in vitro are consistent with previous findings. In addition present results indicate that: (1) mutants may never completely compact, (2) some presumed mutants produce blastocoelic spaces but fail to form well defined blastocoels, (3) the phenocritical period of t^{w32} homozygotes appears to extend over the 66-79 hpc interval.

IN VITRO DEVELOPMENT OF t^{w32} HOMOZYGOUS LETHAL EMBRYOS

INTRODUCTION

The complex T-locus in the house mouse (Mus musculus) provides in utero lethal mutants for studies in mammalian developmental genetics. The earliest acting (ontogenetically) known T-locus mutants (t^{12} and t^{w32} homozygotes) are lethal at the time of morula-to-blastocyst transformation. Apparently developmental events which occur in normal embryos are either greatly retarded or nonexistent in t^{12}/t^{12} and t^{w32}/t^{w32} embryos. According to Bennett and Dunn, 1964 t^{12} and t^{w32} alleles are the same. In contrast, Hillman and Hillman, 1975 state "...the effects of these two alleles (t^{12} and t^{w32}) result in the same or in a closely associated developmental aberration."

Following extensive in vitro observations on control and t^{12}/t^{12} embryos Mintz, 1964b reports that certain changes which normally occur prior to blastocyst formation are either limited or completely absent in t^{12} homozygotes. These normal events include: (1) decrease in cytoplasmic granularity (or increase in cytoplasmic translucency), (2) formation of numerous prominent spherical bodies, and (3) coalescence of spherical bodies to form blastocoelic cavities. Although Mintz, 1964b observed small clear vacuoles or cavities in 2.6% of arrested morulae in experimental crosses, she interpreted these to be non t^{12} allele-induced abnormals. Although Mintz, 1964b observed that the presence of prominent spherical bodies is limited or absent in t^{12}/t^{12} mutants, Calarco and Brown, 1968 using the electron microscope

report the "most prominent cytoplasmic structure in normal and t^{12}/t^{12} morulae is spherical vesicles containing medium density material." They also noted that mutant cells "appeared more rounded and less closely applied" than normal cells.

In vitro observations have revealed differences between t^{12} and t^{w32} alleles. Hillman et al., 1970 and Hillman and Hillman, 1975 report one major difference in phenocritical and phenolethal periods. The t^{12}/t^{12} lethality (phenolethal period) is expressed from 8-cell to early blastocyst stages, with most embryos arresting at late morula (Hillman et al., 1970). The phenolethal period for t^{w32}/t^{w32} embryos is from 8-cell to late morula stages, with most arresting at early morula (Hillman and Hillman, 1975). Hillman et al., 1970 also report that embryos arresting early (8-12 cell stage) are usually normally (round) shaped; but when embryos arrest later (early to mid morula stage) blastomeres may be "either round or misshapen." Although Dunn and Bennett, 1964 could not distinguish t^{12} from t^{w32} homozygotes on the basis of flushing and observing (with no culture), Hillman, 1975 does indeed review data supporting the notion that t^{12} and t^{w32} are separate alleles.

Further information on generation of the lethal syndrome in t^{w32} homozygotes should be obtained by culturing and observing embryos from control and experimental crosses. Objectives of the present study were to: (1) recover and culture mouse embryos from $\male T/t^{w32} \times \female T/t^{w32}$, $\male$ICR x $\female$ICR, $\male +/t^{w32} \times \female +/t^{w32}$, and $\male +/t^{w32} \times \female +/T$ crosses from 54 hours post coitum (hpc) to over 100 hpc, (2) compare developmental

chronology of experimentals and controls, (3) determine percentage of developmentally arrested morulae (t^{w32}/t^{w32}) in experimental versus control crosses, (4) describe the genesis of the t^{w32} homozygous lethality and (5) discover new information pertaining to t^{w32} gene expression.

MATERIALS AND METHODS

Experimental crosses were made between proven $+/t^{w32}$ males and $+/t^{w32}$ females. Control crosses consisted of $+/t^{w32}$ males x +/T females. Two females were placed with each male at 8:00 p.m. and removed the following morning at 8:00 a.m. Presence of a vaginal plug indicated that mating had occurred. Embryo age was calculated using 2:00 a.m. (the middle of the dark period) as the time of fertilization. Thus at 8:00 a.m. on the morning of plug discovery, embryos were assumed to be 6 hours post coitum (hpc).

Embryos were recovered from experimental and control crosses at 54-59 hours post coitum (hpc) and cultured in Brinster's Medium BMOC-3 (GIBCO). Oviducts of T/t^{w32}, ICR, $+/t^{w32}$, and +/T females were flushed with BMOC-3 and cultured with 2.0 ml BMOC-3 in small Falcon plastic petri dishes in a gas flow incubator kept at 37^{o} C and gassed with 5% CO_2 in air. Embryos were observed, scored for developmental success, and photographed at given intervals. Careful observations were made during each of the following time periods: 54-59, 60-64, 65-69, 75-79, 80-84, 85-89, and 90-94 hpc. Records of developing embryos were kept for each observation. Photographs of controls and

experimentals were taken with a Nikon inverted phase microscope at 50 and 100x magnifications. Criteria used for staging embryos and assessing developmental progress are comprehensively outlined in Johnson and Granholm, 1976 and Granholm and Brenner, 1976. Embryos were cultured until 50% of the controls had transformed to early blastocysts. At that time, embryos were scored and prepared for microscopy.

RESULTS

Tables 1 and 2 provide chronological data on developmental rates of embryos from control ($\male$+/t^{w32} x $\female$+/T) and experimental ($\male$+/t^{w32} x $\female$+/t^{w32}) matings respectively. The following differences in developmental rates between embryos of control and experimental crosses include: (1) at the 54-59 hpc interval, embryos from experimental crosses were more developmentally advanced than those from control crosses; 53.2% of the experimental group were either partially or completely compacted versus 32.6% of the control group; (2) at the 65-69 hpc interval, developmental rate fluctuations between groups had decreased; 44.7% of the experimental groups were either compacted 8-cell embryos or older, while 48.3% of the control group fell into this category, and (3) at the 75-79 hpc observation interval, the presence of lethal mutants within the experimental population became evident with 43.4% morulae in the experimental group versus 10.8% morulae in the control population.

