THE SPATIAL DISTRIBUTION OF TRANSCRIPTS OF THE SEGMENTATION GENE *FUSHI TARAZU* DURING *COELOPA* EMBRYONIC DEVELOPMENT

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BY

JOSEPH OSEI-FRIMPONG, B. S., M. S.

DENTON, TEXAS

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DENTON, TEXAS

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To the Dean for Graduate Studies and Research:

I am submitting herewith a dissertation written by Joseph Osei-Frimpong entitled "The Spatial Distribution of Transcripts of the Segmentation Gene *Fushi Tarazu* During *Coelopa* Embryonic Development." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Molecular Biology.

T. Schuler

Major Professor

We have read this dissertation

and recommend its acceptance:

Michael J. Kurdick

Linda Elphouse

John E. Kneeskern

Allan Eckstine

T. Schuler

Department Chairperson

R E Collier

Dean of College

Accepted

Leslie M Thompson

Dean for Graduate Studies and Research

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ABSTRACT

THE SPATIAL DISTRIBUTION OF TRANSCRIPTS OF THE SEGMENTATION GENE *FUSHI TARAZU* DURING *COELOPA* EMBRYONIC DEVELOPMENT

JOSEPH OSEI-FRIMPONG

AUGUST, 1988

The expression of the *Drosophila fushi tarazu (ftz)* gene was investigated in the kelp fly, *Coelopa frigida* . The investigation included the detection of the gene in the genome of the kelp fly and the detection of the transcripts of the gene in unfertilized eggs and embryos. The spatial distribution of *ftz* $^{+}$ transcripts during normal *Coelopa* embryonic development was investigated by *in situ* hybridization.

The results obtained in this study have led to the following conclusions:

1. The genome of the kelp fly, *Coelopa frigida* , contains sequences homologous to the *Drosophila ftz* $^{+}$ gene.
2. The *ftz* $^{+}$ gene is probably not expressed maternally in the kelp fly.
3. In the *Coelopa* embryo the *ftz* $^{+}$ transcripts are generally distributed during early cleavage; at these stages the transcripts are of low intensity and there seem to be no regional differences.
4. *Ftz* $^{+}$ transcripts become localized around the periphery of the embryo around the 11th nuclear division (syncytial blastoderm); transcripts after the 12th nuclear division are generally restricted to a broad

region between approximately 10 and 70% of the egg length.

5. After about the 13th nuclear division when cell membranes begin to form and even before cells are formed, the parasegmental pattern of *ftz* $^{+}$ transcript distribution in the seven odd-numbered parasegments becomes more apparent.
6. It appears that by the time segmentation is first obvious and the germ band is fully extended (about 4.5 h of development) the *ftz* $^{+}$ gene is no longer expressed.
7. Similar results have been observed in *Drosophila* wild type embryos. Therefore the embryos used in these experiments were expressing the same dipteran gene at the corresponding morphological stages of development.

Some general conclusions that can be drawn from this study are the following:

1. The data obtained on *ftz* expression graphically illustrate that spatially restricted gene expression can occur even at the syncytial blastoderm stage in the absence of cell membranes.
2. They confirm that a structural association between nuclei and their adjacent cytoplasm exists at this stage.
3. These observations provide direct molecular corroboration for the notion that differences in developmental potential already exist between nuclei prior to the cellular blastoderm completion.

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CHAPTER 1

INTRODUCTION

Developmental biology is the study of the complex morphological and biochemical changes that occur in the life cycle of an organism. All these changes result from the synthesis and expression of gene products during the life cycle of the organism. Almost every multicellular organism is a clone of cells descended from a single original cell, the fertilized egg. Thus the cells of the body , as a rule, are all genetically alike. But phenotypically they are different; some are specialized as muscle, others as neurons, others as blood cells, and so on. The different cell types are arranged in a precisely organised pattern, and the whole structure has a well defined shape. All these features are determined by the genome, which is reproduced in every cell. Each cell must act on the same genetic instructions, but it must interpret them with due regard to time and circumstance, so as to play its proper part in the multicellular society.

Within a day of egg fertilization, insect embryos may comprise many morphologically distinct tissues and organ primordia, the arrangement of which remain essentially unchanged throughout all subsequent development. In each region of the embryo, cells initiate distinct programs of gene expression as they embark on diverse pathways of differentiation, the basic body plan of the embryo being laid down from earliest tissues. As yet, however, almost nothing is known about the molecular mechanisms that underlie regional differentiation in cell fate within the early embryo.

Classical theories suggest that the spatial pattern of early cell differentiation reflects the distribution of cytoplasmic factors or determinants inherited by the blastomeres and that tissue differentiation appears to be mediated by cytoplasmic determinants (see Davidson,1986, for review). These cytoplasmic factors, localized in the egg or cleaving embryos, are selectively distributed to particular embryonic cell lineages where they are thought to direct specific developmental programs. Although the molecular constitution of egg cytoplasmic determinants and their mode of action are largely unknown, informational molecules such as messenger RNA (mRNA) and proteins appear to be likely candidates (see Jeffery, 1983, for review). However, few of these molecules have been demonstrated and none has been purified. The possible role of maternal mRNA in cytoplasmic determination has motivated a search for localized mRNA molecules in a number of different kinds of eggs.

Localization of RNAs by *In Situ* Hybridization

Recent success in molecular cloning of a variety of genetic loci from the *Drosophila* genome has created a demand for methods that permit precise determination of the spatial and temporal limits of gene expression. Perhaps the most direct method for identifying the specific cells of an organism that express a given gene is to localise, by *in situ* hybridization, the RNAs that are specified by the gene within serial sections. Hybridization probes that are used for such analyses are derived from cloned DNA segments which contain coding seqences for the RNA of interest.

The most commonly used technique employs the hybridization of nick translated DNA probes to sections of frozen tissues (Hafen and Levine 1986). The use of optimal conditions for fixation of tissues, radiolabelling of probe and hybridization permits the detection of as few as 10-100 RNA copies in a cell of average dimension (Hafen et al.1983).

Early Development in Flies.

The insect embryo has proved over the years to be a very useful tool in the study of the processes underlying development, becausem embryonic development lasts only a few hours, rather than days or months, as in mammals. Rearing and maintenance of insects require minimal effort compared to those of other animals. In addition, because of the special opportunities some insects offer for genetic analysis, their investigation has provided some key insights into the fundamental problem of how genes specify the differences between cells in different parts of the body. From the study of a number of mutants in insects, different distinct loci of developmental control genes have been identified, and this has greatly facilitated the study of morphogenetic functions of these genes.

Following fertilization, fly development begins with a series of roughly synchronous syncytial mitoses, which occur once every 6-10 min at the cleavage stage (Turner & Mahowald 1976; Foe & Alberts 1983). A cytoskeletal network organizes a zone of cytoplasm around each syncytial nucleus, and together nuclei and cytoplasm go through a programmed series of divisions and movements. During the telophase periods of the eighth and

ninth nuclear division cycles, most of the nuclei migrate to the periphery of the egg to form the syncytial blastoderm. About five nuclei arrive at the surface of the posterior pole of the embryo at cycle nine, cause the egg membrane to bulge outward, and become fully enclosed in cell membranes during the tenth nuclear division. These "pole cells " are the progenitors of the germ cells. Most of the remaining nuclei reach the surface of the egg in cycle ten and undergo four more syncytial divisions at progressively slower rates (9-21 min) before cellularization is completed. Streaming of cytoplasm to and from the anterior and posterior poles of the embryo occurs during the later nuclear divisions (Foe & Alberts 1983). During a 30 min period following the thirteenth division, cell membranes grow in between the nuclei to form individual cells (cellularization stage). Completion of cellularization, at about 2.5 h of development, produces the cellular blastoderm. Although the resulting shell of about 6000 cells has few visibly distinctive features, it is primed to undergo a rapid development of visible pattern during the gastrulation movements . As is discussed below, at least a rough code of spatial information is already present in the cellular blastoderm.

As soon as the cell membranes are formed, gastrulation begins (Turner & Mahowald 1977). The first movements visibly segregate presumptive mesoderm from ectoderm while the endoderm develops more slowly.

Cellular Determination.

Early cleavage nuclei are developmentally totipotent, as tested by

transplantation into an early embryo (Zalokar 1971), but a variety of experimental strategies have revealed that cellular determination, at least for segments and possibly even for the germ layers, has occurred by the time gastrulation begins. These studies have been extended to demonstrate that by about the thirteenth nuclear division, nuclei transplanted with their associated cortical cytoplasm already show a commitment to differentiate anterior or posterior structures (Kauffman 1980). These findings suggest that molecular heterogeneities in cortical cytoplasm arise before cellularization. A particularly elegant demonstration that cells at the blastoderm stage have a firm commitment, at least with regard to segmental determination, was provided by Simcox & Sang (1983). Individual blastoderm cells from the prospective first thoracic segment were transplanted to a region of the blastoderm from which the third thoracic segment arises. Subsequently, donor cells were found intergrated into a third thoracic leg, but, nevertheless, differentiated a sex comb, a structure characteristic of the first leg. This clearly demonstrates that at the time of the transplantation, these blastoderm cells were firmly commited to a specific developmental pathway.

Maternal Contribution to Embryogenesis : The Initial Activation of the Zygotic Genome.

Eggs are unusually large cells and the synthetic capacity of a diploid nucleus appears inadequate to direct the rapid changes that occur in the initial stages of development. Indeed, early embryonic divisions

proceed with little detectable transcription, and activity of the genome appears to be unnecessary for many of the early events. This suggests that early events must be primarily carried out by products previously packaged in the egg. At cellularization, there is an abrupt transition during which transcriptional activity is dramatically increased and the zygotic nuclei begin to take on their normal substantial synthetic responsibilities (Edgar and Schubiger 1986). A critical problem in the understanding of pattern formation is how genes that come to be expressed in spatially specific patterns are initially regulated in the very early embryo.

Oogenesis and Maternal mRNA Synthesis in Meroistic Insects.

Insects with nurse cells that support oocyte growth are said to have meroistic follicles. In these insects, the cluster of oocytes and associated nurse cells is called an egg chamber. Nurse cells appear to provide the developing oocyte with macromolecules and even with ribosomes which are transported from the nurse cells to the oocyte through intercellular bridges, the ring canals (Browder 1984).

One of the processes that occurs during oogenesis is the production of mRNA for utilization during early development. This RNA is part of the informational pool with which the zygote begins its development. The oocyte nucleus is of diminished significance during this phase in meroistic insects, since the nurse cell nuclei assume a major role in production of oocyte cytoplasmic RNA. After determination of the oocyte, the nurse cells

grow and become highly polytene due to extensive replication of DNA without further cell division, and may have up to 1024 times as much DNA as the haploid genome. The newly synthesized RNA is seen to be transported into the oocyte (King 1970). Since each nurse cell has an equivalent of multiple genomes, the oocyte can acquire vast amounts of RNA . It is likely that some of this RNA is utilized in oocyte protein synthesis. Most of the RNA transported to the oocyte is ribosomal RNA (rRNA) that enters the oocyte in the form of ribosomes (see Davidson, 1976 for review). However, evidence from work done on giant silkworms indicates that poly(A) RNA (i.e. putative mRNA) and 4S RNA are also synthesized in the nurse cells and transported into the oocyte (Paglia et al. 1976). Ultimately, the nurse cells inject virtually all of their cytoplasm into the oocyte; the ring canals are then severed, and the nurse cells are sloughed from the oocyte prior to ovulation. It is thus clear that two of the prime functions of oogenesis are the synthesis and accumulation of mRNA.

RNA Synthesis During Early Development of the Kelp Fly, *Coelopa frigida*

In the kelp fly, nuclei of the stages of internal and early superficial cleavage do not reveal nucleoli prior to the 2000 nuclei stage which occurs 105-110 minutes after oviposition (Schwalm and Bender 1973). This observation is consistent with other reports on cleavage nuclei in the Diptera (Mahowald 1963). Since the presence of nucleoli generally indicates rRNA synthesis, we can safely conclude that this species of RNA is not being synthesized during cleavage stages. These observations, seen in conjunction

with reports of limited development in flies which by genetic experimentation have lost the nucleoli from their genome (Beermann 1960), reveal that early development is independent of synthesis of nucleolar RNA. Cytological and biochemical studies lend direct support to this conclusion. Lockshin (1966) has shown protein synthesis autoradiographically in the absence of newly synthesized RNA in the early development of *Leptinotarsa* (Coleoptera). RNA synthesis in the egg of the dipteran *Musca domestica* is resumed after the first 90 minutes of development (Pietruschka and Bier 1972). In all these investigations, RNA synthesis (all species of RNA) is undetectable in the early stages of development. The hypothesis, at the present stage, is that long lived, specifically activated RNA (synthesized during oogenesis) controls whatever protein synthesis is required for the first step of embryonic development.

Early Embryonic Development in *Drosophila* : Establishment of Metameric Pattern.

The generation of a specific number of repetitions of a standard basic unit or segment is a fundamental mechanism underlying the development of most higher organisms. Little is known about the molecular mechanisms involved in the establishment of spatial organization. *Drosophila melanogaster* is a fly in which, after 70 years of genetic studies, genes that control specific aspects of early embryonic development have been identified. One of the central problems in developmental biology is to determine how a developing organism controls the spatial arrangement

and identity of the descendants of a single cell, the fertilized egg, so as to reproducibly form a functional organism. One way to understand how a pattern of body organization becomes established is to identify the genetic elements involved in the control of development. This path has been most profitably followed in the case of *Drosophila*, where a great number of mutants have been isolated that disrupt or alter normal development. The most interesting and instructive of these *Drosophila* developmental mutants are the pattern-formation mutants. Into this class fall both the segmentation mutants and the homeotic mutants (see Mahowald and Hardy, 1985, for review). The division of the body into segments appears to result, at least in part, from the activities of the segmentation genes described by Nusslein-Volhard and Wieschaus (1980), while the specification of segmental identities is known to be controlled by the genes of the *bithorax* complex (BX-C) (Lewis 1978) and some of the genes of the *antennapedia* complex (ANT-C) (Kaufman and Wakimoto 1980). Mutations of segmentation genes result in alterations in the number and polarity of body segments of the fly, while homeotic mutations cause the replacement of one body structure by another which is normally located elsewhere. Homeotic mutant strains of *Drosophila* are occasionally encountered showing bizarre disturbances of the body plan. Wings, for example may sprout from the head where there should be eyes (the mutation *ophthalmoptera*) or legs may grow in place of antennae (the mutation *antennapedia*) (Wakimoto et al. 1984; Bateson, 1984). Thus, the segmentation genes seem to lay down the metameric pattern of the fly, and the homeotic genes specify unique

identities to various elements of the pattern.

The Zygotically Active Segmentation Genes : A Genetic Hierarchy.

The segmentation genes are divided into three phenotypic classes based on the extent of the pattern defects (Scott and O'Farrell 1986). Mutations in four "gap" loci delete large parts of the embryonic pattern; mutations in eight "pair-rule" genes cause deletions of patterns spaced at two segment intervals; mutations in nine "segment polarity" loci affect parts of every body segment. The gap loci can be viewed as coarse dividers of the embryo, the pair-rule loci as functioning to divide the embryo into segmental units, and the segment polarity loci as being involved in forming patterns within each segment.

The localized defects seen in segmentation mutants appear to be well-correlated with the spatial patterns of expression of these genes. For example, *in situ* hybridization of tissue sections shows that as early as division cycle II in the *Drosophila* embryo, RNA from the *Kruppel (Kr)* gap locus is localized in a thick belt around the middle of the embryo (Knipple et al. 1985). The position of the pattern deletion in *Kr* embryos roughly corresponds to the position of the early belt of expression. The pattern of *Kr* expression becomes more complex later in development, spreading into new regions well separated from the first place where the gene was expressed. Thus, this gap gene is expressed very early in development, and the transcripts are localized before the embryo is divided into cells.

Mutations in two extensively studied pair-rule loci, *fushi tarazu (ftz)*

and *hairy (h)*, cause defects in alternate parasegments, which are offset from each other. The *ftz* mutations result in the absence of the maxilla-labral, T1-T2, T3-A1, A2-A3, A4-A5, A6-A7 and A8-caudal (T=thoracic, A=abdominal) segment boundaries (Wakimoto and Kaufman, 1981), while *h* mutations result in the absence of the segment borders that are not affected by *ftz* (Nusslein-Volhard and Wieschaus 1980, Ish-Horowicz et al. 1985). Both *h* and *ftz* transcripts are first detected at about the same time that the *Kr* RNA is first detected (Hafen et al. 1984, Ingham et al. 1985). Initially, *h* RNA is found uniformly throughout the embryo, while *ftz* RNA is restricted to a broad region between 15 and 65% of the egg length (0% is the posterior tip and 65% is approximately the junction of the thoracic and head segments). During the next two nuclear divisions, both of the patterns become more complex, resolving first into several broad bands and then into seven transverse stripes at two-segment intervals (Hafen et al. 1984, Weir and Kornberg 1985, Ingham et al. 1985). Thus the periodic pattern defects seen in the mutant embryos are preceded by spatially restricted expression in a similarly periodic pattern.

Genes of the third class of zygotic segmentation genes, the segment polarity genes, act in every segment. The expression of these genes may be spatially offset; some genes affect posterior compartments of the segments, some affect anterior compartments of segments, and some affect boundaries. RNA (and protein) from one of these genes, the *engrailed (en)* locus, accumulates in fourteen transverse stripes (each about one cell in width) by late division cycle 14, after completion of cellularization

(Kornberg et al. 1985, Fjose et al. 1985). Later, during germ-band elongation, a fifteenth *en* stripe develops near the posterior end of the embryo. The positions of these stripes correspond to the anlagen of the posterior compartment of each segment. *En* has been shown to act in posterior compartment cells to distinguish them from anterior compartment cells, both during embryogenesis and during metamorphosis (Kornberg 1981).

The conclusion from all of these data is that the embryo is progressively subdivided by successive actions of the gap, pair-rule, and segment polarity loci.

More direct evidence for the proposed hierarchy comes from examining the expression of genes in one class in embryos carrying mutations in genes in another class. The few experiments reported up to date are all in agreement with the gap --- pair-rule --- segment polarity gene hierarchy. For example *ftz* (pair-rule) expression is altered by mutations in all four gap loci but is unaffected by mutations in three segment polarity loci, including *en* (Carrol and Scott 1986). Furthermore, in the next tier of the proposed hierarchy, mutations in any of the eight pair-rule loci alter *en* expression. In addition to the regulatory interactions between classes, some of the genes within a class interact. Thus, for three of the seven other pair-rule loci a mutation alters *ftz* expression (Carroll and Scott 1986, Howard and Ingham 1986). Whereas mutations in the pair-rule locus *h* affect *ftz* expression, the pattern of *h* RNA is not affected by *ftz* mutations (Ingham et al. 1985, Howard and Ingham 1986). Therefore, even within the class of pair-rule loci, hierarchial interactions can be defined.

The hierarchy can be extended to genes that are expressed only during oogenesis. Eight "maternal effect" segmentation loci have been shown to alter the pattern of *ftz* expression (Mohler and Wieschaus 1985, Carroll et al. 1986). Some of the maternal effect loci alter all of the *ftz* stripes, while others disrupt only part of the pattern. Some of the maternal gene products may be needed only in certain parts of the embryo; others may be needed throughout most or all of the embryo. The potential complexities are evident; for example, a maternally active segmentation gene could directly control the expression of *ftz*, or its effect could be mediated by all four gap loci and at least the three pair-rule loci that are above *ftz* in the hierarchy.

Expression of the *ftz* gene in the *Drosophila.*

From *in situ* hybridization experiments, Hafen et al. (1984) reported that transcripts of the *ftz* $^{+}$ gene were first detected during nuclear cleavage prior to cell formation in the embryo of *Drosophila.* There seem to be low levels of *ftz* $^{+}$ transcripts which appear to be evenly distributed in the egg at these early embryonic stages. If this is true, then this even distribution and the fact that the first 9 nuclear cleavages in *Drosophila* occur without new RNA synthesis (Mahowald and Hardy 1985), suggest a possible maternal origin of these transcripts. More important , it will be interesting to find out the possible role the protein product(s) of the gene may play in the determination of the progenitor cells of the segmental units in which the transcripts of the gene are detected at the blastoderm stage, where all cells are believed to be determined (if indeed determination

occurs during cleavage). At the blastoderm stage (3.5 h after oviposition), the hybridization pattern consists of 7 bands of cells which are restricted to the region of the egg from which T1-A8 segments arise. There are no detectable levels of *ftz* $^{+}$ transcripts in embryos older than 4 h (Hafen et al. 1984). Thus, it appears that by the time segmentation sets in and the germ band is fully extended, (4.5 h after oviposition) the *ftz* $^{+}$ gene is no longer expressed.

Rationale and Purposes of Study.

If the *ftz* $^{+}$ gene is expressed in the embryo before cellularization (early zygotic expression) and its gene product(s) are directly involved in the early determinative events that lead to the formation of the parasegment primordia, it should be transcribed in the progenitor cells of the segmental units that are missing in the *ftz* mutants. Futhermore, *ftz* $^{+}$ expression could then be used to identify those progenitor cells early in development. It is quite conceivable that *ftz* $^{+}$ transcription in the early embryo is universal but the gene product(s) could be sequestered selectively into progenitor cells of specific parasegments. On the other hand if *ftz* $^{+}$ transcripts are detected before or during nuclear cleavage (possible maternal in origin) prior to cell formation, they could be compartmentalized into progenitor cells early in development. The issue of lineage-specific segregation in *Drosophila* (or the kelp fly) has not been examined, but in ascidians there is suggestive evidence for the preferential segregation of actin mRNA (Jeffery et al. 1983). Results from these experiments suggest

that actin mRNA sequences synthesized during oogenesis are localized in the myoplasm of ascidian eggs, participate in ooplasmic segregation, and are distributed to the mesodermal cell lineage during early embryogenesis. If a similar redistribution of specific types of mRNA and their sequestering by certain cell lines coincides with segment formation in dipteran embryos, one can ask the following questions : Do *ftz* $^{+}$ transcripts, generally distributed in the egg before cell formation, become localized during cytoplasmic rearrangement after oocyte maturation or after oviposition? Is this segregation under the control of maternal determinants? Are these determinants and *ftz* $^{+}$ transcripts preferentially segregated into specific cell lines? The current hypothesis is that the gradual restriction in *ftz* $^{+}$ transcription might be caused by the establishment of positional information during nuclear cleavage. The nature of the signals or gene product(s) that may become localized in the embryo to regulate *ftz* $^{+}$ expression in such a progressive manner is unknown. The best candidates for genes that regulate the *ftz* $^{+}$ expression are maternal effect or early zygotic genes. Do specific messsages (mRNA, proteins) become rearranged or preferentially segregated into specific cell lines which give rise to progenitor cells of the segmental units in which transcripts of the gene are detected at the blastoderm stage? This research will try to answer some of the questions raised above using the kelp fly as a tool.

The early development of *Coelopa* is representative of that of flies in general. Cleavage and blastoderm formation of *Coelopa frigida* has been described by Schwalm and Bender (1973). The zygote nucleus is first

observed 20-30 min after oviposition. Between 35 and 40 min after oviposition (2-4 nuclei stage) cytoplasm concentrates around each cleavage nucleus, forming 'energids'. The following cleavages succeed each other rather rapidly, doubling the number of nuclei every six to seven min until, at 80 min, 512 nuclei populate the egg. At this time, most of the nuclei have migrated to the egg surface. The next nuclear divisions show a slight extension of the generation time for the two subsequent superficial cleavages which produce approximately 2000 nuclei on the egg surface (90-100 min after oviposition). During the last of these rapid superficial cleavages, pole cells are formed and the nuclei which have stayed in the egg interior distinguish themselves as yolk nuclei. The blastoderm maintains synchrony in its nuclear divisions after this step, while presumptive pole cell nuclei and the nuclei remaining in the yolk plasmodium divide at a pace differing from that of the blastoderm nuclei. The production of approximately 3000 nuclei at 110 min (about 2 h) reflects a further increase in generation time of blastoderm nuclei.

