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EXPERIMENTAL DISENGAGEMENT OF MORPHOGENETIC PROCESSES AND ITS EFFECTS ON PRIMORDIAL GERM CELL COLONIZATION OF THE GONADAL RIDGES IN RANA PIPiens

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PROCESSSES AND ITS EFFECTS ON PRIMORDIAL GERM CELL
COLONIZATION OF THE GONADAL RIDGES IN RANA PIPiens

by

Joseph E. Penkala

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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May 1986
ABSTRACT

EXPERIMENTAL DISENGAGEMENT OF MORPHOGENETIC PROCESSES AND ITS EFFECTS ON PRIMORDIAL GERM CELL COLONIZATION OF THE GONADAL RIDGES IN RANA PIPIENS

by

Joseph E. Penkala

During normal embryogenesis in *Rana pipiens* the primordial germ cells (PGCs) undergo a displacement from deep within the ventral endoderm of the Stage 17 tailbud embryo to the dorsal endodermal crest of the hatched Stage 20 larva; eventually they exit the endoderm and populate the paired gonadal primordia. The present study was undertaken to examine the migratory properties of PGCs and obtain evidence for the mechanisms of their displacement. This was accomplished by temporally uncoupling PGC migration from the morphogenetic events that normally accompany it.

The methodology consisted of reciprocally transplanting germ cell-containing endoderm regions between Stage 17 embryos and Stage 20 larvae, where the hosts were UV-irradiated to prevent their contribution to the gonadal germ cell population. Success of migration from the donor graft was determined by direct gonadal germ cell counts at host Stage 25.

Normal Stage 20 PGCs transferred to 2-1/2 day younger
UV-irradiated Stage 17 hosts were able to colonize the gonadal ridges of the heterochronic graft larvae. The number of gonocytes present was equal to approximately 20% of the unoperated control number. The data from these experiments together with the histological study of the heterochronic larvae (Subtelny and Penkala, 1984), convincingly demonstrate that PGCs can be experimentally retained within the endoderm for at least 2-1/2 days and are still able to migrate to the gonadal ridges.

Reciprocal transfers of Stage 17 PGCs into UV-irradiated Stage 20 hosts resulted in the precocious colonization of the host gonadal ridges 2-1/2 days earlier than would normally have occurred had the PGCs remained within the donor embryo. The proportion of PGCs occupying the host gonadal ridges was significant, but restricted, averaging 2% of the unoperated control number of gonocytes. Nevertheless, the results show that 1) intraendodermal PGCs can colonize the gonadal ridges at least 2-1/2 days earlier than they normally would and 2) they do so in the absence of the main events of embryogenesis that occur during intraendodermal migration (Stages 17 to 20).

The data from this study in conjunction with the histological analysis of the reciprocal heterochronic graft larvae (Subtelny and Penkala, 1984) indicate that under conditions of temporally disengaged morphogenesis the PGCs in either form of heterochronic graft emerge from the endoderm during a restricted time of development of the
host, corresponding to Stage 22+/23−. This emergence is intimately associated with the movements of mesodermal cells of the host lateral plates involved in dorsal mesentery formation. The findings support the hypothesis that morphogenesis plays a major role in the migration of PGCs from the endoderm to the gonadal ridges.
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BACKGROUND FOR THE STUDY

I. Introduction

Morphogenetic cell movements provide the fundamental machinery by which embryonic cells become organized into a three-dimensional array, and thereby assume the specific spatial patterns which define the tissues of an organism. In this regard, morphogenesis may be viewed as the quintessential feature of vertebrate embryogenesis. In general, the morphogenetic movements of embryonic cells occur in two different manners: cells migrate \textit{en masse} in sheets or clumps, forming a cohesive surface, cord, or plate of coordinately functioning cells; this process is clearly visualized during gastrulation and neurulation. Alternatively, cells migrate individually through cellular interstices, often to a site quite distant from the point of origin. This latter mode of cellular translocation is characteristic of the primordial germ cell (PGC), a cell that has its origin in vertebrates and certain invertebrates at a distant extragonadal site, and only through a series of cellular movements comes to reside within the gonadal epithelium, the environment in which the PGC transforms into the mature reproductive cell.

The migration of anuran germ cells during embryo-
genesis, which has been studied by a number of laboratories may best be described as occurring in five stages (Fig. 1A-E): A) **Presumptive primordial germ cell phase**: subcortical vegetal pole germ plasm is partitioned into select vegetative blastomeres via the cleavage process to become presumptive primordial germ cells (pPGCs); B) **Intraendodermal phase I**: the pPGCs behave similarly to the neighboring yolk-laden blastomeres and via the morphogenetic movements of gastrulation become seated deep within the ventral endoderm of the early neurula; C) **Intraendodermal phase II**: the initiation of a series of presumably active movements, whereby the PGCs translocate dorsally and accumulate within a median dorsal crest atop of the posterior endoderm mass; D) **Endodermal-exiting phase**: the germ cells emerge from the endoderm concomitant with the formation of the intestinal mesentery and become positioned at the dorsal root of the mesentery in the so-called "median germ ridge"; E) **Gonadal colonization phase**: the median ridge of PGCs becomes partitioned bilaterally whereby the paired gonadal rudiments are formed.

Clearly, during its embryonic history, the anuran germ cell is exposed to different microenvironments and is subject to a spectrum of morphogenetic events, including segmentation, gastrulation movements, morphogenesis of the dorsal mesentery from the dorsal lateral plate mesoderm, and formation of the gonadal anlagen. Before discussing these
Figure 1. Stages in germ cell migration in *Rana pipiens*.

A. **Presumptive Primordial Germ Cell Phase**: germ plasm is distributed to selected cleavage blastomeres in the vegetal hemisphere.

B. **Intraendodermal Phase I**: germ plasm-containing cells are shifted by morphogenetic movements to deep within the endoderm mass of the neurula.

Figures 1A and 1B are representative sections through embryos for each given stage. Germ plasm is represented by dashed lines or black dots.
FIGURE 1. STAGES IN PGC MIGRATION IN RANA PIPiens

A. Presumptive Primordial Germ Cell Phase
   - Fertilized Egg
   - Two-Cell
   - Blastula

B. Intraendodermal Phase 1
   - Gastrula
   - Neurula
C. Intraendodermal Phase II: active intraendodermal migration from the deep endoderm to the dorsal median endoderm crest.

Figure 1C shows representative embryos (top drawings) for each of the developmental stages presented and corresponding cross sections (bottom drawings) taken at the level indicated by the vertical line. Cells labelled by black dots are primordial germ cells.
C. INTRAENDODERMAL PHASE II

TAILBUD
STAGE 17

HEART BEAT
STAGE 19

HATCHING
STAGE 20
D. Endodermal Exiting Phase: germ cell emergence from the endoderm and displacement to the median genital ridge at the root of the dorsal mesentery.

E. Gonadal Colonization Phase: lateral displacement of PGCs from the base of the intestinal mesentery to the paired gonadal ridges, thus forming the gonadal rudiments.

Figures 1D and 1E show representative embryos (top drawings) for each of the developmental stages presented and corresponding cross sections (bottom drawings) taken at the level indicated by the vertical line.
aspects in detail, a background will be presented first on
the origin, identification, and differentiation of PGCs
during their migration to the gonadal ridges (Section II).
Then attention will be given to the mechanisms of germ cell
migration from the endoderm to the gonad, providing a
current review of the research conducted (Section III);
finally, the aim and rationale for the experiments conducted
and reported in this thesis will be presented in Section IV.

II. Historical Review: Identification, Origin, and
Differentiation of Primordial Germ Cells

A. Early Identification of Primordial Germ Cells in
Anurans

Prior to 1900, the germinal elements in Amphibia were
considered to originate in the region of the gonadal anlage,
either deriving from the cells of the gonadal epithelium,
once the rudimentary gonad had formed (Waldeyer, 1870), or
existing as a separate germ line distinct from the somatic
cells of the embryo and continuous throughout development
(Nussbaum, 1880).

The first detection of extragonadal germ cells in
Amphibia, prior to their localization in the genital folds,
was reported by Bouin (1900) in *Rana temporaria*. In
cross-sections of a 10mm tadpole in the region of the caudal one-third of the body, he discovered an unpaired median mass, lying between the cardinal veins, beneath the dorsal aorta, and above the attachment of the mesentery to the dorsal body wall. This "ebauche genitale" was comprised of yolk-rich cells (the PGCs), which Bouin suspected of originating from the yolky endoderm mass below by way of the dorsal mesentery. Alternatively, he postulated that these cells may have arisen from peritoneal or mesenchymal cells of the axial region.

Subsequent work by Allen (1907) with *R. pipiens* supported Bouin's first interpretation. Allen observed the presumptive sex cells to be localized within a dorsal median endoderm crest overlying the archenteron and continuous with the endoderm proper. During a phase of germ cell development that he referred to as the "important period", Allen observed the germ cells within the endodermal crest to be "pinched off" from the remainder of the endoderm mass as the dorsal mesentery was being formed beneath them. However he postulated that an amoeboid movement was involved in the displacement of the germ cells from the endoderm mass.

The identification and localization of the PGCs in the endoderm crest have been further substantiated by several workers: King (1908), in *Bufo americanus*; Kuschakewitsch (1908, 1910), in *R. esculenta*; Witschi, in *R. temporaria*
(1914) and in *R. sylvatica* (1929); Swingle (1921), in *R. catesbeiana*; Bounoure (1924), in *R. temporaria* and *B. vulgaris*; Humphrey (1925), in five different species, reconfirming works in *R. pipiens*, *B. americanus*, and *R. sylvatica* as well as new reports in *R. palustris* and *R. clamitans*; Cheng (1932), in *R. cantabrigensis*. In all of these works, the germ cells were traced back to the median dorsal endodermal crest in hatched larvae with gill circulation. Due to the large amounts of yolk present in both the germ cells and somatic endodermal cells as well as the vagueness of definition of cell boundaries and the lack of distinction of germ cells from somatic endodermal cells, other than their position in the endodermal crest, it was impossible to identify the germ cells within the endoderm mass itself at earlier stages of development. Nevertheless, several workers perceived the germ cells as arising amidst the endoderm cells (King, 1908; Witschi, 1914; Humphrey, 1925; Cheng, 1932), and it was even speculated that the PGCs had their origin from the same region of the uncleaved egg that gave rise to the endoderm.

Meanwhile Bounoure (1925), investigating *R. temporaria* and *Triton alpestris*, reported the detection of cytologically distinct germ cells in the endoderm along either side of the archenteron cavity in the late tailbud stage embryo, and their accumulation within the dorsal
crest of the endoderm in the hatching stage larva. Perle (1927) reported a similar localization of PGCs in *B. vulgaris* using the same cytological criteria as Bounoure for their identification: 1) large cell size and yolky cytoplasm (in contrast to the mesenchymal cells), 2) a faintly staining, sparsely pigmented cytoplasm (in contrast to other yolky cells in the endoderm), 3) a poorly staining nucleus, exhibiting lack of basophilia and containing two or more small nucleoli. Unfortunately, the reliability of these cytological criteria for unequivocal identification of PGCs cannot be extended to earlier stages of development and may be somewhat suspect as it has since been determined that germ cells in the genus *Triton*, and urodeles in general, arise from the lateral mesoderm and not from the endoderm.

B. Origin of PGCs Within the Endoderm and Their Differentiation During Embryogenesis

A much more convincing demonstration that germ cells originate from the endoderm was first published by Bounoure in 1929, whereby he utilized a modification of Altmann's stain (Volkonsky, 1928) and discovered a basophilic cytoplasmic inclusion consisting of a high density of mitochondria and devoid of yolk platelets that was characteristically present in select tailbud and gastrula
stage cells within the ventral endoderm, as well as those cells adjacent to the archenteron and those comprising the dorsal endodermal crest of hatching stage larvae. This "germinal plasm", as Bounoure has termed it, became a convenient marker for germ cell identification (Bounoure, 1929); and cells within the endoderm containing this cytoplasmic marker in gastrulae, tailbud embryos, and hatching larvae could be followed in histological sections of older animals to enter the gonadal ridges. Bounoure has since reported on this technique (1934, 1939, 1964), describing the presence of germ plasm even in the uncleaved fertilized egg.

Bounoure (1934, 1939) was able to map the localization of the "plasm" within select cells, the primordial germ cells (PGCs), in the embryo at successive developmental stages in R. temporaria. It was first detectable in the subcoelomic region of the vegetal pole of uncleaved eggs. During cleavage it became incorporated into a select number of blastomeres (pPGCs). During gastrulation to early tailbud stage, germ plasm-bearing cells were detected deep within the endoderm, later distributing dorso-laterally on either side of the enteron, and finally accumulating above the enteron in the dorsal endodermal crest, at which point the cells no longer showed a differential stainability either because the plasm had undergone some qualitative
change or lost its affinity for the stain.

Bounoure's initial observations in *R. temporaria* were later confirmed by Blackler (1958) who also examined embryos of *B. bufo* and *Xenopus laevis*. Studies on the latter species had been initiated by Nieuwkoop and Faber (1956) who observed germinal plasm in sections of the fertilized egg and cleavage stage embryos. Again, Blackler (1958) identified the germ line cells from the time of fertilization until the time of colonization of the gonadal ridges. He also looked at *R. esculenta*, but was unable to detect germ plasm in this species. However, to date, this technique is by far the most useful for light microscopical identification of germ cells in several anuran species (Blackler, 1958; Gipouloux, 1962; Czolowska, 1969; Whittington and Dixon, 1975; Kamimura et al., 1976). Thus, the early cytologists were able to detect germ cells within the dorsal endodermal crest of hatched larvae by morphological criteria, but then with Bounoure's application of the Altmann stain there seemed to be an opportunity to experimentally determine the origin of the PGCs, to establish the role of germ plasm as a cytoplasmic determinant for germ line cells, and to establish whether or not the germ line was continuous throughout embryogenesis as well as from generation to generation.

It is now well established that gonadal germ cells of anurans are derived from PGCs originally within the endoderm
during early embryogenesis. This evidence is based on grafting experiments between genetically marked embryos at the neurula (Blackler, 1962, 1966, 1970; Blackler and Gecking, 1972 a, b) or tailbud stage (Subtelny and Carrethers, 1975). In the former instance, the endoderm region of host neurulae, shown earlier histologically to contain the germ plasm-bearing cells, was replaced by the same region from neurula donors. The donor cells were genetically marked in some experiments by means of the Oxford nuclear marker in X. laevis (Blackler, 1962), by making intraspecific grafts between two subspecies of Xenopus (Blackler, 1966) in other experiments, or by making interspecific grafts between X. laevis and X. mulleri (Blackler and Gecking, 1972a,b) in yet other experiments. In every case, subsequent transmission of the resultant sex cells in animals maturing from grafted embryos yielded offspring bearing the genetic marker of the donor. Similarly, Subtelny and Carrethers (1975) transferred endoderm regions corresponding to those containing the germ plasm bearing cells from R. sylvatica tailbud donors into UV-irradiated R. pipiens tailbud hosts with the result that the developing froglets contained R. sylvatica germ cells in their gonads as clearly shown by their distinct LDH isozyme pattern on starch gels.

There are several types of indirect experimental evidence that the germ plasm is a cytoplasmic determinant
for germ line cells. Each type of experiment relies on the fact that the specially staining germinal cytoplasm is present in the vegetal cortex of the unfertilized and fertilized egg and that a perturbation of this region, which in effect disrupts the germinal plasm, results in the absence or reduction in the number of germ cells present in the gonads of treated embryos that complete development. One example is to expose the vegetal cortex of uncleaved, fertilized eggs of *R. temporaria* to ultraviolet radiation (Bounoure, 1939; Bounoure et al., 1954). This leads to partial or total sterility in metamorphosing froglets which develop from eggs exposed to UV light. These results have been confirmed by Aubry (1953a, b) in the same species, and by Padoa (1963) in *R. esculenta*. More recent experiments by Smith (1966) convincingly show 100% sterility in feeding stage *R. pipiens* larvae that were exposed to 18,000 ergs/mm² of UV radiation at the two-cell stage. In one part of this study, Smith varied the wavelength of UV radiation to which the two-celled embryos were exposed and found that a wavelength of 253.7 nm resulted in the highest percentage of sterile larvae developing from the irradiated embryos. This particular wavelength is known to induce dimer formation in nucleic acids and it is suggested that perhaps one of the ways that UV radiation affects the formation of germ line cells is to damage critical ribonucleic acids (RNAs), possibly messenger RNAs, within the germinal plasm.
of treated embryos. Ikenishi et al. (1974) reported morphological alterations within the germ plasm of *Xenopus* embryos examined shortly after irradiation at the two-cell stage. The mitochondria appeared swollen and vacuolated and the germinal granules were fragmented. In contrast, Smith and Williams (1975, 1979) and Williams (1978) were unable to detect this disruptive effect of UV radiation on the elements of the germ plasm in *Rana* or *Xenopus* at early stages of development, but did note that gonadal PGCs in larvae developing from UV-treated embryos apparently had abnormal looking mitochondria associated with nuage material whereas mitochondria in untreated controls were normal in appearance. The latter authors feel that the effect of UV radiation on the germ plasm immediately after treatment as reported by Ikenishi et al. (1974), could be observed in control animals as well and was probably artificial (Smith and Williams, 1979). Quantitative evidence that the germ plasm may play a determinative role in the formation of PGCs comes from experiments by Tanabe et al. (1974) where *Xenopus* embryos at the two-cell stage were subjected to UV radiation, while one of the following three parameters was varied: a) the distance of the germ plasm from the plasmalemma at the vegetal pole, by centrifugation b) the total area of the vegetal pole exposed to UV radiation, by shielding, or c) the total dose of UV radiation
administered. In each case (a, b, c) the parameter varied had an inverse relationship to the proportion of gonadal germ cells in the irradiated tadpoles, implying that the more protected the germ plasm is from UV radiation, i.e. a) the more internalized the germ plasm is in centrifuged eggs, b) the less relative area of the vegetal hemisphere containing the germ plasm that is exposed in shielded eggs, or c) the less overall dose of UV radiation to which the germ plasm containing vegetal hemisphere of the egg is exposed, then the greater the number of PGCs that colonized the gonadal ridges of treated larvae. Thomas et al. (1983) found a similar dose response curve of germ cell number versus UV dose in the same species. In addition, Iriji and Kotani (1976) found that eggs vary in their sensitivity to UV light, according to the developmental stage at which they are exposed, such that UV treatment of unfertilized and fertilized eggs gives rise to embryos with maximum sterility, whereas at later cleavage stages, the UV treatment is no longer able to induce sterility, and it is presumed that the distant position of the germ plasm from the vegetal pole within the later cleavage blastomeres "protects" the plasm from UV radiation. Finally, Ikenishi and Kotani (1979) observed a definite effect of UV treatment on the position of PGCs within the endoderm. At the time the PGCs in nonirradiated controls are in the dorsal endoderm at late tailbud stages, they are still deep within the
ventral endoderm in the UV-treated animals. After this stage, they are no longer detectable in the endoderm. The delay in intraendodermal migration in the UV-treated embryos, suggests an effect of UV on the germ plasm-bearing cells.

Another type of experiment designed to perturb the germ plasm-containing region of the egg and measure the impact of the perturbation on the formation of germ-line cells involves the deletion of vegetal cytoplasm from fertilized eggs and the determination of the subsequent effect on gonocyte numbers in the resultant larvae. Early studies to this effect were somewhat equivocal. Nieuwkoop and Suminski (1959) in *Xenopus* and Fischiarolo (1960) in *Discoglossus* found that deletion experiments did not substantially affect the number of gonocytes in the larvae that developed from pricked eggs, whereas Librera (1964), using the same technique as Nieuwkoop and Suminski (1959) found in *Discoglossus* a marked reduction in the number of gonocytes in tadpoles following the removal of the vegetal cytoplasm at the four-cell stage. In none of the above experiments did the authors verify that the cytoplasmic exovate deleted from the vegetal pole of the egg did indeed contain the germinal cytoplasm. The most convincing study in this regard was conducted by Buehr and Blackler (1970) who found either complete sterility or a substantial reduction in the
number of gonadal PGCs following deletion of vegetal cytoplasm at the two-cell stage in *Xenopus*. The investigators examined histological sections of eggs that had been pricked in order to confirm that the germ plasm was extruded from the vegetal pole of the egg into the exovate. Furthermore, examination of the pricked eggs at blastula stage revealed that 33% of the embryos did not contain germ plasm which correlates well with the finding that 32% of the treated tadpoles were sterile. Apparently, there is a relationship between the removal of germ plasm-containing exovate from eggs by pricking and the absence of germ plasm in blastomeres in one-third of the blastulae and the complete absence of gonadal germ cells in one-third of the tadpoles. These results were later confirmed by Gipouloux (1971, 1975) in *Discoglossus*. However, the exact manner by which sterility is induced by pricking the egg cannot be stated. It is tempting to say that the formation of PGCs relies on the presence of the germinal plasm, but other factors, such as mechanical disruption of the plasmalemma are also introduced by pricking the egg.