Data presented in Table 3 show 36.3% presumed t^{w32}/t^{w32} lethal mutants. Interestingly of 194 embryos from 18 control crosses which

Table 1. Control data. Developmental staging *in vitro* of ♂+/t^{w32} x ♀+/T control embryos over the 54-94 hpc interval expressed as number of embryos and percent of embryos per developmental stage per hours *post coitum*.

	Hours Post Coitum															
	54-59		60-64		65-69		70-74		75-79		80-84		85-89		90-94	
Stage	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
4-cell	30	32.6														
8-cell uc	32	34.8	25	13.6	11	7.6										
8-cell pc	24	26.1	113	61.4	64	44.1	7	26.9	2	1.1						
8-cell c	6	6.5	46	25.0	70	48.2	19	73.1	10	5.4						
Morula									20	10.8	4	3.2				
Early blastocyst									105	56.8	47	37.9				
Mid blastocyst									44	23.8	50	40.3	21	53.8	29	24.6
Definitive blastocyst									4	2.2	23	18.5	18	46.2	89	75.4
Total embryos	92		184		145		26		185		124		39		118	

Table 2. Experimental data. Developmental staging *in vitro* of ♂+/t^{w32} x ♀+/t^{w32} experimental embryos over the 54-94 hpc interval expressed as number of embryos and percent of embryos per developmental stage per hours post coitum.

	Hours Post Coitum															
	54-59		60-64		65-69		70-74		75-79		80-84		85-89		90-94	
Stage	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
4-cell	17	13.7														
8-cell uc	39	31.4	42	20.2	7	5.3	1	8.3								
8-cell pc	36	29.0	104	50.0	64	48.5	5	41.7	2	1.8	1	0.4				
8-cell c	30	24.2	49	23.6	47	35.6	6	50.0								
Morula			4	1.9	3	2.3			49	43.4	70	29.3	14	25.9	25	28.7
Early blastocyst			7	3.4	9	6.8			57	50.4	101	42.2	8	14.8	1	1.1
Mid blastocyst									4	3.5	47	19.7	22	40.7	33	37.9
Definitive blastocyst									1	0.9	20	8.4	10	18.5	28	32.2
Other	2	1.6	2	1.0	2	1.5										
Total embryos	124		208		132		12		113		239		54		87	

Table 3. Development *in vitro* of embryos from ♂+/t^{w32} x ♀+/t^{w32} in comparison with non-lethal control (♂+/t^{w32} x ♀+/T) matings.

Mating	No. of females	Total no. of embs.	Avg. no. embs per female	Dev't. to blasto-cyst	Dev't. blocked at morula	
Experimentals +/t^{w32} x +/t^{w32}	24	223	9.2[1]	141(73.2%)	82(36.8%)	36.3% presumed t^{w32}/t^{w32} lethal mutants
Controls +/T x +/t^{w32}	18	194	10.8	193(99.5%)	1(0.5%)	

[1]In one experiment (Apr 7612), four females were flushed 24 hours late at 84 hpc versus 60 hpc, and only 14 embryos were recovered from the four females. By discounting those four females and their 14 embryos, the average number of embryos per female becomes 209/20 or 10.4.

Table 4. Development *in vitro* of embryos from T/t^{w32} x T/t^{w32} matings in comparison with non-lethal controls from ICR x ICR crosses.

Mating	No. of females	Total no. of embs.	Avg. no. embs per female	Dev't. to blasto-cyst	Dev't. blocked at morula	
Experimentals T/t^{w32} x T/t^{w32}	7	57	8.1	31*(58.5%)	22*(41.5%)	40.1% presumed t^{w32}/t^{w32} lethal mutants
Controls ICR x ICR	7	74	10.1	73(98.6%)	1(1.4%)	

*Lost four embryos.

were flushed and cultured at approximately 60 hpc, only one or 0.5% of this control population failed to undergo cleavage, compaction, morula formation, and cavitation. In another set of experiments using tailless Brachyury stock as parents (σT/t^{w32} x φT/t^{w32}) and ICR mice as controls (σICR x φICR) 40.1% of the embryos in experimental crosses were presumed to be t^{w32} homozygotes. Again, only a very low percentage (1.4%) of control embryos failed to develop to blastocyst stages.

Cultural observations on control preimplantation mouse development afforded the following information. The embryo seen in Fig. 1a represents a 6-cell embryo between 36-48 hpc. In Fig. 1b the embryo has 8-12 blastomeres, and its developmental age is approximately 60 hpc. Fig. 1c represents a 16-32 cell morula. It is at this stage or slightly earlier that t^{w32} homozygotes undergo developmental arrest. Figs. 1c-1f illustrate the differentiation of embryonic blastomeres into two distinct cell types, ICM and trophoblast, during the morula-to-blastocyst transformation. An early blastocyst (EB) is shown in Fig. 1d; note the presence of two prominent blastocoelic spaces which will eventually coalesce to form a midblastocyst (MB) similar to Fig. 1e. Finally Fig. 1f shows a well differentiated blastocyst of 84-96 hpc with a typical signet ring profile. Such definitive blastocysts soon hatch from their surrounding zona pellucida, expand, and begin to implant at 96 hpc. One can clearly distinguish inner cell mass (ICM) at the lower left of Fig. 1f and trophoblast cells (T) which completely cover the entire blastocyst.

When embryos from experimental crosses (♂+/t^{w32} x ♀+/t^{w32}) were examined at intervals during the 60-94 hpc culture period a number of abnormalities were observed. Of the total progeny (223) from 24 experimental crosses analyzed, 82 (36.8%) embryos failed to develop into blastocysts and remained as arrested morulae (see Table 3). Data on each of the experimental crosses is presented in Table 5. Percent of arrested morulae within individual litters ranged from 0% in June 7613-E2 to 66.7% in June 7611-E1.

