

The zebrafish early arrest mutants

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SUMMARY

This report describes mutants of the zebrafish having phenotypes causing a general arrest in early morphogenesis. These mutants identify a group of loci making up about 20% of the loci identified by mutants with visible morphological phenotypes within the first day of development. There are 12 Class I mutants, which fall into 5 complementation groups and have cells that lyse before morphological defects are observed. Mutants at three loci, *speed bump*, *ogre* and *zombie*, display abnormal nuclei. The 8 Class II mutants, which fall into 6 complementation groups, arrest development before cell lysis is observed. These mutants seemingly stop development in the late segmentation stages, and maintain a body shape similar to a 20 hour embryo.

Mutations in *speed bump*, *ogre*, *zombie*, *specter*, *polter-*

geist and *troll* were tested for cell lethality by transplanting mutant cells into wild-type hosts. With *poltergeist*, transplanted mutant cells all survive. The remainder of the mutants tested were autonomously but conditionally lethal: mutant cells, most of which lyse, sometimes survive to become notochord, muscles, or, in rare cases, large neurons, all cell types which become postmitotic in the gastrula. Some of the genes of the early arrest group may be necessary for progression through the cell cycle; if so, the survival of early differentiating cells may be based on having their terminal mitosis before the zygotic requirement for these genes.

Key words: cell cycle, cytokinesis, mitosis, cell lethal, maternal-zygotic transition

INTRODUCTION

What are the total number of zygotic genes that are necessary for embryonic survival through the first day of development and what phenotypes do these genes produce when mutant? The accompanying papers in this volume describe many of the genes that are required for specific morphogenetic processes. This paper focuses on the remainder of the early genes, those that, when mutant, have general effects on morphogenesis.

Many of these general phenotypes may result from mutations in genes that are autonomously required for cell survival, e.g., genes necessary for cell maintenance. Because it is likely that such zygotic housekeeping functions are a continuation of maternal functions, the time of onset of these phenotypes may help to delineate the transition of cellular functions from maternal control to zygotic control, termed the maternal-zygotic transition.

This transition begins at the beginning of another transition, the zebrafish midblastula transition (Kane and Kimmel, 1993). During the midblastula transition, from cycle 10 until cycle 13, the cell cycle lengthens and transcription begins. Throughout this period, the cell cycle length correlates with the ratio

of nucleus to cytoplasm, a type of maternal control. Then, in early cycle 14, the cell cycle begins to lose its correlation with the nucleocytoplasmic ratio and mitotic domains appear that correlate with the forming morphological subdivisions (Kane et al., 1992). The deep cells of the blastoderm, which form the embryonic anlagen, acquire cycles shorter than that predicted from the nucleocytoplasmic ratio. The enveloping layer cells of the blastoderm acquire a longer cell cycle than that predicted by the nucleocytoplasmic ratio. However, the most dramatic departure from nucleocytoplasmic ratio is found when the yolk cell enters a mitotic arrest in cycle 14. Thus, as in the mitotic domains of *Drosophila* at the equivalent stage (Foe, 1989), we see in zebrafish indications of zygotic control of the cell cycle.

Shortly after the emergence of the mitotic domains, individual deep cells leave the cell cycle as they become committed to their fates. These terminal 'birthday' divisions begin in the midgastrula at 8 to 10 hours postfertilization (Kimmel et al., 1994). Division 15 produces notochord cells and a few somitic muscle cells; division 16 produces the large primary motoneurons of the spinal cord and many somitic muscle cells. Thus, only one cell cycle separates the last

divisions of the midblastula transition from the terminal divisions of differentiated cells.

This report describes mutants isolated from the Tübingen screen for new zebrafish mutants that die or 'arrest' in the first 24 hours; these mutants are termed the Early Arrest Group mutants. These, together with the remainder of the Day 1 Group mutants form a statistical picture of the types of genes that are necessary for survival through 24 hours; this report summarizes that distribution. We suggest that a portion of the Early Arrest Group genes may be necessary for progression through the cell cycle.

MATERIALS AND METHODS

Production and isolation of mutations: the Tübingen screen

The detailed procedures for the mutagenesis and isolation of new and novel zebrafish mutants are described elsewhere (Mullins et al., 1994; Haffter et al., 1996). Briefly, male zebrafish were mutagenized with ethyl nitrosourea and outcrossed; their progeny were raised to adults en masse, producing the F₁. Random incrosses between the F₁ progeny produced individual families; these F₂ families were raised to adults. Random incrosses within each family produced F₃ embryos which were screened for the presence of mutants. If a family was carrying a particular mutation, one quarter of the incrosses, on the average, had mutant embryos; in each such cross, one quarter of the embryos were mutant. Individual pairs of parents that produced mutant embryos were outcrossed to carry the mutations to subsequent generations.

Stocks

Fish were maintained as described elsewhere (Westerfield, 1993; Mullins et al., 1994). Stocks of all the mutants of the Early Arrest Group were maintained as outcrosses against, in alternate generations, Tübingen and TL, both stock lines maintained in Tübingen.

Embryos

Eggs were produced for experiments by natural crosses and initially maintained in E3 medium (Mullins et al., 1994). For most experiments, embryos were dechorionated manually and incubated at 28±2°C in E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.150 mM KH₂PO₄, 0.050 mM Na₂HPO₄, and 0.7 mM NaHCO₃, pH 7.1-7.4).

DAPI staining

Embryos were fixed in 4% buffered paraformaldehyde for 2 hours at room temperature, washed in PBST, and incubated in a solution of 0.01% DAPI for 1 to 2 hours. Photographs were taken within 2 hours of staining using a Hoechst filter set.