Smith (1966) examined the determinative nature of the vegetal cytoplasm by a type of rescue experiment, whereby *R. pipiens* eggs treated with a sterilizing dose of UV radiation (18,000 ergs/mm²) were injected at the vegetal pole with cytoplasm from normal eggs from the same region. Such eggs, when allowed to develop, were found to contain gonadal germ
cells at feeding tadpole stage, whereas noninjected UV irradiated controls or UV irradiated eggs injected with saline or animal pole cytoplasm gave rise to sterile larvae. Similar studies have been reported by Wakahara (1977, 1978) in *R. chensinensis*. The injection of a crude homogenate of vegetal pole cytoplasm into UV-irradiated eggs resulted in the development of tadpoles that had considerable numbers of PGCs (67% relative to the untreated controls). Unfortunately, none of the UV-irradiated controls in these experiments were devoid of germ cells and, on the average, contained 23% of the PGCs found in untreated controls. In addition, Wakahara (1977) showed that the germ cell-inducing cytoplasm could be pelleted at 15,000 g, and ultrastructural analysis of the pellet revealed the presence of membranous structures, mitochondria, and aggregates of electron-dense granules resembling germinal granules. In 1978, Wakahara induced supernumerary PGCs in *Xenopus* eggs by injecting non-irradiated eggs with vegetal pole cytoplasm that had been pelleted at 20,000 g. The number of gonocytes in the resultant larvae were 30% greater than in the untreated controls.

In all of the above studies, an attempt was made to establish a link between the integrity of the germinal cytoplasm, as a localized cytoplasmic determinant in the egg, and the formation of PGCs. The experiments were of
three basic designs: a) UV-irradiation studies, b) deletion studies, and c) rescue studies. Changes in gonadal germ cell number were detected following these various manipulations of the germ plasm-containing region of the zygote, and it was therefore logical to assume that the germ plasm was the target of these manipulations, and because its integrity was required for germ cell formation, that it must indeed be the cytoplasmic determinant for these cells. However, these conclusions are yet premature, and to date there has been no direct experimental evidence that demonstrates the germinal plasm in anurans is the determinant of the germinal cell. It may further be stated that alternate hypotheses have been proposed to accommodate the data from these studies. Even back in 1947, Nieuwkoop questioned the validity of Bounoure's conclusions based on UV studies (Bounoure, 1935, 1937) that germ plasm was the target of UV action and was, therefore, the determinant required for PGC formation. Instead, Nieuwkoop felt that general embryogenesis was being affected by the UV-irradiation, and that deviation in the normal pattern of development, rather than the destruction of the cytoplasmic determinant of germ cells, was the cause of reduction in gonadal PGC numbers in treated larvae. That UV-irradiation of zygotes can induce developmental anomalies, in particular, those corresponding to defects in primary axis formation, has been documented by Grant (1969), Grant and
Wacaster, (1972), and Malacinski, et al. (1975). However, it should be pointed out that the regimen for UV-treatment in these studies were considerably different than those used to induce sterility in embryos: the time of irradiation during the first cell cycle was earlier and the level of total irradiation was considerably higher in the the studies designed to disrupt primary axis formation. Beal and Dixon (1975) and Zust and Dixon (1975) discovered that UV-irradiation of embryos between first and second cell division impaired cleavage furrow formation in the vegetal pole region of the zygote. Absence of cleavage with continued karyokinesis gave rise to a syncytium of nuclei within the vegetal hemisphere of the blastula which resulted in either aborted development or resumption of cytokinensis and completion of development. The authors concluded that UV-radiation acted primarily on early embryogenesis and that this could account for the absence or delayed migration of PGCs. It should again be noted that the doses of UV-radiation, usually exceeding 11,0000 ergs/mm² were in considerable excess to those doses normally used to attain sterility of the gonadal primordia in Xenopus (7800 ergs/mm²; Thomas et al., 1983). In fact, Thomas et al. (1983) have shown that at the lower UV doses, gonadal sterility is induced, but yet there is no detectable alteration in the cleavage planes or maintained change in
rate of cleavage in the UV-irradiated embryos which suggests to the authors that the UV effects on germ cell formation and cleavage furrow formation are two independent effects. Independent studies by Smith and Williams (1979) and Subtelny (1980) have shown that 100% sterile larvae can develop from batches of UV-treated eggs in both *Rana* and *Xenopus* without any defects occurring in cleavage or embryogenesis in general. Furthermore, Subtelny (1980) has shown that in parabionts formed between normal and UV-irradiated tailbud embryos, in which the dorsal structures of the normal parabiont were removed, PGC migration takes place in a normal fashion to the gonadal ridges of the UV-host. The degree of migration is equivalent to that obtained in normal-to-normal parabionts, demonstrating that the endoderm environment of the UV-irradiated embryo supports normal germ cell migration. It would therefore appear that the target of UV radiation which results in the absence of germ cells, and that responsible for normal cleavage and primary axis formation, are separate and distinct. This reasoning is supported by Thomas et al. (1983) whose experiments with *Xenopus* embryos clearly show that there is no correlation between the UV disruption of cleavage and the number of PGCs formed, and that rotation of the egg 90° shortly after UV irradiation, which rescues the embryo from having defects in primary axis formation (see Scharf and Gerhart, 1980), will not prevent a reduction in
the number of germ line cells. Furthermore, the UV-induced defects in cleavage and primary axis formation occur at doses of radiation in considerable excess to that required for inducing 100% sterility in feeding tadpoles.

Ultrastructural studies have identified and characterized the germ plasm in the vegetal pole region of unfertilized eggs. The plasm arises as a series of subcortical islets of yolk-free, mitochondria-rich cytoplasm (Czolowska, 1972; Mahowald and Hennen, 1971; Williams and Smith, 1971; Ikenishi et al., 1974). Ultrastructural evidence of germ plasm was first presented by Balinsky (1966) who observed electron-dense bodies associated with mitochondrial aggregates in the vegetal region of fertilized eggs of the South African frog, Phrynobatrachus natalensis, that appeared analogous to the basophilic cytoplasmic inclusions observed at the light microscope level. Later work with R. pipiens (Kessel, 1971; Mahowald and Hennen, 1971; Williams and Smith, 1971) and X. laevis (Czolowska, 1972; Kalt, 1973; Ikenishi et al., 1974) confirmed this discovery. The germ plasm consists of electron-dense bodies, or germinal granules (Williams and Smith, 1975), which measure 0.17 to 0.25 μm in diameter; the latter are composed of "granular" electron-dense foci ranging from 10 to 20nm in diameter, as well as finer components (2 to 8 nm in diameter) that appear fibrillar in nature. The electron-
dense bodies are always associated with large aggregates of mitochondria and abundant particles about the size of ribosomes. At the level of the electron microscope, the character of the germ plasm and the integrity of the germinal granules does not change appreciably during cleavage (Williams and Smith, 1971; Mahowald and Hennen, 1971). At the 16-cell stage, there is an approximate doubling in diameter of the germinal granules to 0.4 to 0.5 μm, suggesting coalescence of two or more of these dense bodies (Williams and Smith, 1971; Mahowald and Hennen, 1971).

The presence of abundant ribosomes in close proximity to the electron dense bodies has lead to the suggestion that the germinal granules may be involved in protein synthesis during early embryogenesis (Mahowald and Hennen, 1971). However, the possibility that the germinal granules in anurans possess RNA has not been resolved. Ultrastructural studies by Mahowald and Hennen (1971) suggest that germinal granules in *R. pipiens* stain moderately with the indium trichloride procedure. On the other hand, Kalt (1973) attributes the faint staining of the dense bodies in *Xenopus* to background density rather than the presence of nucleic acids. In addition, RNAse digestion, which partially disrupts ribosomes, has no effect on the integrity of the germinal granules (Smith and Williams, 1971; Kalt, 1973), whereas pepsin digestion results in considerable degradation of the dense bodies. The effective wavelength of UV-irradiation
used to produce sterility in anuran embryos is indicative of a nucleic acid as the target of the UV light (Smith, 1966). However, as Smith (1966) pointed out, the UV target still could be a cytoplasmic entity other than the germinal granule. Autoradiography after microinjection of $^3$H-leucine into fertilized eggs of *X. laevis* shows that blastomeres containing germ plasm synthesize protein as do the other blastomeres during cleavage. Newly synthesized, labelled protein accumulates in germ plasm as well (Hogarth and Dixon, 1976). Similar results from an autoradiographic study of germ plasm in *R. pipiens* were obtained at the ultrastructural level (Smith and Williams, 1979). The data support the view that the germ plasm is either a site for protein synthesis or for protein accumulation. The former interpretation of the autoradiographic data would lend strong support for the idea that the germ plasm functions as a cytoplasmic determinant, whereas the latter interpretation is more equivocal.

The morphogenetic movements of gastrulation result in an endodermal mass within the newly oriented ventral region of the neurula and tailbud embryo. Positioned within this mass of yolky cells are the primordial germ cells (Fig. 1B), again conspicuous by the perinuclear halo of germ plasm. The ultrastructural view of the germ plasm during gastrulation has not changed appreciably from that of the cleavage stages. However, certain changes within the PGC, apparent by
late blastula to mid-gastrula stages, suggest the possibility that the PGC has begun to differentiate from the surrounding endoderm cells. At the light microscope level the most conspicuous change in *Xenopus* and *Rana* (Bounoure, 1934; Blackler, 1958; Di Berardino, 1961; Whittington and Dixon, 1975) is the intracellular shift of the germinal plasm, which occurs rather synchronously in all PGCs at the late blastula and early gastrula stages of development. During gastrulation, the germ plasm undergoes a cytoplasmic shift from the periphery of the cell to a juxtanuclear position (Bounoure, 1934, 1939; Blackler, 1958; Di Berardino, 1961; Whittington and Dixon, 1975), a movement that apparently depends on microtubule function (see Section IIIIB). This event is intriguing, since it occurs at a time when embryonic cells are being determined and differential gene expression is beginning to take place; thus, considerable speculation has been made regarding the perinuclear localization of germ plasm and its role in the determination of PGCs. Blackler (1958) proposed two putative roles for the plasm as a cytodeterminant: 1) to protect the PGC nucleus from the molecular information that regulates the determination and differentiation of other cells of the endoderm; 2) to inhibit any further mitotic activity in PGCs prior to entering the gonadal ridges. The latter notion was dispelled by Dziadek and Dixon (1977) who demonstrated in *X. laevis* that roughly two to
three cell divisions take place in PGCs from the time of endoderm phase I to gonadal colonization (see Fig. 1). This approximation also holds true for *R. pipiens* (Subtelny, 1980). Currently, it has been hypothesized that the germ plasm is involved in the expression of the migratory phenotype of PGCs (Smith and Williams, 1979; Dixon, 1981; Smith et al., 1982).

In *Xenopus*, the nucleus in germ plasm-bearing cells becomes distinguishable morphologically from the nuclei of surrounding somatic cells by a lobulated appearance that is initially detected as a slight indentation at the end of the late blastula stage; this progressively becomes more prominent such that by mid-tailbud stage the nucleus has a highly lobulated form and it is pale and vacuous in appearance (Ikenishi and Kotani, 1975; Whittington and Dixon, 1975; Dixon, 1981).

After gastrulation, the PGCs also differ biochemically from endoderm cells in DNA synthetic activity (Dziadek and Dixon, 1977). *Xenopus* embryos were pulse labelled at successive stages of development by microinjection of tritiated thymidine. The overall results obtained from the autoradiographic data indicate that germ plasm-bearing cells synthesize DNA, as do neighboring endoderm cells, throughout gastrulation, neurulation, and tailbud formation, but cease to do so after the late tailbud stage (Stage 36); i.e.,
shortly before they emerge from the endoderm mass. They do not initiate DNA synthesis and replication again until after at least 1 to 2 weeks residence in the gonadal ridges in feeding tadpoles (Kalt and Gall, 1974; Iriji and Egami, 1975; Dziadek and Dixon, 1977). On the other hand, the endoderm cells continue to synthesize DNA and replicate throughout the later stages of embryogenesis into the feeding tadpole stage without interruption. The fluctuations in pattern of $^3H$-thymidine incorporation by the germ line cells, together with data on PGC numbers, and observations of mitosis at successive stages of development, suggest that between late blastula and the time the PGCs exit from the endoderm at intraendodermal phase II (Figs. 1B, C, D), they undergo two to three cell cycles. Kamimura et al. (1976) and Whittington and Dixon (1975) obtained estimates of PGC numbers from sectioned embryos between the gastrula and late tailbud stages. They compared these counts with estimates of the number of endoderm cells present during a similar period of embryogenesis obtained by Woodland and Gurdon (1968) and Graham and Morgan (1966), respectively. The former authors conclude that the cell cycle times for PGCs and the other endoderm cells are of the same order of magnitude, but the germ line cells may cycle slightly more rapidly than somatic endoderm cells (Kamimura, et al., 1976; Dziadek and Dixon, 1977; Dixon, 1981). From a comparison of PGC numbers at the late blastula to the early gastrula stage
(DiBerardino, 1961), with the gonadal germ cell numbers in Stage 25 *R. pipiens* larvae (Subtelny, 1980) the data indicate that in this species, too, the germ line cells undergo two to three cell divisions between blastula and the time they emerge from the endoderm. In addition, from gonocyte counts made on triploid larvae (Ladner, 1977), haploid tadpoles (Stettner, 1979) and tetraploid animals (Subtelny, unpublished) in this laboratory it appears that the germ line cells behave just as do the adjacent somatic endoderm cells with respect to cell division cycles and their respective ploidies. There is no evidence from estimates of cell numbers and cell size in the animals with the different chromosome constitutions that PGCs undergo a special replication cycle during their intraendodermal residence different from that of the adjacent somatic endoderm cells.

With respect to RNA synthesis, in *Xenopus* Dziadek and Dixon (1977) found that there is a gradient along the animal-vegetal axis in the initiation of RNA synthesis among the cells of the early to late gastrula. For example, germ plasm-bearing cells close to the blastocoel begin RNA synthesis at early gastrula, as judged by $^3$H-uridine incorporation in the autoradiograms, whereas germ plasm-bearing cells in the yolk plug region begin RNA synthesis at late gastrula. However, when RNA synthesis was observed to begin
in germ line cells, it always did so in concert with neighboring endoderm cells. Thus, PGCs and somatic endoderm cells initiated RNA synthesis at the same time, subject to their position in the endoderm mass as a whole. It is noted, however, that none of the identifiable PGCs in animals from mid-tailbud stage (Stage 24) to the feeding tadpole stage (Stage 45) synthesized RNA, whereas all the other endoderm cells continued to do so during this period of development. So, after gastrulation PGCs differ dramatically from somatic endoderm cells in their transcriptional activity and are virtually quiescent.

Interestingly, all of the $^3$H-uridine label incorporated into germ plasm-bearing cells at pre-tailbud stages appeared to be confined to the nucleus, and there was no evidence for the transport of newly-synthesized RNA to the cytoplasm in the PGCs during the stages studied. The same pattern of $^3$H-uridine incorporation was evidenced in the germ line cells of embryos bearing the O-nu mutation (which lacked nucleoli) and in the genetically wild type animals (Dixon, 1981; unpublished). It thus appears that the nucleolus is not required for the observed newly synthesized RNA and that the latter is likely to be mRNA. In an attempt to more directly determine whether the RNA present in germ cells during tailbud to feeding stages was indeed mRNA, Wakahara (1982) employed in situ hybridization with $^3$H-poly U as a probe to detect the presence of poly A+ RNA. However, he was unable
to identify PGCs within the endoderm, and it was not until they had already exited the endoderm and were situated at the dorsal root of the intestinal mesentery that he was able to locate them and examine them for poly A+ RNA. Once the PGCs had accumulated in the root of the dorsal mesentery at Stage 43/44, moderate $^{3}$H-poly U binding was noted. This activity disappeared after Stage 47, and was not detectable until Stage 52, the time during which gonadal PGCs begin their proliferation and overt sexual differentiation of the gonad takes place.

Ultrastructural studies reveal that beginning with the late neurula or early tailbud embryo in *Xenopus*, the structure of the germ plasm undergoes modifications (Ikenishi and Kotani, 1975). An irregular string-like body (ISB), fibrillar in nature, and measuring 20 nm in diameter, appears to derive from the original germinal granule, and this transformation becomes more common as development proceeds.

From the aforementioned morphological and biochemical characteristics, it is difficult to determine precisely when the PGCs initially become determined and undergo differentiation distinct from that of the somatic endoderm cells. However, since the shift in the germ plasm to a juxta-nuclear position at the early gastrula stage results in its equal distribution to both daughter cells, in what can
be regarded as clonal divisions of PGCs, and since morphological changes in the nucleus itself are detected as well, these characteristics support the hypothesis that differentiation of the PGCs may be initiated by the time of gastrulation. Certainly, this would seem to be so by the mid-tailbud stage, just before the onset of dorsal intraendodermal migration, when a distinct cessation in RNA synthetic activities is clearly discernible.

Following the onset of gastrulation, the germ plasm progressively undergoes a decrease in staining reaction, as observed with the light microscope, indicating that some change in this cytoplasmic structure is taking place. First, there is a loss in the staining reaction for RNA (Blackler, 1958). Also, the stain for germ plasm, in general, progressively decreases such that with the translocation of the PGCs to the dorsal crest of the endoderm, the germ plasm becomes very faint indeed, and is totally absent by the time the germ line cells are in the gonadal ridges (Bounoure, 1934; Blackler, 1958; Whittington and Dixon, 1975). However, at the EM level, the germ plasm can be identified throughout this period of development as a distinct cytoplasmic entity that undergoes changes in morphology. Thus an analysis of the germ plasm by electron microscopy during the active migration of PGCs provides the following scenario: From the early tailbud stage to the onset of gill circulation in the hatched larva, PGCs undergo a
dorsal translocation within the endoderm mass (Figure 1 C). During this period, the germinal plasm remains juxtanuclear in position, but undergoes changes in morphology as detected ultrastructurally (Ikenishi and Kotani, 1975). The major alteration in germ plasm morphology during this developmental period in *Xenopus* is the gradual dissociation of the distinctive germinal granules and the increased presence of irregular string-like bodies (ISBs). The authors interpret these observations to be a transformation of the former into the latter structures such that by hatching stage (Stage 40, in *Xenopus*) the ISB is the predominant form of the electron-dense material and the germinal granules are correspondingly very few in number in PGCs accumulated at the dorsal crest of the endoderm mass.

The PGCs then separate from the endoderm mass and become displaced during dorsal mesentery formation to a medial cord (the "median germ ridge") along the anterior-posterior length of the dorsal mesenteric root (see Fig. 1 D). Subsequently, the PGCs move bilaterally together with coelomic epithelial cells and become associated with paired gonadal ridges (Fig. 1 E). By the time the PGCs are in the gonadal ridges, the characteristic form of the juxtanuclear germ plasm is no longer evident. Instead, a fibrogranular, electron-dense material, termed "nuage", is present and has been reported in both *Rana* (Mahowald and Hennem, 1971) and *Xenopus* (Kalt, 1973). Both authors believe that the nuage
is derived from the original germinal granules, which are no longer observed by the time the nuage appears. The fibrogranular material is associated with numerous mitochondria, but the inclusions believed to be ribosomes and glycogen in the germ plasm at earlier stages of development are seldom observed, or absent. Ikenishi and Kotani (1975) believe that the ISBs, which seem to derive from the germinal granules, in turn transform into the fibrogranular nuage-like material. It is interesting to note that Delbos et al. (1982), in their study with *R. dalmatina* and *B. bufo*, report no such alterations in the germinal granules within PGCs in the endoderm crest, the dorsal mesentery, or the gonad. If the fibrogranular nuage-like material is indeed a further elaboration of the germinal granules, then a continuity would exist in the germ plasm between the unfertilized egg and the onset of gametogenesis.

There is a marked difference in replication patterns in the PGCs between *Xenopus*, and *Rana*, once they have attained the gonadal ridges. In *Xenopus*, the gonadal germ cells are mitotically inactive for a considerable period of time (Kalt and Gall, 1974; Iriji and Egami, 1975; Zust and Dixon, 1977; Dixon, 1981) and then appear to initiate proliferation about 1 to 2 weeks later. The rate of proliferation depends on the sex of the individual, whereby gonial cells in
females multiply more rapidly during this initial period than gonial cells in males (Zust and Dixon, 1977). In *Rana*, although the PGCs are never entirely quiescent mitotically, it is virtually so at the time they exit from the endoderm and enter the gonadal ridges. Mitotic figures among PGCs during this period in development are very rare, indeed. However, estimates of PGC numbers when they separate into bilateral gonadal ridges (Stage 24), and on successive days thereafter, indicate that the germ line cells initiate a period of proliferative cycles within 2 to 4 days after colonization of the gonadal ridges has terminated (Stage 25) and before they enter gametogenesis. Gonadal germ cell mitosis is initiated randomly and not simultaneously among individuals from a given artificially inseminated cross and among individuals from different matings (Subtlelny, unpublished).