The experiment identified as June 7614-E1 serves to illustrate the pattern of developmental events observed in experimental crosses. Seven partially compacted and one compacted 8-cell embryo (eight total) were flushed at 61 hpc and scored as normal. Development could not be detected to be abnormal until 78 hpc when the population of embryos consisted of four early blastocysts (EB), two morulae (M), and two lagging but apparently compacted 8-cell embryos. At 83 hpc each of the eight embryos was photographed and scored as four midblastocysts (MB), one EB, and three uncompacted morulae. This latter group (three uncompacted morulae) were definitely abnormally retarded and presumed to be t^{w32} homozygous lethal embryos. Figs. 2a-2h illustrate normal and mutant embryos under discussion. Embryos in Figs. 2a and 2d appear to represent either late morulae or early blastocysts. Internally their blastomeres are numerous and well compacted as judged by the difficulty in distinguishing individual cells. However peripheral blastomeres appear abnormally rounded, and in both cases, the embryo proper is retracted or pulled away from the surrounding zona pellucida. Four of

Table 5. Summary of embryo culture in each experimental ($\sigma^{\prime}$ +/t^{w32} x ♀ +/t^{w32}) cross.

Experiment identification	Total embryos	Dev't. to blastocyst	Dev't. blocked at morula	% arrested morulae
Apr 7612[1]	14 (4 ♀'s)	8	6	42.8
Apr 7613-E1	11	6	5	45.4
" -E2	9	7	2	22.2
" -E3	9	5	4	44.4
" -E4	11	7	4	36.4
Apr 7614-E1	13	8	5	38.5
Apr 7615-E1	11	4	7	63.6
" -E2	11	6	5	45.4
May 7611-E1	11	5	6	54.5
" -E2	16	13	3	18.8
May 7613-E1	12	10	2	16.7
June 7611-E1	12	4	8	66.7
June 7613-E1	10	7	3	30.0
" -E2	13	13	0	0.0
" -E3	8	4	4	50.0
June 7614-E1	8	5	3	37.5
June 7616-E1	14	9	5	35.7
" -E2	10	7	3	30.0
June 7617-E1	11	7	4	36.4
" -E2	9	6	3	33.3
Totals	223	141	82	36.8

[1] In one experiment (Apr 7612), four females were flushed 24 hours late at 84 hpc versus 60 hpc, and only 14 embryos were recovered from the four females.

the eight embryos resemble Fig. 2c, or midblastocysts, which possess: (1) definite internal blastocoels, (2) small cells no longer individually recognizable, (3) relatively large volumes, (4) partition of cells in ICM and trophoblast components, and (5) continuity between the embryo proper and surrounding zona pellucida. In contrast, Figs. 2b and 2e are presumed t^{w32}/t^{w32} mutants. Note the presence of large rounded blastomeres, retraction of embryo from zona, and smaller relative embryo sizes. At the final observation period (91 hpc), five of the eight embryos had developed into healthy definitive blastocysts. Two of the three arrested morulae, in a more advanced state of degeneration than at 83 hpc, are shown in Figs. 2f and 2g.

Representative embryos (presumed mutants) of experimental crosses were observed to either fail to undergo compaction or to become uncompacted as 16-cell embryos or early morulae. For example the embryo in Fig. 2h at 79 hpc has obviously become uncompacted or dissociated. Fig. 2i represents another 8-16 cell presumed mutant at 80 hpc demonstrating a degree of dissociation not seen in 80 hpc control embryos. Finally, some presumed mutants were observed to form blastocoelic vesicles and small blastocoelic spaces.

DISCUSSION

Present results on the generation of t^{w32} homozygous lethalities *in vitro* are consistent with previous findings. The 36.3% and 40.1% presumed t^{w32}/t^{w32} lethal mutant figures of Tables 3 and 4 respectively, agree well with the classical findings of Smith, 1956, Mintz, 1964, and

others with regard to the t^{12} homozygote and with the more contemporary findings of Bennett and Dunn, 1964, Hillman, 1975, and Hillman and Hillman, 1975 with respect to the t^{w32} homozygote. If, as Hillman and Hillman, 1975 suggest, t^{12} and t^{w32} are in fact separate alleles, then this study together with those of Hillman, 1975, Hillman and Hillman, 1975, and Bennett and Dunn, 1964 represent the only attempts to characterize t^{w32} homozygotes in vitro as they undergo developmental arrest.

Observations on the developmental success of embryos from ♂ T/t^{w32} x ♀ T/t^{w32} matings indicate that lethal T/T embryos do not undergo arrest until after 94 hpc. Smith, 1956 and others who have investigated t^{12} and t^{w32} homozygotes did not use tailless mice (T/t^{12} or T/t^{w32}) as parents to produce lethal homozygotes, because "...the appearance of the t^{12}/t^{12} (or t^{w32}/t^{w32}) homozygote and the time of its death were unknown," and the "...offspring would have included the T/T homozygote and, possibly, abnormals of unknown causes arising as a result of inbreeding homozygosity." Notwithstanding these reasons, we were interested in the in vitro analysis of t^{w32}/t^{w32} embryos derived from ♂ T/t^{w32} x ♀ T/t^{w32} matings. Regarding inbreeding homozygosity, by con-ducting a sufficient number of matings and analyses, one may be able to "iron out" the randomness due to depression. Secondly, T/T embryos which do not undergo abnormal development until day eight (Chesley, 1935 and Bennett and Dunn, 1964) probably do not contribute significantly to the number of arrested morulae in ♂ T/t^{w32} x ♀ T/t^{w32} matings. Thus abnormalities due to: (1) inbreeding homozygosity and (2)

precocious expression of the T/T lethality probably do not contribute significantly to the total number of arrested morulae produced from T/t^{12} parents. There may be advantages to the *in vitro* analysis of t^{w32} homozygotes derived from T/t^{w32} parents. For example, the BALB/c background of those mutants derived from σ^{+}/t^{w32} x $\varphi +/t^{w32}$ matings could influence the expression of the t^{w32} allele. Mutants derived from T/t^{w32} parents would possess a different genetic background than those derived from $+/t^{w32}$ parents. Accordingly one might be able to detect phenomena of the t^{w32} allele more readily in this different genetic background. In addition, *in vitro* analyses of t^{w32}/t^{w32} embryos from tailless parents (T/t^{w32}) also allows one to critically examine the descriptive criteria of Mintz, 1964a and 1964b for determining t^{12}/t^{12} mutants from controls *in vitro*.