Transplantations

Transplantations were done as described by Ho and Kane (1990). Usually cells from two donors, one labeled with rhodamine-dextran and one labeled with fluorescein-dextran (both dyes from Molecular Probes), were transplanted into one host.

Microscopy and time lapse recordings

Observations were made on either a Zeiss Axioskop or Axiophot microscope equipped with Nomarski differential interference contrast optics and UV epilumination. Viewing chambers were constructed of two 60×24 mm no. 1 coverslips separated with three pairs of 18×18 mm no. 1 coverglass spacers, or with tape spacers of the same thickness; the chamber edges were sealed with Vaseline to prevent evaporation. Low light images were captured on an CCD Camera

attached to an Intensifier (Videoscope KS-1381) and computer enhanced before recording.

For time lapse recordings, the microscopes were equipped with a motor driven focus controller and motor driven shutters for the white light and UV sources (Applied Scientific Instrumentation, Eugene, Oregon). Embryos were immobilized with 0.1% agarose + 0.01% agar in E2 medium. A high resolution camera (Newvicon, model VS2000N, from Videoscope) was used to record single frame images to a Laser Videodisc Recorder (SONY, model LVR-4000P) using a computer to control the plane of focus, UV and white light shutters. A Quadra Mac II Computer (Apple Computer Corporation) equipped with a digitizing board (Perceptics) and an auxiliary monitor output (RasterOps) running AxoVideo 2.0 software was used for time lapse control, image enhancement, and generating playback sequences. All figures were produced using Adobe Photoshop 3.0.

RESULTS

Because of the numbers of the mutants collected in the Tübingen screen, over two-thirds of all the mutants isolated were discarded; most of these discarded mutants had the general morphological phenotypes that have been described (Mullins et al., 1994; see also Furutani-Seiki et al., 1996). However, regardless of phenotype, all mutants having phenotypes visible before 24 hours were saved; the total collection of these mutants is termed the **Day 1 Group**. The majority of these mutants had specific developmental defects; such mutants are described in the other reports in this volume. This report characterizes mutants having general morphological defects visible before 24 hours; the total collection of these mutants is termed the **Early Arrest Group**.

For complementation testing and subsequent analysis, the Early Arrest Group was subdivided into **Class I** mutants, those

Table 1. Genes, alleles and phenotypes for the Early Arrest Group

Gene (abbreviation)	Alleles	Stage when visible	Major phenotypic features
Class I			
<i>speed bump</i> (<i>spb</i>)	<i>ti279</i> <i>to43</i> <i>te374</i>	80% epiboly	Cellular lysis, mitotic bridges
<i>zombie</i> (<i>zom</i>)	<i>te317</i> <i>ta94</i> <i>ta285</i>	Early segmentation	Cellular lysis, mitotic arrest
<i>ogre</i> (<i>ogr</i>)	<i>ta52a</i>	Early segmentation	Cellular lysis, lack of cytokinesis
<i>specter</i> (<i>spr</i>)	<i>ta214</i> <i>tu21</i>	Middle segmentation	Cellular lysis
<i>poltergeist</i> (<i>plt</i>)	<i>tb216</i>	Middle segmentation	Cellular lysis; rapid embryonic death at 20 to 22 hours
Class II			
<i>troll</i> (<i>trl</i>)	<i>ts225</i>	Late segmentation	Arrested development
<i>banshee</i> (<i>ban</i>)	<i>tf221</i>	Late segmentation	Arrested development
<i>kasper</i> (<i>kap</i>)	<i>ty236</i>	Late segmentation	Arrested development
<i>nirvana</i> (<i>niv</i>)	<i>tg229</i>	Late segmentation	Arrested development, immotile.
<i>ghoul</i> (<i>ghl</i>)	<i>tu274</i> <i>to52b</i> <i>tq251</i>	Late segmentation	Arrested development
<i>harpy</i> (<i>hrp</i>)	<i>ti245</i>	Late segmentation	Arrested development

that showed gross cellular abnormalities before morphological changes, and **Class II** mutants, those that showed morphological abnormalities before cellular abnormalities. Within the Class I mutants, the mutant phenotype was a reasonably accurate predictor of the complementation group. This was not true for the Class II mutants, all of which look very similar. The complementation groups of the Early Arrest Group, their alleles, and an abbreviated description of their phenotypes, are listed in Table 1.

Given the severe phenotype of the mutants of the Early Arrest Group, alleles of such mutants could be statistically under-represented in the Tübingen screen. For example, since the mutants are early lethals, the mutant embryos themselves might have not been present when the embryos were first screened. Alternatively, if the mutants were examined late, they might have been classified as 'late arresting' phenotypes and also discarded. These incorrect classifications would be reflected by a lower than normal number of alleles recovered per locus identified, and, indeed, such a possibility was suggested by the large number of complementation 'groups' in the early arrest group with single alleles. However, in a comparison of the allele frequencies of the Early Arrest Group with the Day 1 Group (loci of which are defined and listed in Table 2) or with the total loci identified in the Tübingen screen (Haffter et al., 1996), we found no statistical differences

Table 2. Day 1 Group genes, defined as those that were identified by mutants with morphological phenotypes visible before 24 hours