The fate of the dense nuage material throughout oogenesis is not clear. In *R. pipiens*, investigations have described the presence of dense fibrogranular material associated with mitochondria and adjacent to the nucleus in oogonia and small oocytes (Kessel, 1969; Eddy and Ito, 1971). Williams and Smith (1971) reported the presence of dense nuage-like material in oocytes 500 um in diameter, or about one-third the maximum size. However, the structures are no longer evident by the time yolk deposition begins in the larger oocytes. After a gap during growth of the
oocyte, small electron-dense masses, similar to nuage, within clusters of mitochondria, are identified just beneath the cortex of the vegetal hemisphere (Williams and Smith, 1971). It is not until germinal vesicle breakdown occurs and during oocyte maturation that the electron-dense masses associated with ribosomes within mitochondrial aggregates become prominent. Then electron-dense masses gradually become more compact and take on the appearance once again characteristic of germinal granules (Smith and Williams, 1975). In *Xenopus*, dense nuage-like structures within mitochondrial aggregates and adjacent to the nucleus have been observed in gonadal PGCs and small oocytes. These cytoplasmic entities persist into meiotic prophase of previtellogenic oocytes and the nuage-like materials seem to increase in amount during this period (Al-Mukhtar and Webb, 1971; Kalt, 1973). The nuage-mitochondria aggregates have been observed in early vitellogenic *Xenopus* oocytes, about 600 um in diameter (Stage III, Dumont, 1972). However, these structures disappear during later growth of the oocyte. Williams and Smith (1971) and Smith and Williams (1975) were not able to observe nuage-like material in full grown oocytes (Stage VI), but electron-dense bodies in the vegetal hemisphere identical to the germinal granules appear during oocyte maturation, following germinal vesicle breakdown. The reappearance of the definitive germinal
granules at the subcortical region of the vegetal pole takes place in maturing, enucleated oocytes at the same time as it occurs in nucleated oocytes. Therefore, the event is a cytoplasmic maturation phenomenon in which "precursors" - nuage-like materials - at the vegetal pole appear to coalesce and give rise to definitive germinal granules.

More recent studies by Heasman et al. (1984) implicate the mitochondrial cloud of previtellogenic *Xenopus* oocytes as the source of the germinal granules in unfertilized eggs. In the smallest oocytes (less than 70 um in diameter, Stage I; Dumont, 1972) mitochondria, interspersed with electron-dense granules, were identified in a large dense mass by using a specific fluorescent label, rhodamine 123. The mitochondrial cloud as a single dense mass of mitochondria becomes most prominent in the stage I previtellogenic oocytes, 200-300 um in diameter. At the edge of the mitochondrial cloud the mitochondria are embedded in an electron-dense nuage which appears as granulofilibril material (GFM). In stage II oocytes, 300-450 um in diameter, the mitochondrial cloud breaks down and disperses as small islands of mitochondria with GFM, and these collect in the subcortical region during stage III. In stage IV and V, the GFM is localized in the subcortical vegetal pole region and is similar in position and appearance to the GFM in mid-stage III oocytes. Heasman et al. (1984) postulate that the mitochondrial cloud in previtellogenic oocytes accumulates
and localizes the GFM, which becomes dispersed together with
the mitochondria to the vegetal pole during vitellogenesis.
The GFM then serves as precursors to the definitive germinal
granules of the full-grown, mature egg. Whether the overall
interpretation from electron microscopical images of the
germinal granules being transformed into the nuage-like
material, which in turn increases during oogenesis and
becomes dispersed to the vegetal pole region of oocytes to
give rise to the precursor of the definitive germinal
granules of the unfertilized egg, is, in fact, the true
sequence of events remains to be investigated.

III. Mode of PGC Migration from the Endoderm to the
Gonadal Ridge

A. Presumptive Primordial Germ Cell Phase

Two kinds of movements are important during the early
development of the PGC: 1) the intracellular displacement
of the germinal cytoplasm during segmentation, 2) the
intercellular displacement of the germ plasm-bearing
blastomeres within the vegetal hemisphere of the embryo. It
has generally been agreed that the early movements of the
germ plasm during segmentation and the minor displacements
of the plasm-bearing cells can be accounted for solely by
the dynamics of the cleavage process and the general shuffling of blastomeres during a period marked by abbreviated mitotic cycles and rapid cell proliferation (Bounoure, 1934; Blackler, 1958; Whittington and Dixon, 1975).

The movements of the germ plasm highlight what are considered to be important stages in the establishment of the lineage of PGCs. The germ plasm undergoes several distinctive movements during the course of PGC development. First, in the unfertilized egg, the germ plasm exists as subcortical islets which subsequently undergo aggregation following fertilization or activation of the egg to form large patches that accumulate at the vegetal pole and then become distributed to four blastomeres by second cleavage. This aggregation behavior depends on microtubules but is independent of the cleavage process (Dixon, 1981; Ressom and Dixon, 1984). Aggregation is believed to be important in the partitioning of the plasm between the first four blastomeres. Second, the plasm becomes internalized from the vegetal pole inwards. This movement of the germ plasm along the cleavage furrows of the two- and four-blastomere stage embryo (Blackler, 1958; Whittington and Dixon, 1975) seems to be analogous to the movements of dye particles injected into the egg in this region during early embryonic cell division as observed by Schechtman (1934) and Ballard (1955). It thus appears that the ingestion of the germ plasm along the
cleavage furrows is subject to the general pattern of inward cytoplasmic streaming during embryonic cleavage.

When the germ plasm is internalized within the first four blastomeres, it is positioned at the cell periphery, adjacent to the plasma membrane. This asymmetrical distribution of germ plasm within the cells of the early morula segregates the plasm to only one of the two daughter blastomeres during subsequent cleavage divisions, with few exceptions. This mitotic behavior limits the number of pPGCs in the blastula to 4 to 15 (DiBardino, 1961; Whittington and Dixon, 1975) as compared to the 5000 or more somatic cells that comprise the embryo at this time (Resson and Dixon, 1984). The mechanism underlying the restricted distribution of the germ plasm is believed to consist of its "capture" by cleavage asters, which ensures its unequal segregation to one of the two daughter blastomeres with each cell division (Resson and Dixon, 1984).

Finally, at late blastula stage and with the onset of gastrulation, the germ plasm shifts intracellularly to a perinuclear position (Bounoure, 1934; Blackler, 1958; DiBardino, 1961; Whittington and Dixon, 1975). The manner in which this cytoplasmic shift occurs is unknown, but it may depend on the integrity of microtubules (Dixon, 1981). This shift occurs at a stage in development when blastomeres are generally becoming determined and it is concurrent with what
appears to be the initiation of differentiation in the germ cell (Dixon, 1981), suggesting that the juxtanuclear shift in germ plasm may be important in the transition from pPGC to PGC. The germ plasm remains in its juxtanuclear location until the end of endodermal phase II, at which time it is no longer detectable by differential staining (Bounoure, 1934; Blackler, 1958; Whittington and Dixon, 1975).

The germ plasm-bearing cells themselves undergo slight displacement among the vegetative blastomeres during cleavage, eventually becoming distributed with the majority located within the upper-third of the vegetal hemisphere in the case of Rana (Bounoure, 1934; Blackler, 1958; DiBerardino, 1961), and within the lower-third in the case of Xenopus (Whittington and Dixon, 1975).

B. Intraendodermal Phase I

During the late blastula stage in Rana, the PGCs are mainly positioned within the upper-third of the vegetal hemisphere, and they are often readily visible in histological sections just beneath the blastocoel floor (Bounoure, 1934; Blackler, 1958). The PGCs are distinguished by the juxtanuclear crescent of germ plasm which now becomes distributed to both daughter cells during subsequent mitotic divisions resulting in the formation of a founder clone of true PGCs. Also, at least in Xenopus, the germ-line cells
appear to exhibit certain signs of differentiation (Dixon 1981; see Section II B). However, in all other respects, these cells have the cytological characteristics of the neighboring somatic cells of the endoderm mass. Subsequently, the PGCs in both Xenopus and Rana undergo the typical displacement of endoderm cells as a result of the morphogenetic movements that take place during gastrulation such that they are carried to the ventral side of the embryo, deep within the endoderm mass of the late gastrula. During neurulation, when the embryo begins to elongate in the anterior-posterior direction, the PGCs are displaced to the posterior part of the endoderm mass (Bounoure, 1934). However, Whittington and Dixon (1975) report that there is also a slight posterior displacement of PGCs within the endoderm along the anterior-posterior axis in Xenopus. They also ascribe this subtle shift to the changes in shape of the embryo. Thus the overall picture, as presented in Figure 1B, is that the PGCs remain contiguous with the neighboring endoderm cells, and the shifting in position of the progenitor sex cells conforms to the passive displacements of the somatic endoderm cells in general, which are subject to the morphogenetic movements occurring in these early stages of embryogenesis.

C. Intraendodermal Migration Phase II

At the completion of neurulation, the germ plasm-
bearing cells have been commonly reported to be situated deep within the ventral endoderm mass of the early tailbud embryo. Actually, recent extirpation experiments indicate that there is an increasing dorsal to ventral gradient in PGC numbers within the endoderm mass of R. pipiens embryos. Comparisons of gonadal germ cell numbers were made between normal untreated controls and larvae at the feeding stage that had the ventral half or ventral three-fourths of the endoderm mass removed at an earlier stage in development. The comparisons suggest that the majority of the PGCs (70%) are situated in the ventral half of the endoderm in the tailbud embryo, whereas 30% are located in the dorsal half of the endoderm mass. Of the latter, about 10% appear to have assumed a position within the upper one-fourth of the endoderm mass (Subtelny, 1980). In the ensuing stages from tailbud to the hatched larva, the germ-line cells undergo a series of dramatic positional changes. In longitudinal sections the germ plasm-bearing cells have been observed to rise up little by little within the endoderm mass at each successive stage in development to attain the level of the archenteric cavity and then to assemble within the median roof of the archenteron, at the extreme dorsal border of the endodermal crest. This dorsally-directed movement of PGCs within the endoderm mass has been followed in histological sections by a number of workers,
using the germ plasm marker. The pathway of migration is quite similar in most anuran species examined with respect to both spatial and developmental aspects (Bouyoucos, 1934; Blackler, 1958; Gipouloux, 1962; Whittington and Dixon, 1975; Kamimura et al., 1976; Ikenishi and Kotani, 1979). However, Kamimura et al. (1976) have observed that the PGCs initially tend to move in a lateral direction towards the periphery of the endoderm mass and then dorsally toward the roof of the archenteron. These authors postulate that, in *Xenopus* at least, there may be an influence of the splanchnopleure of the lateral plate on the guidance of germ cells during their intraendodermal movements. An exception to the usual developmental pattern of intraendodermal migration also appears to exist in *R. japonica* (Shirane, 1981), where the PGCs apparently initiate a dorsal shift precociously relative to other species, at the neurula stage, and are already at the dorsal endodermal level of the archenteron by tailbud stage.

This evidence of a dorsal translocation of PGCs within the endoderm obtained by observations of histological sections at the light and electron microscope level also has been recently experimentally confirmed in *R. pipiens* by a series of extirpation experiments between the tailbud embryo (Stage 17) and the hatched larva (Stage 21) in this laboratory. It has been found that at Stage 17,
the majority of the PGCs are in the ventral half of the endoderm, with approximately 30% in the anterior half and 70% in the posterior half. Between Stages 18 and 18+, 90% of the germ cells are in the posterior half of the embryo, still ventrally located. Then, between Stages 18+ and 19+, a dramatic shift in the germ cell distribution takes place such that 90% of them are localized in the dorsal one-half to one-fourth of the posterior endoderm mass (see Subtelny, 1980).

It is generally believed that the dorsal displacement of the PGCs to the crest of the endoderm is an active movement on the part of the germ cells themselves. It has often been stated in the literature that this is so since no apparent displacement of endoderm, mesoderm, or ectoderm tissue takes place during this period in embryogenesis that could account for a passive dorsal displacement of the germ plasm-bearing cells (Blackler, 1958; Whittington and Dixon, 1975; Kamimura et al., 1976). However, there has been no experimental verification of this statement.

Bounoure, who first described the presence of germ cells within the endoderm at the light microscope level in 1929 in R. temporaria, reported that they possess pseudopodial extensions, suggesting an independent and active amoeboid locomotion of germ cells within the endoderm. Although Blackler (1958) was unable to confirm the presence of the amoeba-like processes on intraendodermal PGCs, he
postulated that they must actively move since no existing morphogenetic movements of the endoderm existed that could account for the positional changes undertaken by these cells. In their 1975 paper, Whitington and Dixon strongly advocated a mechanism of active cell migration to account for the dorsal intraendodermal displacement of germ cells. The only morphogenetic movements that could possibly accompany the translocation of PGCs within the endoderm were certain rearrangements reported to take place within the posterior midgut and anterior hindgut regions in the formation of the small intestine (Takahara and Nakamura, 1961; Harris, 1967; Ballard, 1970); but the occurrence of such morphogenetic movements, which Whitington and Dixon (1975) were unable to detect, would only be initiated after Stage 38, in *Xenopus*, at which time the PGCs have accumulated in the dorsalmost region of the endodermal crest, above the enteron, and translocation within the endoderm is normally completed. So, Whitington and Dixon are thoroughly convinced that no passive mechanisms exist to account for the displacement of PGCs within the endoderm, which, by default, must indicate an active mechanism is operating.

Direct experimental evidence for independently migrating germ cells is lacking. However, there is rather convincing histological evidence to support this
proposition. Kamimura et al. (1976), examining thick sections of Xenopus embryos in epon, afford some histological evidence of subtle differences in germ cells, as compared to endodermal cells, in morphology (PGCs are more rounded), in their separation from somatic endoderm cells (PGCs are found in relatively large interstices between endoderm cells; see also Bounoure, 1934), and in orientation (PGCs are more dorsally-directed); these features are characteristic of active, individual migratory cells. At the ultrastructural level, intraendodermal germ cells display a polymorphism, suggestive of the transitional appearances of migratory cells (Kamimura et al., 1980). The cells are structurally polarized, exhibiting a dorsalwards orientation. Large intercellular spaces characteristically separate them from endoderm cells, and, unlike the somatic endoderm cells which surround them, PGCs contain large mitochondria, microtubules radiating from a centriole (thought to be involved in intracellular polarization and pseudopod formation; see Maleck et al., 1977, who describe the role of microtubules in pseudopod formation and cellular polarity in human neutrophils), and cell surface phenomena such as filopodia, all indicative of active cell locomotion. Certain of these features (cellular polarity, filopodia, and intercellular spaces) have been independently reported by Ikenishi et al. (1979) and Delbos et al. (1981, 1982). The latter authors
have extended these studies to the PGCs of the endodermal crest in *X. laevis* as well as in *R. dalmatina* and *B. bufo*. At this time the PGCs possess single, large pseudopodia at their "dorsal" surface, associated with an extracellular matrix, as revealed by lanthanum dioxide or ruthenium red staining. FITC-lectin binding studies at the ultrastructural level and the staining of lectin binding sites with lactosyl-ferritin show D-galactose and N-acetyl-galactosamine to be present on the pseudopodial surface of PGCs as well as in the accompanying extracellular space and disseminated throughout the germ cell cytoplasm during intradendodermal phase II of PGC migration (Delbos et al., 1982; 1984); no specific lectin binding was shown to be associated with the neighboring somatic cells of the endoderm nor with PGCs once they had invaded the gonadal ridges. It has previously been shown that the sugar residues α-methyl-mannoside and N-acetyl-glucosamine are present on the surface of migratory chick PGCs (Lee, 1978). In this latter case, a role has been postulated for glycoconjugates in the mechanism of PGC migration in chick, partly based on inhibition studies with concanavalin A and wheat germ agglutinin (Lee, 1978). In *Bufo*, *Rana*, and *Xenopus* (Delbos et al. 1982; 1984), a similar role has been proposed and this receives experimental support from studies which show that daily injections of PNA lectin (from
Arachis hypogea) into the endoderm of Xenopus embryos during intraendoderm phase II, inhibits migration to the gonadal ridges of up to 90% of the PGCs when compared to noninjected controls and controls which received injections of phosphate buffered saline or PNA lectin plus galactose. The interpretation of these results is that the injected PNA lectin associates with membrane galactosyl residues of PGCs and/or with those of the ECM, and in this way interferes with the function of the glycoconjugates in the intraendodermal migration of PGCs.

The descriptive morphology of intraendodermal germ cells provides rather convincing evidence for individually migrating PGCs moving in a dorsal direction to the roof of the archenteron. Experimentalists have tried to verify this interpretation, while addressing the following questions: 1) Is migration active? 2) Is the path of migration fixed? 3) What factors direct this movement? A number of experiments provide indirect evidence that a) the displacements of the PGCs are by active migration, b) the direction of migration within the endoderm mass is not fixed, and c) the directionality of migration is probably cued by a diffusible chemotactic substance(s) emanating from the dorsal mesodermal structures.

Sabbadin (1959) initially demonstrated that intraendodermal germ cell migration was not dependent on a fixed endodermal route. By inverting dorso-ventrally the
orientation of the lateral plate in tailbud embryos, in certain cases the germ cell pathway was diverted to a more ventro-lateral position when a mesentery also formed there. In an extensive investigation examining five species of anurans, Gipouloux (1970) rather convincingly showed by various experimental manipulations that the direction of germ cell migration was not fixed: a) after parabiosis of two tailbud embryos in the ventral abdominal region, and b) by 180° rotation of the endoderm mass along the dorsal-ventral axis in the tailbud embryo. These experiments altered the intraendodermal migratory path of PGCs, but did not prevent these cells from reaching their gonadal destination; thus the experiments suggest that the PGCs are capable of active movement to the gonadal ridges independent of the orientation of the endoderm mass. Later parabiosis experiments by Subtelny (1980) in R. pipiens have reaffirmed Gipouloux's findings. In this case, the pair of ventral abdominal parabions were a UV-irradiated tailbud host and a normal tailbud donor from which the doral axial structures were removed the day following grafting. Autopsy of the developed larvae revealed a considerable number of PGCs (approximately 50% of the unoperated control number) within the gonadal ridges of the UV-irradiated host parabiont. The unoperated UV controls were completely sterile, confirming the donor origin of the PGCs. Moreover,
the PGCs had to have reversed their original direction of migration, which would support the notion that migration is effected by an active independent movement on the part of the PGCs. Giorgi (1974) also contributed to this contention by grafting studies in B. bufo. The anterior or the posterior part of the dorsal axial structures from a normal tailbud donor were grafted to the ventral abdominal body wall of a normal host at the same stage of development. In either case, the number of PGCs populating the host gonadal ridges was considerably reduced and in some cases, zero. Histological sections revealed, however, an accumulation of PGCs in the gonadal ridges of the ventral graft. Grafts of posterior dorsal axial structures had a significantly higher number of associated PGCs than anterior grafts. Recent investigations (Wakahara et al., 1984) on the developmental response of the Xenopus egg to rotation along its animal-vegetal axis reveal that when the fertilized egg is inverted, the germ plasm remains in the vegetal cortex (now uppermost), but since the large yolk platelets sediment with gravity to the bottom of the egg (the original animal pole), the upper "vegetal" hemisphere forms micromeres; some of the latter blastomeres receive a distribution of germ plasm and eventually come to lie long the roof of the blastocoel at the blastula stage. PGCs can still populate the gonadal ridges under these conditions indicating that they have accomplished this
migration by an unorthodox pathway. Although a thorough analysis of these findings is still forthcoming, they support the hypotheses that germ cell migration is active, follows a novel route, and is likely to be guided by a chemotactic stimulus (see Gipouloux, 1970).

Studies on the actual guidance mechanism(s) for dorsally-directed intraendodermal PGC migration have been chiefly undertaken by Gipouloux (1963a, b; 1964; compiled in 1970). Initially he extirpated the dorsal axial mesodermal tissues (somites, notochord, pronephros) and found that when either somites plus notochord, or somites plus Wolffian ducts, or somites plus notochord plus Wolffian ducts were present, normal dorsal migration occurred; i.e., the gonadal ridges of the operated embryos were populated by a normal complement of PGCs compared to the controls. The presence of somites alone allowed for partial migration of the PGCs to occur, and Wolffian ducts or only part of the somites resulted in migration of very restricted numbers of PGCs. Implants of these dorsal tissues into the ventral region of the endoderm mass (Gipouloux, 1970) appeared to exert an attraction on host PGCs, as determined by decrease in the number of host gonadal PGCs and an accumulation of PGCs in the region of the ventral graft, as revealed in histological sections. Based on the accumulation of PGCs in the vicinity of the graft, a ranking of PGC-attracting
activity could be assigned to the dorsal tissues as follows, in order from strongest to weakest: 1) notochord plus Wolffian ducts plus somites, 2) notochord plus somites or notochord plus Wolffian ducts, and 3) somites. In conclusion, Gipouloux proposed that the dorsal mesodermal organs influence the dorsal migration of PGCs chemotactically by means of a molecular substance(s) that diffuses from these organs, producing an increasing ventral to dorsal gradient. Preliminary studies reveal that when agar strips impregnated with crude extracts of the dorsal mesodermal tissues were implanted into the ventral endodermal region of tailbud embryos, germ cells were detected in histological sections to be associated with the implant at feeding tadpole stage (Gipouloux 1964, 1970). Initial biochemical studies (Gipouloux, 1970) reveal that a substance of a diameter of less than 100 Å, and sensitive to heat and proteinases but insensitive to ether or chloroform, could be extracted from the dorsal tissues of tailbud embryos. This substance exhibited germ cell-attracting activity by virtue of the ventral implantation procedure referred to above. Although these properties are characteristic of a protein, the study was very preliminary and the preparations were somewhat crude and nonhomogeneous. Certainly a more extensive study to isolate the putative germ cell-attracting factor(s) from these tissues
is warranted.