Cultural observations on lethal t^{w32} homozygotes from σ^{+}/t^{w32} x $\varphi +/t^{w32}$ matings reveal the following important points: (1) mutants may never completely compact, (2) some presumed mutants produce blastocoelic spaces but fail to form well defined blastocoels, and (3) in our experimental system the phenocritical period appears to extend over the 66-79 hpc interval. Hillman and Hillman, 1975 report that most t^{w32} mutants die as early morulae (16-cell stages). Our data which indicate that some presumed mutants do not undergo normal compaction at 8-cell stages are consistent with Hillman, 1975 who stated that the t^{w32} homozygous phenocritical period begins with the 8-cell stage.

Correlation of t^{w32} expression with abnormal 8-cell embryo compaction would indeed be interesting. In addition to investigating

developmental genetics of the T-locus, the investigation of t^{w32} homozygotes might also yield productive information on the compaction response. Continued *in vitro* observations should provide more specific information on the precise timing of the lethal t^{w32} allele and on morphological aspects of t^{w32} allele expression.

Fig. 1. Normal preimplantation ICR mouse development

a. Cleavage stage embryo of six cells, 36-48 hpc. Note the two large blastomeres in the lower right which will soon divide to form the 8-cell embryo (X650).

b. Cleavage stage embryo of 10-12 cells, 54-60 hpc, a morula stage embryo (X650).

c. Late morula to early blastocyst embryo of 16-32 cells, 60-72 hpc. Note that the individual character of the constituent blastomeres is lost except at the periphery of the early blastocyst embryo (X650).

d. Early to mid blastocyst, 72-84 hpc. Note the presence of two large blastocoelic vesicles which together with other such vesicles will coalesce to form the definitive blastocoel. Trophoblast cells are forming at the periphery of the embryo, while inner cell mass cells are developing internally (X650).

e. Mid blastocyst of 80-90 hpc. The microscope was focused on the rear trophoblast wall, and one can distinguish cell borders of the flat, squamous-shaped trophoblast cells lining the blastocoelic cavity (X650).

f. Definitive blastocyst of 84-96 hpc. The blastocoel occupies about 4/5 of the blastocyst's total volume. The inner cell mass, located at the lower left, will develop into the embryo proper. Trophoblast cells located at the periphery of the entire embryo are involved principally with the implantation process (X650).

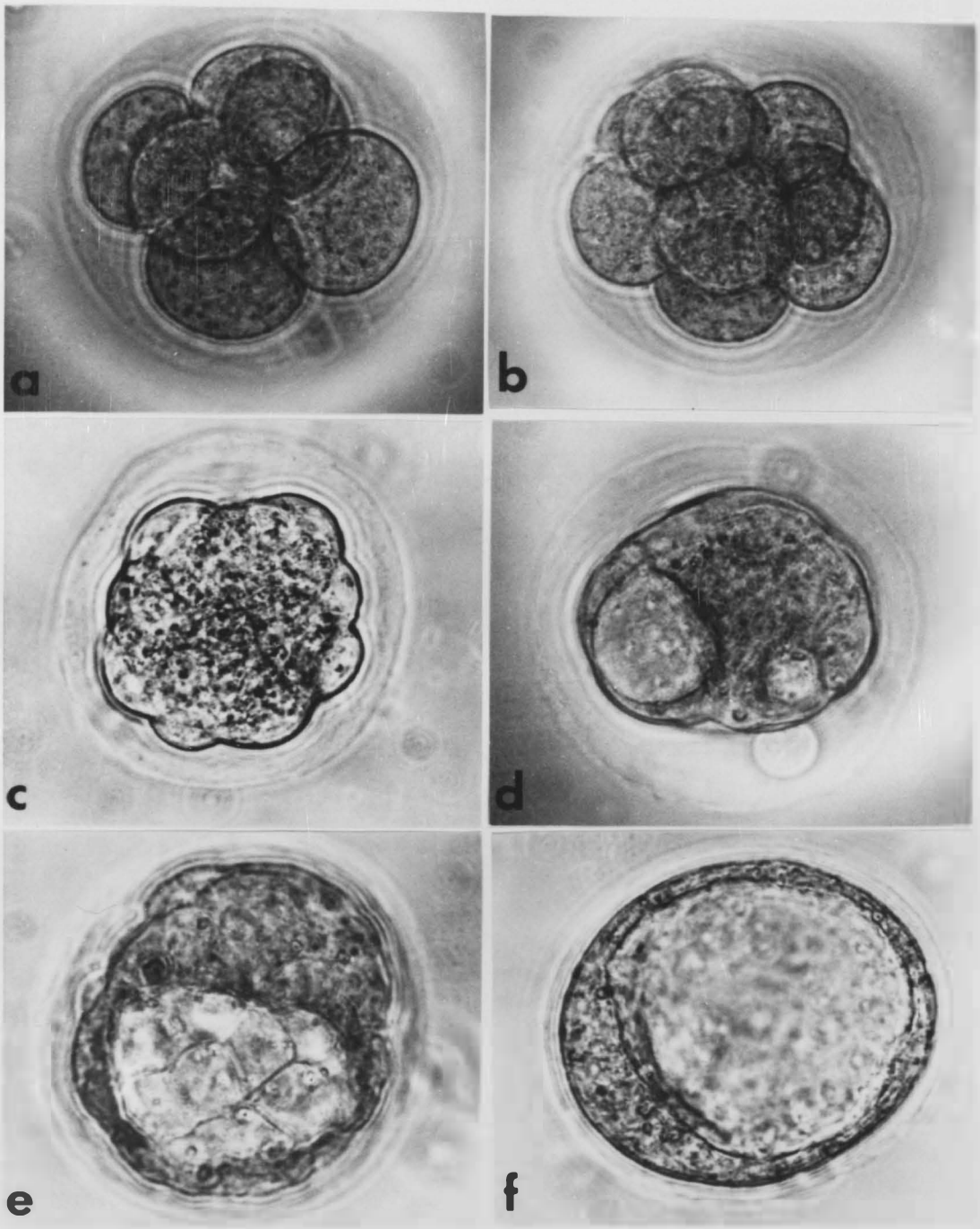
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Fig. 2. Preimplantation embryos from experimental ($\male +/t^{w32}$ x $\female +/t^{w32}$) matings

a,d. Apparently normal late morulae or early blastocysts of June 7614-E1 at 83 hpc, X525.