Group	Gene (abbreviation)	References
Epiboly	<i>half baked (hab)</i> ; <i>avalanche (ava)</i> ; <i>lawine (law)</i> ; <i>weg (weg)</i>	a
Dorsal specification	<i>snailhouse (snh)</i> ; <i>swirl (swr)</i> ; <i>piggytail (pgy)</i> ; <i>somitabun (sbn)</i> ; <i>lost-a-fin (laf)</i>	b
Ventral specification	<i>mercedes (mes)</i> ; <i>dino (din)</i>	c
Gastrulation	<i>sancho panza (sch)</i> ; <i>pipe tail (ppt)</i> ; <i>one-eyed-pinhead (oep)</i> ; <i>spadetail (spt)</i> ; <i>trilobite (tri)</i> ; <i>biber (bib)</i>	d
Notochord formation	<i>no tail (ntl)</i> ; <i>floating head (flh)</i> ; <i>momo (mom)</i> ; <i>doc (doc)</i>	e
Early Arrest, Class I	<i>speed bump (spb)</i> ; <i>zombie (zom)</i> ; <i>ogre (ogr)</i> ; <i>poltergeist (plt)</i> ; <i>specter (spr)</i>	f
Somite formation	<i>fused somites (fss)</i> ; <i>beamter (bea)</i> ; <i>deadly seven (des)</i> ; <i>after eight (aei)</i> ; <i>sonic-you (syu)</i> ; <i>you-too (yot)</i>	g
Neural tube	<i>silberblick (slb)</i> ; <i>masterblind (mbl)</i> ; <i>acerebellar (ace)</i> ; <i>cyclops (cyc)</i> ; <i>chameleon (con)</i> ; <i>white tail (wit)</i>	h; i; j
Notochord differentiation	<i>sleepy (sly)</i> ; <i>grumpy (gup)</i>	e
Motility	<i>sloth (slo)</i> ; <i>fibrils unbundled (fub)</i> ; <i>nicotinic receptor (nic)</i> ; <i>sofa potato (sop)</i> ; <i>relaxed (red)</i> ; <i>frozen (fro)</i> ; <i>heart attack (hat)</i> ; <i>herzschlag (hel)</i>	k
Skin	<i>dandruff (ddf)</i>	l
Early Arrest, Class II	<i>troll (trl)</i> ; <i>kasper (kap)</i> ; <i>banshee (ban)</i> ; <i>nirvana (niv)</i> ; <i>harpy (hpy)</i> ; <i>ghoul (ghl)</i>	f

References: a, Kane et al., 1996; b, Mullins et al., 1996; c, Hammerschmidt et al., 1996a; d, Hammerschmidt et al., 1996b; e, Odenthal et al., 1996; f, this report; g, van Eeden et al., 1996b; h, Brand et al., 1996; i, Heisenberg et al., 1996; j, Jiang et al., 1996; k, Granato et al., 1996; l, van Eeden et al., 1996a.

(Table 3), suggesting that the Early Arrest Group mutants are equitably represented among the total distribution of the Day 1 Group mutants.

For the initial phenotypic descriptions of mutants of the Early Arrest Group we studied live mutant embryos using normal DIC microscopy, examining the notochord, somite muscle, neural tube, pronephric duct, hatching gland, and other tissues that have unique and easily discernible cellular morphologies. In many cases we examined DAPI-stained embryos to visualize the nuclear morphology. A further general strategy was to transplant cells from the mutant embryos into wild-type hosts. This powerful method allows the detailed analysis of mutant cells, for we can study their phenotypes for many days after the cells would have died in situ.

Early Arrest Group mutants manifesting early cellular abnormalities: the Class I phenotypes

Ten mutants display gross cellular abnormalities before morphological changes in tissue or body shape are apparent; these fall into 5 complementation groups: *speed bump (spb)*, *zombie (zom)*, *ogre (ogr)*, *poltergeist (plt)*, and *specter (spr)*. Two of these, *ogr* and *plt*, are each represented by a single allele. All phenotypes segregate as simple Mendelian recessives.

Fig. 1 shows photographs of the mutants at 14 to 17 hours and at 22 to 26 hours. In all cases, mutant embryos are distinguished during segmentation stages by the presence of dark regions in the embryo; these dark regions are caused by the appearance of spherical cells some of which appeared to be dying. Nevertheless, all mutants with the exception of *plt* survived for 10 to 20 hours after the onset of cell death and developed at least some tissues that seemed not to have the mutant phenotype. Embryos mutant for *plt* died at 20 to 22 hours postfertilization.

The mutant *speed bump*: the earliest phenotype

The *spb* phenotype is one of the earliest identified in the Tübingen screen. At 80% epiboly, *spb* embryos develop dark regions lateral to the neural keel; these dark regions spread rapidly throughout the embryo (Fig. 2B). As wild-type siblings enter segmentation stages, mutant embryos remain arrested at tailbud stage (Fig. 1C). At this point the phenotype begins to become variable, due more to clutch-to-clutch variation than

Table 3. Comparison of allele frequency of Early Arrest Group genes, the Day 1 Group genes, and the total genes

Alleles per gene	Early Arrest Group genes*	Day 1 Group genes†	Total genes
1	7 (63%)	27 (49%)	204 (58%)
2	1 (9%)	12 (21%)	59 (17%)
3	3 (27%)	7 (13%)	30 (9%)
4-5	0	4 (7%)	25 (7%)
6-10	0	5 (9%)	22 (6%)
over 10	0	0	11 (3%)
Total	11	55	351‡

*Class I and Class II genes together.

†Early genes, listed in Table 2, that have mutants with morphologically recognizable phenotypes before 24 hours.

‡Statistical analysis for the entire table: $\chi^2 = 14.0$ (10 degrees of freedom); $P < 0.9$.

Pairwise sub-analysis between all combinations of columns were also statistically not significant.