To this end, current experiments in Gipuloux's laboratory now focus on the nature of the putative chemo-attractant responsible for the dorsal intraendodermal guidance of PGCs. The results suggest that grafts of chordamesoderm attract PGCs as do grafts of individual dorsal mesodermal tissues (Gipouloux, 1970; Gipouloux et al., 1979; Giorgi, 1974). From these studies it has been hypothesized that a putative chemo-attractant, emanating from the dorsal mesodermal structures and released during intraendodermal phase II, diffuses into the endoderm, establishing an increasing ventral to dorsal gradient; it stimulates the PGCs chemotactically to migrate in a dorsal direction along the gradient.

There has been accumulating evidence that cyclic 3',5' adenosine monophosphate (cAMP) serves as a diffusible chemo-attractant for migratory cells in a several different systems. In the slime mold Dictyostelium discoideum, which exhibits positive chemotaxis during the amoeboid stage of its life cycle, cAMP has been shown to direct migrating amoebae in their movements to form aggregates (Konijn et al., 1967; Bonner et al., 1968; for review, see Newell, 1981). Evidence that cyclic AMP may also direct the migration of vertebrate embryonic cells has been shown in the chick both in vitro and in vivo. In vitro, both
isolated cells and cell sheets (Robertson, 1977), as well as disaggregated cells (Gingle, 1977), are attracted by a microelectrode source of cAMP. In vivo, it has been shown by Gingle and Robertson (1979) that microelectrode sources of cAMP will induce the following reaction in the tissues of the chick embryo: a) bending of the embryonic axis towards the source, b) attraction of cells on the ventral surface of the embryo towards the source, and c) disruption of the blastodisc. Furthermore, Reporter and Rosenquist (1972) have found regional differences in cAMP levels within the chick embryo, with maximum levels occurring in the mesoderm forming region.

Gipouloux and coworkers have therefore examined the possibility that cAMP might also be responsible for germ cell chemotaxis in anuran amphibians. Preliminary studies (Gipouloux et al., 1978) were conducted in which whole embryos were exposed to exogenous cAMP in an attempt to disrupt the purported endogenous gradient of the PGC chemoattractant within the embryo. The marked reduction in gonadal PGCs observed in the treated embryos, although open to numerous interpretations, followed the prediction of the cAMP hypothesis. An attempt was then made to establish an artificial gradient of cAMP by making ventral endodermal implants of cAMP impregnated agar blocks. The assay for germ cell attraction was the same as described above for grafting studies of notochord, somites, and Wolffian ducts.
(Gipouloux, 1970). The implants of cAMP appeared to attract PGCs in a similar fashion as ventral grafts of chordamesoderm. Furthermore, this germ cell attraction by chordamesoderm or cAMP appeared to be blocked by 3′,5′-cyclic nucleotide phosphodiesterase, an enzyme responsible for the breakdown of cAMP to AMP. From these studies conducted on Bufo, Rana, or Xenopus, Gipouloux and coworkers postulate that cAMP is the diffusible chemo-attractant produced by the chordamesoderm and is involved in PGC chemotaxis.

The enzyme adenyl cyclase, which acts on an ATP substrate to synthesize cAMP, has been localized in the chordamesoderm at the ultrastructural level (Delbos et al., 1980). This activity appears on the plasma membrane of peripheral cells of the notochord, at the junction of these cells with the muscle mass, on the plasma membrane of connective tissue cells in the notochordal-myotomal space, and lightly on perichordal collagen. The deposition on the exterior surface of cell membranes suggests to the investigators that the production of cAMP is probably exogenous, which fits well with the hypothesis that an exogenous cAMP is produced by the chordamesoderm and diffuses ventrally within the endoderm to form a gradient for PGC chemotaxis. It is rather encouraging that the adenyl cyclase activity is restricted to the period of
development corresponding to intraendoderm phase II of PGC migration (Delbos et al., 1980). This specific developmental period corresponds to Stages 24 to 39 in *R. dalmatina* and stages 33/34 to 41 in *X. laevis*. Furthermore, PGCs appeared to "respond" to the chemotactic stimulus by the production of an endogenous cAMP, indirectly evidenced by the appearance of adenyl cyclase activity on their membranes, during the same period of development. This latter finding is analogous to the chemotactic response of *Dictyostelium* amoebae to exogenous cAMP; they in turn synthesize their own cAMP for external release, which results in an enhancement of the attractive signal eliciting aggregation (Newell, 1981). Adenyl cyclase activity was not associated with neighboring endoderm cells.

Finally, secondary catecholamines could be responsible for initiating and sustaining the synthesis of cAMP in the chordamesoderm. These biogenic amines have been detected exclusively in the notochord region during chick embryonic development (Kirby and Gilmore, 1972; Strudel, 1977; Strudel et al., 1977). It is known that catecholamines can stimulate adenyl cyclase activity (McMahon, 1974). Since notochord is derived from chordamesoderma tissues, which appear to be the source of the PGC chemo-attractant, and since this attractant is believed to be cAMP, Rais et al. (1981) analyzed this region in *Xenopus* and *Rana* for the presence of catecholamines by fluorescence microscopy and
examined the developmental stages of their production. The analysis revealed catecholamine localization in the notochord that appeared during the same developmental period as adenyl cyclase activity. The treatment of embryos with L-dopa, a precursor of catecholamines, enhanced chordal fluorescence whereas α-methyl-para-tyrosine, an inhibitor of catecholamine synthesis reduced fluorescence under the same conditions. Finally reserpine, a drug that interferes with catecholamine secretion, inhibits the levels of catecholamines in the notochord as well as interferes with PGC migration when embryos are exposed to it in solution (Rais et al., 1981 a,b).

The overall picture that Gipouloux and coworkers present is that catecholamines produced in the notochord stimulate adenyl cyclase activity in notochordal and possibly adjacent tissues (somites and Wolffian ducts), resulting in the production of cAMP, the proposed molecular agent that conveys directional information to PGCs in a gradient fashion. However, an attempt to link these systems to germ cell migration in a definitive manner seems premature. Gipouloux (1970) earlier demonstrated that notochord alone could not direct dorsal migration of PGCs; in fact, neither removal of notochord nor transplantation of it to the ventral region of a neurula embryo had any marked influence on PGC migration to the gonadal ridges. Somites
on the other hand, and Wolffian ducts to a lesser extent, exhibited germ cell attracting-activity in the same types of ablation and grafting experiments mentioned above (Gipouloux, 1970). However, no adenyl cyclase activity has been demonstrated in Wolffian ducts and only slight activity associated with the perichordal region of the somites during the migratory period of PGCs through the endoderm. It therefore does not seem justified to postulate a central mechanism in the dorsal guidance of PGCs based on a model of primarily notochord tissue. If this model were correct, then one would expect notochordal tissue to be absolutely necessary for dorsal migration of PGCs to occur. Also, cAMP or adenyl cyclase activity has not been detected in marked quantities in somite tissue, which has been shown to exhibit the greatest potency of the chordamesodermal tissues in PGC attraction.

D. Endodermal Exiting Phase

Perhaps one of the most widely investigated aspects of gonadal migration of PGCs in anurans of recent times is their translocation from the dorsal endodermal crest to the median germ ridge. The latter occupies a region within the dorsal root of the intestinal mesentery, subjacent to the dorsal aorta, and between the two lateral subcardinal veins
(see Fig. 1D). In reviewing the evidence for the mechanism(s) of endodermal exiting of the PGCs in anurans, it is important to distinguish between those works involving a descriptive analysis versus those supported by experimentation.

From the turn of the century until the studies of Sabbadin in 1959 all examinations on the mode of germ cell migration from the endoderm were descriptive in nature; also, these earlier studies dealt primarily with Rana (Allen, 1907; Kuschakewitsch, 1908; Witschi, 1914; Humphrey, 1925; Cheng, 1932; Bounoure, 1934; 1939) and Bufo (King, 1908; Humphrey, 1925), with the exception of Blackler (1959), who also examined Xenopus embryos. In general, there was a debate among these investigators as to whether the dorsal movement of germ cells out of the endoderm was a dependent process, passively relying on the morphogenetic movements of dorsal mesentery formation or an active process based on the independent ability of the PGCs themselves to become positioned in the median germ ridge.

The most detailed observations on this phase of PGC migration were carried out by Allen (1907), Kuschakewitsch (1908), King (1908), and Humphrey (1925) in Rana and Bufo. Humphrey believed that the evidence favors a morphogenetic role of dorsal mesentery formation in the passive translocation of PGCs from the endoderm. Unfortunately, the
conclusions are based primarily on descriptive studies in thick paraffin sections. As illustrated in Figure 1C, the PGCs at Stage 20-21 are situated dorsalmost in the roof of the endoderm crest that is elevated from the top of the main endoderm mass. With the separation of the lateral plate mesoderm into the splanchnopleure and the somatopleure to form the coelom at Stage 22 (Fig. 1D), the inner folds of the splanchnopleure undergo an ingressation at the level of the dorsal endoderm crest actually penetrating the endodermal ridge as the dorsal mesentery forms underneath the localization of closely grouped PGCs, essentially pinching off the dorsalmost portion of the crest of cells "en masse" and lifting it up to now give rise to the anterior-posterior elongated median germ ridge, situated just beneath the dorsal aorta.

Cheng (1932) notes from his observations with the light microscope that throughout the process of shifting from the endodermal crest to the median germ ridge during dorsal mesentery formation, the PGCs exhibit no morphological signs that they are capable of moving independently. Thus his findings are in general accord with Humphrey's (1925). Actually, Vannini and Giorgi (1969) pointed out from their observations on B. bufo that the position of the uppermost part of the endoderm crest at a stage just prior to the formation of the dorsal mesentery (Fig. 1C) is already subjacent to the dorsal aorta such that when its separation
occurs by the approximation of the folds of the lateral plates of mesoderm underneath the closely aggregated PGCs accumulated there, the germ-line cells are essentially at the site of the future median germ ridge, and they have actually undergone very little, if any, "migration per se. These observations have been confirmed by Subtelny and Penkala (1984) for *R. pipiens* (Compare Figs. 3A, 3D, F and 5C). Thus the PGCs accumulated within the uppermost portion of the dorsal endoderm crest appear to be separated from the main portion of the endoderm as a more or less uneven cord of closely aggregated cells along the anterior-posterior axis, immediately prior to the formation and elongation of the dorsal mesentery underneath them. Occasional PGCs may be seen singly or in groups of two's or three's within the recently formed, short dorsal mesentery, and they are sometimes polarized in the dorsal-ventral direction, and thus appear to be in the process of active intramesenteric migration (Subtelny and Penkala, 1984; see Fig. 5B). However, by the time the elongated, definitive dorsal mesentery forms upon retraction of the gut from the dorsal body wall, and when the vast majority of PGCs are situated in the paired gonadal ridges of feeding larvae, identification of PGCs within the extended dorsal mesentery is a rare event.

There is some experimental evidence that morphogenesis
of the mesentery does play a role in the exiting of PGCs from the endoderm. Sabbadin (1959) removed the entire lateral plate mesoderm region with overlying ectoderm from the pronephric bulge to the base of the tail in the anterior-posterior direction, and from the base of the somites to below the midbody region in the dorsal-ventral direction in the Discoglossus tailbud embryo. This piece was inverted by rotating it 180° about the dorsal-ventral axis and transplanted to the opposite side of a recipient embryo. In unilateral and bilateral transplants of this type the mesentery tended to form towards the midline in the ventral site where several PGCs were identified in association with it. Sabbadin concluded from this that the PGCs must have undergone a passive displacement determined by the directed movements of the lateral plate mesoderm during the formation of the mesentery. In another type of experiment, Giorgi (1974) transplanted the axial cranial half and the caudal half of the dorsal mesodermal organs to the ventral abdominal region of Bufo tailbud embryos. PGCs emerged from the endoderm in this ventral site to occupy the gonadal ridges of the graft. From such findings this investigator concluded that the results support Gipouloux's hypothesis for the directed intraendodermal migration of PGCs under the influence of a chemotactic stimulus emanating from the dorsal mesodermal organs (Section IIIC). However, Giorgi also noted that 83% of the
PGCs identified as having exited from the endoderm mass are associated with a well-formed mesentery, indicating a role for morphogenesis in the process of separation of germ cells from the endoderm to the median genital ridge. Recently, Subtelny and Penkala (1984) examined the process of germ cell exiting in *R. pipiens*. Utilizing reciprocal grafts of germ cell-containing endoderm regions between various stage embryos, it has been determined histologically that the graft PGCs emerge from the endoderm in accordance with the time of development of the host dorsal mesenteric tissue. This finding leads to the proposition that dorsal exiting of the PGCs in *Rana* is primarily under the control of local morphogenetic movements involved with the formation of the dorsal mesentery (see Results and Discussion).

Humphrey (1925) referred to the penetration of mesenchyme cells into the upper portion of the dorsal endoderm crest, prior to its separation. These same observations were earlier illustrated by Kuschakewitsch (1908) in *R. esculenta*. However, no particular significance was attributed to this event by either author. Based on current observations (Subtelny and Penkala, 1984), these mesenchyme cells intimately associate with the closely-grouped PGCs and appear to remain associated with them before, during, and after their translocation to the median germ ridge, and subsequently to the bilateral gonadal
primordia. Both King (1908) and Cheng (1932) describe the association of mesenchymal cells with the closely-grouped PGCs within the median germ ridge and during the translocation of germ cells to the gonadal ridges, but not at earlier stages of development. This population of individual mesenchyme cells evidently derives from the lateral plate mesoderm. They migrate over the roof of the archenteron and invade the dorsalmost portion of the endoderm ridge just prior to the infolding of the lateral plates of mesoderm that form the dorsal mesentery rudiment. These morphogenetic events separate the uppermost dorsal crest region containing the PGCs from the region of the endoderm mass below (Subtelny and Penkala, 1984; see Figs. 3C, D, and F). This interaction of germ cell-mesenchyme cell may be of direct significance for the emergence of PGCs from the endoderm and their migration to the gonadal ridges.

Contemporary studies with *Xenopus laevis* embryos (Whittington and Dixon; 1975; Kamimura et al., 1976) indicate that primordial germ cells emerge from the endoderm in this anuran species in an active and independent manner. At the time the PGCs exit the endoderm in *Xenopus* at Stage 41, mesenchyme cells from the medial edges of the lateral plate ensheath the dorsal surface of the endodermal crest. The lateral plates then approach medially above the endodermal crest in the formation of the dorsal intestinal mesentery. This latter event is in contrast to what occurs in *Rana,*
where the lateral mesodermal plates fold inward and fuse beneath the dorsalmost portion of the PGC-containing endodermal crest, which is essentially already in the position of the future median germ ridge subjacent to the dorsal aorta, so that when this portion of the endodermal ridge separates from the main endodermal mass, there is no extensive "migration" of PGCs *per se* to the median germ ridge. In *Xenopus*, the PGCs actively penetrate the overlying sheet of mesenchymal cells, as suggested by Whittington and Dixon (1975; Fig. 4) and Kamimura et al. (1976; Fig. 3B). These authors conclude that the mechanism of PGC emergence from the endoderm in *Xenopus* is strictly active. The translocation, then, of the PGCs to the median ridge at the base of the dorsal mesentery is viewed by Whittington and Dixon (1975) to be partly due to the active movements of the PGCs and partly due to the process of the narrowing and elongation of the dorsal mesentery. Kamimura et al. (1976), on the other hand, interpret the change in position of PGCs in histological sections to indicate an active and independent dorsal movement of these cells up the forming mesentery.

Heasman and Wylie (1983) have remarked that the formation of the dorsal mesentery in *Xenopus* is an imprecisely timed event, and that often it will occur several hours before the emergence of PGCs from the endoderm
crest at Stages 42 and 43, so that an active migration of individual germ cells takes place along the length of the dorsal mesentery between the two splanchnopleural sheets which comprise it. It is this phase of PGC migration, the "intramesenteric" phase (Delbos et al., 1982), that Wylie and coworkers have investigated extensively in vitro, and compared it with in vivo observations obtained by SEM and TEM on fixed embryos (Heasman and Wylie, 1978; Wylie and Heasman, 1979; Heasman et al, 1981). The model derived from these studies invokes the concept that PGCs actively migrate dorsally as individual cells, in a directed fashion, following an oblique course caudo-cephally along the dorsal mesentery. They are guided by a substratum of highly polarized coelomic epithelial cells (CECs), which exhibit a corresponding oblique orientation. At the junction of the dorsal mesentery and the dorsal body wall, the CECs exhibit an abrupt change in polarity, now orienting at right angles to the dorsal mesentery and thereby providing a lateral alignment which guides the PGCs laterally to the regions of the paired gonadal ridges. The isolated intramesenteric PGCs are observed to move in vitro on outgrowths of adult mesentery by extending a filopodium in the direction of substrate orientation. Similar morphological features have been observed in thin sections under TEM and also on fixed tissue under SEM (Heasman and Wylie, 1979). The filopodium contains abundant microfilaments that are otherwise sparse
in the germ cell cytoskeleton, with the exception of the cortical region. The PGC establishes focal contacts (point contacts) with the substratum and these appear to serve as anchorage points for cell movement. The focal contacts, and hence the elongation of the PGC itself, follow a pattern of alignment that corresponds to the arrangement of microfilament bundles within the substrate cells. These bundles are numerous and highly organized and create a pattern of stress fibers in the surface of the substrate cells. It is this pattern of cell surface contours that Wylie et al. believe guide the PGCs in their migration. The mechanism of contact is suggested to be mediated by fibronectin (Heasman et al., 1981), which is abundantly present on substrate cells in vitro and in vivo, and which is organized according to the pattern of microfilament alignment. Antibodies specific to fibronectin inhibit PGC migration on a mesenteric substrate in vitro (Heasman et al., 1981; Wylie and Heasman 1982). Furthermore, Heasman and Wylie (1983) indicate that the morphological features reported for intraendodermal PGCs (Kamimura et al., 1980; Delbos et al. 1982) are similar to those exhibited by intramesenteric PGCs, with the exception that elongated PGCs have not been observed within the endoderm, and suggest that similar mechanisms may be involved in the migration of PGCs within the endoderm as have been proposed for
intramesenteric PGCs in *Xenopus*.

E. Gonadal Colonization Phase

At the time of opercular fold formation (Stage 22/23) in *R. pipiens*, the closely grouped germ cells are situated in a mid-saggital plane as an uneven cord, the median germ ridge, at the dorsal root of the mesentery, subjacent to the dorsal aorta and between the two subcardinal veins. The PGCs are then bilaterally partitioned to either side of the dorsal mesentery at Stage 24 to form paired gonadal ridges, concomitant with the fusion of the subcardinal veins to form the posterior vena cava. By feeding tadpole stage (Stage 25; see Fig. 1E) the PGCs occupy longitudinal thickenings comprised of coelomic epithelia cells (CECs) on either side of the dorsal mesentery (the gonadal ridges) just medial to the Wolffian ducts. In *Xenopus*, this event occurs prior to the completion of the median ridge formation, such that in the posterior fertile region there are paired gonadal ridges; more anteriorly, there is a single median ridge, and further anteriorly germ cells are within the mesentery in the process of dorsal "migration" (Wylie and Heasman, 1976; see Figs. 3A, B, C).

Three different views have been offered to explain the lateral displacement of the PGCs from the median germ ridge to the paired gonadal ridges on either side of the dorsal
mesentery: (1) the germ cells are passively displaced by continued morphogenetic movements of related tissues, (2) the PGCs actively move and are attracted bilaterally by a new chemotactic input originating from the gonadal rudiments, (3) the angular alignment of the CECs to the dorsal midline provides lateral "tracts" that guide the actively migrating PGCs to their final destination.