The early superficial cleavages produce temporary folds in the egg surface between the blastoderm nuclei. During subsequent divisions, the folds between nuclei become permanent, growing deeper into the thick layer of periplasm, and forming blastoderm cell membranes. These membranes reach the yolk plasmodium between 160 and 175 min (about 3 h) of development, and separate blastoderm from yolk plasmodium incompletely.

Thus the stages of early development in the kelp fly can be divided into two distinct phases: the stage of internal cleavage, providing the egg with

128-256 nuclei, and the stage of superficial cleavage and blastoderm formation, during which the number of nuclei is increased to approximately 5000. The beginning of morphogenetic movements, with the formation of the proctodeal invagination, terminates the second phase.

One of the main aims of this investigation is to find out whether the *ftz* $^{+}$ gene is expressed in the kelp fly similarly to its expression in *Drosophila* during those first few hours after oviposition, when this organism progresses through the corresponding stages of cleavage and segmentation.

There are several advantages of studying the *ftz* gene in the kelp fly. This fly produces 80-100 eggs synchronously and can therefore be used for analytical procedures that require large amounts of material with identical genetic and developmental conditions. Biochemical assays of eggs with the same genetic condition are limited in *Drosophila*, because *Drosophila* produces a limited number of eggs simultaneously. In addition to this quantitative advantage, these studies will provide us with a comparative look at the spatial expression of the *ftz* gene in another family of the dipteran order.

Thus the specific aims of this study are the following:

(1) To find out if the genome of the kelp fly has sequences homologous to the *ftz* gene sequences isolated from the *Drosophila* genome.

(2) To investigate whether or not the *ftz* $^{+}$ gene is expressed maternally and if not, to find out the exact stage in which *ftz* $^{+}$ transcripts are first detected after oviposition.

(3) To investigate the spatial distribution of *ftz* $^{+}$ transcripts during the first few hours after oviposition, while the blastoderm forms in the kelp fly embryo.

CHAPTER II

MATERIALS AND METHODS

The organism used for the experiments described below was *Coelopa frigida* (Diptera), commonly known as the kelp fly. A culture of this organism was maintained on an artificial medium of cornmeal-agar-baby cereal at 25 ^{o}C in half pint milk bottles in the laboratory at Texas Woman's University as described by Surver and Bender (1973). Dobson (1977) has described the natural habitat and ecology.

To determine whether the genome of the kelp fly contains homologous sequences to the *Drosophila ftz* $^{+}$ gene, DNA was extracted from the kelp fly, dotted on nitrocellulose filters, and a ^{32}P-labelled anti-sense *ftz* $^{+}$ RNA probe (synthesised by transcription of the *ftz* $^{+}$ gene from a hybrid plasmid with the *ftz* $^{+}$ gene insert) hybridized to the filters.

To determine whether the *ftz* $^{+}$ gene is expressed maternally, RNA was extracted from the kelp fly mature eggs, dotted on nitrocellulose filters, and a ^{32}P-labelled anti-sense *ftz* $^{+}$ RNA probe hybridized to the filters.

To obtain precise information about the spatial distribution and the time of appearance of *ftz* $^{+}$ transcripts during normal *Coelopa* development, I have used *in situ* hybridization of a ^{35}S-labelled anti-sense *ftz* $^{+}$ RNA probe to RNA contained in whole embryos and in tissue sections of the wild type embryo.

Extraction of High Molecular Weight DNA of the Kelp Fly for Dot Hybridization .

The procedure used to extract DNA from adult kelp flies was as described by Maniatis et al. (1985). Flies (1g) were ground in a mortar with a pestle in liquid nitrogen. The powdered tissue was transferred to 10 ml of lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.5 mM spermidine, pH 8.0,) in a 30 ml McCartney bottle with plastic lid. The mortar and pestle were washed with 5 ml of lysis buffer and this was added to the in the McCartney bottle. Proteinase K was added to the preparation to a final concentration of 100 μg/ml (150 ml of 10 mg/ml in 50% glycerol stock solution). The bottle was incubated at 37 ^{o}C for 2 h, occasionally inverting it gently. One volume of re-distilled and equilibrated phenol (pH 8.0) was added and the bottle was inverted gently several times. The mixture was spun in a bench centrifuge for 5 min at low speed in a 50 ml centrifuge tube. With a wide bore pipette the top aqueous layer was pipetted into a fresh tube leaving as much debris at the interphase as possible. The phenol extraction was repeated. Half volume of phenol and half volume of chloroform/isoamyl alcohol (24:1) was added. The tube was gently inverted several times and spun for 5 min at low speed. The top aqueous layer was pipetted into a fresh tube. One volume of chloroform/isoamyl alcohol was added and the tube inverted and spun as before. The aqueous layer was again pipetted and placed in a dialysis bag and dialyzed extensively against a solution of 50 mM Tris-HCl,10 mM EDTA and 10 mM NaCl, (pH 8.0) at 4 ^{o}C for at least two days. After dialysis the

solution was treated with 100 µg/ml RNase A for 60 min at 37 ^{o}C. The solution was then extracted once more with phenol/chloroform (1:1), and dialyzed extensively against 1X TE (10 mM Tris-HCL, pH 8.0; 1 mM EDTA). The concentration of the DNA solution was estimated by utraviolet (UV) absorption and its size was determined by agarose gel electrophoresis.

Spectrophotometric Determination of the Concentration of DNA.

The approximate concentration of the DNA was determined by the spectrophotometric measurement of the amount of UV light absorbed by the bases (Maniatis et al. 1985). This involved diluting 10 µl of the DNA to 300 µl and taking an absorbance (A) reading at wavelengths of 260 nm and 280 nm. The reading at 260 allows calculation of the concentration of the DNA in the sample. The ratio between the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the DNA.

Estimation of the Size of the DNA by Gel Electrophoresis.

The procedure used to estimate the size of the genomic DNA extracted was a modification of a procedure described by Maniatis et al. (1985). A 0.5% agarose was made in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) in a 250 ml flask. Using a 10 ml pipette, 12 ml agarose was poured directly onto the dry plate of the electrophoresis unit with comb in place. The agarose was left to solidify, the comb removed gently and 400-450 ml 1X TAE was added to the unit, covering the gel (2 mm above the top edge of the gel). DNA samples were prepared as follows: the kelp fly DNA sample

consisted of 25 µl DNA (5 µg)+ 4 µl tracking dye; while the DNA marker was made up of 0.5 µl lambda DNA (1 µg) + 10 µl H_20 + 4 µl tracking dye. Each sample was mixed and loaded into slots in the gel. The unit was run at 40V for 2 h and the gel stained with ethidium bromide (0.5 µg/ml in water) for 15 min. The gel was examined under UV light and photographed with a Polorid camera.

Synthesis of Single Stranded RNA Probe of the *Drosophila ftz*$^+$ Gene.

Single stranded *ftz*$^+$ RNA probes were synthesised *in vitro* at high yields from a hybrid plasmid containing the *ftz*$^+$ gene flanked by an SP6 promotor. A 0.39 kb PvuII-BglII *Drosophila* DNA fragment (see Figure I), derived from p523B (McGinnis et al. 1984) and containing the homeobox region of the *ftz* gene (Kuroiwa et al. 1984, see Figure 2) was inserted into the HincII-BamHI sites of pSP65 (by reversing the orientation of the cloned DNA insert, downstream of an SP6 RNA polymerase promotor) such that transcription from the promotor produces anti-sense RNA which hybridizes to both the *ftz*$^+$ gene and its transcripts. The plasmid (total plasmid size is 3.39 kbp) containing most of the coding region of the *Drosophila melanogaster fushi tarazu* homeotic gene was supplied by Amersham Corporation, Arlington Heights, IL. (see Figure 3 for restriction map). The linearized recombinant plasmid served as the template in the transcription reaction (Figure 4).

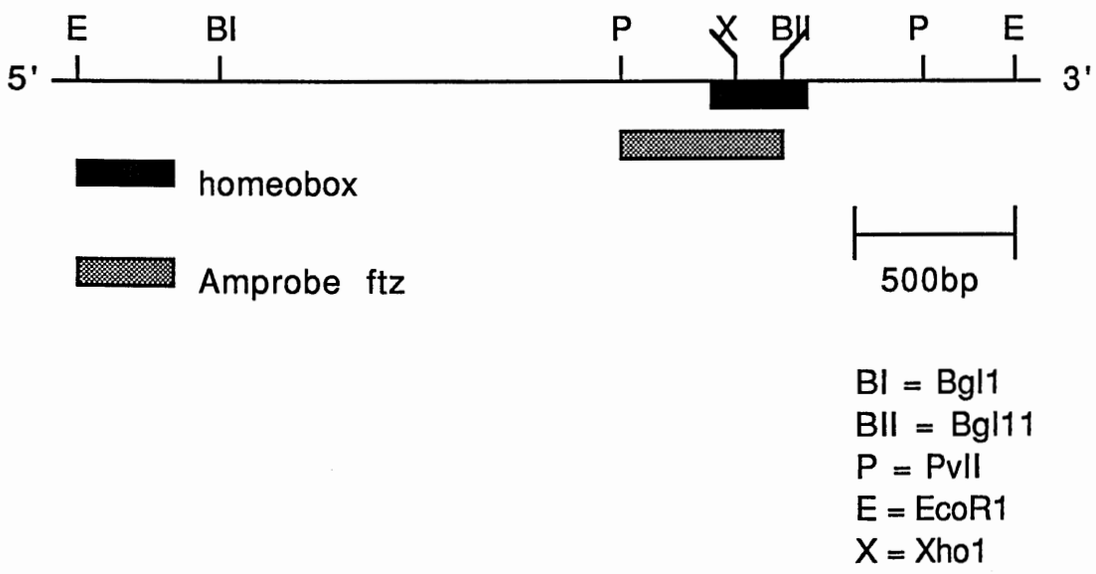


Figure 1. Structure of *Drosophila* DNA in the region of the *fushi tarazu* gene (McGinnis 1984).

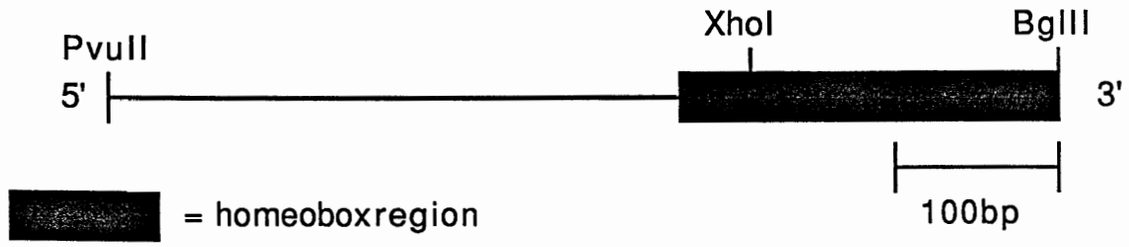


Figure 2. Restriction map of the *ftz* gene (McGinnis 1984).

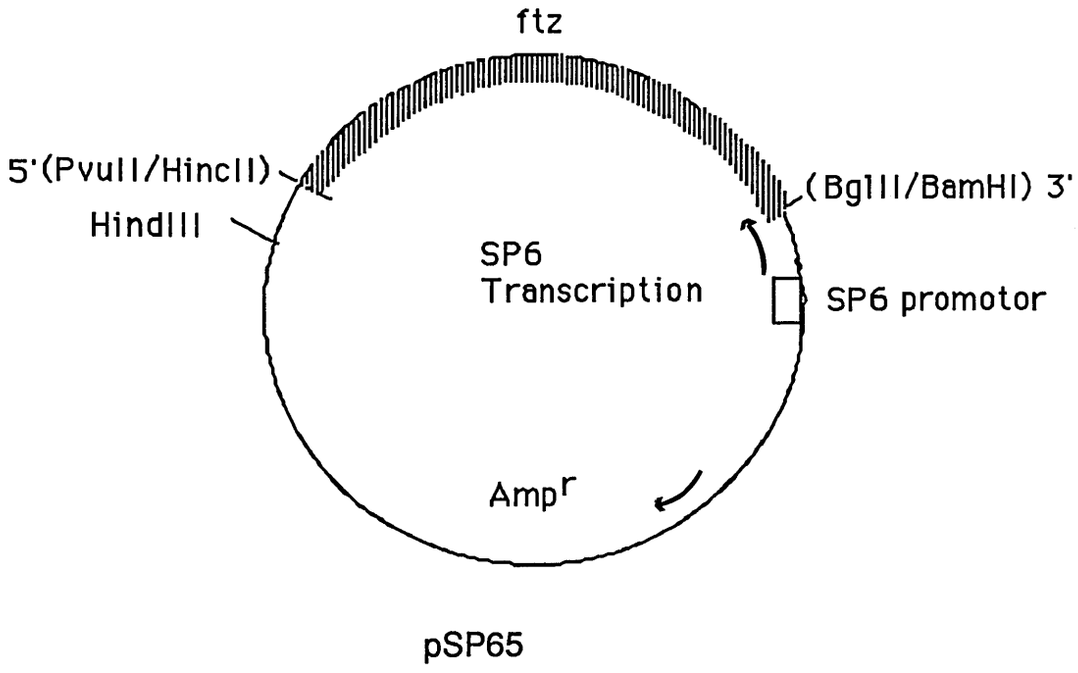


Figure 3. Restriction map of hybrid plasmid with *ftz* gene insert (McGinnis 1984).

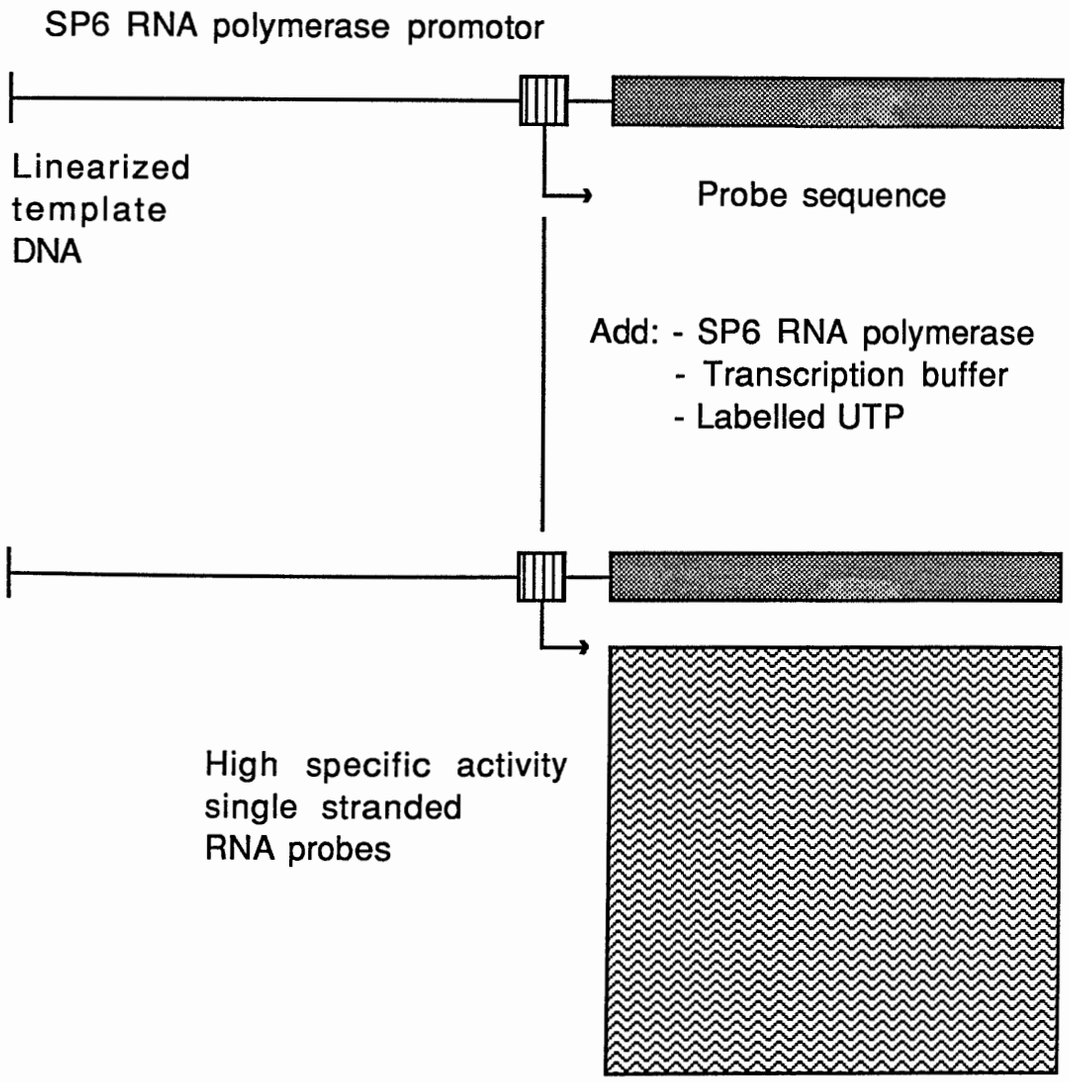


Figure 4. A linearized plasmid serves as the template for transcription.

Synthesis of ^{32}P-labelled *Ftz* $^{+}$ RNA Probe for Dot Hybridization.

The protocol used to synthesise single stranded RNA probes from the *ftz* $^{+}$ gene was provided by Amersham (the manufacturer of the hybrid plasmid). The procedure involved synthesis of single stranded probes of the gene by transcription in the presence of labelled [^{32}P]-UTP (800 Ci/mmol). The following reagents were mixed at room temperature (in the order given) in a microfuge tube : 2 µl transcription buffer (200 mM Tris HCl, pH 7.5; 30 mM $MgCl_2$; 10 mM spermidine; 50 mM DDT; 2.5 mM ATP; 2.5 mM CTP; 2.5 mM GTP; 0.5 mg/ml bovine serum albumin; 5 units/µl human placental ribonuclase inhibitor.), 2 µl (l µg) probe DNA template, 5 µl labelled UTP, 0.5 µl 400 mM DDT and l µl (4.5 u) SP6 polymerase (kept on ice). The lid of the tube was closed and the contents mixed gently but thoroughly. The mixture was incubated for 1 h at 40 ^{o}C and the reaction stopped by the addition of 2 µl stop buffer (0.5 EDTA, pH 8.0).

Synthesis of ^{35}S-labelled *Ftz* $^{+}$ RNA Probe for *In Situ* Hybridization

The *ftz* $^{+}$ RNA probe was synthesised by transcription as outlined above. For *in situ* hybridization [^{32}S]-UTP (850 Ci/mmol) supplied by Amersham was used in place of [^{32}P]-UTP (800 Ci/mmol).

Dot Hybridization of Genomic DNA Using a ^{32}P-labelled *Ftz* $^{+}$ RNA Probe.

The dot hybridization procedure used to detect homologous sequences of the *Drosophila ftz* $^{+}$ gene in the genome of the kelp fly was as described by Amersham (1986). Kelp fly genomic DNA samples were heated at 95 ^{o}C

for 5 min, chilled on ice for 2 min and 5 µl (about 1 µg) of samples spotted onto nitrocellulose membrane in approximately 2 µl aliquots, allowing the spots to dry between application of each aliquot. 1 µg of the hybrid plasmid and 1 µg of plasmid without the *ftz* gene insert were also spotted to serve as positive and negative controls respectively. The membrane was moistened in a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 min and transferred to a neutralizing solution (1.5 M NaCl, O.5 M Tris-HCl pH7.2, 1 mM EDTA). It was then blotted dry with filter paper and air dried. The membrane was baked in a vacuum oven at 80 ^{o}C for 2 h and soaked in 2X SSPE (1XSSPE = 0.18 M NaCL, 10 mM $NaH_2PO_4.H_2O$, 1 mM Na_2EDTA, pH 7.4). It was then added to10 ml prehybridization solution (5X SSPE, 50% formamide (v/v), 5X Denhardts (100X Denhardts=2% each(w/v) of bovine serum albumin, polyvinylpyrollidone and Ficoll), 5% SDS and 100 µg/ml calf thymus DNA) in a heat sealable bag, and incubated at 42 ^{o}C with shaking for at least one h. After incubation, 5-10 ng/ml of RNA probe was added directly to the prehybridization solution contained in the heat sealable bag with the filter and incubated overnight at 42 ^{o}C with shaking. The filters were washed at 42 ^{o}C with shaking following the regimen below:

Wash No.	Solution	Duration
1	2X SSPE + 0.1% SDS	15 min
2	" "	"
3	1X SSPE + 0.1% SDS	30 min
4	0.1X SSPE	15 min
5	"	"

6	10 µg/ml RNase in 2X SSPE	15 min

The filters were then rinsed in 2X SSPE.

Autoradiography of Dot Hybridization Filters.

After washing, the filters were taped to a Whatman 3MM paper (backing sheet) and wrapped in Saran Wrap while still damp. In the darkroom, the sample was placed in an X-ray film holder and covered with a sheet of X-ray film (Kodak X-Omat AR). The film and sample were taped securely in place and exposed overnight. The film was developed according to the protocol below :

Kodak liquid X-ray developer	4 min
3% acetic acid stop bath	30 sec
Kodak rapid fixer	4 min

The film was then dried in a warm cabinet for 1 h.

Synthesis of ^{32}P-labelled *Ftz*$^{+}$ DNA Probe of the *Drosophila ftz*$^{+}$ Gene. by Nick Translation.

The nick translation labelling protocol used was as provided by BRL (1986) and was as follows. The following reagents were mixed briefly in a 1.5 ml microfuge tube (sitting on ice) in the order given : 5 µl transcription solution (BRL solution A2 containing dATP, dGTP, dTTP), 2 µl (l µg) probe DNA template (Amersham), 8 µl [^{32}P]dCTP and 30 µl sterile H_20 (BRL solution E) in a total reaction volume of 45 µl. Five microliters of nick translation Grade Pol. I (solution C containing 0.4 u/µl DNA Polymerase I, 40

pg/μl Dnase, 50 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 50% (v/v) glycerol, 100 μg/ml nuclease free BSA), were added, the lid of the tube closed, and the contents mixed gently but thoroughly. The tube was then centrifuged briefly (15,000g for 5 sec), and incubated at 15 ^{o}C for 1 h. The reaction was stopped by adding 5 μl stop buffer (BRL solution D, 300 mM EDTA). The probe was precipitated by adding the following to the tube in the order given: 3 μl of 20 mg/ml tRNA, 55 μl 4 M ammonium acetate and 300 μl 100% ETOH. The tube was placed in a dry ice acetone bath for 10 min and centrifuged at 15,000g for 5 min. The supernatant was discarded and 50 μl of TE-NT (10 mM Tris-HCl, 10 mM EDTA), 50 μl 4 M ammonium acetate and 300 μl of 100% ETOH were added to the precipitate. The precipitation procedure was repeated once as outlined above. The precipitate was resuspended in 100 μl of NT-TE(10 mM Tris-HCl, 10 mM EDTA). The amount of radioactivity incorporated into the probe was determined by removing a 2 μl aliquot and diluting it to 20 μl with sterile distilled $H_2$0. A 2 μl aliquot of this was counted in 10 ml beta scintillation fluid.

Dot Hybridization of Genomic DNA Using a ^{32}P-labelled *Ftz* + DNA Probe.

A piece of nitrocellulose membrane was placed in distilled water for 1 min after numbering it. The water was discarded, 20X SSPE was added to the filter and incubated at room temperature for 30 min. The filter was baked at 80 ^{o}C for 30 min. Kelp fly DNA samples were denatured by heating at 100 ^{o}C for 2 min and 5 μl spotted on the nitrocellulose filter. Control

DNA was also applied as above (p26). Prehybridization at 68^{o}C was done overnight in prehybridization solution (0.1 mg/ml calf thymus DNA, 5.0 ml H_2O, 3X SSPE, 2X PAF). The DNA probe (1.89 X 10^7 cpm/ml) was added directly to the prehybridization solution and the filter hybridized at 68 ^{o}C for 16 h. The filter was washed with shaking in 300-600 ml of 1X SSPE at 68 ^{o}C for 30 min and dried at 80 ^{o}C for 1 h. Hybridization was detected by autoradiography.