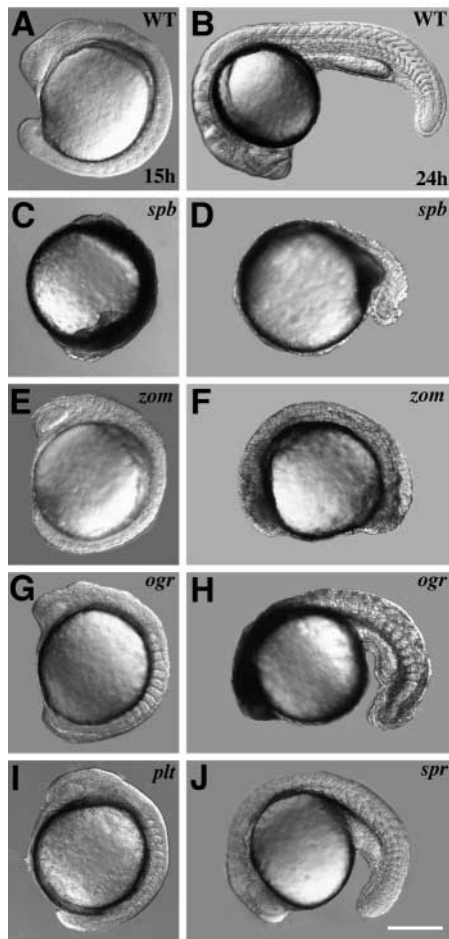


Fig. 1. Early Arrest Group Class I phenotypes. The names of the Class I mutants, with the exception of *spb*, are derived from their appearance at 24 hours. (A) Wild type, 15 hours. (B) Wild type, 24 hours. (C) *spb*, 15 hours. (D) *spb*, 24 hours. (E) *zom*, 15 hours. (F) *zom*, 24 hours. (G) *ogr*, 15 hours. (H) *ogr*, 24 hours. (I) *plt*, 15 hours (*plt* dies at 20 to 22 hours). (J) *spr*, 24 hours (*spr* phenotype looks normal at 15 hours). Scale bar, 300 μ m.

allele strength or genetic background (data not shown). In some clutches, the mutant embryos begin to die at 15 hours; in others, they continue to develop past 24 hours and develop a stunted little tailbud (Fig. 1D).

Examination of the dark regions reveals that cells are rounding up, appearing superficially similar in morphology to mitotic cells. Fig. 2C-G shows samples taken from a time lapse recording that started at 80% epiboly – shortly after the *spb* phenotype was recognized – and focused on the anlagen of the notochord and paraxial mesoderm. Within a period of 2 hours more than 95% of the cells lateral to the notochord round up and arrest. At the end of epiboly the anlage of the notochord, which can be distinguished in mutant embryos among the rounded cells, appears morphologically normal and appears to undergo the thinning and extension movements of normal development. In the early segmentation stages normal nuclei can still be found in the enveloping layer of the blastoderm and in the yolk syncytial layer (data not shown). If the *spb* embryos survive to 20 hours, the notochord is always present (Fig. 3D) and careful examination of the animal pole reveals hatching gland cells (Fig. 3C). In mutant embryos that survive until 35 hours, one or two melanocytes may appear (data not shown). Although difficult to find, muscle cells must be present, for some mutant embryos twitch. This twitching may be controlled by a rudimentary nervous system, for the twitching is not the early random spontaneous movements of the late segmentation stage embryo (caused presumably by

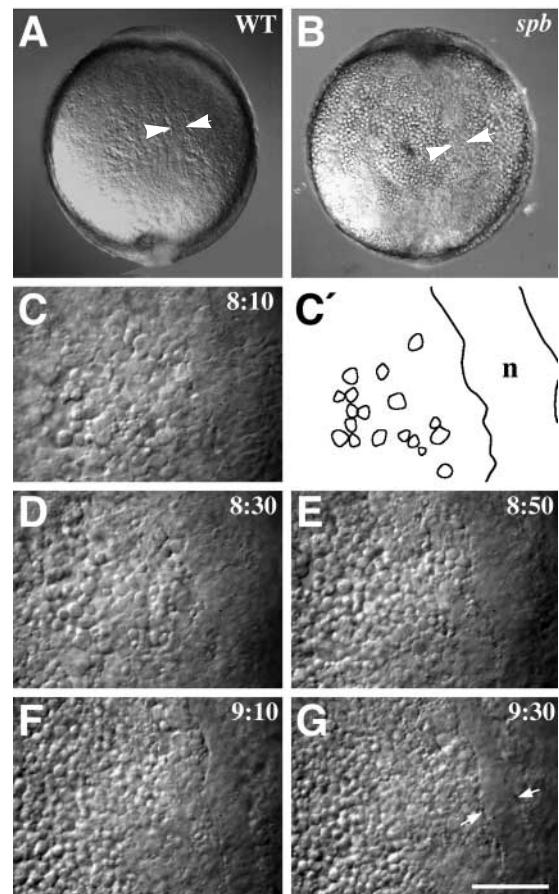


Fig. 2. The *speed bump* phenotype is visible in the epiboly stage gastrula. The name is derived from the appearance of the 'speed bump'-like notochord in the 24-hour mutant embryo. (A) View of dorsal side of a normal embryo at 100% epiboly at 10 hours. (B) View of dorsal side of *spb* embryo at same stage as A. Arrowheads in A and B indicate the width of the notochord anlage. (C-G) Video pictures from a time-lapse recording of the paraxial mesoderm and notochord anlagen of a *spb* mutant embryo at a focal plane 10 μ m above the yolk cell, showing the morphogenesis of the notochord and the rounding up of cells lateral to the notochord. (C) 8:10 hours postfertilization at 80% epiboly, 15 minutes after the phenotype was recognized on a dissecting microscope. (C') Drawing of C, showing the notochord anlage marked as 'n'. The outlined cells indicate spherical cells having no visible nucleus. (D) 8:30 hours. (E) 8:50 hours, about 90% epiboly. (F) 9:10 hours. (G) 9:30 hours. The thinning of the notochord (arrows) is roughly similar to the notochord anlage in normal embryos (not shown). Scale bar, 300 μ m for A and B; 50 μ m for C-G.

muscle cells responding to the first contacts of outgrowing motoneurons) but the rhythmic regular movements of a 22-hour wild-type embryo.