Passive displacement of PGCs during their terminal localization was first proposed by Humphrey (1925), who concluded from observations on sectioned material under the light microscope that the movements of PGCs after leaving the endoderm were attributable to the general growth processes of surrounding tissues. Cheng (1932) arrived at the same conclusion from his descriptive studies on R. cantabrigensis. He reported that the lateral displacement of PGCs from the median germ ridge is coincidental with, and may possibly be brought about by, the medial movement of the two subcardinal veins to form the posterior vena cava at the site formerly occupied by the median germ ridge. Vannini and Giorgi (1969) noted that the positioning of the PGCs laterally from the median ridge parallels the morphogenesis of the vascular system in B. bufo. They also concluded that the formation of the posterior vena cava results in the splitting of the median germ cell mass and the lateral distribution of the PGCs. Moreover, they conducted an
experiment which convinced them that this lateral movement of PGCs is exclusively of a passive, mechanical nature. Following unilateral extirpation of the pronephric duct territory of the tailbud embryos, the posterior cardinal vein was also reduced in size or missing on the operated side in the later developing larvae. From estimates of gonadal germ cells between the two sides in the larvae, these authors found a great difference in PGC numbers in favor of the unoperated side. They interpreted this to mean that the morphogenetic movements of the vascular tissue and formation of the posterior vena cava in the region of the median germ ridge is responsible for the passive lateral distribution of the germ cells to the paired gonadal anlage.

Recently, Hamaguchi (1982) made a descriptive study at the light and electron microscope level in the medaka, Oryzias latipes. In the embryo of this teleost, PGCs situated in the somatopleure of the lateral plate mesoderm are in close association with one another. During their interstitial migration from this position to the gonadal ridges, they are entirely surrounded by closely adhering, small mesenchymal cells. Since the germ cells themselves show no morphological evidence of cells in movement, the author concludes that the displacement of these cells is likely to be passively accomplished by the morphogenetic movements of the surrounding tissues.

The other two views encompass the notion that the PGCs
actively migrate laterally to the paired gonadal ridges, but differ according to the precise mechanism involved in the terminal localization of the germ cells. From a series of experiments Gipouloux (1970) concludes that chemotaxis is involved and that factors originating and emanating from the gonadal regions "attract" or "guide" the germ cells from the median ridge to the latter structures. As mentioned earlier (Section III C), Gipouloux's experiments support the view that dorsal mesodermal structures emit a chemotactic stimulus and the intraendodermal PGCs respond to this signal to attain the median germ ridge. To explain the new direction of movement, which is no longer dorsal but now lateral, this investigator first attempted to experimentally determine whether the PGC migration in the latter direction also involves a chemotactic signal from the dorsal mesodermal structures. To this end, he made unilateral additions of somites to host parabionts. In another type of experiment the notochord primordium was removed from an early neurula and at tailbud stage this same embryo received a notochord grafted to the dorsal lateral region at the level of the somites. In both types of experiments estimates of the gonadal PGC numbers were made to determine whether the germ cells were preferentially distributed to the gonadal ridges on the side that the additional dorsal mesodermal structures were transplanted. He found that the
PGCs distributed bilaterally in almost equal numbers and that this distribution is not influenced by the presence of additional dorsal mesodermal organs localized to one or the other side of the embryo. Thus, the putative chemotactic stimulus responsible for the dorsal intraendodermal migration of PGCs is not responsible for the directed bilateral movements to the paired gonadal primordia. Finally, Gipouloux extirpated the notochord and a pronephric duct from one side of the embryo. During later development, a genital crest formed unilaterally in the operated embryos adjacent to the intact pronephric duct, and all the PGCs that were capable of leaving the dorsal mesentery distributed unilaterally to the unoperated side. Gipouloux had determined earlier that the pronephric ducts had no chemotactic attractive power towards the PGCs. Thus this investigator concluded that it is the presence of the gonadal ridge that is the determining factor in the active migration in the lateral direction, probably mediated by a different and stronger chemotactic signal than the one emanating from the dorsal mesodermal organs.

These findings confirm the results obtained earlier in the chick. Dubois (1964, 1965) placed the trunk region of the chick embryo containing sterile gonadal ridges with either the germinal crescent or a gonadal ridge containing gonocytes. In both types of experiments the germ cells appeared to emigrate into the sterile genital crests,
suggesting that the gonadal epithelium exerts a chemotactic attraction on the active movements of the sex cells.

Based on studies in *Xenopus*, Wylie and coworkers visualize the lateral displacement of PGCs from the root of the dorsal mesentery to paired bilateral ridges to be active and to be directed by the orientation of substrate cells, the CECs of the dorsal body wall, which in this case are aligned transversely to the plane of the dorsal mesentery. The PGCs apparently elongate, extruding a filopodium which coinaligns with the shape of the underlying CECs, or their cytoskeleton, consisting of microfilament bundles, or both (Heasman and Wylie, 1981; Wylie and Heasman, 1982). Then much in the same manner as described above for intramesenteric PGCs (see Section III D), the filopodium attaches by means of adhesion plaques, the trailing end of the PGC retracts as the cell moves forward, and the PGC draws up into a rounded form. These observations have been recorded primarily *in vitro* with dissociated PGCs from Stages 44 to 48 larvae that were seeded onto outgrowths of adult mesentery cells (Wylie et al., 1979). However, the main features of migratory PGC morphology and orientation and the character of the CECs observed *in vivo* have been verified by scanning and transmission electron microscopy on fixed samples taken from live embryos during the same developmental period (Heasman and Wylie, 1981; Wylie and
Heasman, 1982). The attachment of the PGCs to the substrate cells is possibly mediated by fibronectin which has been detected by immunohistochemistry on the surface of the CECs along the dorsal body wall (Heasman et al., 1981). The 90° shift in polarity of the CECs of the lateral dorsal body wall with respect to the orientation of the CECs of the dorsal mesentery is believed to allow the PGCs to assume a lateral migratory path via contact guidance once they reach the dorsal body wall. No specialized junctions are noted between PGCs and CECs prior to Stage 45 (Wylie and Heasman, 1976). By this time, the paired gonadal ridges are beginning to form in the posterior region of the larva and the appearance of desmosomes as well as 300 nm junctions filled with electron dense material are noted between germ cells and the somatic cells of the coelomic epithelium. It is suggested that this association may be important in establishing the structure of the gonad. By Stage 46, SEM studies reveal the formation of a tight band of CECs which form a tight interlocking plait, are oriented craniocaudally, and surround the now lateral PGCs to form the gonadal epithelium (Wylie et al., 1976). Although Wylie et al. (1976) feel that the distribution of PGCs to the paired gonadal ridges is active, they do not rule out the possible involvement of morphogenesis of the coelomic lining in this translocation, especially since it is easy to conceive of the medially-situated PGCs being displaced laterally by
forces such as the CECs that condense to form the paired ridges, and the dorsal mesentery that elongates and narrows beneath the PGCs. The condensation of the CECs to form the paired gonadal ridges has been observed to be independent of the presence of PGCs by examination of the same event in UV-sterile embryos. Finally, the model of contact guidance for the lateral PGC migration does not exclude the possible involvement of a chemotactic attraction stimulus.

IV. Rationale and Objectives of the Study

Morphogenetic cell movements provide the fundamental machinery by which cells become organized into tissues and organs within the developing embryo. In this regard, morphogenesis may be viewed as the quintessential feature of vertebrate embryogenesis. PGC migration represents one instance among many whereby cells move from one place to another destination where they comprise an organ rudiment, in this case the gonadal ridges, together with mesoderm cells of the coelomic lining.

The purpose of the present experiments was to obtain information on the migratory properties of the PGCs in the anuran Rana pipiens and to obtain additional evidence for the mechanisms involved in their displacement from their
site of origin, the endoderm, to the paired gonadal ridges. To this end, the experimental design involved the temporal disengagement of the movements of intraendodermal PGCs and morphogenesis of the somatic tissues within the developing embryo to determine its effects on PGC colonization of the gonadal ridges. Recent research indicates that anuran PGCs can colonize paired gonads of UV-irradiated larvae appreciably later than they would during normal development (Zust and Dixon, 1977; Smith and Williams, 1979; Subtelny, 1980), and that this may result from a retardation in their intraendodermal migration (Ikenishi and Kotani, 1979). One of the aims in the present study was to determine whether normal PGCs can be experimentally delayed in their migration and still attain the gonadal ridges. This was achieved by transplanting intraendodermal PGCs from Stage 20 swimming larvae into younger Stage 17 tailbud embryos. The data obtained from these experiments, together with the histological study of the heterochronic germ cell graft larvae (Subtelny and Penkala, 1984), provide convincing evidence that normal PGCs can be held in abeyance within the endoderm for at least an additional 2-1/2 days and they still retain the ability to migrate to the gonadal ridges. Another aim of this study was to determine whether intraendodermal PGCs can precociously migrate to the gonadal ridges. The experimental procedure involved transplanting PGCs from Stage 17 tailbud embryos into Stage 20 swimming
larvae, thus circumventing the major period of their intraendodermal migration. The data obtained from these experiments reveal for the first time that intraendodermal PGCs can colonize the gonadal ridges at least 2-1/2 days earlier than they would during normal development.

The combined data from these experiments, together with the histological analyses of the reciprocal heterochronic graft larvae reported earlier (Subtelny and Penkala, 1984), disclose that separation of the graft PGCs from the endoderm in Rana Pipiens is correlated with the morphogenetic movements of the host lateral plate mesoderm cells that are associated with the formation of the dorsal mesentery. This suggests that morphogenesis plays a major role in the migration of the PGCs from the endoderm to the gonadal ridges.
MATERIALS AND METHODS

I. Adult Animals

Mature *Rana pipiens* of the Northern variety were obtained in the Fall from various suppliers. The frogs were maintained at 4°C in two-quart aquarium bowls containing "aged" water (dechlorinated, aerated tap water). The water was changed three times per week. Adults could be kept viable and reproductively stable in this manner until the next Fall.

II. Embryonic Material

The methods for obtaining gametes and artificial inseminations followed routine procedures that conformed to those of Rugh (1934) and DiBerardino (1967, 1977). Briefly, females were induced to ovulate following intraperitoneal injections of two female adenohypophyseal glands in 0.5ml Amphibian Ringer's (AR) medium and 0.2ml of progesterone (2.5 mg/ml) suspended in AR medium. The injected animals were kept at 18°C and tested for ovulation. Artificial fertilizations were performed 48 hours later. Sperm suspension was prepared from a freshly sacrificed male. The pair of testes was removed, placed in 10ml of 1:10 AR solution and finely dissected with watchmaker forceps. About
10 to 15 minutes were allowed for activation of sperm. To fertilize, about 100 to 150 eggs were stripped onto clean glass microscope slides. The eggs were covered with sperm suspension using a Pasteur pipette and allowed to stand for 10 minutes. The excess sperm suspension was then decanted from the slides, which were placed in individual Petri dishes (100 x 15mm) containing 1:10 AR medium with penicillin (100u units/ml) and streptomycin (100 ug/ml) added. The embryos were allowed to develop at 18°C and were characterized according to the serial stages of Shumway (1940). Since all embryos for a particular graft experiment were siblings from the same cross, different aged donors and hosts, together with their controls, were obtained by placing a portion of them at 4°C when they had reached early tailbud stage (Stg. 16+), and maintaining them at that temperature (for 2-1/2 days) until their sibling counterparts at 18°C had reached Stage 20 (hatching), at which time both groups were prepared for microsurgery. A few experiments were arranged in such a way that both donor and hosts were maintained at 18°C before grafting. The results obtained were essentially the same as those in which embryos were kept at 4°C. Moreover, comparisons of gonadal germ cell counts in embryos kept at 4°C for one week with siblings kept at 18°C indicated that the lower temperature did not result in any decrease in gonocyte numbers.
III. Preparation of Irradiated Hosts

Smith (1966) found that UV treatment of *R. pipiens* embryos at first cleavage resulted in the total absence of gonadal germ cells at Stage 25, although the larvae were normal in all other respects. Based on his procedure, we have devised the following scheme for preparing germ cell-free sterile embryos to serve as hosts in germ cell transfer experiments.

Following rotation (ca. 45 minutes post-fertilization at 18°C), fertilized eggs were mechanically dejellied with watchmaker forceps. Groups of 30 eggs were placed on a quartz slide and oriented with the vegetal hemisphere downwards. Excess fluid was drained off with a Pasteur pipette, and then the eggs were sandwiched with another slide until slightly flattened, the distance between the two slides being fixed by a piece of clay placed at either end (see Fig. 2). At 120 to 180 minutes after fertilization, the slides were placed over a short-wave (253.7nm) UV source (Mineralight Model, R25, Ultra-violet Products, Inc.), exposing the vegetal hemispheres of the eggs for 6 to 7-1/2 minutes to UV light, at a dose rate of 40 ergs/mm²/sec. This resulted in a total UV dose of 14,400 ergs/mm² to 18,000 ergs/mm² (measured with a J-225 short wave meter, Ultra-violet Products, Inc.). We selected this particular range of total UV dose on the basis of data from seven
Figure 2. Method for preparing UV-sterile hosts for grafting experiments (as modified from Smith, 1966). Groups of fertilized eggs were dejellied and placed between two quartz slides with the vegetal hemisphere downwards at 120 to 180 minutes after fertilization. In this manner, the embryos were placed over a 253.7nm short-wave UV source, thus exposing the vegetal hemispheres for a period of 6 to 7-1/2 minutes, resulting in a total UV dose of 14,400 to 18,000 ergs/mm².
experiments, which compared the response of embryos to varying doses of UV radiation as determined by their gonocyte numbers at Stage 25 (see Fig. 3). Total UV dose administered varied because egg sensitivity to UV light leading to developmental abnormalities and arrest at gastrulation was found to vary, particularly with females used at the beginning and at the end of the experimental season. Following UV treatment, embryos were returned to 1:10 AR medium (with antibiotics added) for development until grafting. The development of a proportion of the UV-treated embryos from some crosses was slightly retarded (approximately 6 to 12 hours) when compared to untreated normal controls at the feeding tadpole stage. However, with the exception of the absence of gonadal germ cells, as determined by visual examination of autopsied larvae at Stage 25 (Fig. 4), the UV-treated animals proceeded to develop normally in every other respect. In the experiments reported here, 297 out of 300 (99%) UV-treated controls were sterile at Stage 25. The occasional experiment in which one UV-treated animal had a few gonocytes could be attributed to a technical error in the UV-irradiation procedure. Thus, we can say with increased confidence that all, or virtually all, of the gonadal germ cells in the graft larvae at Stage 25 were contributed by the donor transplant.
Figure 3. Response of embryos to varying doses of UV radiation as determined by their number of gonadal germ cells at Stage 25. Seven experiments, each utilizing sibling embryos from a different female, are represented by the seven curves. Each data point is an average of germ cell counts from 10 embryos. Based on these experiments, the region labeled "host irradiation" was selected for preparing hosts for grafting experiments because it consistently resulted in embryos without gonadal germ cells, regardless of the range in the average number of PGCs, which varied five-fold among the untreated embryos between the seven different crosses.
Figure 4. A. Paired gonadal ridges of a normal untreated Stage 25 larva, apparent as two more or less continuous thick white cords of large yolk cells. The embryo was anesthetized, dissected, and the viscera removed.

B. Gonadal ridges of a Stage 25 larva that was UV-irradiated (18,000 ergs/mm²) in the vegetal hemisphere at the onset of the two-cell stage. Notice the conspicuous absence of any detectable large yolk PGCs. (Scale bar represents 500 μm.)
IV. Transfers of Germ Cell-Containing Endoderm Regions to UV-Irradiated Hosts

Two series of heterochronic germ cell grafts were performed:

1) In one experimental series, normal Stage 20 larvae were used as donors for heterochronic germ cell grafts into 2-1/2 day younger Stage 17 UV-irradiated hosts (N Stg. 20 -> UV Stg. 17). The vast majority of the primordial germ cells in the Stage 20 larva were previously determined to be localized in the upper half of the endoderm mass and in the vicinity of the enteron or above it (Subtelny, 1980). Therefore, the horizontal incision through the donor endoderm mass was made at the level just below the dorsal endoderm crest, actually passing through the region where a large proportion of the PGCs were accumulated; the graft was then transplanted into the middle-third of the host Stage 17 endoderm mass (see Fig. 5).

2) In another series of experiments, normal Stage 17 tailbud embryos served as donors for heterochronic germ cell transfers into the endoderm of 2-1/2 day older Stage 20 host larvae that had been previously exposed to UV light at the two-cell stage (N Stg. 17 -> UV Stg. 20). The graft included virtually the entire germ cell-containing region of the endoderm of the Stage 17 donor (see Fig. 6). The vast
Figure 5. Germ cell graft from a normal Stage 20 hatched larva into a UV-irradiated Stage 17 tailbud embryo. A region consisting of roughly three-fourths of the ventral endoderm mass in the dorsal-ventral direction and the middle third of the endoderm in the anterior-posterior direction was transferred to approximately the middle third of the endoderm of a UV-irradiated Stage 17 recipient. The dorsal horizontal incision in the donor either passed through or above the enteron, leaving a small dorsal region of endoderm remaining in the donor.

Isochronic grafts of N Stage 17->UV Stage 17 and N Stage 20->UV Stage 20 were prepared in a similar fashion. The Stage 17 donor region (for the isochronic grafts) was the same as that described for the N Stage 17->UV Stage 20 grafts in Figure 6. The Stage 20 donor region for the isochronic grafts corresponded to that described for the N Stage 20->UV Stage 17 heterochronic grafts in Figure 5.
Normal Stage 20 Donor

UV Stage 17 Host

donor region

graft region
Figure 6. Germ cell graft from a Stage 17 tailbud embryo into a previously UV-irradiated Stage 20 hatched larva. Approximately two-thirds of the ventral endoderm mass, in the dorsal-ventral direction, and the middle third of the endoderm in the anterior-posterior direction from the Stage 17 donor was transplanted into the middle third of the endoderm of the UV-irradiated Stage 20 recipient. The dorsal incision in the host was made at the level just below the enteron, leaving several layers of host endoderm cells, including the archenteron roof, between the graft and the host dorsal endoderm.

Isochronic grafts of N Stage 17→UV Stage 17 and N Stage 20→UV Stage 20 as described in the legend to the previous figure (Fig. 5) were also included with these experiments.
Normal Stage 17 Donor

donor region

UV Stage 20 Host

graft region
majority of the primordial germ cells were previously determined to be localized in the ventral-half, and middle-fourth of the endoderm mass at this stage (Subtelny, 1980).

For each of the heterochronic germ cell graft series, control isochronic germ cell grafts were also performed. They included transfers of normal Stage 17 germ cell-containing endoderm into UV-irradiated Stage 17 embryos, and transfers of normal Stage 20 germ cells into UV-irradiated Stage 20 hosts. Additional controls for each experimental series included unoperated normal embryos and UV-irradiated embryos.

V. Surgical Procedures

Microsurgery was performed on sibling embryos with a Circon microknife and Dumont fine watchmaker forceps under semi-sterile conditions, using a Wild-Heerbrugg M-5 dissecting microscope. The grafts were basically a modification of the procedures used by Blackler and Fischberg (1961) and Cipouloix (1970). Previous extirpation experiments had delineated the regions within the endoderm that contained primordial germ cells in R. pipiens at successive stages of development between tailbud Stage 17 and the swimming larva at Stage 21 (see Subtelny, 1980).

Donor and host embryos were decapsulated, then rinsed three times in sterile Niu-Twitty (NT) medium (Niu and
Twitty, 1953) containing penicillin (100 u units/ml) and streptomycin (100 ug/ml). They were then transferred to another dish in which 0.01% MS222 (ethyl m-aminobenzoate methane sulfonate) was added to the NT medium to anesthetize the animals. Experimental and control embryos were maintained in anesthetic 5 to 10 minutes prior to surgery. The experimental animals were transferred to a plasticine-lined operating dish containing the same medium. The actual microsurgery consisted of cutting out the germ cell-containing endoderm region (together with the covering ectoderm and mesoderm layers) from the normal donor embryo and inserting this plug of tissue into the UV-irradiated recipient from which a corresponding region of endoderm had been removed. The host was placed ventral side up in a small depression in the plasticine and the graft was inserted, taking care to maintain its proper anterior-posterior orientation. Immediately following the operation, a stainless steel clip was used to immobilize the grafted tissue (Figs. 7 and 8). After 15 to 20 minutes, grafted embryos were transferred to fresh sterile NT medium containing the antibiotics and anesthetic. After 5 to 6 hours, when sufficient healing had taken place in the graft individuals, the experimentals and controls were transferred to sterile 1:10 AR medium (containing antibiotics) in small (60 x 15mm) plastic Petri dishes until they reached Stage 25, when counts of gonadal germ cells were made.
Figure 7. N Stage 20-UV Stage 17 heterochronic germ cell graft embryo, immediately after Stage 20 graft transplantation (A), 30 minutes after the operation (B), and 5 to 6 hours after microsurgery (C). In Figure A, the Stage 17 UV host has just received a graft from a normal Stage 20 donor hatched larva and is shown here with its ventral side up seated in a depression in plasticine and held in position with a wire clip. The embryo is then transferred to a sterile petri dish containing 1:10 Ringer's medium and anesthetic and antibiotics. As shown in Figure B, epidermal overgrowth has been initiated and the borders of the wound have begun to heal. By 5 to 6 hours (Fig. C), epidermal fusion has occurred; at this point the grafted embryo is removed from anesthesia and transferred to 1:10 AR medium containing antibiotics. Note the distinction between donor and host pigmentation, indicative of the difference in epidermal differentiation between Stages 20 and 17. (Scale bar represents 1mm.)
Figure 8. A N Stage 17→UV Stage 20 heterochronic germ cell graft larva depicted immediately after (A), 30 minutes after (B), and 5 to 6 hours following microsurgery (C). (Scale bar represents 1 mm.)
VI. Germ Cell Counts

To assess the results of the experiments in this study in terms of gonadal germ cell migration, direct germ cell counts were made at larval Stage 25. This simple assay developed by our lab (Subtelny, 1980) does not require the analysis of sectioned material, but permits direct assessment of germ cell numbers in anesthetized embryos under the dissecting microscope.