Collection of Mature Eggs by Hand Dissection of Ovaries.

Ovaries were obtained from mature female flies by hand-dissection and collected in a single drop of sterile distilled H_2O on a coverslip. The coverslip was picked up with forceps and slowly lowered into dry ice. The frozen drop was then rapidly inverted over a microfuge tube. The top surface was touched to warm the frozen drop and the coverslip was immediately slid away allowing the frozen drop to fall into the tube. The lid of the tube was immediately closed and the tube refrozen. When ready for extraction, the ovaries were thawed in a 50 ml round bottom centrifuge tube and homogenized in a solution containing 1% glucose in a phosphate buffer containing 2.26% (v/v) NaH_2PO_4 and 2.52% (v/v) NaOH, at low speed (Polytron setting of 3, Brinkman Instruments) to release mature eggs. The eggs fall to the bottom of the tube and are collected with a Pasteur pipette and quickly frozen in dry ice for RNA extraction.

Extraction of RNA from Mature Eggs.

The hot phenol/chloroform method used for RNA extraction was as described by Jowett (1986). To about 300-500 frozen mature eggs (obtained as above) 4 ml of phenol saturated with 0.2 M sodium acetate (pH 5.0) at 65 ^{o}C was added. The embryos were homogenized in a Tissumizer (Tekman) at a power setting of 70 after which 3 ml of 0.2 M sodium acetate (pH 5.0) and 0.8 ml of 10% SDS were added to the homogenate. The mixture was incubated at 65 ^{o}C for 5 min and vortexed . It was then cooled to room temperature and 4 ml of chloroform were added. The mixture was vortexed and spun in a bench centrifuge at 1000 g. The lower organic phase were removed and discarded. Two volumes of saturated phenol and 2 ml chloroform were added to the aqueous layer, vortexed and spun. The lower organic phase was removed and discarded again. To the aqueous layer 4 ml of chloroform was added, vortexed and re-spun. The aqueous phase was decanted into a fresh tube leaving behind the lower organic phase and the interphase. Two and a half volumes of absolute ethanol was then added to the aqueous phase and left overnight at -20 ^{o}C. It was removed from -20 ^{o}C and spun at 10,000g for 10 minutes. The pellet was washed in 70% ethanol, dried and redissolved in diethylpyrocarbonate (DEPC) -treated H_2O. To remove DNA, the pellet was washed two times with 3 M sodium acetate (pH 6.0). The precipitate was smeared on the inside of the tube to aid the dissolution of the DNA. The sample was re-spun, the supernatant removed and the pellet redissolved in 0.1 M sodium acetate (pH 7.0). Two milliliters of ethanol were added, reprecipitated and spun. The pellet was washed,

dried and redissolved in DEPC-treated H_2O. The resulting RNA was used for dot hybridization.

Preparation of Gelatinized Slides (Subbed Slides)

One half of a gram of Knox gelatin was added to 99.5 ml distilled H_2O in a 125 ml Erlenmeyer flask. The flask was covered with parafilm and put into an incubator at 60 ^oC. The gelatin was allowed to imbibe and swell. After the gelatin was completely dissolved, the flask was taken out of the incubator and put at room temperature to cool. After cooling, 0.5 ml of 10% chrome alum (chromium potassium sulfate : $CrK(SO_4)_2.12H_2O$) was added. This is "chromed-gel" (1% gelatin and 1% chrome alum).

Using Coplin jars, slides were processed through a cleaning solution (100 ml conc. H_2SO_4 added to 3.5 ml saturated $KCrO_20_{7)}$. Slides came out clean in this solution after 3 min. The slides were transferred into an empty jar and put under running tap water and washed until the yellow color was gone (about 5 min). Slides were then transferred from the tap water to a Coplin jar containing distilled water and held for dipping into "chromed-gel". Slides were dipped into the chromed-gel at room temperature. The slides were then placed in an upright position and allowed to air dry before they were stored in a slide box.

Preparation of Siliconized Tubes.

Clean 1 ml 10X50 glass tubes were soaked with a 1% solution of

dichlorodimethylsilane in chloroform. They were then washed a minimum of three times with methanol and baked at 180 ^{o}C for 3 h before use.

Limited Alkaline Hydrolysis of Probe

The RNA probe labelled with [^{32}P]-UTP (see p.19) was hydrolyzed to smaller lengths by limited alkaline hydrolysis, as described by Cox et al. (1984). This involved hyrolysis of the probe in 0.1 M NaOH at 0 ^{o}C for 20-60 min. The hydrolysis reaction mixture consisted of: 0.2 µl 10 M NaOH, 10 µl of the ftz^{+} probe synthesis reaction mixture and 8.8 µl DEPC-treated H_2O in a total reaction volume of 20 µl. Aliqouts (4 µl) were removed after 20, 30, 40, and 60 min and neutralized by adding sodium acetate to 2 M and HCl to 0.1 N. Each neutralizing reaction mixture consisted of 4 µl of the hydrolysis reaction mixture, 2 µl 1M sodium acetate and 4 µl 0.25 N HCl in a total reaction volume of 10 µl.

Determination of Probe Size by Gel Electrophoresis through an Agarose Gel Containing Formaldehyde.

Unhydrolysed and the partially hydrolysed probe samples were run on a formaldehyde agarose gel to estimate the size of the probe after 20, 30, 40, and 60 min of hydrolysis. The procedure used was as described by Maniatis et al. (1985). A 2.5% agarose gel was prepared by melting agarose in water, cooling it to 60 ^{o}C and adding 5X gel running buffer (0.1 M morpholinopropanesulfonic acid (MOPS) pH 7.0), 50 mM sodium acetate, 5 mM EDTA pH 8.0) and formaldehyde to give 1X and 2.2 M final

concentrations respectively (one part of stock formaldehyde solution was diluted with 4.6 parts of agarose solution). The RNA samples were prepared by mixing the following in a sterile Eppendorf tube : 4.5 µl RNA, 2.0 µl 5X gel running buffer, 3.5 µl formaldehyde and10 µl formamide. Samples were incubated at 55 ^{o}C for 15 min. To each sample were added 2 µl of sterile loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol). The samples were loaded onto the gel and the unit was run at 40 V until the bromophenol blue had run two-thirds down the gel.

After electrophoresis, the gel was removed from the unit and wrapped in Saran Wrap. Autoradiography was performed as outlined above for filters (p27) using a paper film holder instead of a metal film holder.

Collection of Embryos and Preparation of Tissue Slides from Early Embryonic Stages of *Coelopa.*

Adult flies were collected from culture tubes and anesthetised with diethyl ether. The flies were sexed and combined into groups of 20, each group consisting of 5 males and 15 females. Each group was put into a culture tube with fresh food and a rolled paper towel. Bottles were then placed in the refrigerator at 4 ^{o}C for 2-3 days. The bottles are removed from the refrigerator and put at room temperature after which they were inspected for laid eggs about every 15 min. If and when eggs were found, they were removed and their approximate ages determined as described below.

Staging of Embryos.

The mophological criteria used for identifying approximate ages of living embryos during cleavage and blastoderm stages was as described by Wieschaus and Nusslein-Volhard (1986). Freshly laid eggs were immediately collected from the mating bottle and placed on a moist paper towel. Two or three embryos were removed and dechorionated ,as described below, while the rest were allowed to continue to develop at room temperature on moist paper towel. The dechorionated embryos were immediately immersed in parrafin oil and viewed under a stereomicroscope. Once their chorions became transparent, they were transferred to a drop of oil on a glass slide, viewed under a light microscope and their approximate age determined. The rest of the eggs were timed from then on until the approximate cellular blastoderm stage was reached (160-175 min after oviposition). Embryos were removed at approximate ages of 0-35, 35-40, 80-90, 105-120 and 160-175 min old. They were then dechorionated, the vitelline membrane was removed and the embryos were fixed, embedded and sectioned as described below. Photographs were taken of 1-2 living embryos at each of the above stages and kept as a permanent record of that particular batch of eggs.

Prefixation of 0-4 h Embryos: Dechorionation and Removal of the Vitelline Membrane.

Embryos were dechorionated according to a protocol developed by Michison and Sedat (1983) for the fixation of embryos prior to

immunolocalization analyses on whole-mount preparations. Staged embryos were rinsed with a solution containing 0.4% NaCl and 0.03% Triton X-100 in a 5 mm sterile Petri dish. The solution was removed and the embryos were dechorionated in 50% household bleach for 2-3 min at room temperature. Embryos were agitated gently during this process. After dechorionation the embryos were extensively washed in H_20 and rinsed as above. They were transferred into a 15 ml siliconized and baked flask and blotted free of excess liquid. The vitelline membrane was removed as described previously (Mitchison and Sedat 1983). Briefly, a mixture of 10 ml heptane, 9 ml methanol and 1ml 0.5 M EGTA that had been pre-cooled to -70 ^{o}C in a dry ice bath was added to the embryos in a 15 ml siliconized and baked flask. The mixture was agitated vigorously in a flask at -70 ^{o}C surrounded by dry ice for 10 min. The embryos were rapidly warmed to room temperature by shaking the flask under a stream of hot tap water. After warming to room temperature the flask was swirled for another 2-5 min. The embryos, which now sank to the bottom of the lower methanol phase, were removed with a Pasteur pipette and rinsed twice with a solution of 5 ml 90% methanol and 50 mM EGTA. Embryos were rehydrated through two 5 ml changes each of 70, 50 and 25% methanol-EGTA solutions for 2 min each. Embryos were finally rinsed several times with a solution containing 0.4% NaCl and 0.1% Triton X- 100.

Fixation and Washes of Embryos.

The embryos were placed in 1 ml siliconized and baked10x50 mm glass

tubes. They were then prefixed according to a modification of a procedure used for whole imaginal discs (Kornberg et al. 1985). This included fixation on ice for 15 min in PBS (PBS is 130 mM NaCl, 7 mM Na_2PO_4, 3 mM NaH_2PO_4) containing 4% paraformaldehyde. The solution was removed and the embryos fixed for an additional 15 min at room temperature in 4% paraformaldehyde, 0.1% Triton X-100 and 0.1% deoxycholate in PBS. The embryos were then incubated for 10 min in PBSM (PBS with 5 mM $MgCl_2$) and then 10 minutes in 0.5 M Tris-HCl, pH 7.5, 0.1 M glycine.

Storage of Fixed Embryos before Hybridization.

For embryos to be stored until hybridization, the fixative was removed and 0.4 ml 0.3 M ammonium acetate added. An equal volulme of 100% ETOH was added dropwise over 10 min. Embryos were washed in 100% ETOH, stored in 100% ETOH at 4 ^{o}C, and moved to -20 ^{o}C for longer storage. The ETOH was removed prior to hybridization, and the embryos rehydrated in PBSM. The embryos were then washed as above for hybridization. If storage was unnecessary, the washes directly followed fixation as outlined above.

In Situ Hybridization of Whole Embryos and Washes.

A standard hybridization condition (Hafen and Levine 1986) was modified to include 10 mM DTT to maintain a reducing enviroment for the [^{35}S]-label, 1% SDS to reduce non-specific probe retention, and 10% polyethylene glycol(PEG) to accelerate hybridization (Jorgensen and Garber

1987).

The procedure used for hybridization of whole embryos was a modification of a procedure described by Jorgensen and Garber (1987). The last wash solution was removed and the prehybridization solution (50% formamide, 0.6 M NaCl, 10 mM Tris-HC, pH7.5, 1 mM EDTA, 1% SDS, 10 mM DTT, 0.25 mg/ml tRNA, 1X Denhardt's, 10% PEG-6000) was added. The tube was sealed with parafilm and incubated at 50 ^{o}C overnight. Probe concentration of 60 ng/ml/kb complexity was added directly to the prehybridization solution and the embryos were incubated overnight at 50 ^{o}C. After hybridization, 0.3 ml of PBSM was added and the solution removed. Embryos were incubated twice in PBSM for 5 min each. The embryos were then treated for 30 min in 20 µg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, at 37 ^{o}C. Embryos were incubated at 37 ^{o}C for an additional 30 min in a solution containing 0.5 M NaCl and 10 mM Tris-HCl (pH 8.0). The embryos were washed once according to the following regimen:

a) 2X SSC, 50% formamide, 10 mM DTT, at 50 ^{o}C for 30 min
b) 1X SSC, 50% formamide, 10 mM DTT, at 50 ^{o}C for 30 min
c) 1X SSC, 50% formamide, 10 mM DDT, 0.05% Triton X-100 at 37 ^{o}C for 30 min.

The embryos were embedded in plastic, sectioned, mounted on subbed slides and autoradiographed.

Dehydration, Embedding and Preparation of Sections of Embryos after *In Situ* Hybridization.

The hybridized embryos were dehydrated in increasing concentrations of ethanol in 0.1% SSC (15 min each in 50, 80, 95% and 30 min twice in 100% ethanol). After dehydration embryos were placed in an Immuno-Bed (Polysciences, Inc.) infiltration solution (100 ml solution A, 0.9 g catalyst) and infiltrated for a minumum of 3 h at room temperature with gentle agitation. After infiltration embryos were embedded in an Immuno-Bed mixture (1 ml solution B, 25 ml catalyzed solution A) on ice. The embedding procedure was as follows: using a disposable pipette, BEEM capsules were filled to 2/3 full with Immuno-Bed embedding mixture. Embryos were placed into the container (one in each capsule) and oriented. The BEEM capsules were then completely filled and closed tightly to exclude air. The medium was allowed to auto-polymerise overnight at room temperature. Two micron sections were cut on a Porter-Blum microtome, floated on subbed slides (sections were placed within the lower fourth of the slides to facilitate covering with a small amount of emulsion during autoradiography) and baked at 60 ^{o}C for 3 h. The plastic was removed by soaking the slides 5 min each, twice in Xylene, and twice in 100% ETOH, and then air dried. The sections were then ready for autoradiography.

Autoradiography and Processing: Liquid Emulsion Dipping Method.

Ten milliliter aliquot of 1:1 NTB2/ distilled H_2O emulsion was melted in the darkroom in a water bath at 45 ^{o}C. It took about 20 min for the

emulsion to melt. A dipping chamber was filled with the liquid emulsion and the slides dipped individually into the emulsion 3 times within a period of 5 sec, slides were removed completely from the emulsion each time. The back of the slides were wiped with tissue paper and the excess emulsion drained off by holding the slide vertically on tissue paper. The dipped slides were placed horizontally on a pre-cooled glass plate (plate sitting on a tray containing ice) to quickly solidify the emulsion on the slide. They were then placed in a vertical position in a slide holder to drain onto a paper towel and left to dry in a light-proof place for at least 2 h at room temperature. Sometimes a non-sparkling fan was used to reduce this time to 1 h. After drying, the slides were placed in a slide box containing a drying agent (Drierite), the edges of the box were sealed with tape and the boxes were wrapped in aluminum foil. The slides were exposed at 4 ^{o}C for 6-8 days.

Developing Autoradiographs and Histological Staining.

The slide box containing the slides was allowed to warm up to room temperature before opening in the darkroom. The slides were placed in a rack and immersed into a solution containing Kodak D-19 developer equilibrated to 15 ^{o}C and incubated in the darkroom under red safelight conditions for 2 min. The slides were transferred to a solution containing 2% acetic acid (stop solution) pre-cooled to 15 ^{o}C and incubated for 30 sec. Afterwards, they were fixed in Kodak fixer pre-cooled to 15 ^{o}C for 5 min and rinsed in 3 changes of distilled water pre-cooled to 15 ^{o}C for a total of

15 min. At this stage the slides were taken out of the darkroom. They were then immersed in a freshly prepared solution containing 10 mM $NaPO_4$ (pH 6.8) and 0.5% Giemsa stock solution (the water used to prepare the staining solution was pre-equilibrated to 15 ^{o}C) and incubated in this solution for 30 sec. The slides were covered from dust and allowed to air dry overnight. Tissues were mounted under permount and #1 coverslip, and the coverslip weighted for 2 days. The sections were photographed underbright-field to show morphology and under dark-field to illuminate the silver grains.

Control Hybridizations.

As a control, embryos were hybridized to RNA probes synthesized from a human *K-ras* (cDNA) proto-oncogene. A 1.1 kb fragmant containing full length human *K-ras* -2 cDNA (Figure 5) was inserted into the Bam HI-PstI sites of pSP65 (McCoy et al.1984, Figure 6) such that transcription from the SP6 promotor produces anti-sense RNA, which hybridize to *K-ras* mRNA. A linearized plasmid (supplied by Amersham) served as the template for the probe synthesis. Synthesis of the probe; hybridization, washes, embedding, and sectioning of embryos; and autoradiography of the sections were as described above.

In Situ Hybridization of Tissue Sections.

Dechorionation, removal of the vitelline membrane and fixation of 0-175 min embryos prior to embedding was as described above. After fixation the embryos were dehydrated in increasing concentrations of ethanol (50, 80,

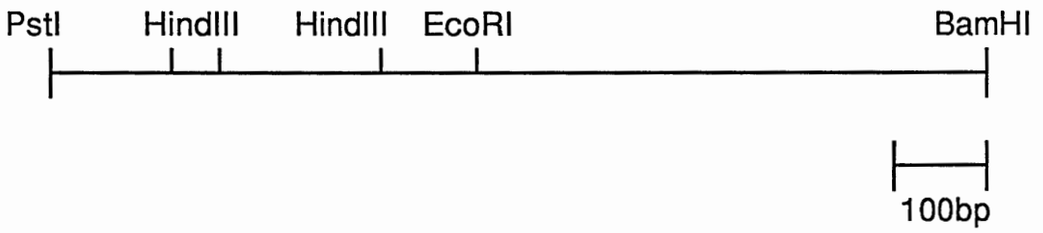


Figure 5. Restrictiom map of the *K-ras* gene (McCoy et al. 1984).

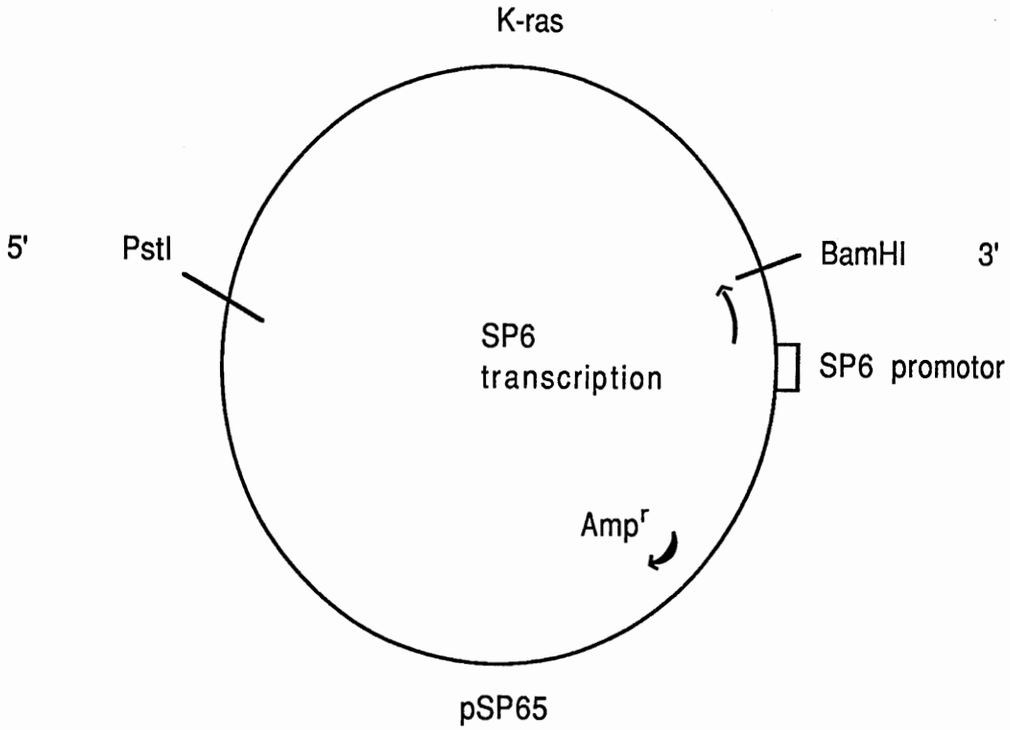


Figure 6. Restriction map of hybrid plasmid with *K-ras* gene insert (McCoy et al. 1984).

95 and 100%), and embedded in Inmmuno-Bed as described above.

Preparation of Tissue Sections.

The procedure used was as described by Levine (1986). Sections measuring about 2 um in thickness were prepared and collected on subbed slides and placed on a slide warmer at about 50 ^{o}C for 1-2 min. The sections were allowed to air-dry at room temperature for 1 to 2 h. The slides were placed in a metal rack and immersed in a solution containing 4% paraformaldehyde in PBS and incubated at room temperature for 5 min. They were then washed in PBS, twice, for 5 min each. They were ethanol-dehydrated in a graded series at room temperature as follows: 30% (2 min), 60% (2 min), 80% (5 min), 94% and100% 2 min each. The slides were then air-dried and stored at room temperature before hybridization.

Treatment of Sections for Subsequent Hybridization.

The method used to prepare the slides for hybridization was as described by Roberts (1986). Slides were placed in 0.2 M HCL and incubated at room temperature for 20 min. They were rinsed in 2X SSC for 5 min and transferred into a solution containing 2X SSC pre-heated to 70 ^{o}C and incubated at 70 ^{o}C for 30 min. The slides were rinsed in distilled water for 5 min at room temperature. Excess liquid was drained from slides by holding them vertically on tissue paper and wiping the reverse side. A solution containing 0.25 mg/ml pronase in 50 mM Tris-HCL(pH 7.5) and 5 mM EDTA was layered on the section with a Pasteur pipette and incubated for 10

min at room temperature. Excess pronase solution was blotted from the slides by holding them vertically on tissue paper. Slides were placed in a solution of PBS containing 2 mg/ml glycine for 30 sec at room temperature and then washed twice in PBS for 30 sec each. The sections were fixed in a solution containing 4% paraformaldehyde in PBS for 20 min at room temperature. The sections were then ethanol- dehydrated in a graded series at room temperature as follows: 30% (2 min) 60% (2 min), 80% (5 min), 94% (2 min). The slides were stored at 4 ^{o}C for hybridization.

Hybridization of Tissue Sections.

The procedure used was as described for frozen sections (Amersham 1986) with modifications. The hybridization solution consisted of approximately 0.3 ng/µl of probe in a solution of 50% formamide, 0.6 M NaCL, 10m M tris-HCL, pH7.5, 1m M EDTA, 1% SDS, 10 mM DTT, 0.25 mg/ml tRNA, 1X Denhardt's solution and 10% PEG-6000. The probe mix was applied to the section in 15 µl aliquots and covered with a siliconized cover slip. Air bubbles were avoided by spreading the mixture slowly as the coverslip was lowered. The coverslip was sealed with rubber cement to prevent evaporation. The slides were incubated at 50 ^{o}C in sealed chambers equilibrated with wash buffers (6X SSPE, 50% formamide) overnight. Slides were removed, placed in a slide rack and immersed in wash buffer at 50 ^{o}C for 1 h. The buffer was changed and washed for 1 additional h. Slides were transferred into 0.5 NTE (0.5 M NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA) at 37 ^{o}C and incubated for 5 min. The slide rack was placed in 0.5 NTE

containing 20 μg/ml RNase A (boiled 2 min before use) at 37 ^{o}C for 30 min. Slides were washed in a total of 5 changes of 0.5 NTE at 37 ^{o}C for a total of 1 h. Sections were dehydrated through graded ethanol (30, 60, 85, 95%) in 0.3 M ammonium acetate, 2 min each, and then in 100% ethanol and air-dried.