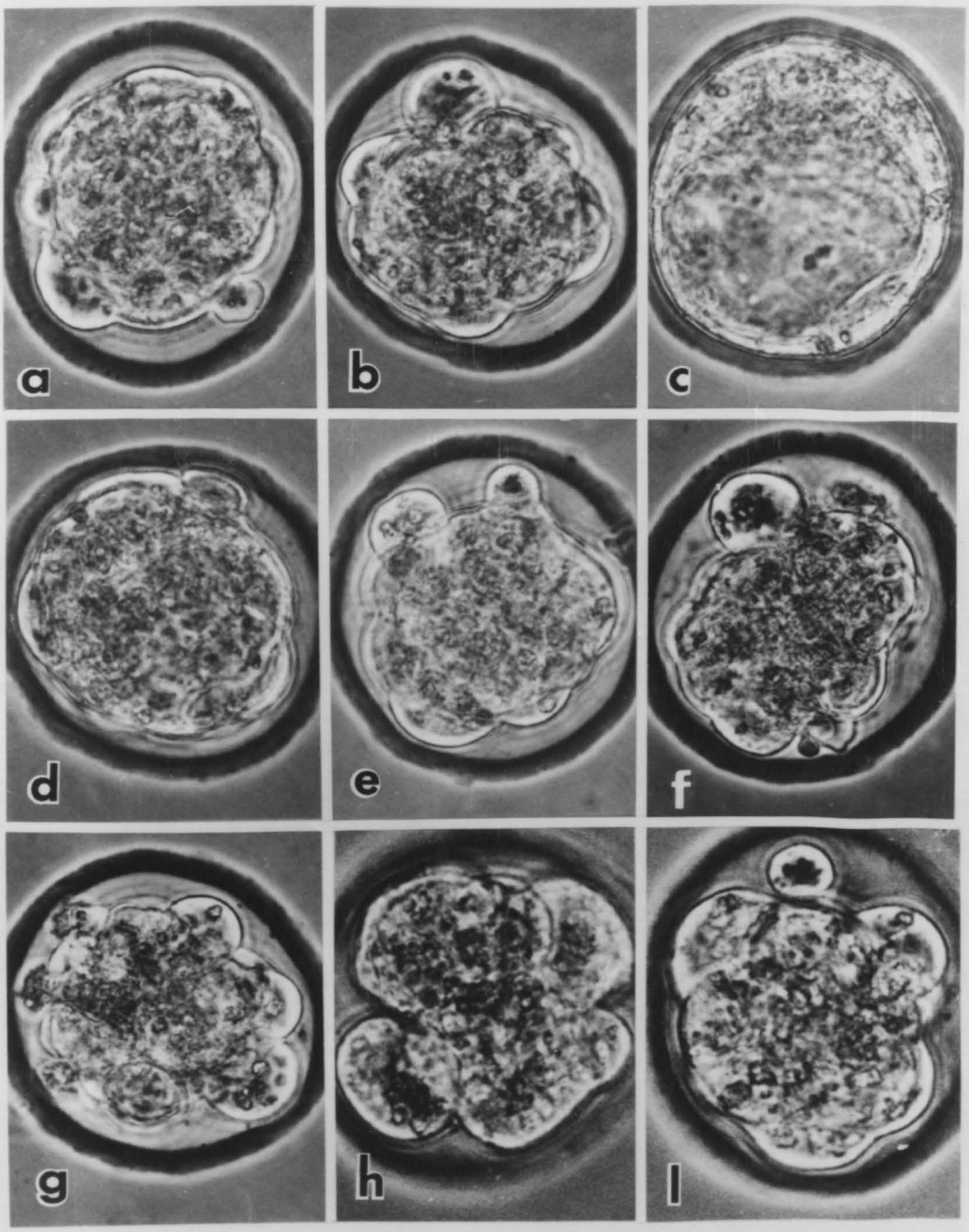
b,e. Presumed t^{w32} homozygote from June 7614-E1 undergoing developmental arrest at the morula stage at 83 hpc, X525.

c. Normal midblastocyst from June 7614-E1 at 83 hpc. Note the presence of a large blastocoel (lower center) and ICM (upper center), X525.

f,g. Degenerating arrested morulae from June 7614-E1 at 91 hpc, X525.

h. Uncompacted 8-cell (presumed mutant) embryo from June 7613-E1 at 79 hpc, X600.

i. Morula of June 7613-E2 undergoing dissociation or becoming uncompacted at 80 hpc, X600.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MORPHOLOGICAL CHARACTERIZATION OF t^{w32} HOMOZYGOTES AND CONTROL PREIMPLANTATION MOUSE EMBRYOS[1]

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RUNNING TITLE: JOHNSON AND GRANHOLM - MORPHOLOGICAL CHARACTERIZATION

ABSTRACT:

Phenotypic expressions of the t^{w32} homozygous lethality include: (1) excess cytoplasmic and intranuclear lipids, (2) binucleate cells, and (3) asynchronous cell death. It also appears that mutant embryos do not completely compact or are unable to maintain a compacted state. Objectives of the present study are to characterize cytoplasmic abnormalities of t^{w32}/t^{w32} mutant embryos and to generate data on cell contact relationships between blastomeres in mutant and control embryos.