To establish the cells or tissues which autonomously required the *spb* gene product for cell survival, we assayed the survival of mutant *spb* cells transplanted into wild-type hosts. In this analysis, most mutant donor cells (>99%) that were transplanted into wild-type hosts lysed by the late segmentation stages. However, many exceptional cells survived, and many of these survivors differentiated into cell types that are present in the mutant embryos. Fig. 4A shows an example of notochord that developed from cells transplanted from *spb*

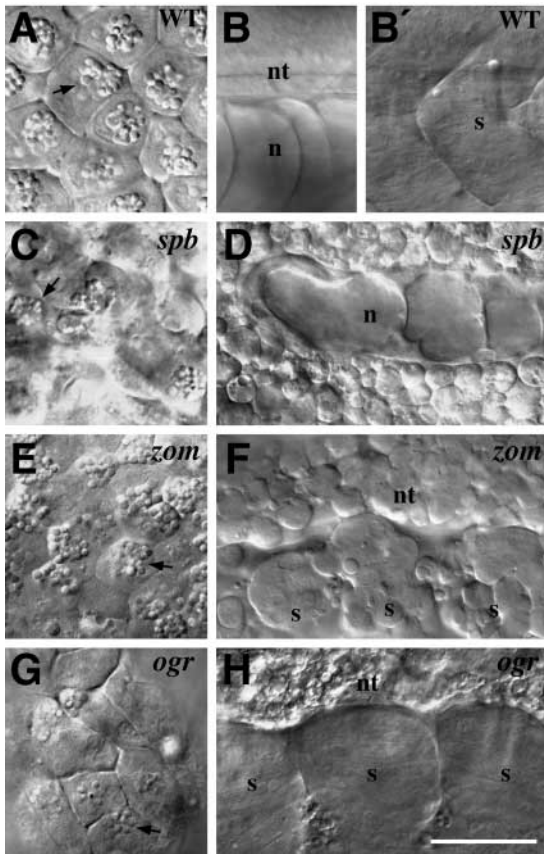


Fig. 3. Tissues that develop in *spb*, *zom* and *ogr* at 20 hours. (A) Normal hatching gland. (B, B') Normal neural tube, notochord, and somite muscle. (C) *spb* hatching gland. (D) *spb* notochord. (E) *zom* hatching gland. (F) *zom* notochord and somite muscle. (G) *ogr* hatching gland. (H) *ogr* notochord and somite muscle. nt, neural tube; n, notochord; s, somite. Arrows in A, C, E and G indicate hatching gland granules. Scale bar, 50 μ m.

embryos; in other cases we recovered muscle cells (data not shown). We followed these cells for about 2 to 3 days in the case of fluorescein-dextran labeled material and about 5 to 7 days for rhodamine-dextran. When cells differentiated, they survived for as long as lineage tracer could be seen in the cells, about 7 days postfertilization.

As seen with Nomarski DIC optics, the nuclei in cells of mutant embryos become invisible as the cells round up, overtly similar to the normal appearance of cells entering mitosis. Often a small 5 to 10 μ m vesicle forms in such cells (Fig. 5A). Some cells, presumably sibling daughter cells, were connected by a long thin strand up to 40 μ m long. DAPI staining of mutant embryos at 80% epiboly showed that many mutant cells displayed abnormally shaped nuclei; some had a single small dense DAPI-positive vesicle (Fig. 5B). Some cells were arranged in pairs, their nuclei attached via long DAPI-positive strands. Nevertheless, the cells did not immediately lyse but remained spherical; they survived in this state for several hours. Vital acridine orange staining, which should label apoptotic cells in living embryos, stained no cells in *spb* embryos until 5 to 10 hours after the phenotype could be identified and the embryos were dying (data not shown). Thus, it seems that, at least initially, the cells are more in stasis than quickly dying.

***zombie* and *ogre* affect mitoses in the embryo**

Both *zom* and *ogre* phenotypes are first visible by the 5- to 10-somite stages; mutant embryos become optically less transparent and large round cells appear in all tissues. In embryos of both mutants, there is usually an accumulation of large cells on the yolk sac lateral to the forming somites. By the 15-somite stage, differences develop between the two phenotypes. In *zom* embryos the somites become difficult to distinguish (Fig. 2E) compared to the more normal *ogr* somites (Fig. 2G). A little later, at the 20-somite stage, the eyes and head of *ogr* embryos become black and necrotic, and by 24 hours there is little, if any, structure present in the head (Fig. 2H); in *zom* embryos, the head, through obviously mutant, seems structurally complete.

Embryos of both mutants develop many tissues that appear morphologically normal. Notochord cells differentiate and begin to expand (data not shown); hatching gland cells appear (Fig. 3E,G). As in *spb* mutants, at least a rudimentary neural musculature system must form because embryos of both mutants first spontaneously and later rhythmically twitch. Embryos mutant for *zom* develop small somites consisting of rather large cells; the neural rod is also filled with large cells (Fig. 3F). Embryos mutant for *ogr* develop remarkably normal somites; however, the neural rod fills with large cells which lyse by 24 hours (Fig. 3H).