Autoradiography, Development of Autoradiographs and Photography.

Autoradiography, developing of autoradiographs and photography were as described above.

CHAPTER III

RESULTS

Determination of the Concentration of Kelp Fly Genomic DNA Extract

To determine the concentration of the DNA sample extracted, A_{260} and A_{280} were taken. Results were as shown in Table 1. The concentration of the DNA is given by the relationship: concentration = A_{260} X 30 X 50. The results thus gave a DNA concentration of 106.5 μg/ml. The purity of the sample was determined by the ratio A_{260}/A_{280}. This gave a ratio of 1.42, a ratio that indicates the presence of contaminants, such as eye pigments (which co-purifies with DNA), in the nucleic acid solution.

Determination of the Size of Kelp Fly Genomic DNA Extract

The standard method used to separate, identify and purify DNA fragments is electrophoresis through agarose gels. The technique is simple, rapid to perform and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other sizing procedures such as density gradient centrifugation. Furthermore the location of DNA in the gel can be determined directly. After electrophoresis, bands of DNA in the gel were stained with low concentrations of the fluorescent dye, ethidium bromide (Sharp et al. 1973). Figure 7 shows a photograph of the bands obtained after electrophoresis on an agarose gel to determine the size range of the

Wavelength	Absorbance (A)
260	0.071
280	0.050

Table 1. Determination of the concentration of DNA.

The concentration of DNA is given by A_{260} x 30 x50 = 106.5 μg/ml.

The purity of the DNA is given by A_{260}/A_{280} = 1.42

Figure 7. Agarose gel electrophoresis of *Coelopa frigida* genomic DNA.

Photograph of agarose gel after electrophoresis and staining with ethidium bromide. Lane (a)represents lambda DNA as marker while lane (b) represents kelp fly genomic DNA

extracted *Coelopa* genomic DNA. Under similar conditions of agarose concentration, conformation of the DNA and applied current, the electrophoretic migration rate of DNA through agarose gel is dependent on the molecular size (Aaij and Borst 1972). Results obtained show that *Coelopa* genomic DNA was successfully extracted in the form of high molecular weight DNA, since the extracted DNA (lane b) is moving at a slower rate than the 23 kb lambda DNA marker (lane a). DNA travels through agarose gel matrices at rates that are inversely proportional to the logarithm_{10} of their molecular weight (Helling et al. 1974).

Dectection of Homologous Sequences of the *Drosophila Ftz* Gene in the Genome of *Coelopa frigida*

To determine whether the genome of the kelp fly has sequences homologous to the *Drosophila ftz*$^+$ gene, the high molecular weight genomic DNA extracted from kelp fly was spotted on nitrocellulose filters. An anti-sense *ftz*$^+$ RNA probe was synthesised from the *Drosophila ftz*$^+$ gene inserted into the Hinc11- Bam Hi site of an SP65 plasmid by transcription in the presence of [^{32}P]-UTP. The probe was hybridized to the filters dotted with the kelp fly genomic DNA. A *ftz*$^+$ DNA probe was also synthesised by nick translation of the hybrid plasmid and hybridized to separate filters dotted with kelp fly genomic DNA. The sensitivities of the two probes were compared.

Dot Hybridizations

There are many methods available to hybridize radioactive probes in solution to DNA or RNA immobilized on nitrocellose filters. These methods differ in the following aspects: the solvent and temperature used; the volume of solvent and the length of hybridization; the degree and method of agitation; the concentration of the labelled probe and its specific activity; the use of compounds such as dextran sulphate, that increases the rate of reassociation of nucleic acids and the stringency of washing following the hybridization.

Presence of the *ftz*$^+$ gene in the kelp fly genome was demonstrated by annealing a homologous nucleic acid probe (^{32}P labelled *ftz*$^+$ RNA) to unique sequences on the target DNA (kelp fly genomic DNA) immobilized on nitrocellulose filter.

The *ftz*$^+$ RNA probe used for the dot hybridization reactions was synthesised by transcription of of a hybrid plasmid with the *ftz*$^+$ gene insert in the presence of high specific activity [^{32}P]-UTP. Probe specific activity was 1.33×10^9 dpm/μg. To ascertain whether radioisotopes were being incorporated into the anti-sense RNA synthesised, an aliquot of the probe synthesis reaction mixture was analysed by electrophoresis on formaldehyde agarose gel. Figure 8 shows the result of the autoradiography of the gel after electrophoresis. The figure shows that radioisotope had been incorporated into the synthesised RNA and that most of the transcripts were full length transcripts (approximately 400 bases).

Hybridization of the filter dotted with kelp fly genomic DNA was

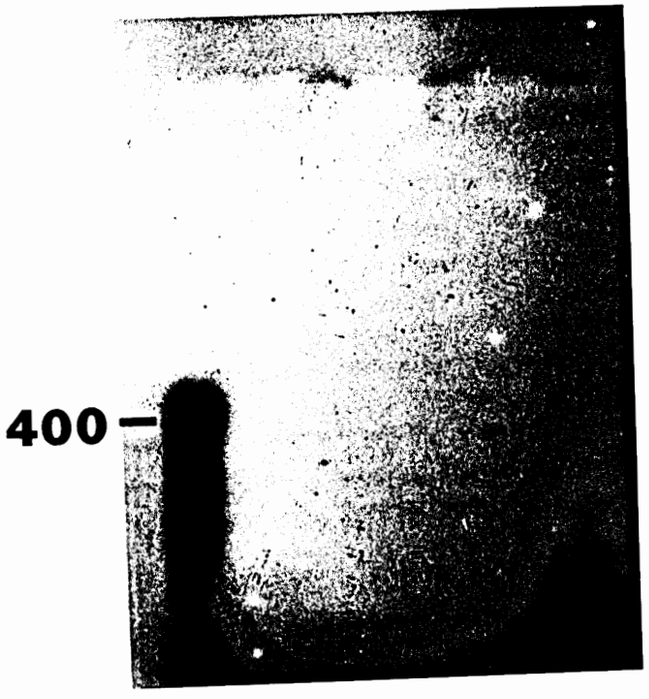


Figure 8. Formaldehyde agarose gel electrophoresis of ftz^{+} RNA.

Autoradiography of gel after electrophoresis of ^{32}P labelled *ftz* $^{+}$ RNA probe. Exposure was for overnight.

performed at 42 ^{o}C in 50% formamide in a sealed bag. This method presented less of an evaporation problem and is less harsh on the filters than is hybridization at 68 ^{o}C (Maniatis 1985). Formamide is a mild nucleic acid denaturant and works by disrupting hydrogen bonding. It was used for fractionation of single-stranded nucleic acids so that secondary structures did not interfere with the hybridization. Denhardts solution was added to accelerate the rate of association of the nucleic acids by excluding the nucleic acids from the volume of the solution occupied by the polymer (Wahl et al. 1979). A probe concentration of 5-10 ng/ml (approximately equivalent to 5 µl of a standard probe synthesis reaction mixture) was used in a hybridization solution volume of 10 ml. The filter was washed at 42 ^{o}C and treated with RNase A to remove unhybridized probe. Figure 9 shows the results obtained after autoradiography of the filter. The results show that kelp fly DNA was successfully retained on the nitrocellulose filter. Lane (1), dotted with hybrid plasmid DNA as positive control, shows a strong positive reaction. Since the probe was prepared from the hybrid plasmid was expected to hybridise to the denatured hybrid plasmid DNA. Thus the positive reaction was as expected. Lane (2) also shows a positive reaction which shows that the kelp fly genome contains sequences homologous to the *Drosophila ftz* $^{+}$ gene. The weak signal (compared to Lane (1)) was as expected, since the gene is very much diluted in the genomic extract. Lane (3) which was dotted with plasmid DNA without a *ftz* insert was used as a negative control. The positive reaction was therefore not expected. Obviously a short sequence of the pasmid (between the *ftz* insert and the

Figure 9. Dot Hybridization of *Coelopa frigida* Genomic DNA using *ftz*$^+$ RNA probe.

Autoradiography of filter after hybridization of a *ftz*$^+$ RNA probe to kelp fly genomic DNA. Exposure was for overnight. (1) represents hybrid plasmid DNA (template DNA) as positive control, (2) represents kelp fly genomic DNA, while (3) represents plasmid DNA without insert.

SP6 RNA promotor, see Figure 3) is transcribed and this explains the positive reaction obtained. The dot hybridization was repeated using a DNA probe synthesised by nick translation. Results are shown in Figure 10. Once again Lane (1) dotted with hybrid plasmid DNA as positive control, shows a strong positive reaction. The spot spotted with kelp fly DNA, Lane (2), shows a negative reaction. This result indicates that for the same concentration of the kelp fly genomic DNA, the presence of the gene in the kelp fly genome could not be detected by the DNA probe. Results for the lane spotted with pasmid DNA without insert (lane (3) was the same as described for Figure 9 above.

Since the RNA probe was succesfully transcribed at high yields from the plasmid containing the desired insert, and proved to be more sensitive than the DNA probe, I opted to use the RNA probe for in *situ* hybridizations.

Detection of Transcripts of the *Ftz*$^+$ Gene in Oogenic RNA Pool

To determine whether the *ftz*$^+$ gene is expressed maternally, mature oocytes were collected by hand dissection of ovaries and the ovaries were homogenized at low speed to release eggs. RNA was extracted from mature eggs thus obtained and dot hybridization reactions performed as outlined above for detection of specific RNA sequences.

The key to efficient RNA preparation is to inactivate the endogenous nucleases as quickly as possible and avoid subsequent contamination with RNase from solutions or glassware. All solutions were autoclaved and treated with 0.1% DEP where possible. All glasswere was treated by baking

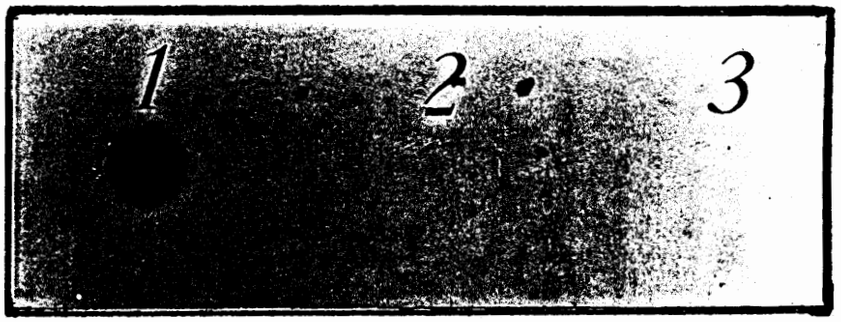


Figure 10. Dot Hybridization of *Coelopa frigida* Genomic DNA using *ftz* $^{+}$ DNA probe.

Autoradiography of filter after hybridization of a *ftz* $^{+}$ DNA probe to kelp fly genomic DNA. Exposure was for overnight. (1) represents hybrid plasmid DNA (template DNA) as positive control, (2) represents kelp fly genomic DNA, while (3) represents plasmid DNA without insert.

for 4 h at 250 ^{o}C. Gloves were worn at all stages of the preparation of RNA to prevent contamination with ribonucleases. To achieve RNase inactivation as quickly as possible the hand dissected ovaries were first frozen in liquid nitrogen as soon as they were dissected from flies. The hot phenol method (Jowett 1986) was used to extract RNA. To optimize RNA yields the phenol extractions were done at an elevated temperature (65 ^{o}C), acid pH (5.0) and in the presence of chloroform. At 65 ^{o}C DNA is partitioned into the organic phase, at acid-pH RNA is preferentially partitioned in the aqueous phase. In the presence of chloroform, phenol does not retain any aqueous phase thus minimizing the loss of RNA. Figure 11 shows an agarose gel after electrophoresis of the RNA extract showing most of the RNA (lane b) smaller than 2 kb lambda marker. Dot hybridization to determine whether the RNA pool contained *ftz* $^{+}$ transcripts was performed on nitrocellulose filters as outlined above for gene detection using an RNA probe. Figure 12 shows the result obtained from the assay. As expected a positive signal was obtained from the hybrid plasmid (1). The results showed a negative reaction for the RNA sample (3). If there were any *ftz* $^{+}$ transcripts in the RNA extract it could not be detected by the assay used here. Lane (4) was dotted with plasmid DNA without *ftz* $^{+}$ insert and the reason for the positive result obtained is as explained for result for lane (3) in Figure 9 above.

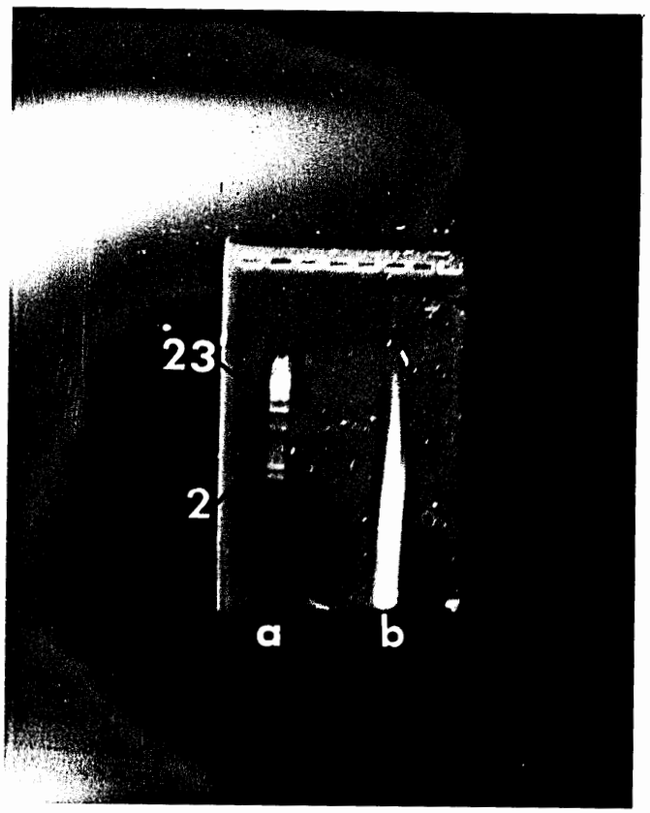


Figure 11. Agarose gel electrophoresis of *Coelopa frigida* oogenic RNA.

Photograph of gel after electrophoresis of RNA and staining with ethidium bromide. Lane (a) represents Lambda DNA as marker while lane (b) represents kelp fly mature egg RNA.

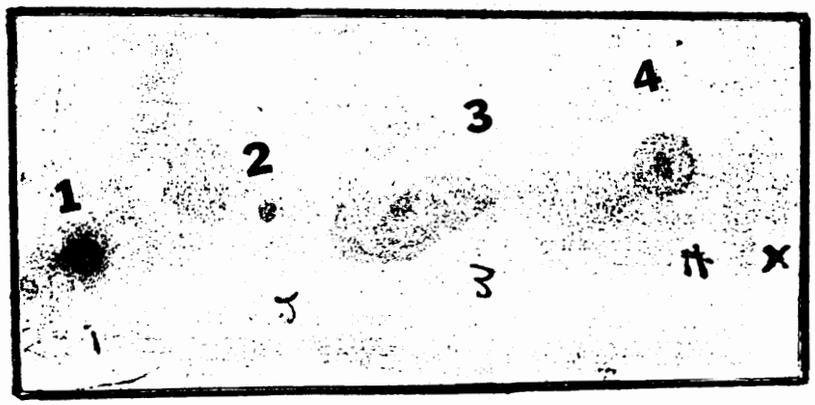


Figure 12. Dot hybridization of *Coelopa frigida* matured egg RNA using *ftz*$^+$ RNA probe.

Autoradiography of filter after hybridization with a *ftz*$^+$ RNA probe. Exposure was for overnight. (1) represents hybrid plasmid DNA (template DNA) as positive control, (2) represents kelp fly genomic DNA, (3) represents kelp fly mature egg RNA while (4) represents plasmid DNA without insert.

Detection of *Ftz*$^+$ Transcripts in Kelp Fly Embryos by *In Situ* hybridization

(a) Staging of Embryos.

Flies were mated and different stages of embryos were obtained at different times. Embryos to be fixed for hybridization were staged by inspection. Figure 13 shows pictures of embryos at different stages (approximate ages). The following scheme of staging proved to be very useful: freshly laid eggs (0 to 15 min) - cytoplasm of the embryo is homogenous; early cleavage (15 to 50 min) - at the posterior end of the embryo, the egg contracts away from the vitelline membrane. A cup of clear cytoplasm becomes visible at the posterior pole: this 'polar plasm' thins out during each cleavage cycle and forms again during interphase, the remainder of the eggs is covered by a thin layer of clear cytoplasm; early syncytial blastoderm formation (60 -90 min) - the layer of cytoplasm on the surface becomes thicker and inhomogeneous due to the migration of nuclei to the cortex, at the posterior pole, the cap of polar plasm thins out and becomes irregularly shaped, buds appear at the surface, enlarge and finally pinch off and form pole cells; late syncytial blastoderm (90-120 min) - the cortical cytoplasm becomes more clearly delimited from the underlying yolk. To test the above scheme of staging based on inspection of living embryos, embryos were selected at various stages and fixed, embedded and sectioned.

Figure 14(i-iii) shows the appearance of sections prepared from embryos obtained at different stages of development. These pictures show that the gross morphological criteria used in staging reflect accurately the cytological events of nuclear distribution and cellularization of the

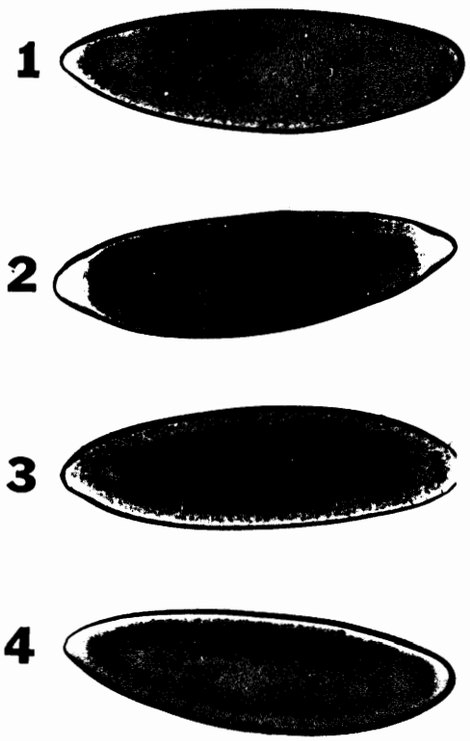


Figure 13: Staging of *Coelopa frigida* embryos.

1. **Freshly laid eggs (0 to 15 min) -cytoplasm of the embryo is homogeneous.**
2. **Early cleavage (15 to 50 min) - at the posterior end of the embryo, the egg contracts away from the vitelline membrane. A cup of clear cytoplasm becomes visible at the posterior pole.**
3. **Syncytial blastoderm formation(60 to 90 min) - the layer of cytoplasm on the surface becomes thicker and heterogeneous due to the migration of nuclei to the cortex, at the posterior pole, the cap of polar plasm thins out and becomes irregularly shaped, buds appear at the surface, enlarge and finally pinch off and form pole cells.**
4. **Syncytial blastoderm (90 to 120 min) - the cortical cytoplasm becomes more clearly delimited from the underlying yolk.**

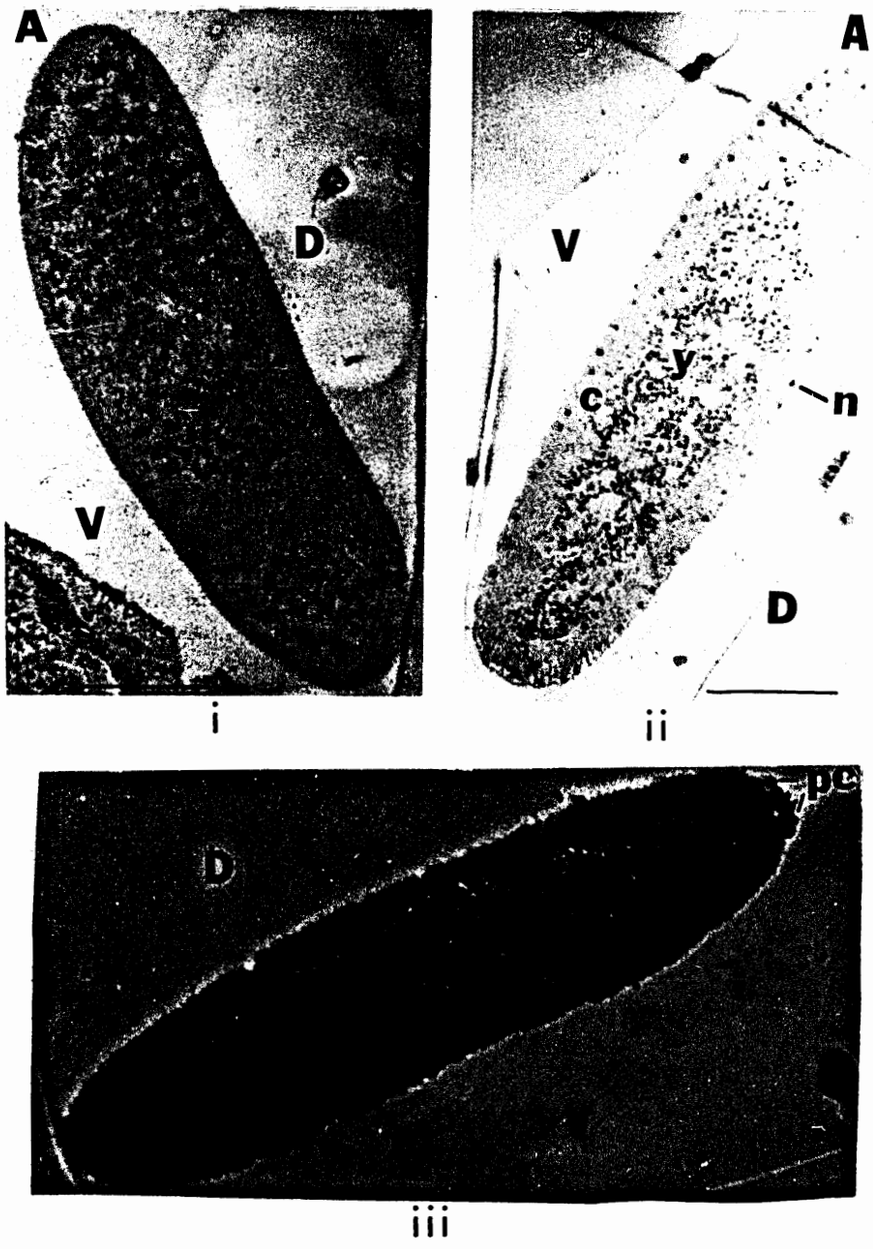


Figure 14. Different stages of early *Coelopa frigida* embryos.
(i) Freshly laid eggs, embryo 0-15 min old. (ii) Syncytial blastoderm, approximately 90 min old embryo, about 250 nuclei stage (iii) Cellular blastoderm, approximately 165-170 min old embryo, cell membranes formed. Abbreviations: n,nuclei; p.c.,pole cells; c,cytoplasm; y,yolk; P,posterior; A,anterior; D,dorsal; V,ventral.

blastoderm during the respective time intervals.

(b) Preparation of Probes of Different Sizes by Partial Hydrolysis.