Embryos were anesthetized in 1:10 AR medium containing 0.01% MS222 (see Fig. 9 for outline of procedure). Dissections were then performed in 1:10 AR medium to remove gut and other viscera, thus exposing the bilateral gonadal ridges on either side of the dorsal root of the median intestinal mesentery. Embryos were then rinsed in 1mM EDTA (ethylenediamine-tetraacetate) in modified (Ca\(^{++}\)- and Mg\(^{++}\)-free) Niu-Twitty (MNT) medium to remove excess divalent cations and transferred to a plasticine-lined syracuse dish containing 1mM EDTA in MNT medium. Wire clips were used to pin down embryos. Then 5 to 10 minutes were allowed for the thin, transparent germinal epithelium to dissociate, thus freeing the large, yolky germ cells for counting (see Fig. 10). Counts were made using a glass microneedle to remove individual germ cells under a dissecting microscope.
Figure 9. Procedure for dissociating Stage 25 gonadal PGCs for making direct counts (after Subtelny, 1980). Stage 25 larvae are anesthetized in 0.01% MS222 and the ventral body wall is dissected open to expose the gut. The viscera is carefully removed, revealing two white more or less continuous cords of yolky cells (PGCs) within the gonadal ridges. Each gonadal ridge is on either side of the mesentery which is attached to the dorsal body wall in the midline, and just beneath the somites in the posterior region of the abdomen. Embryos are then rinsed briefly in Ca\textsuperscript{++}-, Mg\textsuperscript{++}-free MNT medium containing 1mM EDTA and exposed to the same medium for 5 to 10 minutes until the germ cells round up, indicating their dissociation from the gonadal epithelium. It is now possible to make direct counts of the PGCs using a glass microneedle.
STAGE 25

ANESTHETIZATION

DISSECTION

EVISGERATION

DISSOCIATION OF PGCs
Figure 10. A. Paired gonadal ridges of a Stage 25 larva evident as two more or less continuous, thick white cords of cells. The larva has been anesthetized in 0.01% MS222 and dissected to remove the gut.

B. The same gonadal ridges, following a 10 minute exposure to 1mM EDTA in Ca
\(^{++}\), Mg
\(^{++}\)-free MNT medium (Subtelny, 1980). The gonocytes have dissociated from the thin transparent gonadal epithelium, and now appear as individual large, white yolky cells, whose number is easily counted under a dissecting microscope with the aid of a glass microneedle. (Scale bar represents 200 um.)
VII. Statistical Analysis of Germ Cell Counts

The data in the form of numbers of gonadal germ cells were analyzed with the SAS (1982) statistical packet for an IBM main-frame computer. In most experiments the differences between experimental groups were tested for by analysis of variance using the General Linear Model Procedure (SAS, 1982). Individual planned comparisons were then made by a Least Squares Mean Analysis. In certain experiments, comparisons of gonadal germ cell numbers in Stage 25 larvae with post-Stage 25 larvae (see Results, Section IIIC; Tables 8 and 9) were made using a two-way nonparametric analysis. In all of the above tests, a difference was considered significant at a probability level of $p < 0.05$. 
RESULTS

To assess the influence of heterochronic germ cell grafts on gonadal germ cell numbers, certain requirements had to be met:

1) It was necessary to determine gonadal germ cell numbers in many experimental and control animals. This was accomplished by direct counting of individual, dissociated gonocytes in autopsied Stage 25 larvae; i.e., after germ cell migration to the gonadal ridges is normally terminated and at a time when gonocyte numbers remain essentially stable prior to entering intensive mitosis (Subtelny, unpublished).

2) The graft recipients should be sterile and devoid of host germ cells. This was established by examination of the gonadal ridges of UV-irradiated control siblings at Stage 25 in each experiment. As mentioned in the Materials and Methods (Section III), 99% of the UV-irradiated controls were sterile and devoid of gonocytes.

3) A knowledge of the localization of the intraendodermal PGCs was needed at Stages 17 and 20 used as donors for the heterochronic graft experiments. This was earlier determined by extirpation experiments with normal embryos (Subtelny, 1980). The Stage 17 grafts involved endoderm regions surrounding the bulk of the PGCs localized in the ventral half of the endoderm mass (see Fig. 6),
whereas the Stage 20 grafts necessitated cutting through the dorsal endoderm region occupied by the PGCs at this stage in development (see Fig. 5).

4) Finally, preliminary experiments were performed with normal embryos at successive stages of development between Stage 17 and Stage 20, to determine the influence of extirpation and anesthesia on germ cell migration in the graft embryos.

I. Effects of Microsurgery and Anesthesia On Germ Cell Migration

In each of two experiments the germ cell-containing endoderm was removed and reinserted back into the same individual in operating medium a) containing dilute anesthetic and b) lacking anesthetic. In the latter series, the embryos were partially immobilized by clipping the tail and making an incision through the middorsal spinal cord and somite region, which healed rapidly following the grafting procedure. When the experimental and control siblings attained the feeding stage (Stage 25, Shumway, 1940), the animals were sacrificed and the numbers of gonadal PGCs determined according to the procedure outlined in the Materials and Methods (Section VI). The results of these experiments are presented in Table 1.
TABLE 1
Effects of Surgical Procedure and Anesthesia on Germ Cell Migration

<table>
<thead>
<tr>
<th>Stage of grafting</th>
<th>NO ANESTHESIA</th>
<th></th>
<th>ANESTHESIAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of larvae</td>
<td>Germ cells C ( x ± sem)</td>
<td>Percent germ cells of controls</td>
</tr>
<tr>
<td>Controld</td>
<td>30</td>
<td>82 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>29</td>
<td>64 ± 4</td>
<td>78</td>
</tr>
<tr>
<td>18</td>
<td>26</td>
<td>54 ± 4</td>
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</tr>
<tr>
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<td>23</td>
<td>23 ± 4</td>
<td>28</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>26 ± 5</td>
<td>32</td>
</tr>
</tbody>
</table>

a Table represents results of two replicate experiments.

b Embryos in this group were anesthetized in 0.01% MS222 during surgery and for 6 hours thereafter.

c Germ cell counts at Stage 25 on unoperated controls and normal larvae which underwent extirpation and reinsertion of the germ cell-containing endoderm region, expressed as mean ± standard error of the mean.

d Unoperated controls were exposed to the operating medium with or without anesthetic in the same manner and at the same stage of development as embryos grafted at Stage 20.
At Stage 17 microsurgery alone, the simple removal and reinsertion of the germ cell-containing endoderm region, results in about a 20% decrease in gonadal germ cell numbers relative to the unoperated controls regardless of whether the anesthetic, MS222, was included in the operating medium or not (Table 1, row 2). This decrease in mean gonocyte numbers between Stage 17 graft embryos and unoperated controls is significant ($p<0.0001$). Although there is a consistent additional reduction in the gonocyte numbers when the operation is performed at Stage 18 as compared with Stage 17 (Table 1; compare rows 2 and 3), the differences between the means are not significant at the 5% level. Again, the results do not reveal any effect of anesthesia on germ cell migration in the embryos operated on at Stage 17 or Stage 18.

A marked reduction in germ cell migration to the gonadal ridges is observed when the operations are performed on Stage 19 and 20 larvae in medium without anesthetic. The graft larvae possess only 30% of the gonadal germ cell numbers present in the unoperated controls and this difference is highly significant ($p<0.0001$). Interestingly, the operations performed at these two later stages of development in dilute anesthetic consistently resulted in a higher proportion of gonadal germ cells (ca. 50%) as compared with 30% gonocytes in larvae operated on without anesthetic (Table 1, compare rows 4 and 5). The mean
numbers of gonadal germ cells were significantly higher in the experimental groups, operated on at Stages 19 and 20 and briefly reared in medium without anesthetic. These data are summarized in the histogram in Figure 11.

Two conclusions can be drawn from the data: 1) The surgical procedure alone interferes with the intraendodermal germ cell migration to varying degrees, particularly when the dorsal interstitial germ cell migration is well underway during Stages 19 and 20. 2) Maintaining the graft embryos in dilute anesthetic until a brief period following the operation did not interfere with germ cell migration. This is evident from Table 1, row 1, showing little difference in the germ cell counts between the unoperated anesthetized and nonanesthetized control larvae. The controls were placed in medium with or without MS222 at Stage 20 and maintained for the same length of time and under the same conditions as the Stage 20 operated embryos.

The simplest interpretation of the data is that the grafting procedure can interfere with the interstitial migration of the intraendodermal PGCs to varying degrees unless the tissues properly heal following the operation. The significantly higher proportion of gonadal germ cells in the experimental animals operated on at Stages 19 and 20 and briefly reared in MS222, in contrast with the gonocyte numbers in the experimentals reared in medium without
Figure 11. Bar graph summarizing the results of two replicate experiments determining the effects of microsurgery and anesthesia on germ cell migration to the gonadal ridges. The data for these experiments are presented in Table 1.
anesthetic, seems to be ascribed to the influence of the anesthesia in preventing muscle contraction in the experimental animals and in allowing sufficient healing of the wound during a critical period following the microsurgery.

The implication from these data is that intraendodermal PGCs migrate to the gonadal ridges in sufficient numbers under the conditions of the experiment to allow the present study to be carried out.

II. Transfers of Normal Stage 20 PGCs into UV-Irradiated Stage 17 Hosts

One of the objectives of the present experiments, outlined in the Rationale and Objectives of the Study was to determine whether intraendodermal germ cells can be experimentally delayed in populating the gonadal ridges. To this end, transfers of PGCs from normal Stage 20 donors into UV-irradiated Stage 17 hosts were made. In addition to the unoperated normal controls and UV-irradiated controls, isochronic germ cell grafts with sibling embryos (N Stage 17->UV Stage 17, and N Stage 20->UV Stage 20) were carried out with each experiment to distinguish possible experimental differences between isochronic grafts and heterochronic grafts.
A. Development of the heterochronic graft embryos and controls

Figures 7 and 8 in the Materials and Methods (Section V) illustrate the appearance of the experimental larvae after the initial grafts were made. Annealing of the graft endoderm with that of the host occurred within one hour in all operated embryos, but there were differences in the length of time required for the fusion of the overlying ectoderm and mesoderm and complete closure of the borders of the wound (for example, compare Fig. 12A with Fig. 16A). It was observed that the following types of grafts tend to heal completely, beginning with the most rapid, in the following sequence: N Stage 20→UV Stage 20, N Stage 17→UV Stage 20, N Stage 17→UV Stage 17, and N Stage 20→UV Stage 17. In certain instances, small gaps in the fusion of ectoderm and mesoderm at the border of the graft were present after 24 to 48 hours on one or the other side of the embryo. We have attempted to determine whether differences in gonadal germ cell numbers correlated with gross differences in the extent of the wound and the rate of healing of the grafts. In general, there appeared to be no significant or consistent correlation between the delay in complete healing of the wound and variations in gonocyte numbers. Certain individuals with delayed healing may possess more gonadal
Figure 12. Photomicrographs comparing development of heterochronic N Stage 20→UV Stage 17 larva with unoperated control. (Scale bar represents 1 mm).

A. Lateral view of heterochronic graft larva at host Stage 17, one hour after transplantation. The graft tissue has fused intimately with the host along its posterior borders whereas the graft ectoderm and mesoderm tissues have not quite fused with the host along the anterior border.

B. Lateral view of unoperated control at Stage 17.

C. Lateral view of heterochronic graft larva at host Stage 21+/22. Donor tissues are incorporated into host. Note overlying epidermis of graft (arrow) which is advanced in differentiation with respect to the host. The graft larva shows typical external morphology (cf. control (D)).

D. Lateral view of normal unoperated control larva at Stage 21+/22. At this stage the PGCs are situated in the dorsal ridge of the endoderm and will exit within 24 hours.

E. Lateral view of heterochronic graft larva at Stage 23. The transplanted region (arrow) continues to differentiate but in an advanced fashion with respect to the host or unoperated control (F). Otherwise, the larva appears morphologically and behaviorally similar to the control (F).

F. Lateral view of normal unoperated control larva at Stage 23. By this stage, PGCs have exited the endoderm and are concentrated in a medial cord at the base of the dorsal intestinal mesentery.
germ cells than those displaying rapid, complete annealing of the host and graft ectomesoderm. In this respect, the extent or degree of fusion of the host and graft endoderm would appear to play a more important role in the proportion of germ cells that colonize the gonadal ridges than that of complete annealing of the ectomesoderm.

In other respects, the graft embryos overtly develop in a fashion normal to Stage 25 (see Fig. 13), except in the following way. By the time graft embryos attain Stage 21+/22, one does note that the epidermis of the graft takes on a transparent appearance (graft corresponds temporally to that of Stage 24/25), indicating that the graft tissues tend to differentiate according to their own developmental time schedule in the host embryo (see Figs. 12C and 12E, arrows; compare with Figs. 12D and 12F). This has been verified in transverse sections of fixed material at successive stages of development (Subtelny and Penkala, 1984). By the time graft embryos attain Stage 25, this difference is readily apparent as illustrated in Figures 13A and 13B (compare with Fig. 13C). In addition, at Stage 25 the coiling of the gut in the experimental (see Fig. 14C) is not as complete as in the controls (see Fig. 14A). The graft portion of the gut (see Fig. 14C) is advanced in differentiation relative to the normal unoperated controls (see Fig. 14A) or isochronic graft larvae (see Fig. 14B), again a difference that has been verified in sectioned material. However, it should be
Figure 13. Photomicrographs depicting epidermal differentiation in N Stage 20→UV Stage 17 heterochronic graft larva at host Stage 25 and unoperated control larva at Stage 25. (Scale bar represents 1 mm.)

A. Lateral views comparing heterochronic graft larva (upper) and normal unoperated control (lower). The transparent ventral epidermis of the graft larva (arrow) normally does not appear until two days later and is not present in control, which still shows an opaque ventral epidermis.

B. Ventral view of heterochronic graft larva, revealing advanced epidermal differentiation. Note that the digestive tract is easily visible through the transparent body wall and that it is folded whereas in the control the gut is coiled.

C. Ventral view of normal unoperated control larva.
Figure 14. Photomicrographs showing gut morphology in the heterochronic graft larva at host Stage 25 and its respective controls. The larvae have been sacrificed and the ventral abdominal wall removed to expose the digestive tract. (Scale bar represents 1 mm.)

A. Ventral view of normal unoperated control larva showing digestive organs. Note typical intestinal coiling.

B. Ventral view of N Stage 17->UV Stage 17 isochronic graft larva. Digestive tract development is identical to the unoperated control (A).

C. Ventral view of N Stage 20->UV Stage 17 heterochronic graft larva. Digestive tract is continuous and folded. Note hindgut region contains less yolk and bears a highly developed epithelial surface, indicative of its advanced developmental state relative to the controls in A and B.
mentioned that this gut morphogenesis occurs after the PGCs have separated from the endoderm at Stage 22, on their way to the gonadal ridges, and therefore has no apparent effect on population of the gonadal ridges by the PGCs.

When the heterochronic graft larvae attained Stage 25, they were anesthetized and the viscera removed to clearly reveal the large yolky germ cells in the gonadal ridges, as shown in Figure 15. One can note the obvious absence of PGCs in the sibling irradiated control (Fig. 15D) and the abundance of germ cells in the unoperated control sibling (Fig. 15A). The gonocyte numbers in the gonadal ridges in the heterochronic (Fig. 15C) and isochronic (Fig. 15B) germ cell graft siblings are clearly reduced in numbers relative to the unoperated controls. The separation and arrangement of the gonocytes along the anterior-posterior length of the paired ridges also emphasize that they have been displaced from the endoderm as small clusters, rather than cords of cells.

B. Comparisons of gonocyte numbers in heterochronic graft larvae and controls

An initial four experiments involving the transfer of normal Stage 20 germ cells into irradiated Stage 17 hosts (N Stage 20→UV Stage 17) were carried out without the
Figure 15. View of the gonadal ridges of N Stage 20->UV Stage 17 heterochronic graft larva and controls at Stage 25. The larvae were sacrificed, dissected and eviscerated, thus exposing the germ cell region (see Fig. 9). (Scale bar represents 1 mm.)

A. Gonadal ridges of normal unoperated control larva.

B. Gonadal ridges of N Stage 20->UV Stage 20 isochronic graft larva. There are individual and small clusters of germ cells separated along the lengths of the paired gonadal ridges. On the average, the gonocyte population is approximately 20% that of the unoperated control.

C. Gonadal ridges of N Stage 20->UV Stage 17 heterochronic graft larva. The proportion of the unoperated control gonocyte population represented here is again 20%, statistically identical with that of Figure 15B.

D. Sterile gonadal ridges of UV-irradiated control larva. Note the complete absence of gonocytes in this larva. This substantiates the assumption that graft germ cells are strictly of donor origin.
presence of anesthetic in the operating medium. Results of germ cell counts in the heterochronic graft larvae and control siblings for each of the experiments are listed in Table 2.

Between 15 and 30 irradiated controls were reared in each of the four experiments. Of the 85 UV-irradiated control larvae examined by autopsy at Stage 25, 84 (99%) completely lacked gonocytes. Thus the eggs of the four females were sensitive to the UV dosages used to provide sterile host embryos for each of the graft experiments. All unoperated controls possessed gonadal germ cells with the mean germ cell number varying between 47 and 124 in the four experiments. Mean germ cell numbers in the isochronic graft controls (N Stage 17->UV Stage 17) ranged between 34 and 74, which corresponds to between 60% and 100% of the PGCs scored in the unoperated controls in the respective experiments. Of interest, the overall mean germ cell number from the isochronic grafts (70%) is not different from the results obtained with similar Stage 17 germ cell grafts that were removed and reinserted into the same normal embryos, testing the effects of MS222 (Table 1). The data indicate that the UV-irradiation of embryos at the two-cell stage to provide sterile host embryos for the germ cell grafts does not impair migration and population of the gonadal ridges by the transplanted PGCs. The reduction in gonadal germ cell numbers relative to the unoperated controls must be
<table>
<thead>
<tr>
<th>Female number of larvae</th>
<th>Germ cells per larva ( \bar{x} \pm \text{sem} ) range</th>
<th>Number of larvae</th>
<th>Number with germ cells</th>
<th>Germ cells per larva ( \bar{x} \pm \text{sem} ) range</th>
<th>Number of larvae</th>
<th>Germ cells per larva ( \bar{x} \pm \text{sem} ) range</th>
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<tr>
<td>52</td>
<td>129.3 ± 8.0</td>
<td>54-170</td>
<td>12</td>
<td>12</td>
<td>21.1 ± 4.1</td>
<td>4-56</td>
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<tr>
<td>59</td>
<td>52.0 ± 3.2</td>
<td>36-78</td>
<td>23</td>
<td>22</td>
<td>11.6 ± 1.9</td>
<td>1-33</td>
</tr>
<tr>
<td>66</td>
<td>47.1 ± 4.2</td>
<td>10-69</td>
<td>10</td>
<td>10</td>
<td>14.3 ± 3.9</td>
<td>4-37</td>
</tr>
<tr>
<td>72</td>
<td>58.1 ± 3.0</td>
<td>42-84</td>
<td>15</td>
<td>11</td>
<td>10.7 ± 2.0</td>
<td>1-23</td>
</tr>
</tbody>
</table>

* Based on total number of larvae with germ cells in each experiment.
primarily related to the graft procedure itself.

Of 62 total heterochronic graft larvae, 55 possessed gonadal germ cells, and the mean gonocyte numbers varied between 11 and 21, corresponding to between 17% and 30% of the gonocytes scored in the unoperated controls. These results indicate that at least a proportion of the Stage 20 intraendodermal PGCs still retain the ability to populate the gonadal ridges when transplanted back into the endoderm of a 2-1/2 day younger Stage 17 host embryo.