As in the case of *spb*, we assayed the cell autonomy of *zom* and *ogr* using cell transplantation. Most cells (>90%) transplanted from embryos mutant for *ogr* lysed in wild-type hosts. Cells transplanted from embryos mutant for *ogr* lysed especially quickly when transplanted into the anlage of the nervous system (Fig. 4C). Nevertheless, cell survival of both *zom* and *ogr* was better than that of *spb*. Many of the cell types observed forming in the mutant embryos also form from the transplanted cells. Fig. 4F and 4E show, respectively, one of the frequent examples of surviving muscle cells and a single remarkable example of one motoneuron from cells transplanted from *ogr* embryos. Fig. 4B and 4D show, respectively, examples of notochord and muscle from cells transplanted from *zom* mutant embryos. In all cases in which these cells formed, they survived for as long as the lineage tracer could be followed, about 4 to 7 days.

DAPI staining of mutant embryos at 18-22 hours revealed that both *ogr* and *zom* had abnormal nuclei. Embryos mutant for *zom* had a high proportion of cells in metaphase or early anaphase (Fig. 5E); these cells were first apparent over the normal background of mitotic figures in early segmentation stages, 11-12 hours postfertilization; as development continued the proportion of metaphase nuclei increased and cells with DAPI-positive vesicles began to appear (data not shown). In the live mutant embryo, the round cells contained visible plate-like structures, presumably mitotic nuclei (Fig. 5C). Note, however, that mitotic nuclei in normal dividing cells are not normally visible with the microscope objective lenses (Zeiss 40 \times water immersion) used for these observations (Kane and Kimmel, 1993).

DAPI-stained embryos mutant for *ogre* had a high proportion of cells with double nuclei (Fig. 5G). These cells were found in early segmentation stages, 11-12 hours, and as development continued the proportion of cell with double nuclei increased; occasionally cells contained four nuclei (Fig. 5H); as in *zom* and *spb*, cells with DAPI-positive vesicles began to

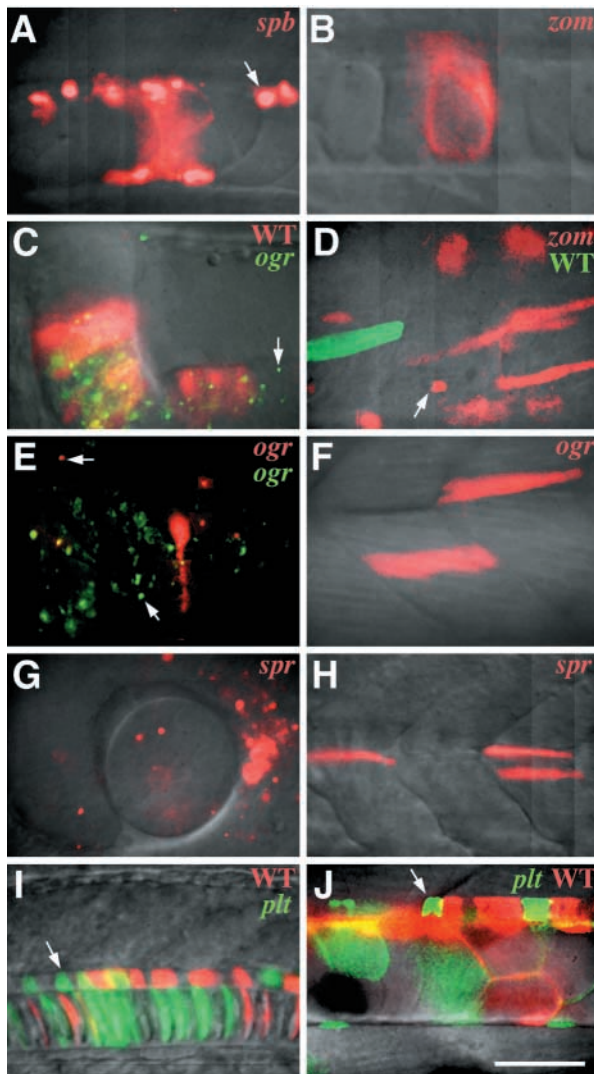


Fig. 4. Cell transplantation analysis of *spb*, *zom*, *ogr*, *spr* and *plt*. All transplantations were made from mutant or wild-type donors into wild-type embryos. (A) Survival of *spb* notochord cell at 30 hours. The labeled material (arrow) alongside the notochord are lysed mutant *spb* cells. (B) Survival of *zom* notochord cells at 30 hours. (C) Lysis of *ogr* cells (green dots, arrow) in the neural tube near the midbrain-hindbrain border at 30 hours. The wild-type cells (red), co-transplanted with the *ogr* cells, span the neural ectoderm. (D) Survival of *zom* (red) and wild-type (green) muscle cells at 30 hours. The out of focus debris are lysed *zom* cells (arrow). (E) Survival of *ogr* neuron (red) in the spinal cord at the level of the first somite. The debris (red and green dots, arrow) are *ogr* cells that have lysed. (F) Survival of *ogr* (red) muscle cells at 30 hours. (G) Lysis of *spr* cells in the eye at 24 hours. (H) Survival of *spr* (red) muscle cells at 30 hours. (I) Survival of *plt* (green) and wild-type (red) notochord and floor plate (arrow) at 24 hours. (J) Same embryo at 100 hours. Scale bar, 50 μ m.

appear, especially in the neural rod. However, many cells survive with multiple nuclei for at least several hours; in the live mutant embryo, many apparently healthy cells contain double nuclei (Fig. 5F).