In order to obtain hybridization probes of optimal size (mean single-stranded length of about150 nucleotides) anti-sense RNA probes prepared as described above were hydrolysed by limited alkaline hydrolysis. The fragments obtained after hydrolysis at different times were electrophoresed through 2.5% agarose containing 2.5 M formaldehyde. Figure 15 shows an autoradiograph of the gel after electrophoresis of an unhydrolysed probe (lane a) and the different size ranges of probes fragments obtained after 20, 30, 40 and 60 min of hydrolysis (lanes b-e). The results show that the average size of the fragments decreased with time. This was as expected. Since no radioactive RNA markers were available to run parallel with the probes to help determine their sizes, hybridizations using the different size ranges of probe fragments obtained after the different hydrolysis times were performed to determine which one gives optimal results; i.e. which size range of the probe gives maximum hybridization and minimum background radiation. Most of the non-specific binding of hybridization probes directly correlates to the size of the probe. Probes with a mean single-stranded length of over 200 nucleotides non-specifically bind to the tissue section. Using probes with a mean single-stranded length of 50-100 nucleotides largely prevents non-specific sticking. Probes with mean single-stranded length of less than 50 nucleotides yield greatly reduced hybridization signals (Hafen and Levine

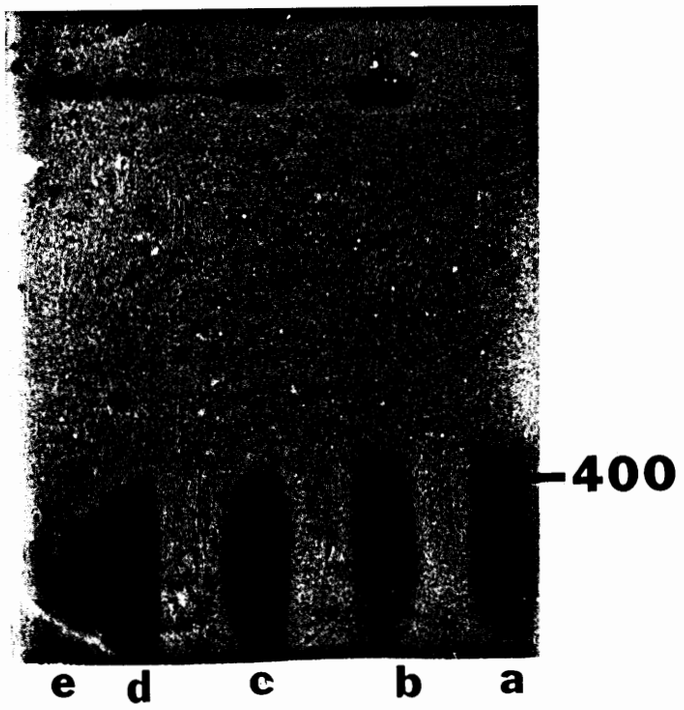


Figure 15. Formaldehyde agarose gel electrophoresis of hydrolysed and unhydrolysed anti-sense ftz + RNA probes.

Autoradiography of gel after autoradiography. Exposure was for overnight. (a) shows the unhydrolysed RNA probe while (b)-(e) shows probe after 20, 30, 40 and 60 min of alkaline hydrolysis respectively.

1986). Thus the preparation and the use of a series of size probes was the safest way to avoid non-specific hybridization.

(c) Hybridization of Whole Embryos.

Whole kelp fly embryos aged 0-175 min old were hybridized with *ftz* $^{+}$ RNA probe labelled with ^{35}S, sectioned and autoradiographed. Before hybridization, staged embryos collected at different times were fixed. The *Coelopa* embryo is protected by the chorion and the vitelline membrane which may block fixative penetration. These membranes were successfully removed by incubation in bleach and by heptane/methanol shock respectively before fixation. Embryos were fixed in two successive stages; first in 4% paraformaldehyde in PBS and then in a solution containing 4% paraformaldehyde, 0.1% Triton X-100 and 0.1% deoxycholate in PBS. Following fixation, embryos were prehybridized overnight and hybridized with ^{35}S-labelled anti-sense *ftz* RNA in siliconised glass tubes at 50 ^{o}C for 16-20 h.

Following *in situ* hybridization and subsequent washes and removal of the nonspecifically bound probe, the embryos were embedded, sectioned and the sections covered with photographic emulsion for autoradiography.

(d) Microautoradiography

The photographic recording of ionizing radiation is used in many research and industrial applications. A major application of this technique, microautoradiography, is used extensively in the research community.

Autoradiography is a photographic method of recording the spatial distribution of radioisotope-labelled substances within a specimen. When a photographic material is placed in direct contact with the labelled specimen, a means for locating and quantifying the radiation emanating from the specimen is provided. The presence of radioactivity in the specimen is manifested by the formation of metallic silver in the photographic material, therefore providing a visible image of the radiation exposure. The photographic material used here was Kodak NTB-2 nuclear track emulsion (Eastman Kodak Co.). Emulsion-coated slides were prepared by dipping slides in melted emulsions at 42 ^{o}C. The slides were then exposed in the dark for 6-8 days. Following exposure the slides were developed and fixed. Some of the slides were stained in Giemsa to show morphology. Photographs of the microscopic image - of the exposed emulsion and of the morphology of the embryo - provided the image for analysis.

(e) Data Analysis

1. Microscopy.

Dried tissues were mounted in permount and a coverslip and the tissue autoradiograms were examined under the light microsope using bright-field and dark-field illumination. In the dark-field the silver grains appear white and are therefore clearly visible above the non-refractile dark tissue. For examination at low magnification (100X or lower) and for clear visualization of weak signals, dark-field illumination was essential, since

at low magnifications the silver grains were hard to observe.

2. Photomicrographs.

Black-and-white photomicrographs were taken using Kodak Technical Pan 2415 (ESTAR-AH Base) film. This film has variable contrast depending on the developer concentration and has a very fine grain. Best results were obtained using a developer concentration of 1 part Kodak developer to 9 parts water (moderate contrast). For bright-field photomicroscopy the automatic control of the camera was used to obtain good contrast negatives. For dark-field photomicroscopy, the correct f-stop was determined empirically to be 8 f-stops. Photomicrographs of sections of hybridized embryos are shown in Figures 16-30.

Early Embryogenesis, Formation of the Syncytial Blastoderm in the Kelp Fly.

After fertilization the zygote nucleus undergoes 13 synchronous divisions without the formation of cell membranes. One hour after oviposition the first four nuclear divisions provide the zygote with 16 nuclei which migrate both anteriorly and posteriorly within the embryo and beneath the surface (Schwalm and Bender 1973). During the next 30 min (90 min after oviposition), nuclei divide rapidly, and after four more divisions (8 nuclear divisions total) approximately 250 nuclei have been formed. After the 7^{th} nuclear division the majority of the nuclei begin to migrate to the periphery, leaving the future yolk nuclei behind. Most of the nuclei become embedded in the cortical layer of periplasm which has

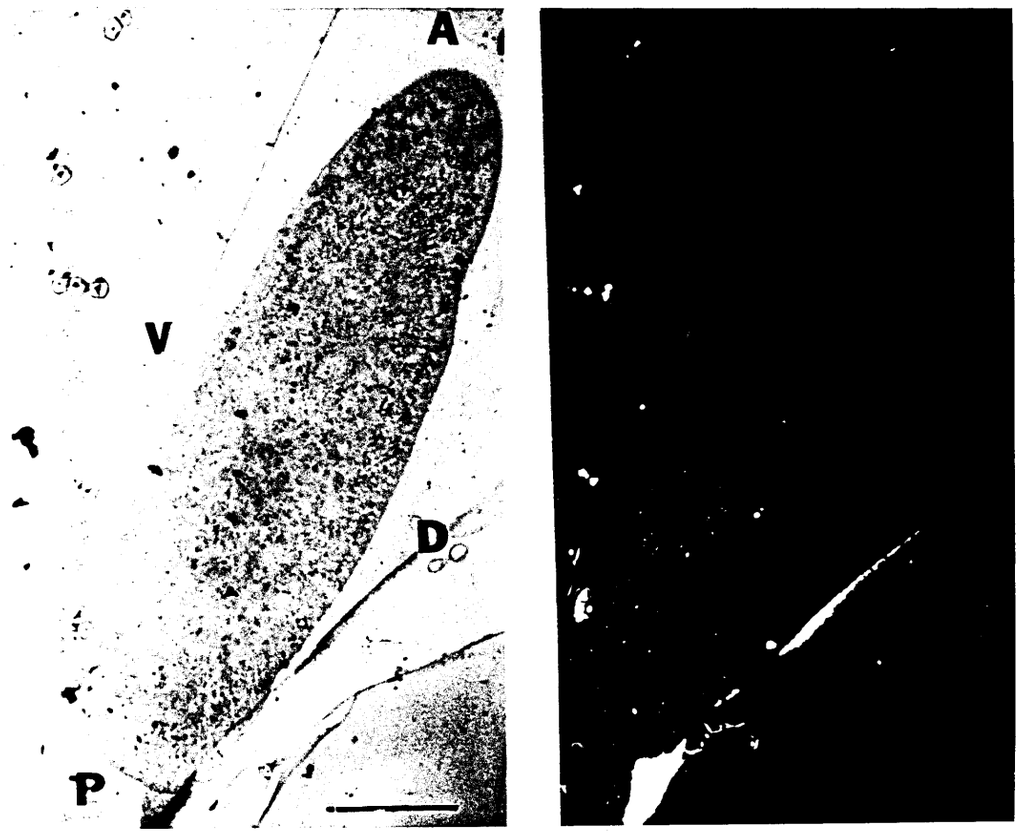
accumulated underneath the surface of the embryo, while yolk and fat droplets concentrate about the central axis of the embryo. Thus a syncytial blastoderm is formed 90 min after oviposition. The first nuclei that reach the posterior pole are enclosed by cell membranes after the 9^{th} division to form pole cells. The remaining nuclei forms a syncytium and undergo four additional divisions. The first visible signs of pole cell formation appear about100 min after oviposition, with the rapid formation of cell membranes between pole cells and the rest of the embryonic syncytium. During the next 20 min the pole cells become completely separated.

The first of the superficial cleavages (9^{th} nuclear division) producing approximately 1000 nuclei, is uniform for the entire egg surface, with the possible exception of the posterior pole, where the separation of the cytoplasm from the egg plasmodium is more pronounced. The first signs of changes in the cleavage pattern appear after the second superficial clevage (10^{th} nuclear division). The number of nuclei is approximately 3000 on the egg surface between 110 and 120 min of development after the 12^{th} division. Nucleoli are observed for the first time in this generation of nuclei (Schwalm and Bender 1973). The generation time of the next division (13^{th} nuclear division) is now extended to 30 min. Permanent cell membranes of the blastoderm begin to grow after nucleoli become clearly discernible (Schwalm and Bender 1973). The process of membrane formation lasts for 30 to 35 min. At 165-170 min after oviposition, blastoderm cells, now columnar and furnished with an oval nucleus, have incorporated most of the periplasm and are separating it from the yolk

plasmodium. This is the cellular blastoderm stage. The spatial distribution of *ftz* $^{+}$ transcripts in the kelp fly embryo has been followed through the stages of internal cleavage, syncytial blastoderm and cellularization.

Spatial Distribution of *Ftz* $^{+}$ Transcripts During Early Clevage (Internal Cleavage) in the Kelp Fly Embryo.

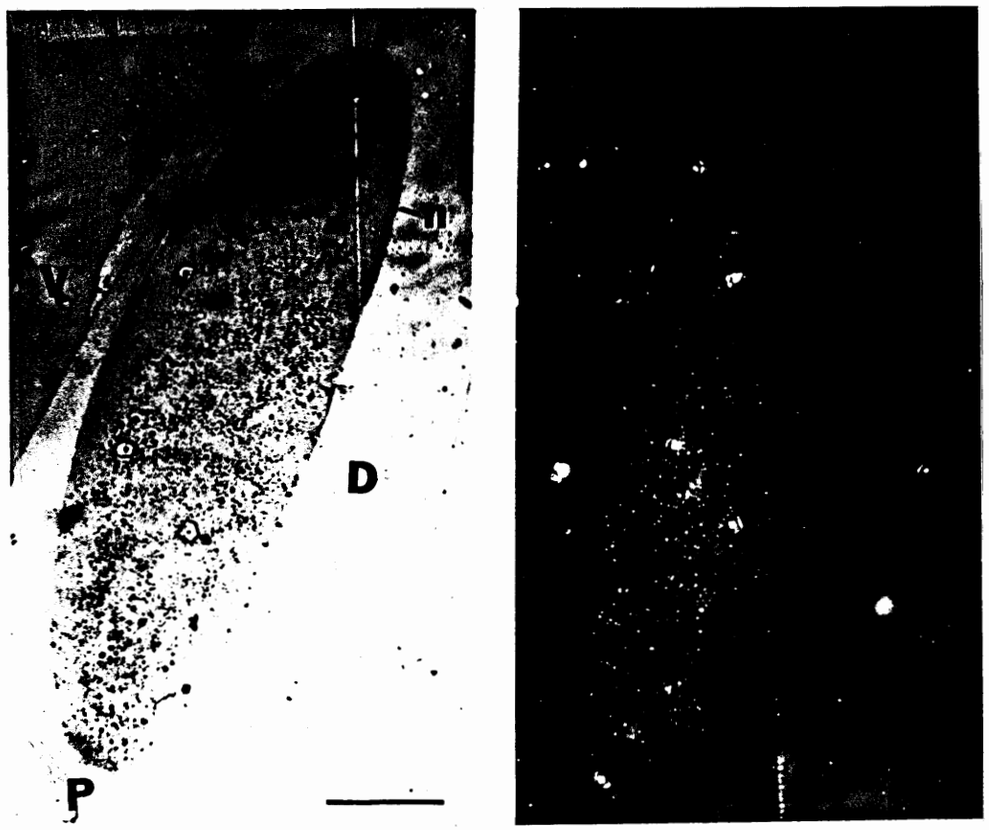
Figures 16a-19a show bright-field photomicrographs of sections through embryos at very early stages of embryogenesis (internal cleavage and syncytial blastoderm formation). These sections were prepared from different embryos hybridized with an ^{32}S labelled anti-sense *ftz* $^{+}$ RNA probe and autoradiographed for 6 days. The same sections are shown in Figure 16b -19b respectively, in dark-field illumination. Figure 16 shows a tissue section of a 30-35 min old embryo, approximately after the completion of the 4th nuclear division. Figure 17 shows a tissue section of a 35-50 min old embryo, approximately after the completion of the 7th nuclear division. Figure 18 shows a tissue section of a 60-90 min old embryo, approximately after the completion of the 8th nuclear division. Figure 19 shows a tissue section of a 60-90 min old embryo, approximately after the completion of the 8th and 9th nuclear divisions. From the signal intensity of silver grains observed in these sections, there seem to be low levels of *ftz* $^{+}$ transcripts in these early embryos. The *ftz* $^{+}$ transcripts appear to be evenly distributed in the egg and no regional differences in grain density can be observed. It can be safely assumed that if transcription of the *ftz* $^{+}$ gene is occuring at this stage, it is of very low



(a) (b)

Figure 16. Distribution of *ftz* $^{+}$ transcripts during internal cleavage in *Coelopa frigida* embryo.

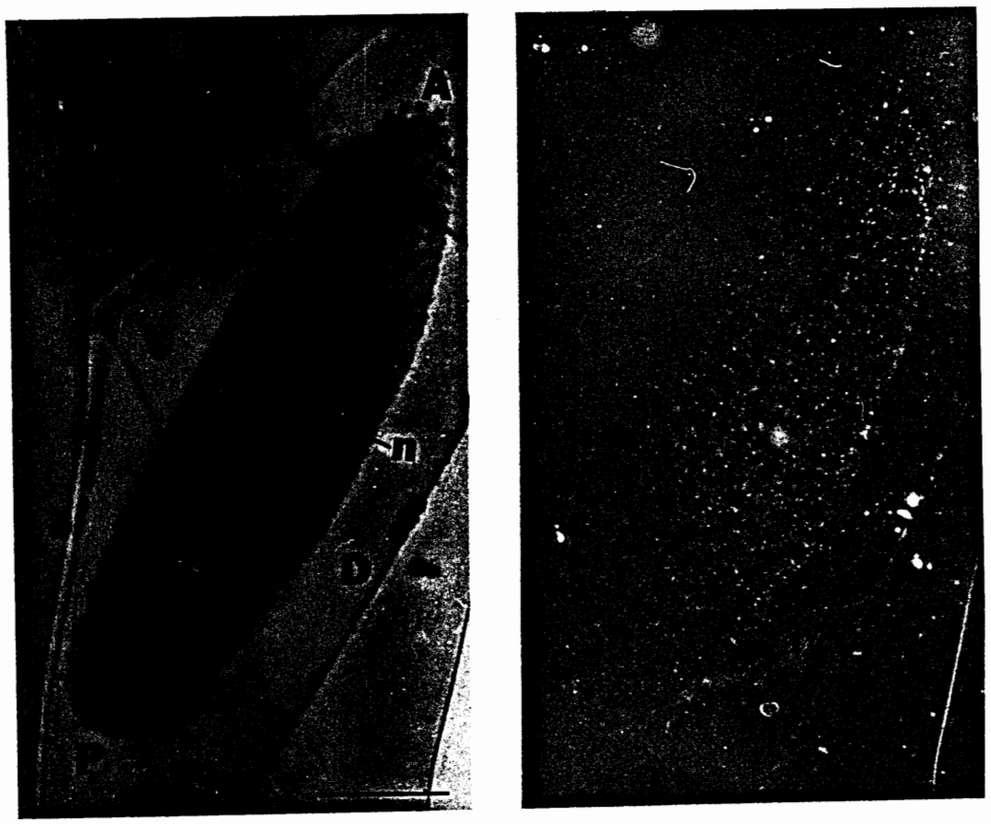
Tissue section of a 30-35 min old embryo, approximately after the completion of the 4th nuclear division; embryo hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a logitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 17. Distribution of *ftz* $^{+}$ transcripts during nuclear migration in *Coelopa frigida* embryo.

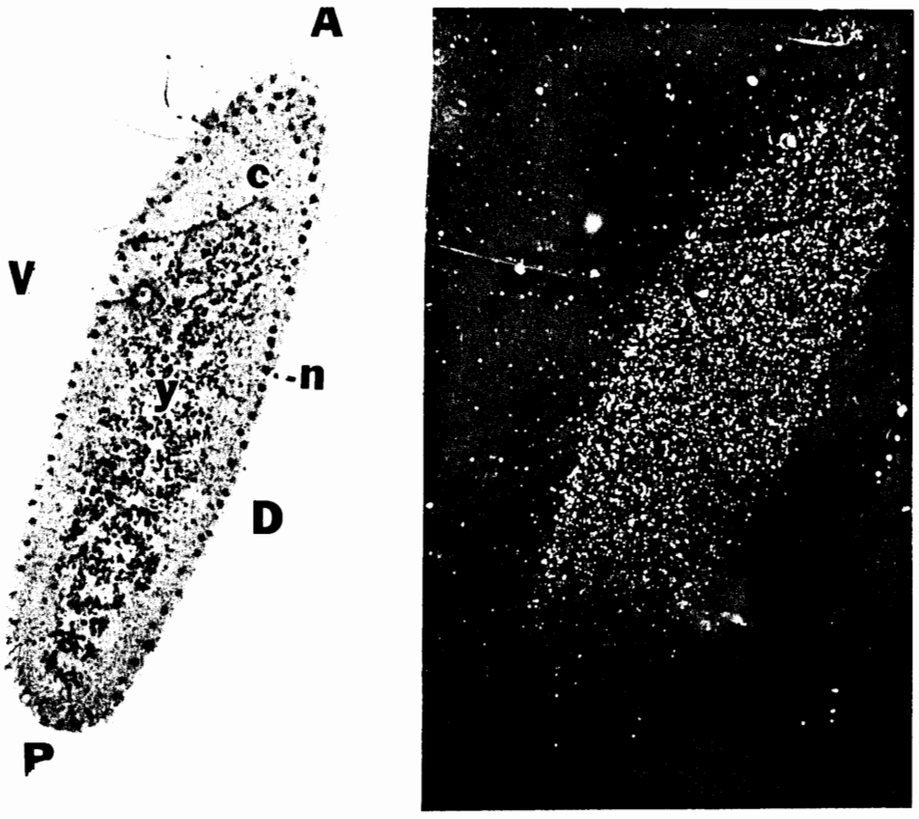
Tissue section of a 35-50 min old embryo, approximately after the completion of the 7th nuclear division; embryo hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a logitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, migrating cleavage nuclei. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 18. Distribution of *ftz* $^{+}$ transcripts during syncytial blastoderm formation in *Coelopa frigida* embryo.

Tissue section of a 60-90 min old embryo, approximately after the completion of the 8th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a logitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, peripheral cleavage nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 19. Distribution of *ftz* $^{+}$ transcripts during syncytial blastoderm formation in *Coelopa frigida* embryo.

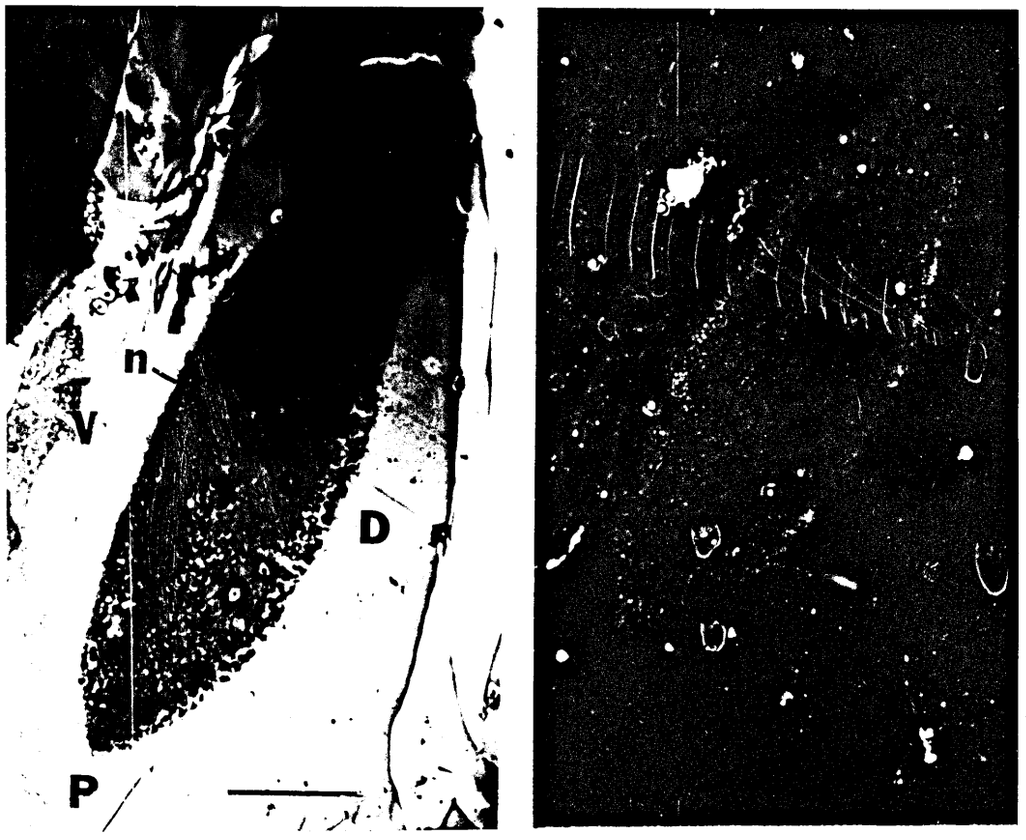
Tissue section of a 60-90 min old embryo, approximately after the completion of the 8th and 9th nuclear divisions; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a logitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, peripheral cleavage nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.

intensity and there are no regional restrictions in transcription.

Spatial Distribution of *Ftz*$^+$ Transcripts During Superficial Cleavage and the Syncytial Blastoderm Stage in the Kelp Fly Embryo.

Figures 20 and 21 show sections of embryos that have completed the 11^{th} nuclear division. These sections were also from embryos that were hybridized with the *ftz*$^+$ probe and autoradiographed for 6-8 days. Figures 20a and 21a show bright-field ilumination photomicrographs while the corresponding dark-field illumination photomicrographs are shown in Figures 20b and 21b. These results show that the peripheral portions of these sections exhibits a greater accumulation of silver grains (compared to Figures 16-19). This signifies restricted transcription and indicates that two nuclear divisions before cells are formed and about 100 min after oviposition the *ftz*$^+$ gene is already expressed in a spatially restricted manner. The accumulation of the silver grains in most cases occurs around the middle portion of the periphery of the embryo.