Embryos from ♂+/t^{w32} x ♀+/t^{w32} (experimental) and ♂+/t^{w32} x ♀ +/T (control) crosses were recovered and cultured until either 50% of all experimental embryos were early blastocysts or until all

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control embryos were definitive blastocysts. Arrested and normal embryos were prepared for microscopic analysis. Approximately half of each embryo selected was serially sectioned (2 µm sections), placed on narrow strips of coverslips, stained, and photographed at 200X and/or 400X. Analysis was made by direct microscopic observations and by photomicrographs.

Present results confirm findings of previous investigators who have characterized the t^{w32} lethality. Excess cytoplasmic lipid clusters were more prevalent in mutant peripheral blastomeres which appeared more rounded, less closely attached, and retracted from zonulae pellucidae when compared to blastomeres of control embryos. Asynchronous cell death was apparent by differences in cell staining, granularity, and organelle content. In this analysis presumptive trophoblast cells appear to be primarily affected by the t^{w32} lethality.

MORPHOLOGICAL CHARACTERIZATION OF t^{w32} HOMOZYGOTES AND CONTROL PREIMPLANTATION MOUSE EMBRYOS

INTRODUCTION

The T-locus of the house mouse (Mus musculus) provides an excellent system for studying normal and abnormal developmental patterns. The t^{12} homozygous embryos are lethal, arresting at the morula-to-blastocyst transformation, making it the earliest acting (ontogenetically) known mammalian lethal. The t^{w32} allele is considered to be the same as the t^{12} allele (Bennett and Dunn, 1964) or different but closely associated. Hillman and Hillman, 1975 state "...the primary affect of these two alleles (t^{12} and t^{w32}) result in the same or in a closely associated developmental aberration." Because the morula-to-blastocyst transformation is considered a primary period of embryonic differentiation, cell relationships of arresting t^{12}/t^{12} and/or t^{w32}/t^{w32} embryos provide interesting phenomena.

Although t^{w32}/t^{w32} embryos arrest during blastocoel formation Hillman and Hillman, 1975 found evidence of t^{w32} expression as early as the two-cell state in the form of excessive cytoplasmic and intranuclear lipids. Another early manifestation of the t^{12} lethality is the presence of nuclear fibrillogranular bodies found as early as two-cell stage (Hillman et al., 1970; Hillman and Hillman, 1975). These agranular bodies appear stellate-shaped because of fibrills radiating out from their dense centers. According to the recent review by Hillman, 1975 fibrillogranular bodies have not been observed in t^{w32}/t^{w32} mutants.

Calarco and Brown, 1968 reported aberrant ribosome arrangements in t^{12}/t^{12} homozygotes. Normal embryos possessed mainly clusters of ribosomes (polysomes), or ribosomes were associated with endoplasmic reticulum at the time of blastocyst formation. Mutant t^{w32} embryos possessed both polysomes and granular endoplasmic reticulum plus greater than control amounts of single ribosomes. These single ribosomes were most often found associated with crystalloid-like material but without the normal crystalline arrangement. In 1968, Calarco and Brown reported no mitochondrial variation between normal and mutant embryos. Hillman and Hillman, 1975 observed two mitochondrial variants in 8-cell, early morulae, and late morulae t^{w32} homozygotes.

Another phenotypic expression of the t^{12} lethality is abnormal nuclear development. During normal blastocyst formation nucleoli assume an elongate shape (Smith, 1956). In t^{12}/t^{12} mutants nucleoli remain round up to time of developmental arrest (Hillman et al., 1970).

Finally, t^{12} and t^{w32} homozygotes possess binucleate cells (Hillman and Hillman, 1975) which are not observed in 2- and 4-cell embryos but increase as the embryos mature. The precise time of mutant embryo death is thought to be a consequence of the ratio of degenerating to healthy cells. Thus asynchronous cell death is also a characteristic of t^{12} and t^{w32} mutant syndromes.

Along with cytoplasmic characteristics of t^{w32} and t^{12} phenotypes, abnormal cell-to-cell relationships have also been suggested. In normally developing embryos a dramatic change in cell association is

observed at the 8-cell stage. Individual blastomeres take on new shapes and maximize their cell surface contacts. Interdigitating microvilli appear indicating a function in adherence of the cells (Calarco and Brown, 1969) and often form focal tight junctions, thought to be a prerequisite for specialized junctions (zonula accludens) necessary for blastocoel formation (Hillman et al., 1970; Calarco and Epstein, 1973; Hillman and Hillman, 1975; Ducibella and Anderson, 1975; Ducibella et al., 1975). Smith, 1956 suggested that t^{12}/t^{12} embryos arrest as blastocysts because of an inability either to elaborate blastocoelic fluid or to form specialized trophoblast cell junctions to maintain blastocoelic fluid. While Calarco and Brown, 1968 stated that t^{12}/t^{12} mutants appeared to be "more rounded and less closely applied" than their normal littermates, they did possess specialized junctional complexes between peripheral trophoblast cells. However, Hillman et al., 1970 found no interdigitating microvilli or junctional specializations in degenerating peripheral cells of t^{12} homozygotes. Such discrepancies in characterizing t^{12} and t^{w32} phenotypes are perhaps a result of the state of mutant degeneration when examined.

Primary objectives of this study were to characterize cytoplasmic abnormalities of t^{w32}/t^{w32} mutant embryos and to generate data on cell contact relationships between blastomeres in mutant and control embryos. It has been shown that there are quantitative intercellular gap distance differences between blastomeres of uncompacted and compacted 8-cell ICR embryos (page 18 this thesis). Such differences are qualitatively apparent at the light microscope level in 8-cell embryos. Mutant

embryos may mainfest cell contact relationships similar to that observed in uncompacted 8-cell embryos. In summary, symptoms of t^{w32} homozygous lethal mutants to be characterized and confirmed by light microscope include: (1) the presence of excess cytoplasmic and intranuclear lipids, (2) the presence of binucleate cells, (3) asynchronous cell death, and (4) the state of compaction of presumed mutant embryos.