specter and poltergeist

Both *spr* and *plt* phenotypes first become visible by the 15 to

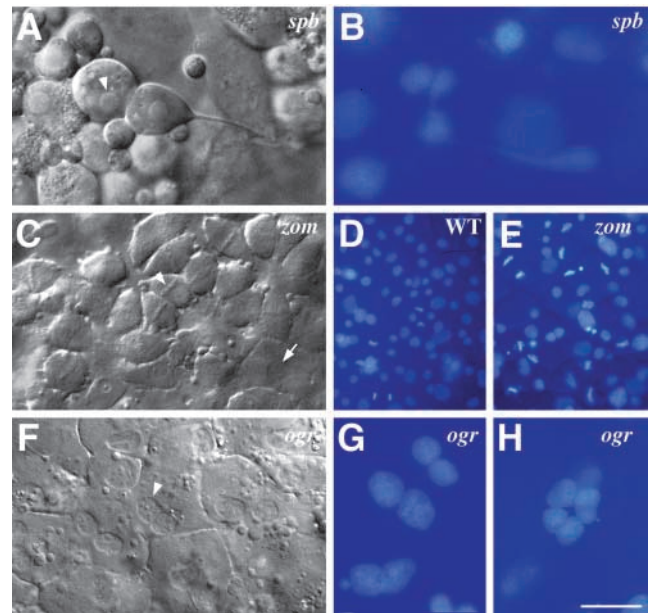


Fig. 5. Abnormal nuclei form in *spb*, *zom* and *ogr*. (A) DIC view of round cells in *spb* at 9 hours. Arrowhead indicates DAPI-positive vesicle. (B) DAPI-stained *spb*, at 9 hours, showing attached conical nuclei. (C) DIC view of round cells in *zom*, showing mitotic 'plates' (arrowhead). Arrow indicates normal nucleus. (D) DAPI-stained low power view of WT and (E) *zom* showing cells in metaphase. (F) DIC view of *ogr* cells with double nuclei (arrowhead). (G) DAPI-stained *ogr* cells with double nuclei and (H) with four nuclei. Scale bar, 30 μ m for A; 20 μ m for B, F, G, H; 50 μ m for D, E.

20 somite stages, as mutant embryos begin to accumulate isolated large round cells throughout the embryo. Embryos mutant for *spr* arrest with the body shape of a 20-hour embryo and, at 24-30 hours postfertilization, massive degeneration begins in the nervous system and spreads to the rest of the embryo. Embryos mutant for *plt* begin to die shortly after mutant embryos can be identified, and are always dead by 22 hours. DAPI-stained embryos of either *spr* or *plt* showed no obvious nuclear phenotype.

Analysis *spr* embryos by cell transplantation showed that, as in *spb*, *zom*, and *ogr*, most (>90%) cells lysed after transplantation from *spr* embryos into wild-type hosts (Fig. 4G). Still, many of the cell types that are present in the mutant embryos survived after transplantation into wild-type hosts. Fig. 4H shows examples of muscles developed from cells transplanted from *spr* mutant embryos. If these cell types formed they survived for as long as we could follow the lineage tracer in the cells, as in the above mentioned mutants.

The analysis of *plt* by cell transplantation produced results different from the remainder of the mutants of the Early Arrest Group, in that all *plt* cells survived until at least 4 days or longer after transplantation into wild-type hosts. Fig. 4I shows an example of notochord and floor plate cells at 24 hours, Fig. 4J shows the same embryo at 100 hours. Muscle cells and neurons also survived (data not shown). Thus, the earliest dying arrest mutant has cells that survive in wild-type hosts.

Mutants of the Early Arrest Group manifesting late cellular abnormalities: the Class II phenotypes

There are 8 Class II mutants which fall into 6 complementa-

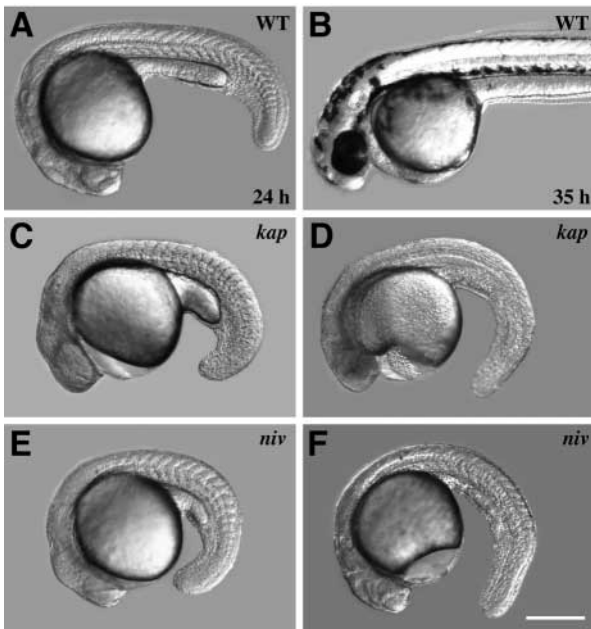


Fig. 6. Class II phenotypes. (A) Wild type, 24 hours. (B) Wild type, 35 hours. (C) *kap*, 24 hours. (D) *kap*, 35 hours. (E) *niv*, 24 hours. (F) *niv*, 35 hours. Scale bar, 100 μ m.

tion groups: *troll* (*trl*), *kasper* (*kap*), *banshee* (*ban*), *nirvana* (*niv*), *ghoul* (*ghl*) and *harpy* (*hpy*); all except *ghl* are represented by a single allele. All the phenotypes segregate as simple Mendelian recessives.

Fig. 6 shows photographs of the mutants at 22-24 hours and at 32-36 hours. The mutants *trl*, *kap*, *ban*, and *niv* have remarkably similar phenotypes. In all cases, mutant embryos first appear retarded during late segmentation stages and by 24 hours, before any cell death is seen, they seem to stop development, retaining the shape of a late-segmentation embryo. The ratio of the width of the ear to the ear-to-eye length, an indication of the embryonic age during the 24-40 hour period, remains constant at the value of a 24 hour embryo. Note that the Class II embryos have the same body shape as embryos mutant for *spr*, however, in the case of *spr* there are dead cells appearing during the segmentation stages, before the body shape arrest, thus placing *spr* in Class I.