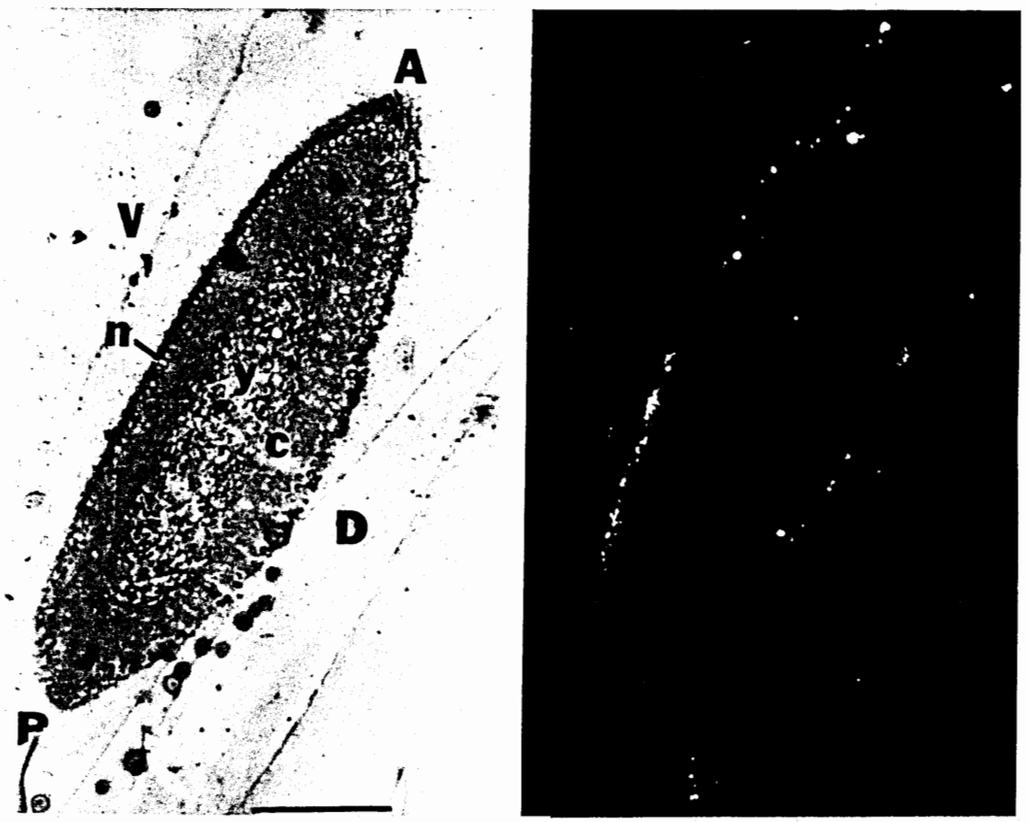
After one further nuclear division *ftz*$^+$ transcripts are still detected around the priphery of the embryo (Figures 22 and 23) and the labelled domain roughly corresponds to the junction of the head region and the first thoracic segment and the abdominal segments. However, regional differences in the signal intensity are observed within the labelled domain. Comparison of the distribution of silver grains in sections of different embryos at the same developmental stage indicates that the position of the most strongly labelled regions are variable within the labelled domain.



(a) (b)

Figure 20. Distribution of *ftz* $^{+}$ transcripts at the syncytial blastoderm stage in *Coelopa frigida* embryo.

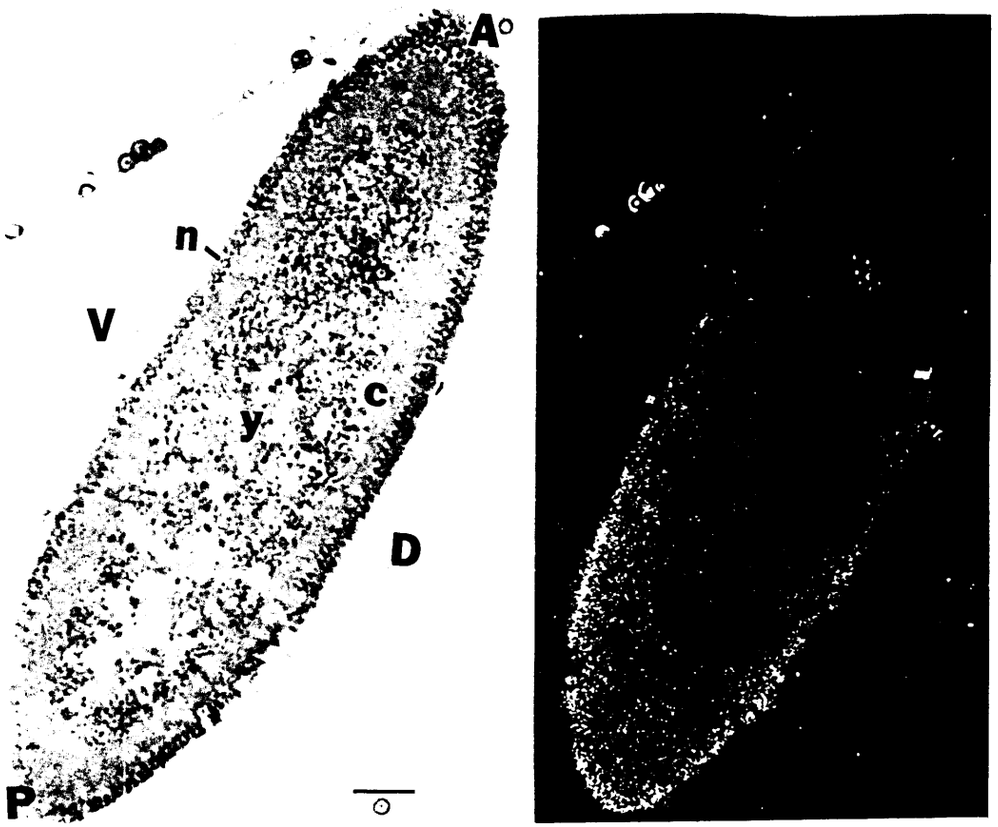
Tissue section of a 100-110 min old embryo, approximately after the completion of the 11th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 21. Distribution of *ftz* $^{+}$ transcripts at the syncytial blastoderm stage in *Coelopa frigida* embryo.

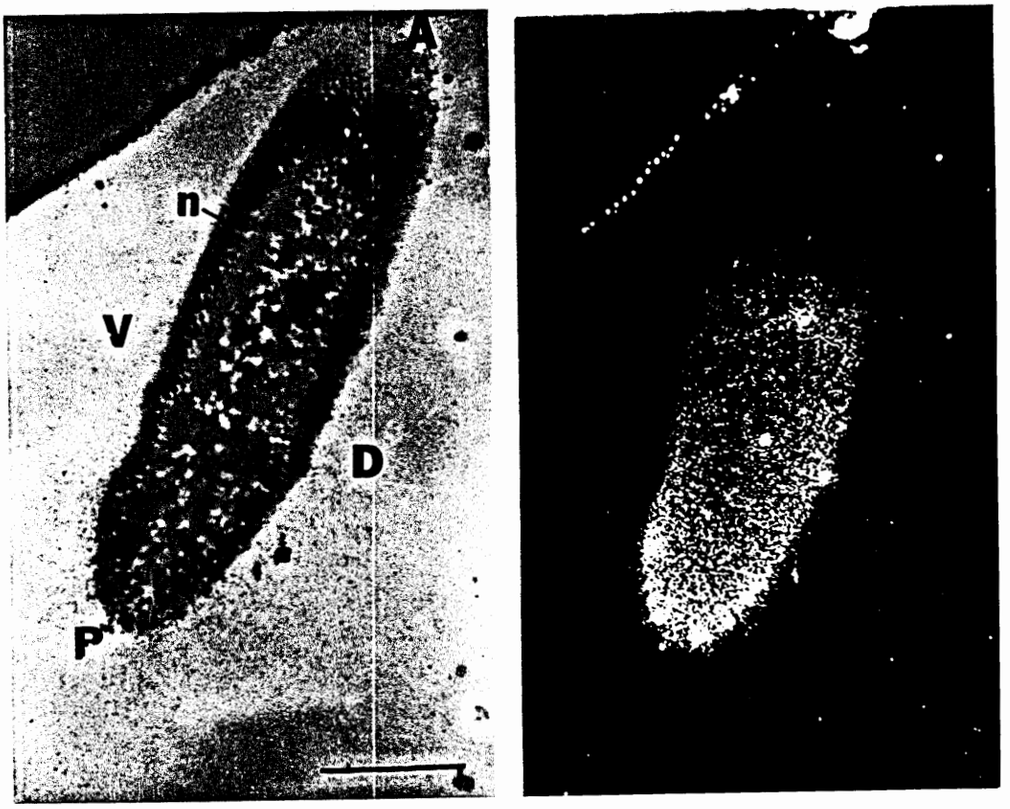
Tissue section of a 100-110 min old embryo, approximately after the completion of the 11th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 22. Distribution of *ftz* $^{+}$ transcripts at the syncytial blastoderm stage in *Coelopa frigida* embryo.

Tissue section of a 110-120 min old embryo, approximately after the completion of the 12th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 23. Distribution of *ftz* $^{+}$ transcripts at the syncytial blastoderm stage in *Coelopa frigida* embryo.

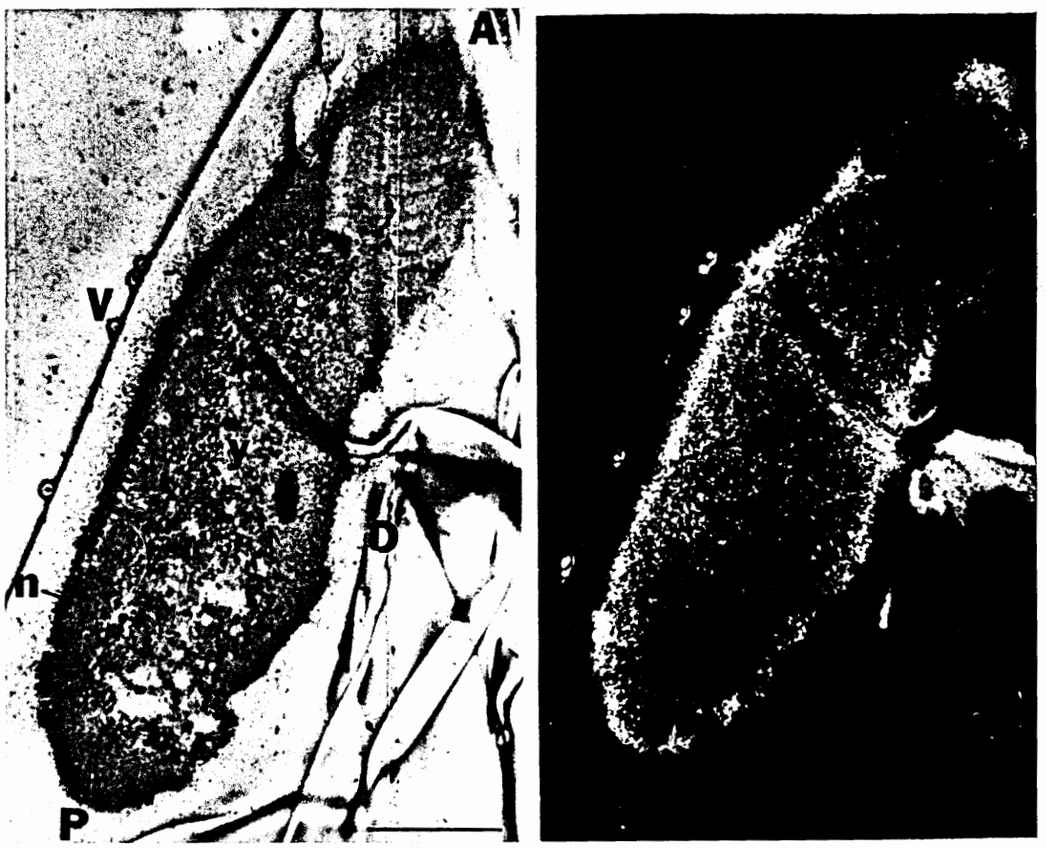
Tissue section of a 110-120 min old embryo, approximately after the completion of the 12th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a sagittal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei; y,yolk; c,cytoplasm. The horizontal bar indicates a length of 0.1mm.

Spatial Distribution of *Ftz*$^{+}$ Transcripts During Formation of the Cellular Blastoderm in the Kelp Fly Embryo.

A longitudinal section through an embryo that has completed the 13th nuclear division is shown in Figures 24 in bright- and dark-field illumination. The segmental pattern of the hybridization signal is more obvious at this stage. Figure 25a shows an unstained section of the same embryo . The signal is less distinct on the dorsal side. Seven distinct clusters of silver grains can be seen on the ventral side of the section and about the same number on the dorsal side. Figure 25b shows a magnified photomicrograph of a portion of the ventral periphery showing the clusters of silver grains. Each distinct cluster of silver grains corresponds to a width of about 5-6 nuclei at this stage. Between the clusters of silver grains, there is considerably less label in the blastoderm cells.

Gastrulation and Germ Band Formation.

Gastrulation and germ band formation in the kelp fly is as described for insects in general. Gastrulation begins as soon as cells on the ventral side of the embryo have completed cellularization. The process is initiated by the formation of a ventral furrow as a longitudinal cleft along the ventral midline of the embryo between 20 and 80% egg length. (Wieschaus and Nusslein-Volhard 1986). The cells at the posterior pole, including the pole cells, migrate dorsally and invaginate to form the posterior midgut rudiment. As the pole cells shift dorsally, the anterior and posterior dorsal folds begin to form. This stage is early gastrulation and occurs between



(a) (b)

Figure 24. Distribution of *ftz* $^{+}$ transcriptsduring the formation of the cellular blastoderm in *Coelopa frigida* embryo.

Tissue section of a 120 min old embryo, approximately after the completion of the 13th nuclear division; hybridized with a*ftz*$^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei. The horizontal bar indicates a length of 0.1mm.

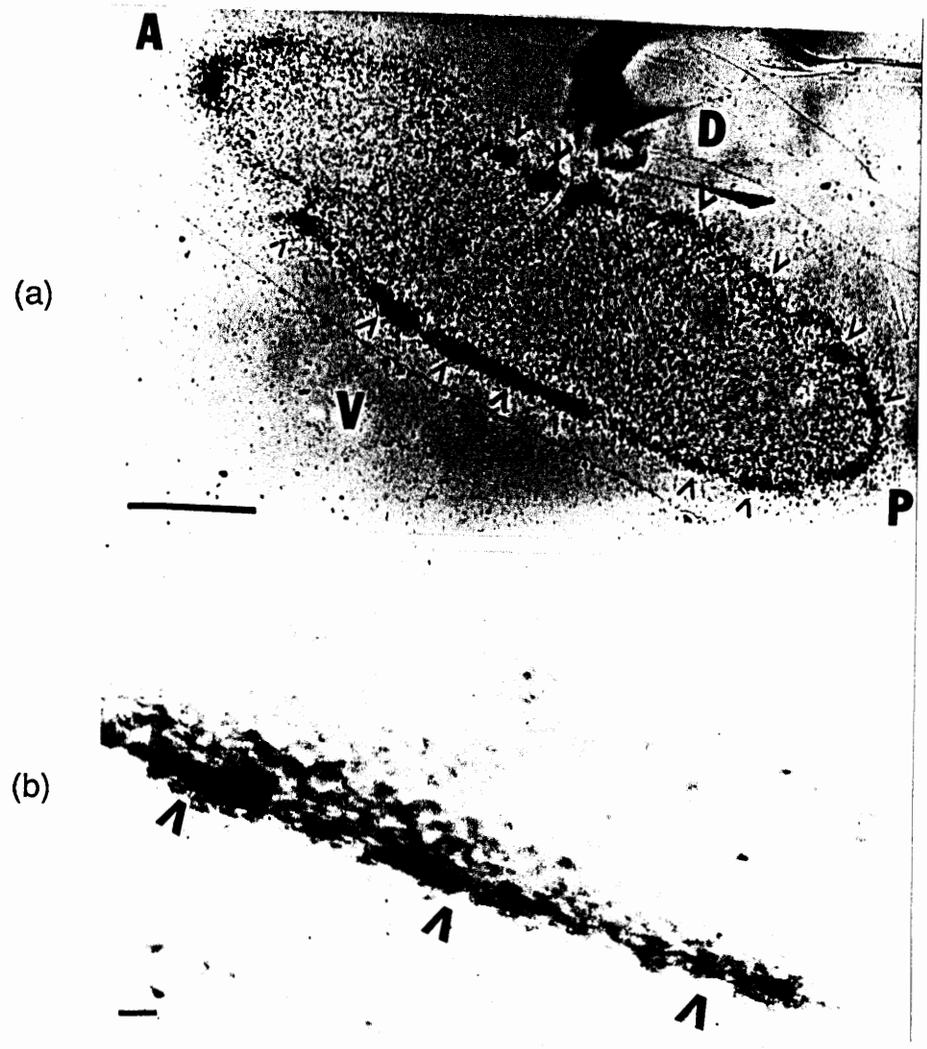
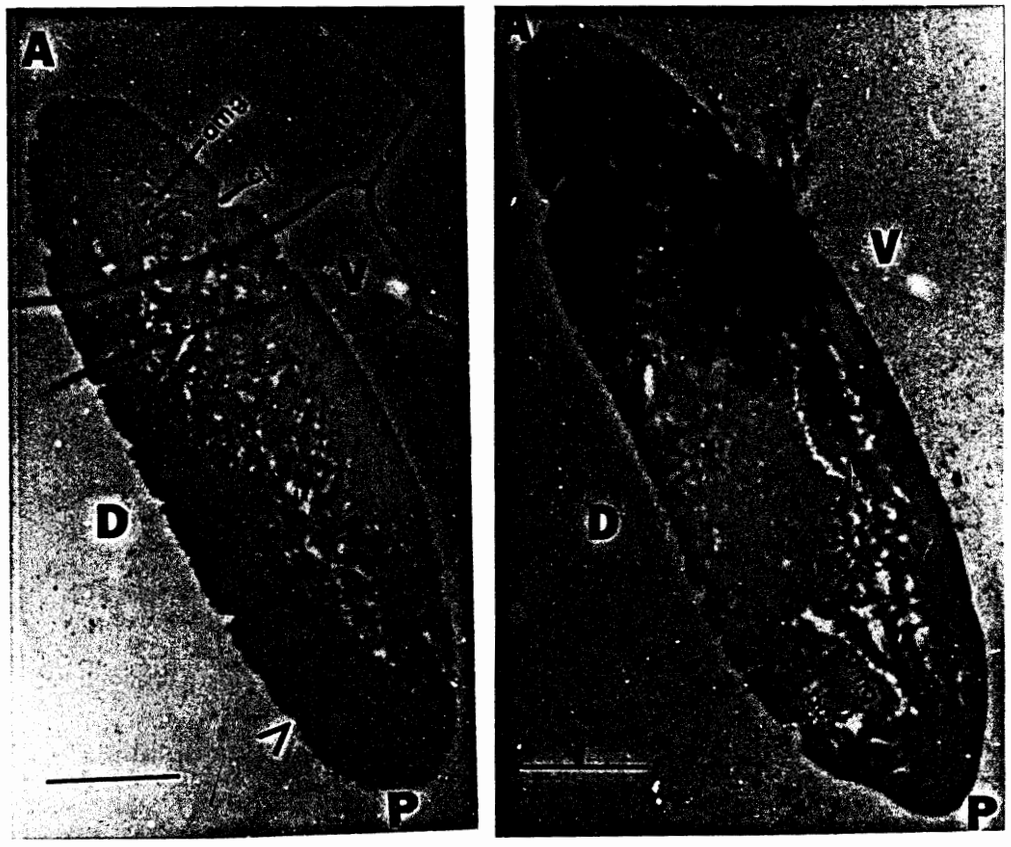


Figure 25. Distribution of *ftz* $^{+}$ transcripts during the formation of the cellular blastoderm in *Coelopa frigida* embryo.

Tissue section of a120 min old embryo, approximately after the completion of the 13th nuclear division; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section. (b) a magnified photomicrograph of the peripheral portion of the ventral aspect of the embryo. Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei. The horizontal bars indicate a length of 0.1mm.

195-215 min after oviposition in the *Drosophila* (Wieschaus and Nusslein-Volhard 1986). Following midgut invagination, a stage characterised by the disappearance of the pole cells into the posterior midgut invagination at 30% egg length, germ band extension begins. The cell layers which have invaginated in the ventral furrow flatten along the ectoderm forming a multilayered band (= the germ band) which curves around the posterior tip of the egg, elongationg along the dorsal side untill the point of posterior midgut invagination reaches the head region at 65% egg length. The cells between the opening of the posterior midgut invagination and the cephalic furrow thin out such that the dorsal folds gradually dissppear. The cephalic folds gradually disappear. The cephalic furrow is no longer easily visible. On the ventral side of the embryo, the germ band buckles into the interior of the embryo at the level of the cephalic furrow producing a transient gap between it and the vetilline membrane. This is the stage of germ band extension and occurs between 3 h 45 min and 4 h 30 min after oviposition (Wieschaus and Nusslein-Volhard 1986).

Figure 26 shows stages of the developing kelp fly embryo during gastrulation and germ band formation.



(a) (b)

Figure 26. Different stages during gastrulation in *Coelopa frigida* embryo.

(a) Lateral sagittal section of an embryo about 3-3.5 h old- arrow indicates bulking caused by forward movement (dorsally) by posterior plate.

(b) Longitudinal section of an embryo about 4-4.5 h old- germ band formation, the posterior midgut rudiment has deepened and extended dorsally,pole cells now in the concavity created by this deepening, the germ band is continous ventrally.

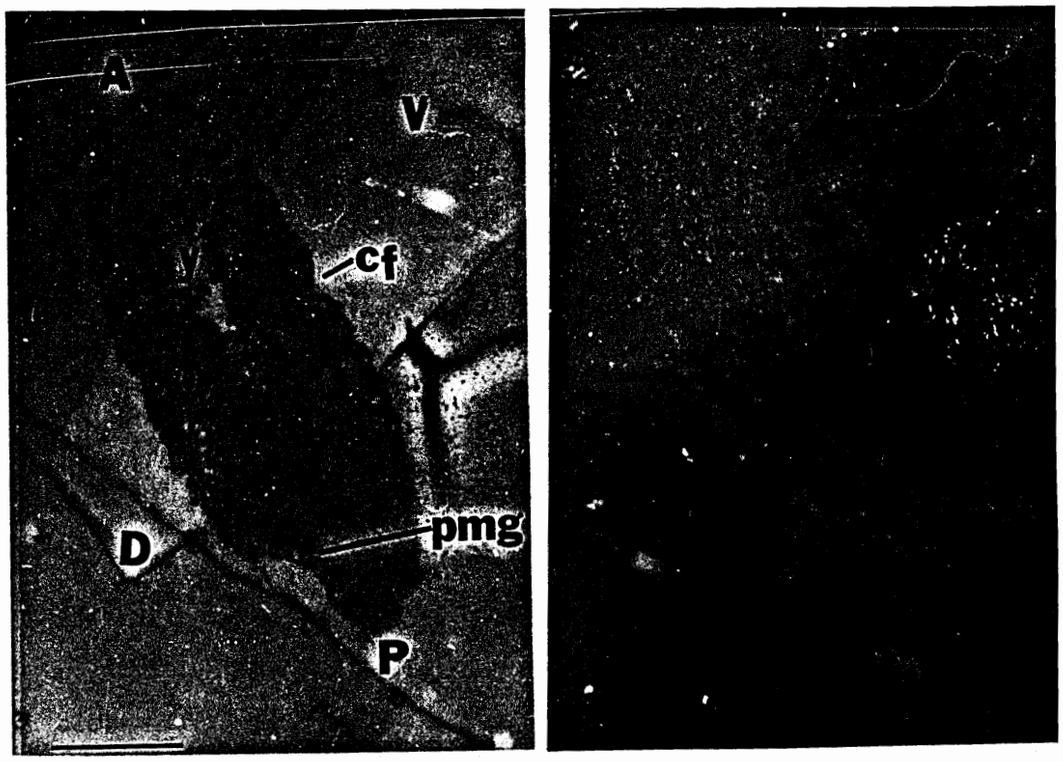
Abbreviations: y,yolk; p.c.,pole cells; cf,cephalic furrow; amg,anterior midgut rudiment; pgm, posterior midgut rudiment; gb,germ band; P,posterior; A,anterior; D,dorsal; V,ventral. The horizontal bar indicates a length of 0.1mm.