The heterochronic graft larvae clearly possessed much fewer gonadal germ cells than the isochronic graft larvae. To obtain some idea whether the reduced gonocyte numbers in the heterochronic graft larvae were related to the inability of a proportion of Stage 20 germ cells to undertake delayed migration to the gonadal ridges, an additional series of experiments were done incorporating isochronic grafts of normal Stage 20 germ cells into irradiated Stage 20 hosts. The experiments were performed with anesthetic in the medium, which was required to immobilize the Stage 20 hosts during the grafting procedure. The results are presented in Table 3 and Figure 15. In addition, this series of experiments included other combinations of grafts; i.e., N Stage 17->UV Stage 20 and N Stage 17->UV Stage 17 (see Table 6).

Virtually all of the UV-irradiated controls examined in
### TABLE 3

Gonadal Germ Cell Numbers in Heterochronic Graft Larvae (N Stage 20 → UV Stage 17)

II. Comparison with Isochronic Graft Larvae (N Stage 20 → UV Stage 20)

<table>
<thead>
<tr>
<th>Female number</th>
<th>Number of larvae</th>
<th>Germ cells per larva</th>
<th></th>
<th>Number of larvae</th>
<th>Number with germ cells</th>
<th>Germ cells per larva</th>
<th></th>
<th>Number of larvae</th>
<th>Number with germ cells</th>
<th>Germ cells per larva</th>
</tr>
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<tbody>
<tr>
<td>40</td>
<td>10</td>
<td>42.2 ± 4.0</td>
<td>23-70</td>
<td>12</td>
<td>11</td>
<td>8.1 ± 1.9</td>
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<td>6</td>
<td>6</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
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<td>19</td>
<td>34.8 ± 2.0</td>
<td>20-58</td>
<td>26</td>
<td>22</td>
<td>11.1 ± 1.7</td>
<td>1-33</td>
<td>16</td>
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<td>10.7 ± 4.2</td>
</tr>
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<td>57</td>
<td>10</td>
<td>44.3 ± 3.3</td>
<td>31-69</td>
<td>20</td>
<td>17</td>
<td>7.0 ± 1.0</td>
<td>1-16</td>
<td>20</td>
<td>19</td>
<td>9.8 ± 1.7</td>
</tr>
<tr>
<td>63</td>
<td>20</td>
<td>56.6 ± 9.8</td>
<td>19-104</td>
<td>7</td>
<td>7</td>
<td>12.3 ± 3.0</td>
<td>2-28</td>
<td>18</td>
<td>17</td>
<td>16.9 ± 2.7</td>
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<tr>
<td>69</td>
<td>20</td>
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<td>23-66</td>
<td>13</td>
<td>13</td>
<td>12.9 ± 3.2</td>
<td>1-37</td>
<td>25</td>
<td>20</td>
<td>11.8 ± 1.2</td>
</tr>
</tbody>
</table>

*a Based on total number of larvae with germ cells in each experiment.
this series (131 out of 132; 99%) were sterile, whereas all the unoperated controls possessed gonocytes with mean germ cell numbers ranging between 35 and 57. Control isochronic N Stage 17->UV Stage 17 graft larvae possessed between 59% and 100% of the gonocyte numbers present in the unoperated controls; a similar range of gonocytes was scored with the Stage 17 isochronic grafts without anesthetic in the previous experiment (compare Tables 2 and 6). Thus the presence of anesthetic in the operating medium did not improve or impair the proportion of PGCs that populated the gonadal ridges in the Stage 17 isochronic graft larvae, and it would not be expected to do so judging from the results in Table 1.

The range in gonocyte numbers scored in the N Stage 20 ->UV Stage 17 heterochronic graft larvae in this experimental series (between 14% and 32% of the unoperated controls, Table 3), is the same as in the previous experimental series (between 17% and 30% of the unoperated controls, Table 2). A comparison of these results with germ cell counts in isochronic N Stage 20->UV Stage 20 graft larvae (between 10% and 28% of the unoperated controls, Table 3) indicates that the Stage 20 germ cell grafts yield the same proportions of gonadal germ cells whether the grafts are made into hosts at the same stage or at an earlier stage in development. This similarity was confirmed statistically by an analysis of variance. Thus, the reduced
number of PGCs populating the gonadal ridges does not appear to be related to the experimentally induced delayed migration in the heterochronic graft larvae. Rather, it seems to be more related to the Stage 20 germ cell graft itself.

C. Estimates of primordial germ cell numbers in Stage 20 grafts

Previous extirpation experiments had localized the PGCs in Stage 20 larvae mainly within the dorsal endoderm, in the vicinity of the archenteron and within the dorsal crest of the endoderm mass (Subtelny, 1980). The Stage 20 graft thus necessitated an incision through the germ cell-containing region (see Fig. 5). The marked reduction in gonadal germ cells resulting from the Stage 20 donor grafts could be accounted for, at least in part, by the fact that the Stage 20 grafts possessed fewer PGCs than the Stage 17 grafts (compare Fig. 5 with Fig. 6). Experimental evidence consistent with this notion was acquired in the following manner: To obtain some idea of the number of PGCs present in the donor grafts, the endoderm regions corresponding to the graft regions were extirpated from normal animals at Stages 17 and 20. The wounds healed rapidly and the animals were allowed to continue development to Stage 25 at which
time they were anesthetized and gonocyte counts were made. The results are presented in Tables 4 and 5. From the triplicate experiments in Table 4 it can be inferred that the Stage 20 donor grafts possess a maximum of 26% to 60% of the total PGCs present in the endoderm of the Stage 20 larvae. Similar extirpation experiments on normal Stage 17 embryos yielded an estimate of 90% to 97% of the total number of PGCs to be present in the donor graft region (see Table 5). In addition, one must also take into account that the microsurgical operation itself on normal Stage 17 embryos results in a reduction of approximately 20% of the PGCs that colonize the gonadal ridges (see Table 1 and Fig. 11). Based on these considerations, it would appear that the majority of the Stage 20 PGCs possess the ability to undergo delayed migration to the paired gonadal ridges when transferred back into 2-1/2 day younger Stage 17 hosts.

III. Transfers of Normal Stage 17 PGCs into UV-Irradiated Stage 20 Hosts

A. Development of the heterochronic graft larvae and controls

The experiments in the third and final part of this study were conducted to determine whether the
### TABLE 4
Determinations of Gonocyte Numbers in Donor Tadpoles That Had the Germ Cell Graft Region Extirpated at Stage 20

<table>
<thead>
<tr>
<th>Female number</th>
<th>Number of larvae</th>
<th>Germ cells per larva $\bar{x}$ + sem</th>
<th>range</th>
<th>Number of larvae</th>
<th>Germ cells per larva $\bar{x}$ + sem</th>
<th>range</th>
<th>Percent germ cells of control</th>
</tr>
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<tbody>
<tr>
<td>43</td>
<td>19</td>
<td>$34.8 \pm 2.0$</td>
<td>24-58</td>
<td>12</td>
<td>$26.0 \pm 1.7$</td>
<td>15-33</td>
<td>74</td>
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<td>65</td>
<td>19</td>
<td>$67.0 \pm 4.2$</td>
<td>38-97</td>
<td>15</td>
<td>$37.6 \pm 5.2$</td>
<td>5-71</td>
<td>57</td>
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<tr>
<td>75</td>
<td>9</td>
<td>$68.3 \pm 5.7$</td>
<td>41-87</td>
<td>10</td>
<td>$26.0 \pm 5.7$</td>
<td>1-51</td>
<td>39</td>
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</table>
TABLE 5
Determinations of Gonocyte Numbers in Donor Tadpoles That Had the Germ Cell Graft Region Extirpated at Stage 17

<table>
<thead>
<tr>
<th>Female number</th>
<th>Number of larvae</th>
<th>Germ cells per larva $\bar{x} \pm$ sem</th>
<th>Number of larvae</th>
<th>Germ cells per larva $\bar{x} \pm$ sem</th>
<th>Percent germ cells of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>19</td>
<td>34.8 ± 2.0 24-58</td>
<td>5</td>
<td>3.9 ± 1.3 0-9</td>
<td>11</td>
</tr>
<tr>
<td>65</td>
<td>19</td>
<td>67.0 ± 4.2 38-97</td>
<td>5</td>
<td>1.9 ± 0.6 0-12</td>
<td>3</td>
</tr>
<tr>
<td>75</td>
<td>9</td>
<td>68.3 ± 3.7 41-87</td>
<td>9</td>
<td>1.9 ± 0.7 0-4</td>
<td>3</td>
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</table>
intraendodermal PGCs in tailbud embryos could exhibit precocious migration to the gonadal ridges when transplanted into 2-1/2 day older swimming larvae (N Stage 17→UV Stage 20). Figure 8 in the Materials and Methods (Section V) illustrates the appearance of the experimental larvae after the initial grafts were made. The photomicrograph in Figure 16A (compare with Fig. 16B) depicts the heterochronic graft larva at Stage 21, one day following the operation. The graft (arrow) is completely healed and clearly demarcated by its more darkly pigmented epidermis. This distinction persists in the more advanced larva at Stage 23 (see Fig. 16C; compare with Fig. 16D). Thus the rather autonomous development of the graft with respect to the host tissues, which was reported earlier for the N Stage 20→UV Stage 17 heterochronic graft larvae, was again evident, in reciprocal fashion, in the N Stage 17→UV Stage 20 heterochronic graft individuals. At host Stage 25 the 2-1/2 day younger graft could still be easily identified in the posterior one-fourth to one-half of the ventral abdomen (Figs. 17A, C; arrows; compare with Fig. 17B) as a more heavily pigmented and opaque region, clearly indicating the less advanced state of differentiation of the transplanted epidermis. In addition, when the larvae were sacrificed and the abdominal wall removed, the digestive tracts were readily available for more detailed examination under the dissecting
Figure 16. Photomicrographs showing development of a N Stage 17→UV Stage 20 heterochronic graft larva (left) and unoperated control (right). (Scale bar represents 1 mm.)

A. Lateral view of heterochronic graft larva at the host Stage 21 (arrow). Note darkly pigmented epidermis delineating healed graft. The graft tissues are completely incorporated into host by this time, although as is evident from the epidermis, the degree of differentiation is less advanced in the graft. Otherwise, this morphology is typical of a Stage 21 larva.

B. Lateral view of a normal Stage 21 unoperated control larva. The PGCs at this stage are located within the dorsal crest of the endoderm and will emerge within the next 24 hours.

C. Lateral view of heterochronic graft larva at host Stage 23. Graft region still contains dark embryonic pigment in contrast to the light pigmentation present in the more differentiated epidermis of the host and unoperated control (Fig. D).

D. Lateral view of Stage 23 unoperated control larva. By this stage PGC migration to a medial gonadal ridge is completed.
Figure 17. Photomicrographs depicting epidermal differentiation in N Stage 17→UV Stage 20 heterochronic graft larva (host Stage 25) and control (Stage 25). (Scale bar represents 1 mm.)

A. Lateral view of a normal unoperated control (upper) and a heterochronic graft larva (lower). The N Stage 17→UV Stage 20 graft larva exhibits a slightly darker pigmentation in the posterior ventral abdominal region (arrow) in comparison with the unoperated control.

B. Ventral view of the normal unoperated control larva. Note the relatively transparent abdominal wall which reveals the coiled gut within it.

C. Ventral view of the heterochronic graft larva more clearly reveals the heavily pigmented and more opaque region of the graft in the posterior region of the abdomen (arrow). Note that the graft region obscures the view of the intestine within the caudal portion of the abdominal wall.
microscope. In the heterochronic graft larvae they were never as coiled as in the controls (compare Figs. 18C with Figs. 18A, B). The integrated graft portion of the gut was also more yolky in appearance than that of the host tissue. In addition to this retarded differentiation of the graft portions of the ectodermal and endodermal derivatives, the delayed separation of the lateral plate mesoderm into the splanchnopleure and somatopleure (with the formation of the coelom) was verified in transverse sections of individuals fixed at successive stages of development (Subtelny and Penkala, 1984).

After removal of the viscera, the large yolky germ cells in the paired gonadal ridges stand out prominently, as shown in Figure 19. Notice the total absence of any identifiable PGCs in the sibling UV-irradiated control (Fig. 19D) and the abundance of germ cells in the paired gonadal ridges of the unoperated control sibling (Fig. 19A). The germ cell number and distribution in the gonadal ridges of the N Stage 17→UV Stage 17 isochronic graft larva (Fig. 19B) is similar to that of the unoperated control, whereas very few gonocytes are encountered in the N Stage 17→UV Stage 20 heterochronic graft larvae. An example of the latter is shown in Figure 19C, where one gonocyte is in the right gonadal ridge and three are in the left gonadal ridge.
Figure 18. Photomicrographs showing gut morphology in the heterochronic graft larva (N Stage 17→UV Stage 20) at host Stage 25 and its controls. The larvae have been sacrificed and the ventral abdominal wall removed to expose the digestive tract. (Scale bar represents 1 mm.)

A. Ventral view of normal unoperated control showing the digestive tract. Note typical intestinal coiling.

B. Ventral view of a N Stage 20→UV Stage 20 isochronic graft larva. The digestive tract is nearly identical to that of the normal unoperated control.

C. Ventral view of a N Stage 17→UV Stage 20 heterochronic graft larva. The digestive tract is continuous, but poorly coiled. Under the dissecting microscope it is readily apparent that the graft portion of the intestine contains more yolk than the control counterparts, indicating its retarded state of differentiation with respect to the host. It appears as a late Stage 23 or early Stage 24 gut.
Figure 19. Gonadal ridges of a N Stage 17→UV Stage 20 heterochronic graft larva and control at host Stage 25. (Scale bar represents 1 mm.)

A. Gonadal ridges of a normal unoperated control containing a full complement of gonocytes.

B. Gonadal ridges of a N Stage 17→UV Stage 17 isochronic graft larva. Note the presence of a more or less continuous cord of PGCs in each of the pair of gonadal ridges, similar to that in the unoperated controls. On the average, this gonocyte population represents approximately 59-100% of the unoperated control number.

C. Gonodal ridges of a N Stage 17→UV Stage 20 heterochronic graft larva. Four germ cells are detectable within the transparent gonadal epithelium. On the average, this type of heterochronic graft larva contained 2% of the unoperated control number of gonocytes.

D. Gonodal ridges of an unoperated UV-irradiated control larva. No gonocytes are present.
B. Comparison of gonocyte numbers in heterochronic graft larvae and controls.

Two series of experiments involving transfers of normal Stage 17 PGCs into UV-irradiated Stage 20 larvae (N Stage 17 → UV Stage 20) were performed with dilute anesthetic in the operating medium, since it was necessary to immobilize the hosts during the graft procedure. The first series consisted of five experiments and the gonocyte counts in the N Stage 17→UV Stage 20 heterochronic graft tadpoles, the N Stage 17→UV Stage 17 isochronic graft larvae, and the unoperated control siblings at Stage 25 are presented in Table 6. From a total of 58 Stage 17 isochronic germ cell transfers into irradiated hosts at the same stage of development, the gonadal ridges of all the N Stage 17→UV Stage 17 graft tadpoles possessed PGCs, whereas 131 of 132 (99%) of the UV-irradiated controls for these experiments were sterile (compare Fig. 19B with 19D). The gonadal germ cell counts in the isochronic graft larvae varied between 57% and 100% of the gonocyte numbers in the unoperated controls in the respective experiments. An identical range in PGC numbers (60% to 100% of the unoperated controls) were obtained with the same type of Stage 17 isochronic grafts in another experimental series in which the operations were performed without anesthetic in the medium (see Table 2). Thus the overall reduction in gonadal PGC numbers in the
### TABLE 6
Gonadal Germ Cell Number in Heterochronic Graft Larvae (N Stage 17 → UV Stage 20)

I. Comparison with Isochronic Graft Larvae (N Stage 17 → UV Stage 17)

<table>
<thead>
<tr>
<th>Female Number</th>
<th>UNOPERATED CONTROLS</th>
<th>HETEROCHRONIC GRAFT LARVAE: N STAGE 17 → UV STAGE 20</th>
<th>ISOCHRONIC GRAFT LARVAE: N STAGE 17 → UV STAGE 17</th>
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<tr>
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<td>Number of larvae</td>
<td>Germ cells per larva</td>
<td>Number of larvae with germ cells</td>
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<td></td>
<td></td>
<td>( X \pm \text{sem} ) range</td>
<td>( X \pm \text{sem} ) range</td>
</tr>
<tr>
<td>40</td>
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<td>42.2 ± 4.0 25-74</td>
<td>6</td>
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<td>43</td>
<td>19</td>
<td>34.8 ± 2.0 24-58</td>
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<td>57</td>
<td>10</td>
<td>44.3 ± 3.3 31-68</td>
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<td>56.6 ± 4.8 19-104</td>
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<td>39.8 ± 2.4 23-66</td>
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<td>13</td>
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</table>

\* The mean number of gonadal germ cells was based on the total number of larvae with germ cells.
graft larvae (approximately 25% of the unoperated controls) can not be ascribed to the addition of anesthetic to the operating medium. Moreover, the results are similar to those obtained with Stage 17 germ cell transplants made by extirpating and immediately reinserting the graft region back into the same normal, nonirradiated embryos (see Table 1). Consequently the reduction in gonocyte numbers in the isochronic Stage 17 graft tadpoles can be ascribed to the microsurgical operation itself rather than to grafts made into UV-irradiated host embryos.

When Stage 17 PGCs were transferred to irradiated Stage 20 hosts there was a drastic reduction in gonadal germ cells in the heterochronic graft larvae relative to the unoperated controls (Table 6). This reduction was evident in two ways: (1) Overall, only 37% of the heterochronic graft larvae at Stage 25 possessed PGCs. In two experiments none of the graft larvae had detectable large, yolky gonocytes whereas in three experiments 45% to 70% of the graft larvae possessed PGCs. (2) Among the latter larvae, the gonocytes were limited in numbers (compare Figs. 19A and 19C). The mean number of gonadal germ cells varied between 2% and 19% of those present in the unoperated controls in the respective experiments. Yet the donor grafts apparently possessed virtually all of the PGCs (approximately 90% to 97%) present in the Stage 17 endoderm that could potentially
migrate to the gonadal ridges (see Table 5).

To determine whether germ cell transfers into Stage 20 hosts as opposed to Stage 17 hosts could account for the restricted numbers of Stage 17 graft PGCs that colonize the gonadal ridges, a second series of heterochronic graft experiments was done and the results compared with isochronic Stage 20 germ cell grafts made into irradiated hosts at the same stage of development. The results in Table 7 disclose that 20% to 50% of the heterochronic graft tadpoles at Stage 25 possessed gonocytes, and of these, the gonadal ridges contained between 3% and 17% of the PGCs scored in the unoperated controls in the respective experiments. Between 9 and 20 irradiated controls were reared in each of the 6 experiments. Of the 83 UV-irradiated controls for these experiments, 82 (99%) were found to lack gonocytes. By contrast, 53% to 100% of the Stage 20 isochronic graft larvae possessed PGCs, and of these, the gonadal ridges contained between 14% and 34% of the PGC numbers in the unoperated controls in the respective experiments. In general, these values could be approximately doubled since the Stage 20 donor grafts were estimated to contain about half of the total number of PGCs in the endoderm mass of the hatched larvae (see Table 4). On the other hand, Stage 17 donor grafts were estimated to possess nearly all of the PGCs in the endoderm mass of the tailbud embryos (Table 5). The results suggest that
| Female Number | Number of larvae | Germ cells per larva | | | Number of larvae | Number with germ cells | Germ cells per larva | | | Number of larvae | Number with germ cells | Germ cells per larva |
|---------------|-----------------|---------------------|---|---|------------------|---------------------|---------------------|---|---|------------------|---------------------|
| 63            | 19              | 67.0 ± 4.3          | 39-97 | 13 | 2.0 ± 0.6         | 1-3                 | 20 | 16 | 14.1 ± 2.9       | 5-36                 |
| 67            | 10              | 69.8 ± 3.8          | 48-81 | 10 | 11.3 ± 4.0        | 5-18                | 10 | 10 | 22.3 ± 4.5       | 6-30                 |
| 68            | 15              | 50.7 ± 3.7          | 30-70 | 15 | 5.8 ± 1.3         | 2-11                | 15 | 14 | 17.1 ± 2.7       | 6-43                 |
| 69            | 12              | 60.8 ± 3.5          | 38-81 | 15 | 2.0 ± 1.0         | 1-3                 | 15 | 13 | 19.2 ± 4.4       | 3-60                 |
| 70            | 17              | 51.1 ± 3.7          | 29-70 | 13 | 1.5 ± 0.3         | 1-2                 | 15 | 8  | 7.0 ± 1.3        | 1-13                 |
| 75            | 9               | 68.3 ± 3.7          | 41-47 | 9  | 4.0 ± 0.0         | 4                   | 9  | 7  | 17.4 ± 4.0       | 1-27                 |

a The mean number of gonadal germ cells was based on the total number of larvae with germ cells.
although grafts made into Stage 20 hosts do result in a considerable reduction in the numbers of PGCs that colonize the gonadal ridges, the Stage 20 hosts do not entirely account for the limited success in PGC transfer resulting from the donor Stage 17 heterografts. The latter seems to be related, at least in part, in some manner to the heterografts made between individuals at the two different stages of development rather than exclusively to the operation being performed in the Stage 20 hosts per se.