METHODS

Methods of obtaining and calculating age of embryos is comprehensively outlined in Granholm et al., 1976. Embryos were flushed 54-60 hours post coitum (hpc), and cultured either until 50% of all experimental embryos were early blastocysts or until all control embryos were definitive blastocysts. Arrested and normal embryos were fixed for electron microscopy (Enders, 1971) over a time interval of 75-94 hpc.

Presumed t^{w32}/t^{w32} mutants and controls were selected using a table of random numbers. Approximately half of each embryo selected was serially sectioned into 2 µm sections, placed on narrow strips of cover slips, stained, and mounted on clean microscope slides using techniques modified from Roberts and Hutcheson, 1975. The modifications consisted of using toludine blue, instead of methylene blue, for a period of 10-12 minutes. Photographs were taken of serial sections of presumed mutant and control embryos at 200x and/or 400x using a Nikon phase microscope equipped with a 20x or 40x bright field objective lens. Analyses were conducted by means of direct microscopic

observations and photomicrographs of 2 µm sections. Twelve controls and fifteen presumed t^{w32} mutants were analyzed.

RESULTS

Twelve normal and fifteen arrested embryos were sectioned and analyzed. Nuclei were counted to determine approximate cell numbers, although embryos were removed from culture at different times and pooled. The mean number of countable nuclei in presumed mutant and control embryos were 95 and 102, respectively. In some arrested embryos, nuclear counts were difficult due to dense differential staining and fragmentation of some blastomeres.

Normal embryos displayed uniform staining (Figs. A and B) compared to arrested embryos (Figs. B-I) which exhibited differential staining between blastomeres. Such staining did not appear to be limited to any particular region of the embryo. In general, degeneration of arrested embryos appeared to decrease toward interior regions of the embryos (compare Fig. C with Fig. I which is sectioned 22 µm deeper into the embryo).

Intranuclear lipids (Figs. E and F) and cytoplasmic lipid clusters in greater amounts than controls were observed in arrested embryos. Such abnormally increased concentrations of nuclear and cytoplasmic lipids were more prevalent in peripheral (Figs. D, E and F) than internal blastomeres.

Presumptive trophoblast cells of arrested embryos generally appeared more rounded than those in controls; also they were less closely attached to each other and to interior cells (Figs. C-F). Binucleate cells were present in arrested embryos (Fig. E) and most nucleoli in mutant embryos appeared dense and round compared to controls which usually were more irregular or elongate.

Blastomeres of arrested embryos were retracted from zonulae pellucidae and did not present smooth profiles as in controls. Also, differences in cytoplasmic granularity and organelle content were noted in arrested embryos.

DISCUSSION

Obvious differences are apparent between arrested (presumed t^{w32}/t^{w32}) mutants and control embryos. Such differences include cytoplasmic abnormalities, nuclear and nucleolar irregularities, and cell-to-cell relationship abnormalities. These results confirm the findings of previous investigators who have characterized the t^{12} or t^{w32} lethality.

Cellular membranes of some arrested mutant blastomeres are obviously less closely applied to each other than those in controls. Within presumed mutants peripheral blastomeres are round, and in some cases, completely excluded from the embryo. In these mutants peripheral cells rarely flatten to form extensive junctions with internal cells. This latter observation was recorded by Calarco and Brown, 1968. As

sections are analyzed further into the same arrested embryos, blastomeres appear less degenerate and peripheral cells are less rounded. However, peripheral cells are not compacted to the same degree seen in controls (Ducibella and Anderson, 1975). Cytoplasmic abnormalities reported by Hillman and Hillman, 1975 to be characteristic of the t^{w32} lethality including excess cytoplasmic and intranuclear lipids, binucleate cells, and asynchronous cell death were all observed in arrested t^{w32} presumed mutants in the present study. Differential cytoplasmic staining observed in mutant embryos suggests asynchronous cellular death. Differential staining patterns indicate that within the same mutant internal blastomeres are less degenerate than external ones. The likelihood that these patterns result from staining artifacts is not great, since all sections are stained simultaneously. One rather interesting possibility to account for differential staining patterns could be the differential susceptibility of presumptive trophoblast cells to the t^{w32} allele. During blastocyst formation presumptive trophoblast cells become peripheral to the more internal presumptive ICM cells (inside-outside hypothesis). Since peripheral cells of arrested morulae appear more degenerate than internal cells perhaps trophoblast cell anlagen are indeed more susceptible to t^{w32} effects than ICM anlagen.