Spatial Distribution of *Ftz*$^+$ Transcripts During Gastrulation and Germ Band Formation in the Kelp Fly Embryo.

The spatial distribution of *ftz*$^+$ transcripts during gastrulation and germ band formation in the kelp fly embryo was investigated to determine transcript distribution after cellularization. Results obtained are shown in Figures 27 and 28. Figures 27a and 28a shows the bright-field photomicrographs of sections of hybridised embryos undergoing gastrulation and germ band extension while Figures 27b and 28b shows the corresponding dark-field photomicrographs. Figure 27 shows a saggital section section of an embryo about 185-190 min old undergoing gastrulation. As can be seen most of the cluster of silver grains has dissappeared with the exception of few small clusters at both sides of the section. Figure 28 shows a section of an embryo about 3.5-4 h old undergoing germ band elongation. All the clusters of silver grains have dissappeared and there are no detectable *ftz*$^+$ transcripts. Thus it seems that by the time germ band elongation sets in, the *ftz*$^+$ gene is no longer expressed in the *Coelopa* embryo.

Control Hybridizations.

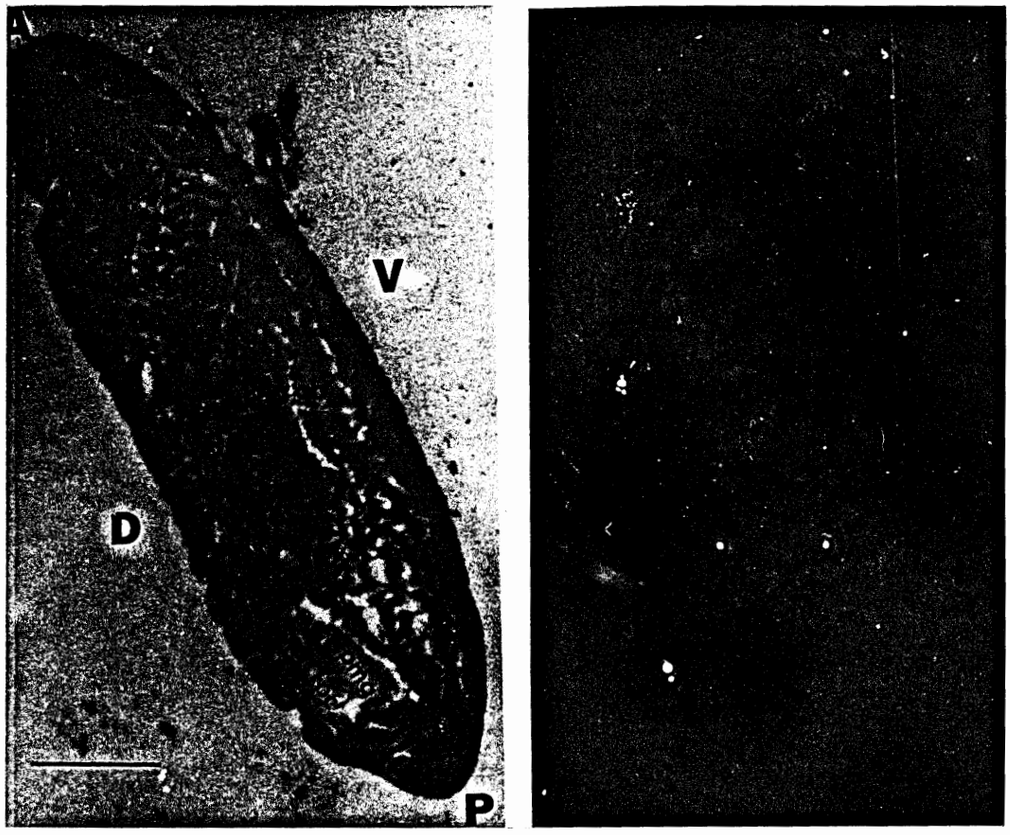
Control hybridizations using an anti-sense RNA probe synthesised from human *K-ras* oncogene were performed on embryos corresponding to those used for the *ftz*$^+$ probe hybridizations. Results obtained are shown in Figures 29 and 30. Figures 29a and 30a show bright-field illumination photomicrographs while the corresponding dark-field illumination



(a) (b)

Figure 27. Distribution of *ftz* $^{+}$ transcripts during gastrulation in *Coelopa frigida* embryo.

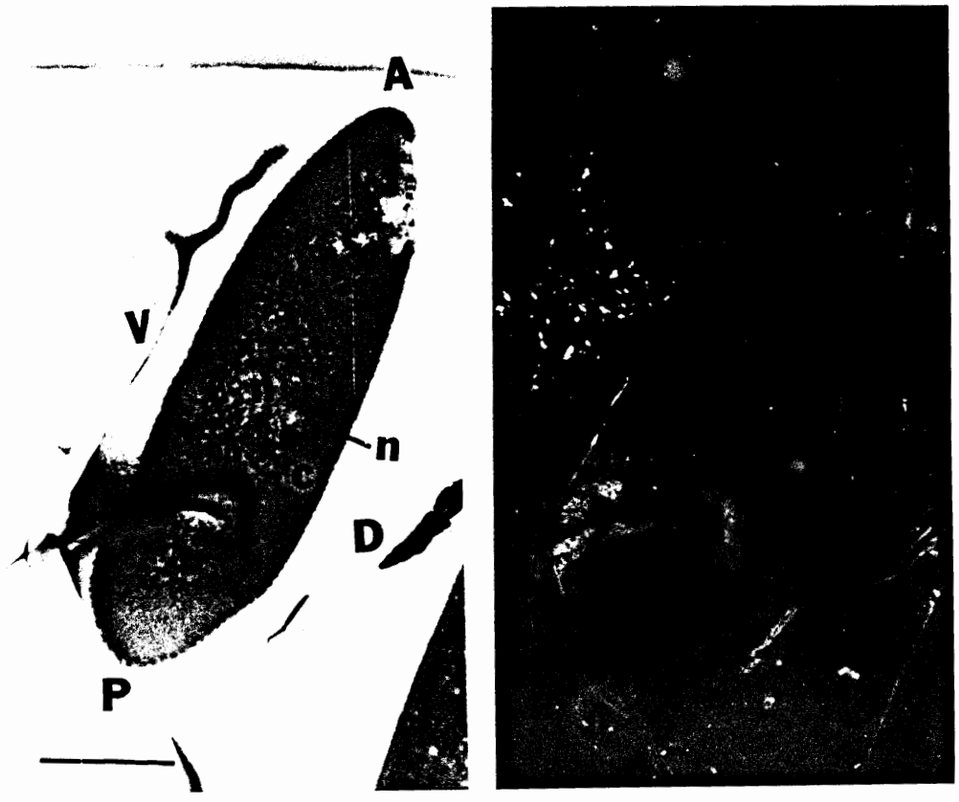
Tissue section of a 185-200 min old embryo; hybridized with a *ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a lateral sagittal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: y,yolk; cf,cephalic furrow; pgm, posterior midgut rudiment; P,posterior; A,anterior; D,dorsal; V,ventral. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 28. Distribution of *ftz* $^{+}$ transcripts during germ band formation in *Coelopa frigida* embryo.

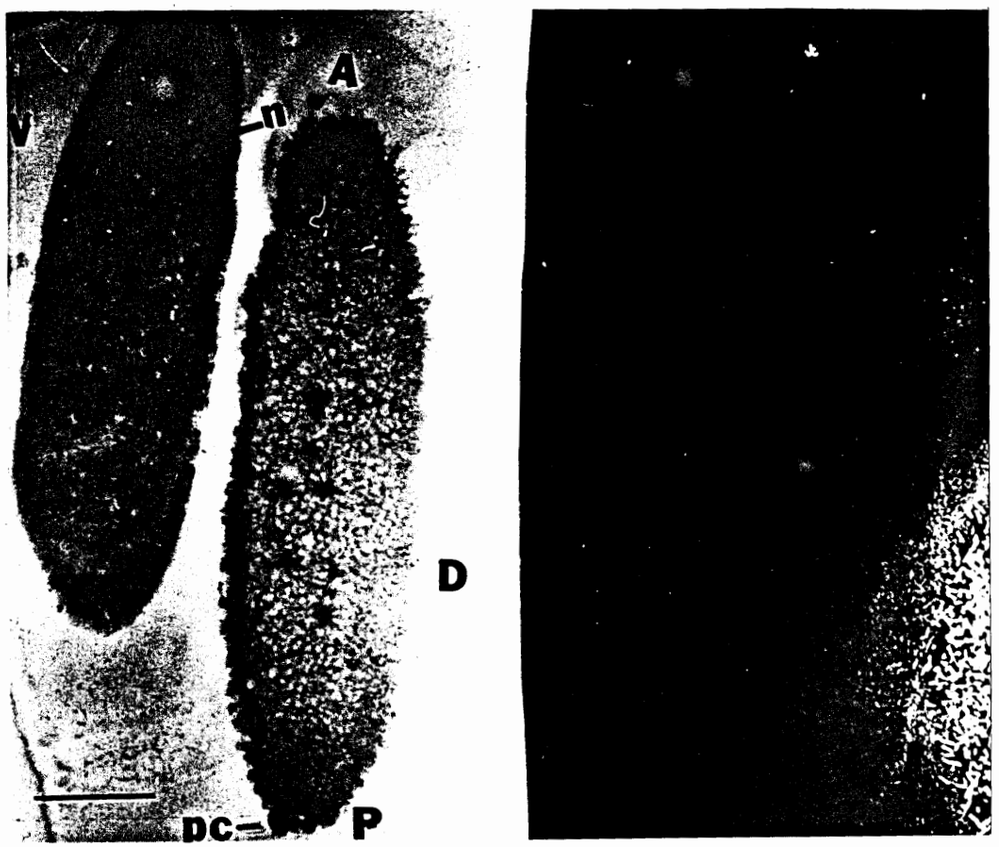
Tissue section of a 4-4.5 h old embryo; hybridized with a*ftz* $^{+}$ probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: p.c.,pole cells; pgm, posterior midgut rudiment; gb,germ band; P,posterior; A,anterior; D,dorsal; V,ventral. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 29. Distribution of Human *K-ras* transcripts during the syncytial blastoderm stage in *Coelopa frigida* embryo.

Tissue section of a 60-90 min old embryo, approximately after the completion of the 9th nuclear division; hybridized with a *k-ras* probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; n, nuclei. The horizontal bar indicates a length of 0.1mm.



(a) (b)

Figure 30. Distribution of Human *K-ras* transcripts before and during the syncytial blastoderm stage in *Coelopa frigida* embryo.

Tissue sections of a 120 and 40-50 min old embryos, approximately after the completion of the 13th and 7th nuclear divisions; hybridized with a *k-ras* probe, washed, embedded, sectioned and autoradiographed for 6 days. (a) A bright-field photomicrograph of a longitudinal section; the corresponding dark-field photomicrograph is shown in (b). Abbreviations: A, anterior aspect of the embryo; P, posterior aspect of the embryo; V, ventral; D, dorsal; p, pole cells, n, nuclei. The horizontal bars indicate a length of 0.1mm.

photomicrographs are shown in Figures 29b and 30b. With the exception of almost undetectable background radiation, these sections show no accumulation of silver grains, evenly distributed or restricted, as observed over corresponding sections when the *ftz* $^{+}$ probe was used.

CHAPTER IV

DISCUSSION

Embryos of many higher organisms are at least partially composed of repeated, morphologically identical units called metameres or segments. The origin and nature of the developmental information required for the formation of metameres, as well as the processes by which the developmental information within each segment functions to establish the future pattern of structures characteristic of each segment, are central issues in developmental biology.

At approximately 10-18 h of development the *Drosophila* embryo consists of a segmented head region, three thoracic segments, eight abdominal segments and a caudal region (Turner and Mahowald 1977). The thoracic and abdominal segments are approximately equal in width (Lohs-Schardin et al. 1979) and can be distinguished from one another late in embryogenesis by the pattern of ventral cuticular structures, such as the setae or denticles, and by the presence of morphological markers such as Keilin's organs (sensory structures which are the only evolutionary remnant of the larval legs and are closely associated with the discs that will construct the adult legs), ventral pits and tracheal pits.

Classical genetic analysis of mitotic clones has shown that each segment is composed of cells from two distinct lineages, the anterior and posterior compartments (Garcia-Bellido et al. 1976). Early in development, close to the blastoderm stage, the main mass of ectoderm is divided up into

alternating anterior (A) and posterior (P) polyclones of cells which will generate the A and P compartments (Garcia-Bellido et al. 1979). One anteriorly-located A and one posteriorly -located P polyclone constitutes the primordium of a segment (Lawrence 1981). In the adult an A/P compartment boundary extends through each segment and divides appendages into two parts. In the larva the A/P boundary cuts right through the Keilin's organs. The precise position of the segment boundary is not so well known, but in the abdominal segments it is near the anterior limits of the denticle bands of the cuticle (Szabad et al. 1979). In the fully developed embryo segments are defined traditionally by structural criteria alone; there is a deep groove in the epidermis near the A/P boundry and longitudinal muscles spanning the segments attach nearby.

A chain of alternating stripes can be grouped into pairs in two ways. In the ectoderm , segments are A/P compartments. The alternate PA pair, where P is the anteriorly located and A is the posteriorly located one, is called a parasegment. The subdivision into compartments and parasegments is important because it has been suggested that these groups of cells are the developmental units that function in pattern-refining steps and in events regulating homeotic gene expression (see Brower, 1985, for review).

Presence of the *Ftz* Gene in Kelp Fly Genome.

The first question that had to be answered in this investigation was whether the genome of the kelp fly has sequences homologous to the *ftz*$^{+}$ gene isolated from the genome of *Drosophila* . A variety of methods based

on nucleic acid hybridization are available to detect whether a particular DNA (or gene) sequence is present in the genome of a particular organism. The techniques do not require that the particular gene is expressed, only that the particular gene sequence is present. Such methods demand that a 'probe', either DNA or RNA, which has extensive sequence homology with the gene is available. The process of nucleic acid hybridization involves the formation of a double helical molecule from two separate populations of denatured preparations. Hybridization is a method of recognizing complementary sequences. DNA-DNA hybridization can be used to evaluate the evolutionary relatedness of different organisms, for recognizing the presence of a sequence of a part of a replicon in another genome and in the mapping of restriction fragments. DNA-RNA hybridizations can be used for probing for the presence of a transcribed strand of DNA with its RNA transcript. DNA-RNA hybridization is also frequently used to measure the number of genes for a particular RNA transcript.

To determine whether the genome of the kelp fly contains sequences homologous to the *Drosophila ftz* $^{+}$ gene, the procedure of choice here was dot hybridization. This involved hybridization of a radioactive probe in solution to denatured DNA immobilised on nitrocellulose filters. High molecular weight kelp fly genomic DNA was succesfully extracted from adult flies, denatured by heating, dotted on nitrocellulose filter, the filter baked to immobilise the DNA and a ^{32}P-labelled *ftz* $^{+}$ RNA probe hybridized to the filter in solution. A ^{32}P-labelled*ftz* $^{+}$ DNA probe was also

hybridized to another filter prepared as above so that the sensitivities of the two probes can be compared. While the presence of sequences homologous to the *Drosophila ftz* $^{+}$ gene was detected in the genome of the kelp fly using the RNA probe, the results obtained (Figures 9 and 10) indicate that the same concentration of the kelp fly genomic DNA extract did not allow detection of the presence of the gene by the DNA probe. This confirms reports that RNA probes are more sensitive than DNA probes (Cox et al. 1984).

Are *Ftz* $^{+}$ Transcripts of Maternal Origin in Kelp Fly Eggs ?

From *in situ* hybridization experiments, Hafen et al. (1984) has reported that transcripts of the *ftz* $^{+}$ gene were first detected during nuclear cleavage prior to cell formation in the embryo of *Drosophila.* There seem to be low levels of *ftz* $^{+}$ transcripts which appear to be evenly distributed in the egg at these early embryonic stages. If this is true, then this even distribution and the fact that the first 9 nuclear cleavages in *Drosophila* occur without new RNA synthesis (Mahowald and Hardy 1985), suggest a possible maternal origin of these transcripts. Thus having confirmed that the genome of the kelp fly has sequences homologous to the *Drosophila ftz* $^{+}$ gene, the next step in this study was to investigate whether the even distribution of *ftz* $^{+}$ transcripts in the egg at these early embryonic stages is due to the synthesis of *ftz* $^{+}$ transcripts during oogenesis.

Detection of RNAs is accomplished by annealing complementary nucleic

acid probes to unique sequences on the target RNA. Probes can be either double stranded DNA or single stranded RNA. Comparisons indicate that single stranded probe hybridizes to target RNA under saturating conditions eightfold more efficiently than double stranded probes (Cox et al. 1984). Single stranded RNA probes offer several advantages. RNA-RNA hybrids are the most stable nucleic acid duplexes (Cox et al. 1984), and therefore wash conditions can be very stringent. Additionally, the tissue can be post-treated with RNase to eliminate unhybridized probe RNA, and thus leading to reduction in background and an increase in the detection sensitivity.

To determine whether *ftz* $^{+}$ transcripts are synthesised maternally, kelp fly oogenic RNA was extracted from matured eggs, dotted on nitrocellulose filter, the filter baked to immobilise the RNA and a ^{32}P labelled *ftz* $^{+}$ RNA probe hybridized to the filter in solution. The result (Figure 12) showed a negative reaction. If there were any *ftz* $^{+}$ transcripts in the RNA extract it could not be detected by the assay used here. It should however be pointed out that since total RNA was extracted, if the *ftz* $^{+}$ transcripts were in very minute quantities, it is quite possible that they could not be detected, because of their low concentration in the total RNA pool.

Expressive Pattern of the *Ftz* $^{+}$ Gene in *Coelopa : In Situ* Hybridizations.

To obtain precise information about the spatial distribution and the time of appearance of *ftz* $^{+}$ transcripts during normal *Coelopa* development

I have used *in situ* hybridization of an anti-sense *ftz* $^{+}$ RNA synthesised by transcription of the *ftz* $^{+}$ gene in a cell-free system to RNA contained in whole embryos and in tissue sections of wild type *Coelopa frigida* embryos.

Recent success in molecular cloning of a variety of genetic loci from the *Drosophila* genome has created a demand for methods that permit precise determination of the spatial and temporal limits of gene expression. Nucleic acid hybridization provides the necessary specificity for accurately monitoring the expression of specific eukaroyotic genes. Perhaps the most direct method for identifying the specific cells of an organism that express a given gene is to localise, by *in situ* hybridization, the RNAs that are specified by the gene within serial sections.

Different protocols of*in situ* hybridization provide a range of sensivities. The sensitivity of the *in situ* hybridization depends on the extent to which the RNAs are retained during the preparation of the cultured cells or the tissue sections, the specific activity of the radiolabelled probe used for the RNA detection, and the accessibility of the target RNA species for interaction with the hybridization probe. A number of methods have been described for the localization of specific RNA species in cultured cells and in serial tissue sections. DNA and RNA probes used in these studies were labelled with ^{3}H, ^{35}S or ^{32}P. In addition, some protocols involved the use of biotinylated DNA probes (Singer and Ward 1982). Signal detection for radiolabelled probes was achieved by autoradiography, whereas the detection of biotinylated probes involves immunofluorescent or immunohistochemical

stains.

Most studies of the parameters affecting *in situ* hybridization have indicated that similar considerations apply as for hybridization to nucleic acids fixed on nitrocellulose filters. Therefore conditions of ionic strength, temperature, probe concentration and hybridization time were chosen for each experiment in the same way as one would choose them for filter hybridizations. The conditions chosen determine the stringency of the hybridization reaction and set limits on the amount of sequence mismatches that can occur. The factors below were some of the more important ones considered in designing procedures for these experiments using the *ftz*$^+$ RNA probe. (a) Tm: the temperature at which 50% of the hybrid molecules are dissociated. This is higher for RNA-DNA and RNA-RNA hybrids than for DNA-DNA hybrids. (b) $C_o t$: the extent of hybridization under standard conditions of salt concentration, pH and temperature is determined by the product of the concentrations of the sequences involved and the incubation time ($C_o t$). The optimal temperature is approximately 25 ^oC below the Tm, and at this temperature the effects of salt concentration above 0.4 M and pH inside the range of 3.0 to 9.0 are limited. (c) Stringency: the stringency of a hybridization reaction determines how closely related two sequences need to be to form a stable hybrid. (d) Signal to noise: it is obviously important to have good signal to noise ratio in any system. The fact that a single stranded RNA probe was used allowed this ratio to be improved in two ways. Firstly RNA-containing hybrids are more stable than those containing only DNA thus allowing higher temperatures to be used. Secondly, the

enzyme ribonuclease A was used to digest unhybridized probe because it degrades single stranded RNA but not RNA in a hybrid with either DNA or RNA.

In situ hybridization can be carried out either in salt solution at high temperature (e.g. 68 ^{o}C) or in formamide at lower temperatures (e.g. 50 ^{o}C). For hybridization at moderate stringency of sequences of average base composition, hybridization in aqueous salt solution is carried out typically in 0.3 M NaCl, 20 mM Tris-HCl, (pH 6.8) at 67 ^{o}C. Hybridization in formamide solution is typically carried out in 40% formamide (de-ionized) in 4X SSC at 40 ^{o}C. Post-treatment of the preparation to remove non-hybridized probe can be achieved by washing in 2X SSC at a temperature 5 ^{o}C less than the hybridization temperature (if a DNA probe is used) or by treatment with RNAse A at 37 ^{o}C (if an RNA probe is used) at a concentration of 20 ug/ml and rinsing in 2X SSC. These washes can be made more stringent by raising the temperature or by lowering the salt concentration.

In situ hybridization procedures using frozen sections usually involves lengthy pretreatments with HCl, high temperature washes and protease digestion. These treatments are intended to free target RNA for probe hybridization. Incubation of slides in dilute HCl is thought to disrupt polysomes and to partially hydrolyse proteins. Treatment with protease partially digests proteins that may interfere with efficient hybridization. Tissue sections on slides are often lost or damaged in the severe hybridization and wash conditions. Hybridization to whole tissues

followed by sectioning avoids many of these problems and gives excellent morphology. In fact, protein digestion and high temperature washes may be destructive to tissue morphology and, from my own observations, lead to loss of slide-mounted tissues. Thus, eliminating these steps improved the reproducibility of the technique. There are no reports of *in situ* hybridization procedures in the kelp fly embryo in the literature. I therefore decided to use two approaches for *in situ* hybridization both of which I hoped would give better morphology than frozen sections. These were: (a) hybridizing whole fixed embryos, embedding them in plastic and sectioning them for autoradiography; and (b) embedding fixed embryos in plastic, sectioning embryos, hybridizing sections and then performing autoradiography. To my knowledge the second procedure has not been done in any insect embryo or any other tissue.

Fixation for *in situ* hybridization must satisfy two, at times conflicting, requirements. Tissue morphology must be maintained and the mRNAs anchored in position, but proteins must not be so highly crosslinked that probe penetration is obstructed. In *Coelopa*, the chorion and the vitelline membrane may block fixative penetration so they were removed by incubation in bleach and by heptane/methanol shock respectively. Staged embryos were succesfully fixed after removal of the chorion and vitelline membrane. Four percent paraformaldehyde in PBS proved to be the fixative superior to acetic acid/ethanol.