C. Comparison of PGC counts between Stage 25 and post-Stage 25 heterochronic graft larvae and controls

The above data reveal that donor PGCs can precociously populate the host gonadal ridges in the N Stage 17->UV Stage 20 heterochronic graft larvae, albeit in restricted numbers. Since the PGCs were transplanted into 2-1/2 day older larvae, an attempt was made to determine whether additional graft PGCs would colonize the host gonadal ridges 2 days later in development. To this end, a comparison of gonocyte numbers was made between heterochronic graft larvae and controls at Stage 25 and at 2 days after Stage 25. The results are presented in Tables 8 and 9. The data in Table 8 reveal that neither the mean nor the range in gonadal germ cell number differs significantly in the unoperated controls
### TABLE 8
Gonadal Germ Cell Counts in Tadpoles at Stage 25 and 2 Days After Stage 25 (at 18°C)a

I. Unoperated Control Larvae

<table>
<thead>
<tr>
<th>Female number</th>
<th>Number of larvae</th>
<th>Germ cells per larva</th>
<th>Number of larvae</th>
<th>Germ cells per larva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \bar{x} \pm \text{sem} )</td>
<td></td>
<td>( \bar{x} \pm \text{sem} )</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>42.2 ± 4.0</td>
<td>10</td>
<td>44.3 ± 5.4</td>
</tr>
<tr>
<td>49</td>
<td>17</td>
<td>25.4 ± 3.6</td>
<td>15</td>
<td>29.1 ± 3.2</td>
</tr>
<tr>
<td>67</td>
<td>10</td>
<td>64.8 ± 3.8</td>
<td>15</td>
<td>60.8 ± 2.4</td>
</tr>
<tr>
<td>68</td>
<td>15</td>
<td>51.2 ± 3.6</td>
<td>15</td>
<td>57.5 ± 4.3</td>
</tr>
<tr>
<td>69</td>
<td>12</td>
<td>60.8 ± 3.5</td>
<td>15</td>
<td>58.0 ± 4.1</td>
</tr>
<tr>
<td>75</td>
<td>9</td>
<td>68.3 ± 5.7</td>
<td>7</td>
<td>70.0 ± 4.4</td>
</tr>
</tbody>
</table>

\( \bar{x} \pm \text{sem} \) range: 27-62, 6-60, 48-81, 30-70, 38-81, 41-87, 25-74, 13-49, 48-81, 33-84, 30-83, 55-87

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a UV-irradiated controls were also autopsied and examined for gonadal germ cells in each of the six experiments at the two respective stages of development. All 73 Stage 25 irradiated larvae examined were sterile and of 79 post-Stage 25 tadpoles examined, 100\% completely lacked identifiable gonadal germ cells.
TABLE 9
Gonadal Germ Cell Counts in Tadpoles at Stage 25 and 2 Days After Stage 25 (at 18°C)

II. Heterochronic Graft Larvae

<table>
<thead>
<tr>
<th>Female Number</th>
<th>Number of larvae</th>
<th>Number with germ cells</th>
<th>Germ cells per larva $\bar{x} \pm$ sem$^a$</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>6</td>
<td>3</td>
<td>1.0 $\pm$ 0.0</td>
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<tr>
<td>49</td>
<td>12</td>
<td>1</td>
<td>2.0 $\pm$ 0.0</td>
<td>2</td>
</tr>
<tr>
<td>67</td>
<td>10</td>
<td>3</td>
<td>11.3 $\pm$ 4.4</td>
<td>3-18</td>
</tr>
<tr>
<td>68</td>
<td>15</td>
<td>8</td>
<td>5.8 $\pm$ 1.3</td>
<td>2-11</td>
</tr>
<tr>
<td>69</td>
<td>15</td>
<td>3</td>
<td>1.7 $\pm$ 0.7</td>
<td>1-3</td>
</tr>
<tr>
<td>75</td>
<td>9</td>
<td>2</td>
<td>4.0 $\pm$ 0.0</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of larvae</th>
<th>Number with germ cells</th>
<th>Germ cells per larva $\bar{x} \pm$ sem$^a$</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>19.3 $\pm$ 3.1</td>
<td>4-27</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>5.2 $\pm$ 2.5</td>
<td>1-15</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>1.0 $\pm$ 0.0</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3.7 $\pm$ 0.9</td>
<td>2-5</td>
</tr>
</tbody>
</table>

$^a$ The mean number of gonadal germ cells was based on the total number of larvae with germ cells.
at the two stages of development. This has been verified by a two-way nonparametric statistical analysis. This also confirms the results obtained earlier in other experiments, which show that gonocyte numbers in normal developing tadpoles remain essentially stable between Stage 24 and up to 2 days after Stage 25 (at 18°C) before they undergo an extensive proliferation (Subtelny, unpublished). Moreover, in each of the corresponding experiments listed in Table 8, between 9 and 15 UV-irradiated controls were examined for the presence of gonadal germ cells. Among 73 irradiated controls examined by autopsy at Stage 25, none were found to possess gonocytes. Similarly, the 79 irradiated controls examined 2 days after Stage 25 also completely lacked gonadal germ cells. The data confirm the view that there is no significant contribution of host PGCs to the gonadal germ cell counts made on tadpoles at Stage 25 and at 2 days after Stage 25 (at 18°C).

The overall data in Table 9 reveal that an equivalent proportion of post-Stage 25 heterochronic graft tadpoles (78%) still completely lack PGCs relative to the Stage 25 larvae (70%). Thus there is clearly no additional germ cell colonization of the gonadal ridges among the vast majority of heterochronic graft animals between the two stages of development. Based on the total number of larvae, or on the number of experimental larvae that do possess gonocytes, two-way nonparametric statistical analysis indicates no
significant difference in PGC numbers among the individuals at the two stages of development. In only one experiment (female no. 67) is there a statistically significant difference (p<0.01) in the number of gonocytes between the post-Stage 25 tadpoles and the Stage 25 larvae. However, it should be pointed out that it is not possible to distinguish by germ cell counts alone whether this apparent increase in gonocyte number reflects a delayed population of the gonadal ridges or an onset of proliferation of gonocytes already present in the gonadal ridges since Stage 25. Results obtained in other experiments reveal that mean gonocyte numbers in unoperated, normal developing tadpoles remain essentially stable between Stage 24 and up to 2 days after Stage 25 (at 18°C). The gonocytes then enter a proliferation cycle within one week after Stage 25. The onset of the proliferation cycle is not simultaneous among individuals derived from a given clutch of eggs, but random (Subtelny, 1980, and unpublished). Thus the apparent increase in gonocytes in the few post-Stage 25 individuals in experiment no. 67 could possibly have resulted from a random onset of proliferation of gonadal germ cells. In any event, the overall data provide compelling evidence that there is no additional colonization of graft PGCs in the host gonadal ridges between Stage 25 and 2 days after Stage 25 (at 18°C).
DISCUSSION

I. Experimentally Induced Delayed PGC Migration to the Gonadal Ridges

The overall experimental results (Tables 2 and 3) clearly reveal that intraendodermal PGCs transplanted from swimming larvae (Stage 20) into 2-1/2 day younger tailbud embryos (Stage 17) can eventually colonize the gonadal primordia. Histological studies on similar heterochronic graft larvae disclose that the experimentally induced delayed migration results from a retention of the graft PGCs within the endoderm. The PGCs do not emerge from the endoderm until just before dorsal mesentery formation in the host larva; i.e., at the same stage and just as it occurs during normal development (Subtelny and Penkala, 1984). Thus we conclude that intraendodermal PGCs held in abeyance within the endoderm for at least 2-1/2 additional days can belatedly populate the gonadal ridges. Evidently PGCs are still capable of occupying the gonadal primordia when retained within the endoderm for 3-1/2 days. In two experiments, Stage 20 PGCs were transferred to Stage 16 and Stage 14 irradiated hosts. The heterochronic graft larvae possessed 30% and 39% of the gonadal germ cells present in the unoperated controls in the respective experiments.

The irradiated host animals are unlikely to contribute
in any significant way to the gonadal germ cells scored in the Stage 25 graft larvae. In the first place, 99% of the irradiated controls were found to completely lack gonocytes, whereas 100% of the isochronic Stage 17 graft larvae and 75% to 100% of the heterochronic graft larvae possessed gonocytes in the respective experiments (Tables 2 and 3). Secondly, aside from the successful eradication of the PGCs by UV-light, the region extirpated in the Stage 17 hosts to accommodate the germ cell grafts very likely eliminated at least 90% to 100% of the PGCs in the endoderm mass that could potentially migrate to the gonadal ridge (see Table 5).

Kamimura et al. (1976) reported that there is no increase in PGC numbers in *Xenopus* larvae between Stage 33/34 and the time when they are in the gonadal ridges. According to Dixon (1981), PGCs do not incorporate 3H-thymidine between the corresponding stages of development, and the final intraendodermal mitosis takes place at Stages 34 to 37, which would correspond to about Stage 20 in *Rana pipiens*. Similar studies have not been done with *Rana*. However, the present studies provide no clear evidence for the replication of graft PGCs during their residence within the endoderm mass. About the same proportion of PGCs (between 10% and 32% of the unoperated controls) attain the gonadal ridges in the N Stage 20→UV Stage 17 heterochronic
graft larvae as in the N Stage 20-→UV Stage 20 isochronic graft larvae. Although the values seem rather low, it must be noted that the Stage 20 donor grafts apparently possess only 26% to 61% of the total number of PGCs present in the Stage 20 endoderm mass that could potentially migrate to the gonadal ridges. Taking into consideration that the microsurgery alone interferes with intraendodermal PGC migration to varying degrees, the data suggests that probably the majority of the heterochronic graft PGCs can undergo a delayed migration to the gonadal primordia.

II. Effects of UV-Radiation on Development

Much debate continues in the literature as to whether UV-radiation specifically affects PGC development or embryogenesis in general, such that germ cell migration is retarded or incomplete (Smith and Williams, 1979; Dixon, 1981; Thomas et al., 1983). Histological examinations of germ cell graft embryos and irradiated controls do reveal that often irradiated embryos from many, although not necessarily all clutches of eggs, may be retarded as much as 6 to 12 hours at the time the unoperated controls attain the feeding stage. In all other respects, however (aside from sterility in the irradiated controls), the embryos develop normally through larval stages and attain metamorphosis at the same time as the unoperated controls.
(Subtelny, data not presented). In the present experiments, the data in Figure 11 disclose that when microsurgery is performed on normal Stage 17 embryos, they possess an overall mean of 78% of the gonocyte numbers present in the unoperated controls at Stage 25. Interestingly, normal Stage 17 isochronic germ cell grafts made into irradiated Stage 17 hosts yield about the same overall proportion of gonadal germ cells (Tables 2 and 6; 73%), since the donor graft region apparently includes nearly all of the PGCs in the Stage 17 endoderm mass (Table 5). These results indicate that the same proportions of graft PGCs migrate to the gonadal ridges whether the transplants are made into normal embryos or into irradiated embryos. Thus the data support the earlier conclusions derived from parabiosis experiments between normal donor embryos and irradiated host embryos at Stage 17; i.e., that UV-radiation primarily affects PGC development rather than the endodermal environment through which they migrate (Subtelny, 1980).

III. Experimentally Induced Precocious PGC Migration to the Gonadal Ridges

Heretofore there has been very little experimental evidence to indicate that intraendodermal PGCs can undergo an earlier than normal migration to the gonadal ridges. In
1970, Gipouloux reported experiments in which he removed the entire endoderm mass from normal early neurulae and, keeping its original orientation, implanted it into the body cavity of feeding tadpoles. Two days after the operation, PGCs were histologically identified in the periphery of the graft where the latter abutted the dorsal mesodermal organs of the host. This location of the PGCs suggested to Gipouloux that they had undergone a precocious intraendodermal migration in the lateral direction, presumably directed by a chemo-attractant originating from the host dorsal mesodermal organs. This intraendodermal translocation would have normally taken 3-1/2 days if the graft had been left intact in the donor embryo. However, the PGCs were never observed to emerge from the endoderm graft.

We have presented evidence that intraendodermal PGCs from Stage 17 tailbud embryos transplanted into Stage 20 hatched larvae can populate the host gonadal ridges 2-1/2 days earlier than they normally would if left intact within the donor embryo. The germ-line cells are present in the genital ridges of the heterochronic graft tadpoles at a time when they would normally be situated in the archenteron wall of the endoderm and just in the process of separating from it. Between 20% and 70% of the heterochronic graft larvae (in 9 of 11 experiments) and all the isochronic Stage 17 graft controls possessed gonadal germ cells, whereas 99% of the UV-irradiated controls were sterile and completely
devoid of gonocytes. Thus, it is very unlikely that the PGCs in the irradiated hosts could have contributed in any significant way to the gonadal germ cells scored in the graft larvae. Moreover, histological studies further support the experimental findings since PGCs have been observed to emerge from the endoderm within 2 days after transplantation into the irradiated heterochronic graft larvae, albeit in very limited numbers and in relatively few individuals. By contrast, no PGCs have been observed in the irradiated controls at corresponding stages of development (Subtelny and Penkala, 1984). From these results we conclude that it is possible to circumvent the major period of intraendodermal germ cell migration and that a small, but significant proportion of the transplanted PGCs undergo precocious migration to the gonadal ridges.

A puzzling aspect is why so few of the normal transplanted PGCs occupy the paired gonadal ridges in the heterochronic graft larvae. There is no evidence that additional graft PGCs colonize the host gonads two days later in the heterochronic graft larvae (Tables 8 and 9). A possible explanation seems to be provided by a detailed consideration of the mechanisms involved in the emergence of the PGCs from the endoderm in anurans.
IV. On the Mechanisms of Separation of the PGCs from the Endoderm in Anurans

From an extensive series of extirpation and grafting experiments, Gipouloux (1970) concludes that the initiation and dorsally-directed active migration of the PGCs out of the endoderm mass are in response to a chemotactic gradient emanating from the dorsal axial mesodermal structures. Moreover, Gipouloux and coworkers (Gipouloux et al., 1979; Delbos et al., 1980; Rais et al., 1981) propose that the chemotactic substance is cAMP, and they suggest that this signal is produced notably by the chordamesoderm during the entire period of intraendodermal PGC migration, but then ceases with the exiting of the germ cells from the endoderm mass.

On the other hand, there is some experimental evidence to suggest that morphogenesis of the dorsal mesentery does play a role in the separation of the PGCs from the endoderm. Sabbadin (1959) removed the lateral plate mesoderm region together with overlying ectoderm from the **Discoglossus** tailbud embryo. This piece was inverted by rotating it 180° about the dorsal-ventral axis and transplanted to the opposite side of the same embryo. In unilateral and bilateral transplants of this type, the mesentery tended to form towards the midline in the ventral site where several PGCs were identified in association with it. Sabbadin
concluded from this that the PGCs must have undergone a passive displacement determined by the directed movements of the lateral plate mesoderm during the formation of the mesentery. In another type of experiment, Giorgi (1974) transplanted the caudal portion of the dorsal axial structures to the ventral abdominal region of *Bufo* tailbud embryos. PGCs emerged from the endoderm in this ventral site to occupy the gonadal ridges of the graft. The vast majority of the PGCs (83%) that associated with the caudal grafts were present at the same level where a well-developed mesentery was also present. Thus Giorgi suggested that the formation of the dorsal mesentery played a morphogenetic role in the separation of the PGCs from the endoderm. However, in both of the aforementioned experiments dorsal axial mesodermal tissues (postulated by Gipouloux to release a chemotactic stimulus) were also present in the graft tissues. Thus the results are equivocal. It is difficult to ascertain with assurance to what extent chemotaxis, morphogenesis of the dorsal mesentery, or both, play a role in the emergence of the PGCs from the endoderm.

Recently, Subtelny and Penkala (1984) examined the process of germ cell emergence from the endoderm in *Rana pipiens*, utilizing reciprocal grafts of germ cell-containing endoderm regions between embryos at various stages of development. In such heterochronic graft larvae, PGC
migration and dorsal mesentery formation were temporally disengaged. The graft tissues differentiated in accordance with their own developmental sequence after transplantation to the host. Nevertheless when normal Stage 20 germ cell grafts were transplanted into irradiated Stage 17 hosts, the graft PGCs remained within the endoderm at least an additional 2-1/2 days and did not emerge from it until host Stage 22; i.e., immediately before the formation of the dorsal mesentery rudiment, just as it occurs in normal development. By contrast, when normal Stage 17 PGCs (as well as isochronic grafts of Stage 20 PGCs) were transplanted into irradiated Stage 20 hosts, the graft PGCs emerged from the endoderm within 2 days after transplantation, precisely when the dorsal mesentery formed in the Stage 22 hosts. No additional PGCs have been found to exit from the endoderm mass after the formation and elongation of the definitive mesentery, upon retraction of the gut from the dorsal body wall. On the other hand, germ-line cells could be identified left behind and still present in the wall of the intestines in the N Stage 17->UV Stage 20 heterochronic graft larvae several weeks after migration to the gonadal ridges would have normally terminated (Subtelny and Vogel, 1983, and unpublished results). Similar findings with normal developing larvae have been reported by other investigators (Humphrey, 1925; Cheng, 1932; Blackler, 1958). These observations disclose that the separation of PGCs from
the endoderm is dependent upon factors extrinsic to the germ cells themselves. In both types of heterochronic graft larvae the germ cells come out of the endoderm concomitant with the formation of the dorsal mesentery in the Stage 22 host. The results suggest that morphogenetic movements involved with the formation of the dorsal mesentery in some manner play a major role in the emergence of the PGCs from the endoderm mass. For if chemotaxis was solely involved, one might expect the transplanted PGCs in the reciprocal heterochronic graft larvae to emerge from the endoderm at developmental stages earlier than, and later than that limited to the morphogenesis of the dorsal mesentery, but they do not. What is the precise role of the latter in the mechanism of germ cell migration in anurans? It has been proposed that PGCs included in the uppermost portion of the dorsal endoderm crest are "pinched off" from the endoderm mass by the infolding of the lateral coelomic folds (Humphrey, 1925; Cheng, 1932; Blackler, 1958; Vannini and Giorgi, 1969). However, there is reason to believe that this morphogenetic event encompasses much more than a simple mechanical process. For example, PGCs have been observed to emerge from the endoderm under experimental conditions in which the lateral coelomic folds fail to approximate each other in the midline, as it does during normal embryogenesis (Subtelny, unpublished).
In 1925, Humphrey referred to the penetration of mesenchyme cells into the upper portion of the endoderm crest just prior to the infolding of the lateral coelomic mesoderm to form the dorsal mesentery rudiment. These same observations were earlier illustrated by Kuschakewitsch (1908). However, no particular significance was attributed to this morphogenetic event by either author. Based on current observations, this event is most clearly observed in UV-irradiated larvae (Subtelny and Penkala, 1984; see Figs. 3E and 4C). This population of individual mesenchyme cells evidently derives from the lateral plate mesoderm. They migrate over the roof of the archenteron and they become intimately associated with the closely grouped PGCs in this dorsalmost portion of the endoderm crest (Subtelny and Penkala, 1984; see Figs. 3C, D, F). These mesenchyme cells also appear to remain associated with the closely aggregated PGCs during their translocation to the median germ ridge, and subsequently to the bilateral gonadal primordia. Both King (1908) and Cheng (1932) have noted their presence in association with the closely-grouped PGCs in the median germ ridge and in the paired gonadal primordia, but not at the earlier intraendodermal stage of development. We have proposed that this germ cell-mesenchyme cell interaction may be of direct significance in the initiation of the emergence of the closely grouped PGCs from the endoderm crest and in their migration to the paired gonadal ridges (Subtelny
and Penkala, 1984). In this vein, it is of interest that Hamaguchi (1982) recently reported the ultrastructural observations on PGC migration in Oryzias latipes. In this species, the surfaces of closely-associated PGCs were entirely surrounded by mesenchyme cells and their fine cytoplasmic extensions during the displacement of the germ-line cells from the somatopleure to the gonadal primordia.

Returning to the original question posed earlier as to why so few PGCs in the N Stage 17→UV Stage 20 heterochronic graft larvae attain the paired gonadal ridges, the simplest explanation seems to be the following: There is an increasing gradient in the number of PGCs distributed within the endoderm mass of the Stage 17 tailbud embryo in the dorsal to ventral direction (Subtelny, 1980). Thus only the few, more dorsally situated PGCs in the Stage 17 graft probably attain the tip of the endoderm crest before the formation of the host mesentery rudiment. Subsequently, morphogenesis of the elongated, definitive mesentery and/or the cessation of a chemotactic stimulus from the dorsal axial mesodermal structures would preclude further emergence of the PGCs from the endoderm.
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