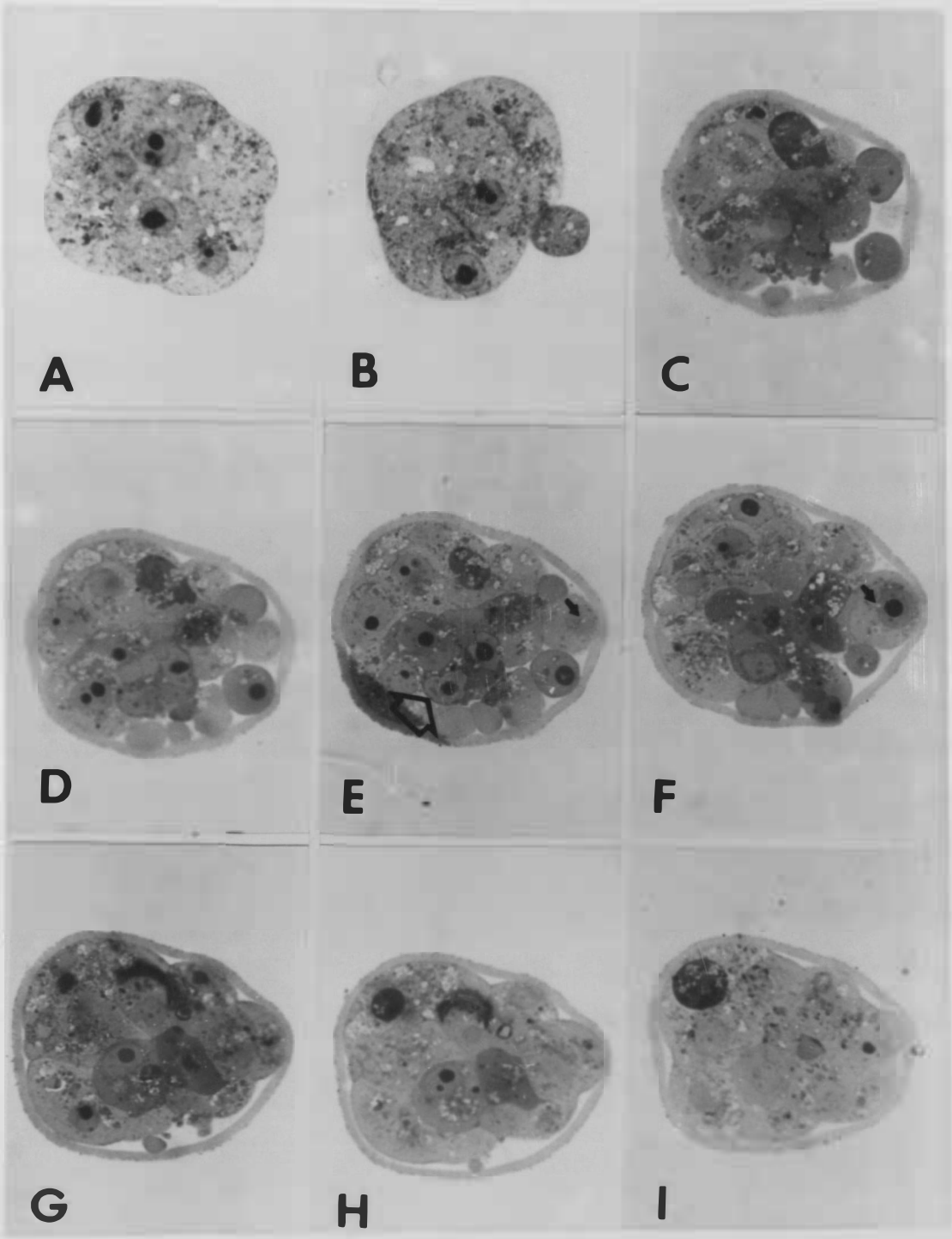
Prior to blastocyst formation in normal embryos nucleoli become irregular-shaped and elongate (Smith, 1956). Hillman et al., 1970 reported that nucleoli in mutants remained round and failed to undergo normal elongation up to the time of developmental arrest. Our analysis

confirms these observations. Nucleoli in arrested embryos remain discrete and round when compared to controls.

Although abnormalities reported by previous investigators and confirmed in the present study characterize the t^{w32}/t^{w32} lethal mutant, all alleged t^{12}- and/or t^{w32}-induced abnormalities (except excess nuclear and cytoplasmic lipids) are found only in arrested embryos. Therefore it is possible that certain characteristics attributed to t^{12} and/or t^{w32} alleles are merely mainfestations of a dying embryo. It is thus essential to separate secondary and tertiary effects from primary t^{w32} defects.

Fig. A and B. 2 μm sections of control morulae. Note smooth profile of embryo inside zonula pellucida and equal staining of all blastomeres. Nucleoli have become irregular and elongated. A polar body is present in Figure B. X900.

Fig. C-I. Selected 2 μm serial sections of arrested t^{w32}/t^{w32} embryo. Note uneven profile of embryo inside zonula pellucida caused by rounded up peripheral cells which are less closely attached to interior cells (C-F); a binucleate cell (E, large arrow) and intranuclear lipids (E and F, small arrows) are present. Large clusters of lipids are seen in peripheral cells of all sections. Note differential staining of blastomeres in mutant compared to control embryos. X900.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DISCUSSION AND CONCLUSIONS

Objectives of the studies presented in this thesis were to characterize and compare normal versus lethal mutant t^{w32}/t^{w32} cellular interactions during preimplantation mouse development. Previous investigators have reported: (1) the phenolethal period for t^{w32} homozygotes extends from 8-cell embryos to late morulae with most arresting at early morula (Hillman and Hillman, 1975), (2) blastomeres of arrested t^{w32}/t^{w32} morulae appear to be less closely applied to each other than those of controls (Calarco and Brown, 1968), and (3) abnormal nuclear and cytoplasmic inclusions as well as other cytoplasmic irregularities are symptoms of the t^{w32} homozygous lethal syndrome (see Hillman, 1975 for current review).

Our investigations confirm these previous findings. Our *in vitro* and light microscope analyses have determined: (1) the phenocritical period of presumed mutant arrest is from 66-79 hpc, (2) blastomeres in some arrested embryos never reach a completely compacted state, or become partially uncompacted as early morulae sometime after arrest, and (3) cytoplasmic evidence of arrest includes binucleate cells, differential blastomere staining indicating asynchronous cell death, abnormal nucleolar shapes, and excess cytoplasmic and intranuclear lipids.

The quantitative analysis of gap distances between blastomeres of uncompacted and compacted 8-cell ICR embryos has shown that there are quantifiable and statistically significant differences between

uncompacted and compacted embryos. This work also provides a foundation for studies on compaction in t^{w32} homozygotes.

Future experimental plans include applying the quantitative technique to litters from ♂+/t^{w32} x ♀+/t^{w32} crosses. If the "less closely applied" state reported by Calarco and Brown, 1968 and the degree of partial-compactedness reported by Granholm et al., 1976 (page 18 this thesis) are investigated quantitatively, this approach may provide identification of t^{w32}/t^{w32} presumed mutants at a time prior to cell degeneration. Until now, characteristics of the t^{w32} lethality, except excessive cytoplasmic and intranuclear lipids which are evident at the 2-cell stage (Hillman and Hillman, 1975), have been identified in degenerating embryos. An analysis of ultrastructure at the 8-cell stage would allow possible separation of secondary and tertiary effects from primary t^{w32} genetic defects.

In return, identification of primary t^{w32} effects could provide data to aid in understanding mammalian gene action in both normal and abnormal mammalian development. Furthermore, such an analysis might provide additional information on the role of the T-locus in controlling mammalian morphogenesis.

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