For embedding I chose to use an immuno-histochemistry plastic embedding medium. Immuno-bed (as it is commercially available) allows

easier penetration of large immunoglobulins through the section for demonstration of antigenic sites. It gives improved results with histochemical stains and 1-2 micron sections offer superior morphological differentiation compared to paraffin or frozen sections (Polysciences, Inc. 1983).

The radioisotopes most commonly used for *in situ* hybridizations are ^{3}H (tritium) and ^{35}S. Tritium offers better resolution than ^{35}S because the mean pathlength is shorter (because of the extremly low energy of the beta particle that is emitted, 0.0181 MeV) than that of beta particles emitted from ^{35}S. These particles travel less than 1 μm through the autoradiographic emulsion, ensuring that the silver grains of the autoradiogram remain closely associated with the site of the radioactive molecule). But the exposure times for rare messages can be prohibitively long when tritium is used. Signal resolution for ^{35}S is satisfactory down to the level of cells but does not localise signals within the cell (Rogers 1979). Since I was interested in labelling cells that contain *ftz* $^{+}$ transcripts the radioisotope of choice here was ^{35}S. In order to obtain hybridization probes of high specific activity, I used ^{35}S nucleotides (UTPs) of the highest available specific activity (850 Ci/mmol).

Before any *in situ* hybridizations were done, staged embryos were fixed, embedded and sectioned. Results obtained (Figure 14) showed that staging, fixation and the embedding procedures I was using yielded good cytological preparations that accurately reflected the cytological events of nuclear distribution and cellularization of the blastoderm during the

respective developmental stages to be analysed for gene expression.

Having decided on methods for fixation, embedding and radioactive labelling, the spatial distribution of *ftz* $^{+}$ transcripts during embryonic development of *Coelopa* was investigated using the two different approaches mentioned above. In the first procedure whole fixed embryos were hybridized, the embryos embedded in immuno-bed and sectioned. In the second approach fixed embryos were embedded in immuno-bed (for plastic sections), then sectioned and the sections hybridized. In both procedures the signal detection was achieved by autoradiography.

Hybridization to whole embryos proved to be a superior and consistent procedure for obtaining good cytological preparations and yielding successful, reproducible hybridizations with minimum background.

In Situ Hybridization in Whole Embryos.

For hybridization to whole embryos standard hybridization conditions (Hafen and Levine 1986) were modified to include 10 mM DTT to maintain a reducing enviroment for the ^{35}S-label probe, 1% SDS to reduce non-specific probe retention, and 10% polyethylene glycol(PEG) to accelerate hybridization (Jorgensen and Garber 1987). The efficiency of hybridization for RNA probes in whole tissues improves sharply with PEG compared to dextran sulphate which is believed to act by concentrating the probe by exclusion from the volume of the solution occupied by the polymer (Pardue 1986).

After trying unsuccesfully to hybridize whole embryos using the probe as

synthesised (probe length of about 400 bases), I decided to use probes of shorter lengths, since I suspected that probe penetration was possibly been obstructed by the length of the probe. To obtain probes of optimal size (mean single-stranded length of about 50-100 nucleotides according to Roberts, 1986), the *ftz* $^{+}$ RNA probe was reduced in size by alkaline hydrolysis and shorter probe lengths used for *in situ* hybridizations. Embryos were hybridized with different sizes of the probes obtained from partial hydrolysis. The probe size obtained after 30 min of partial hydrolysis proved to be superior and gave the highest hybridization signals with minimum background. It was thus assumed that hydrolysis for 30 min produced probes of optimal size (i.e. probe of mean single-stranded length of about 50-100 nucleotides (Roberts 1986)).

Results obtained from hybridization to whole embryos (Figures 16-25) show that the *ftz* $^{+}$ gene is expressed in a segmental manner in the *Coelopa* blastoderm and that this segmental pattern of expression is established gradually during nuclear cleavage.

Coelopa frigida embryos from 30 min to about 90 min of development show an even distribution of *ftz* $^{+}$ transcripts in the egg (Figures 16-19). It can be safely assumed that since there are no regional differences in *ftz* $^{+}$ transcript distribution, if transcription is occurring at these stages, it is not restricted to specific egg regions.

The first sign of restricted transcription was observed in embryos about 100-110 min into development. This is around the 11th nuclear division, the syncytial blastoderm stage. Sections from embryos at this

stage showed a higher accumulation of silver grains around the periphery, mostly around the middle portion of the embryo and little or no label at the poles or in the central yolk (Figures 20 and 21). These transcripts are believed to be newly synthesised transcripts, since they have been observed over nuclei in *Drosophila* (Hafen et al. 1984). It is thus clear that the *ftz*$^+$ transcription pattern is established during the syncytial blastoderm stage in *Coelopa* and that transcripts are detected in the peripheral cytoplasm and over nuclei in the middle portion of the embryo during the two nuclear divisions prior to cell membranes formation. At this stage transcripts appear evenly distributed within the domains where they are found.

After the next nuclear division the *ftz*$^+$ transcripts are still detected around the periphery but the sections exhibit a higher accumulation of silver grains between approximately 10% and 70% of the embryo (the zero point being the posterior pole) (Figures 22 and 23). This corresponds roughly to the boundary between the head region and the first thoracic segment and the abdominal segments. At this stage regional differences in the amount of *ftz*$^+$ transcripts (reflected by differences in the intensity of the signal) are observed within the formerly homogenously labelled domain. Thus the segmental pattern of *ftz*$^+$ transcription is established gradually during nuclear cleavage.

The segmental pattern of the hybridization signal becomes apparent in sections of embryos that have completed the 13th nuclear division and have begun the formation of the cellular blastoderm (Figures 24 and 25). Distinct clusters of silver grains are observed and can be counted both on

the dorsal side and the ventral side of the section. At least seven clusters can be counted on both sides of the section, each cluster encompasses 3-5 nuclei spaced by a stretch of about the same number of unlabelled nuclei.

As stated previously, the peripheral nuclei start to elongate after the completion of the 13^{th} nuclear division and cell membranes grow inward from the surface enclosing the nuclei. A longitudinal section through an embryo in the process of nuclear elongation, before the completion of cell formation is shown in Figure 25a. At this stage the distribution of *ftz*$^+$ transcripts shows a regular pattern of seven clusters on each side of the section separated by blastoderm regions with lower amounts of the transcripts present.

Sections of hybridized embryos during gastrulation and germ band formation show the gradual dissappearance of the clusters of silver grains in the gastrulating embryo and complete dissappearance of *ftz*$^+$ transcripts during germ band elongation (Figures 27 and 28). Thus it appears by the time segmentation is first obvious and the germ band is fully extended (about 4.5 h of development) the *ftz*$^+$ gene is no longer expressed.

Comparision of Patterns of *Ftz*$^+$ Expression in *Coelopa* and *Drosophila.*

It is not known how the spatial coordinates of the egg are defined or what the nature of these determinants of spatial organization is. Transplantation of syncytial blastoderm nuclei with adhering cortical cytoplasm to ectopic sites in another embryo of the same age suggests that these nuclei have acquired at least an anterior versus posterior commitment

(Kaufman 1980).

After fertilization, the fly egg first develops as a syncytium of synchronously dividing nuclei that subsequently migrate to the periphery of the egg, where they forms a monolayer of cells- the blastoderm. Cell lineage (Wieschaus and Gehring 1976), transplantation (Illmensee. 1978), ablation (Underwood et al. 1980), and embryo manipulatation studies (Chan and Gehring 1971; Schubiger et al. 1977) indicate that the developmental fates of embryonic cells are determined at the cellular blastoderm stage (2.5-3.5 h of development), and that cell determination at the blastoderm stage is segment specific. Gynandromorph studies and nuclear transplantation experiments indicate that the cleavage nuclei are totipotent (Sturtevant 1929, Zalokar 1971, Okada 1974, Illmensee 1978). However, when the clevage nuclei reach the periphery and cellularization occurs, the blastoderm cells are determined. This indicates that there is an interaction between the cortical egg cytoplasm and the migrating nuclei which leads to the determination of these nuclei. The process of segmentation which becomes visible only later in development, partitions certain groups of cells into developmentally autonomous units or compartments (see Lawrence, 1981, for review; Garcia-Bellido 1977).

In normal embryos segmentation becomes morphologically visible at the time of maximal germ band elongation at 7-8 h of development (Underwood et al. 1980). However cell lineage studies indicate that determination of segmental boundaries occur as early as the blastoderm stage (3 h) (Wieschaus and Gehring 1976, Steiner 1976, Lawrence and

Morata 1977). The time of *ftz* $^{+}$ expression concides with the time of segment determination during blastoderm formation and gastrulation. This study in *Coelopa frigida* and *in situ* hybridizations in *Drosophila* show that restricted transcription of the *ftz* $^{+}$ gene begins at about the 11th nuclear division; at the blastoderm stage the transcripts have accumulated in seven evenly spaced and sharp stripes, with much less transcript in the cells between them. The studies in *Drosophila* also show that each stripe is about two compartments wide and the registration of the stripes suggests their prospective localization in the larva is closer to parasegments than to segments. By the end of germ-band extension, when the first signs of segmentation are visible, the stripes obtained by hybridization are no longer detectable (Hafen et al.1984). The spatial pattern of *ftz* $^{+}$ transcripts in the wild type embryo correlates well with the mutant phenotype (Hafen et al.1984). The *ftz* $^{+}$ transcript and protein are localized in seven stripes at the cellular blastoderm stage at a double segment periodicity in *Drosophila* (Carrol and Scott 1985, Hafen et al. 1984), and these stripes are coincident with the primordia of the deleted pattern elements in the *ftz* mutant embryo (Wakimoto et al. 1984, Carrol and Scott 1985, Hafen et al. 1984, Ingham and Martinez-Arias 1986). The *ftz* $^{-}$ phenotype consists of the elimination, due to cell death, of seven units which are approximately localised in regions corresponding to parasegments (Nusslein-Volhard et al.1982).

Careful examination of *ftz* $^{-}$ embryos in *Drosophila* (Nusslein -Volhard et al. 1985) has revealed that, with occasional exceptions, each of the

alternating zones that fail to appear in *ftz*$^-$ embryos consists of the anterior portion of an odd numbered segment combined with the posterior portion of the adjacent even-numbered segment. To use the terminology of Martinez-Arias and Lawrence (1985), structures from alternate "parasegments" fail to appear in *ftz*$^-$ embryos. Thus homozygous *ftz*$^-$ mutants lack the posterior part of one segment and the anterior part of the next segment and show a pair-wise fusion of the remaining parts. In *ftz*$^-$ embryos each double-width segment has only one set of denticle belts, with extra rows of satae (Wakimoto et al. 1984). Thus mutations at the *fushi tarazu* locus in *Drosophila* affect both segment number and the pattern of cuticular structures on alternating segments of embryos. Even though the physical process of segmentation and the development of cuticular structures occur between 6 and16 h of embryonic development, the *ftz*$^+$ transcript and the functional gene product are required for the establishment of conditions necessary for later processes such as complete segmentation and cellular differentiation, but are apparently not necessary for the mainteinance of these conditions (Weinet et al. 1984). The *ftz*$^+$ protein is probably only necessary at the blastoderm stage.

The results obtained so far in this study have led to the following conclusions:

1. The genome of the kelp fly, *Coelopa frigida* , contains sequences homologous to the *Drosophila ftz*$^+$ gene.
2. The *ftz*$^+$ gene is probably not expressed maternally in the kelp fly.
3. In the *Coelopa* embryo the *ftz*$^+$ transcripts are generally distributed

during early cleavage; at these stages the transcripts are of low intensity and there seem to be no regional differences.

4. *Ftz* $^{+}$ transcripts become localized around the periphery of the embryo around the 11^{th} nuclear division (syncytial blastoderm); transcripts after the 12^{th} nuclear division are generally restricted to a broad region between approximately 10 and 70% of the egg length.
5. After about the 13^{th} nuclear division when cell membranes begin to form and even before cells are formed, the parasegmental pattern of *ftz* $^{+}$ transcript distribution in the seven odd numbered parasegments becomes more apparent.
6. It appears that by the time segmentation is first obvious and the germ band is fully extended (about 4.5 h of development) the *ftz* $^{+}$ gene is no longer expressed.
6. Similar results have been observed in *Drosophila* wild type embryos, Therefore the embryos used in these experiments were expressing the same dipteran gene at the corresponding morphological stages of development.

Some general conclusions that can be drawn from this study include the following:

1. The data obtained on *ftz* expression graphically illustrate that spatially restricted gene expression can occur even at the syncytial blastoderm stage in the absence of cell membranes.
2. They confirm that a structrual association between nuclei and their adjacent cytoplasm exists at this stage.

3. These observations provide direct molecular corroboration for the notion that differences in developmental potential already exist between nuclei prior to the cellular blastoderm completion.

Pattern Formation.

Pattern formation appears to occur as a sequential process. Thus, a coarse early pattern (the gap pattern) is resolved into a finer (the pair-rule pattern) and finer (the segmental pattern) pattern. The pair-rule gene products regulate the expression of *en* to produce a pattern that is finer (14 stripes) than the spatial pattern of the pair-rule gene products (e.g. 7 stripes of *ftz*). The repeating segment pattern is defined by the persistent expression of *en* and other genes of the 'segment polarity' class. The establisment of this pattern is directed by a transient molecular prepattern that is generated in the blastoderm by the activity of the 'pair-rule' genes. Maternal determinants at the poles of the egg coordinate this prepattern and define the anteroposterior sequence of pattern elements. The primary effect of these determinants is not known, but genes required for their production have been identified and the product of one of these, *bicoid* , is known to be localised at the anterior pole of the egg (see Akam, 1987, for review). One early consequence of activity of maternal genes is to define domains along the antero-posterior axis within which a series of 'cardinal' genes are transcribed.

The activity of the cardinal genes is required both to coordinate the

process of segmentation and to define the early domains of homeotic gene expression. Further interaction between the homeotic genes and other classes of segmentation genes refine the initial establishment of segmental identities. Of 17 genes examined in *Drosophila* that visibly affect embryonic pattern formation, 7 have been shown to influence the initial pattern of *ftz* protein expression at the cellular blastoderm stage of embryogenesis (Carrol and Scott 1986, 1986b). These include all 4 gap loci: *Kruppel* (*Kr*), *knirps* (*kni*), *hunchback* (*hb*) *and giant* (*gt*) *and 3 pair-rule loci: hairy* (*h*), *runt* (*run*) and *even-skipped* (*eve*). Five pair-rule loci: *odd-skipped* (*odd*), *paired* (*prd*), *odd-paired* (*opa*), *sloppy-paired* (*slp*) *and engrailed* (*en*) do not affect the *ftz* pattern. Two segment polarity loci, *hedgehog* (*hh*) and *patched* (*ptc*) and three other genes (unclassified) that affect larval cuticular patterns, *unpaired* (*upd*) *naked* (*nkd*) *and branch* (*bch*) also have no effect on expression of *ftz*. This evidence suggests that *ftz* operates in the segmentation gene hierarchy below or in parallel with the four gap and three of the pair rule genes, but above or independently of the other pair-rule and segment polarity genes. It is also clear that along the length of the embryo, *ftz* expression is not under the control of any single gene. Rather, the expression of *ftz* is regulated by the array of other segmentation genes that are active or inactive in each cell of the embryo. It is also possible that genes regulating *ftz* also regulate each other; thus the observed changes in *ftz* expression could be the outcome of the interaction between the mutant gene and other segmentation genes, and may simply reveal direct

effects on *ftz*.

In *ftz* mutant embryos structures in alternating segments are missing (Wakimoto and Kaufman 1981; Nusslein-Volhard et al. 1982). Comparison of the positions of the blastoderm cells that accumulate *ftz*$^+$ transcripts with the fate map of the segmental anlagen strongly suggests that these cells are the progenitors of the segmental portions that are missing in *ftz* mutant embryos (Hafen et al. 1984). It appears that *ftz*$^+$ expression is required in the progenitors of those structures to ensure their proper development. However, the *ftz*$^+$ gene is not expressed in the intermediate segment primordia which develop normally in the *ftz* mutant embryos. It is possible that there are other genes which exhibit an expression pattern that is out of frame with the *ftz*$^+$ expression pattern. Such genes will be expressed in the intermediate regions where *ftz*$^+$ transcripts are not detected. Good candidates are other members of the pair-rule class of segmentation genes, such as the *even-skipped* (*eve*) gene. Embryos homozygous for *eve* exhibit a cuticular pattern that is complementary to the *ftz* pattern (Nusslein-Volhard and Wieschaus 1980).

Ftz$^+$ is transiently expressed a second time during embryogenesis in *Drosophila* in a segmentally repeated pattern in the developing CNS (Doe et al. 1988). After the *ftz*$^+$ protein disappears from the embryos during germ band expansion, it reappears in a subset of the nuclei in every segment of the developing nervous system (Carol and Scott, 1985). Given the role that this gene may play in cell determination at the blastoderm stage, it is suspected that it might be involved in cell determination during

neurogenesis. In the absence of *ftz* $^{+}$ CNS expression, some neurons appear normal (for example, the aCC, pCC, and RP1), whereas the RP2 neuron extends its growth cone along an abnormal pathway, mimicking its sibling (RP1), suggesting a transformation in neuronal identity (Doe et al. 1988).

The structure of the *ftz* gene has been analysed by S1 nuclease mapping and by restriction mapping of a cDNA clone. The *ftz* gene consists of two exons of 880 and 980 bp respectively and an intron of approximately 150 bp and encodes a single 1.9 kb poly(A)$^{+}$ RNA expressed exclusively from the early blastoderm to the gastrula stage of *Drosophila* embryonic development (Kuroiwa et al. 1984).

Lewis et al. (1980a, 1980b) genetically mapped the *ftz* gene locus in the *antennapedia* complex (ANT-C) between the *Deformed (Dfd)* and *Antennapedia (Antp)* loci. Despite the fact that the *ftz* locus maps within the ANT-C, *ftz* is not a conventional homeotic gene either with respect to the type of pattern alterations caused by mutations at this locus, the period of developmental expression of the gene, or the structure of the *ftz* gene relative to the *Antp* gene in the ANT-C (Garber et al. 1983, Scott et al. 1983) or the *Ubx* gene in the *bithorax* complex (BX-C) (Bender et al. 1983). However the DNA sequence analysis of the homeobox regions in the 3' exons of the *Antp* , *ftz* and *Ubx* genes show that they conserve a homologous protein-coding sequence (McGinnis et al. 1984). The similar protein domain coded for by this homologous sequence is designated the homeo domain. Its conservation in the three pattern-formation genes is interpreted as indication that it serves a common or very similar function(s) in each gene

product. It also implies that the *Antp, ftz* and *Ubx* are derived from a common ancestral gene.

Models of homeotic gene function have postulated that their gene products act as developmental switches by binding to batteries of genes in cells confined to specific compartments (Garcio-Bellido 1977, Lewis 1978). The products of the gene batteries would then give a specific identity to spatially coherent groups of cells, an identity different from that of neighboring groups. This different identity would eventually be revealed in a different spatial arrangement of cells in neighboring compartments.

The protein coded for by the homeo domain contains many basic amino acids and appears to be limited to 60 amino acids in length. Both properties are consistent with a DNA or chromatin binding function (Delange and Smith 1979). The homeobox encodes a protein structure with homology to bacterial DNA-binding proteins (Laughon and Scott 1984) and has been shown to have sequence-specific DNA-binding activity (Desplan et al. 1985). In addition, the gap gene *Kr* encodes a protein product that has structural homology to the 5S ribosomal RNA transcriptional factor IIIA (Rosenberg et al.1986). Thus on the basis of structural evidence, it has been postulated that the homeo domain might serve a DNA or chromatin binding function and that the homeo-box-containing genes may encode regulators of transcription. The control of genes encoding tissue-specific products by segmentation genes has not yet been examined. However, there are indications of a regulatory cascade in which the segmentation genes control

the expression of other regulatory genes. Such a regulatory cascade has been characterized in the case of sex-specific expression of yolk proteins (Belote et al. 1985). I have already discussed the evidence that some segmentation genes control the expression of other segmentation genes. Additionally the segmentation genes appear to contribute to the expression of another class of regulatory genes : the homeotic genes. The *ftz* $^{+}$ protein is localized in the nucleus (Carrol and Scott 1985), an observation which is consistent with its proposed role as a regulatory protein.

The phylogenetic aspects of the homeobox sequence is also of great interest. Evidence shows that the homeobox is extremely conserved in metazoans. It has been found in the genomes of a wide spectrum of animals including annelid worms, frogs, birds, mice and man. In fact the *Xenopus* homeo domain is more similar to the *Antp* domain than the *Antp* homeo domain is to *ftz*. (McGinnis 1984). The extreme conservation of this genetic information between frog and fly (Carraso et al. 1984) indicates that the domain has been present in essentially the same form since the evolutionary branch-point separating the arthropods and the chordates.

These results also suggest the exciting possibility that genes controlling the insect body plan may be homologous to a set of conserved genes controlling the body plan of a wide range of vertebrate and invertebrate animals. The homeobox has not been detected in nematode DNA, in yeast,in *E. coli* or in sea urchin (McGinnis et al. 1984)--all organisms lacking overt segmentation. Perhaps the most exciting way to interpret these results is in terms of the apparent correlation between segmentation and

homeoboxes. All the animals that appear to contain homeoboxes pass through a developmental stage when the body is composed of a linear series of segmental units. For example, all vertebrate embryos form a reiterated pattern of somites from which the basic meristic organization of the skeleton, the nervous system and musculature is ultimately determined. We already know that homeoboxes in insects are part of the mechanism that controls the diversity of segment types. Hence, their homologues in vertebrates might serve a similar function in controlling diversification of the embryonic somites. If correct, this possibility may prove to be a major breakthrough in understanding vertebrate development because it means that a coherent family of highly conserved DNA sequences that control the development of insect segments might have direct counterparts in vertebrate systems. For example, segments in insects are developmental compartments (Lawrence 1981); each arises as a group of blastodem cells all of whose descendants form a precisely defined portion of the larval and adult body. Segments are also the precise realms of action of particular homeotic genes such as those of the *bithorax* and *antennapedia* gene complexes. This intimate relation between cell lineage and homeotic gene action hasled to the proposal that the insect body pattern is initially determined by the irreversible activation of particular homeotic genes in the founder cells of each segment. Accordingly, all the descendant cells inherit the same pattern of active homeotic genes and hence form a polyclone with a common 'genetic address' (Garcia-Bellido et al. 1979).

The discovery of vertebrate homeoboxes now allows us to apply this

'selector gene' or 'compartment' hypothesis directly to vertebrates because it predicts that at least some of the vertebrate homeobox sequences will belong to homeotic genes like those of the *bithorax* and *antennapedia* gene complexes. Thus we can ask whether these genes function in discrete subsets of somites, and even whether somites themselves are developmental compartments. The meristic organization of vertebrates and invertebrates might represent analogous rather than homologous solutions to organizing the body plan and indeed Bateson (1984) has argued that arthropod segment and vertebrate somites evolved independetly, citing evidence from embryological data. While the existence of homeoboxes in a wide spectrum of segmented animals does not, in itself, resolve this issue, it supports the idea for at least one close evolutionary link. What is perhaps more important, however, is that the discovery of homeoboxes provides a precedent as well as a method for looking for homologous functions controlling the development of diverse animal forms. By identifying, comparing and contrasting the roles of such functions we may well gain insights into some general principles that govern development and evolution.

Thus the study of the expression and regulation of developmentally important genes like the *ftz* gene and other genes that may be involved specifically in pattern formation in the kelp fly and other animals and the isolation and characterization of their protein product(s) in the future will hopefully help in our understanding of the development and phylogeny of the animal kingdom.

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