At 24 hours, close examination of the Class II mutants reveals few obvious tissue abnormalities. Notochord, muscles, pronephric duct and central nervous system seem superficially normal. Embryos of all mutants are motile, except for *niv* embryos which respond weakly or not at all to touch. By 30 hours, mutant embryos of *trl*, *ban* and *kap* begin to show a massive degeneration of the central nervous system. Embryos of all mutants never develop melanocytes although some pigmentation develops in the eyes of *niv* and *hpy* embryos. This differs from what occurs in mutants of *spr*, a Class I mutant that has a body shape similar to Class II mutants, as *spr* mutants develop pigment cells at 35 hour.

Of the Class II mutants, only *trl* was analyzed by cell transplantation. As in the Class I mutants, we found that many cells lysed after transplantation from *trl* embryos into wild-type hosts. However, on average, survival was better (>50%). Cells transplanted into the nervous system were dead by 24 hours;

we do not yet know if they differentiated before lysis. Survival outside of the nervous system was good; transplanted mutant cells made muscle and mesenchyme which survived for 7 days.

DISCUSSION

In vertebrates, little is known about the genes that are necessary for early embryonic survival. In this report we consider the genes that are likely to have products that are necessary for the survival of many or all tissues; when these genes, termed the **Early Arrest Group**, are mutant, they cause the embryo to stop developing and die.

Relationship of the Early Arrest Group mutants with the Day 1 Group mutants

The Early Arrest Group comprises one fifth (11/55) of the loci of the **Day 1 Group**, a group defined in this report as that containing all the loci that are identified by mutant phenotypes visible in the live embryo before 24 hours; the Day 1 Group loci are listed in Table 2. Excepting the Early Arrest Group loci, the remainder of the Day 1 Group loci are required for pattern formation or the development of specific tissues. For example, mutants of the Dorsal Specification Group loci affect global patterning of the body plan (Mullins et al., 1996); mutants of the Notochord Differentiation Group loci affect the development of the notochord (Odenthal et al., 1996). Although difficult to make close comparisons because of the differences in screening methods, the great majority of loci identified in the *Drosophila* screen performed at Heidelberg also had specific developmental defects (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984). However, there was a modest number of examples of general type mutants from the Heidelberg screen such as *pebbles* or *string*, genes that are required in most or all cells of the fly embryo. Thus the frequency of mutants in the Early Arrest Group of the Tübingen screen roughly approximates the frequency of the general type mutants of the Heidelberg screen.

The Tübingen screen identified 55 loci in the Day 1 Group. If the screen reached 50% saturation, then there are about 100-150 genes necessary for early development that, when mutant, result in recognizable phenotypes in the 24 hour live embryo. If so then 20-40 of these genes would be expected to have phenotypes similar to those documented in this report. Given the 25 chromosomes of the zebrafish, these approximations suggest there are, on the average, about 4-6 loci of the Day 1 Group and 1-2 loci of the Early Arrest Group on each chromosome. The high proportion of genes with developmentally specific effects suggests that screening for new developmental loci using systematic deletions might be partially successful, for only rarely would the analysis be occluded by the generally required genes such as those described in this report. Such an approach could be valuable in confirming the degree of saturation for the Tübingen screen, similar to that done for the Heidelberg screen (Wieschaus et al., 1984).

The cellular function of the genes of the Early Arrest Group

The lethality of the mutants of the Early Arrest Group, especially that of the Class I mutants, seems very different from that of the remainder of the Day 1 Group mutants. While most

of the Day 1 Group mutants die by 3 days of development, their death seems not to be the direct result of general cellular lysis but rather the secondary effect caused by the physiological imbalances due to miss-proportioned tissues or non-functional organs. In contrast, the Class I mutants exhibit early cellular lysis before any morphogenetic defects are apparent in the embryo; this cellular lysis is presumably the direct cause of embryonic death. In the initial stages of our analysis, we presumed that the early arrest mutations were in genes necessary for general cell maintenance. This hypothesis was tested by monitoring the survival of mutant cells which were transplanted into wild-type hosts, similar in strategy to the method used in the initial analysis of early mutants of the Mexican axolotl (Armstrong, 1985). If the genes were autonomously necessary for cellular survival, the homozygous mutant cells should lyse even in a wild-type host. For example, in *Drosophila*, the *Minute* locus is thought to code for products necessary for normal cellular maintenance, such as tRNAs (Ritossa et al., 1966). When clones of cells homozygous for any of the *Minute* loci are produced by mitotic recombination, the homozygous mutant cells disappear from the embryo (Stern and Tokunaga, 1971). When this type of analysis is extended to general samples of genes in *Drosophila*, about 10% of all lethal point mutations are cell lethal (Ripoll and Garcia-Bellido, 1979).

The transplantation results support the idea that, in general, the genes of the Early Arrest Group are autonomously required in all cells. This is especially true in the cases of *spb*, *ogr*, *zom*, and *spr*, where there was massive cell death of transplanted mutant cells in all tissues. However, in all cases, some cells survived. These surviving cells, even though rare, are extremely informative. Surviving cells are seen in the notochord, in muscles and, in rare cases, in the large motoneurons of the spinal cord. All these cell types are known to have their terminal mitosis in the mid to late gastrula (Kimmel et al., 1994).

Given that mutants of *spb*, *zom*, and *ogr* have abnormal nuclei, a likely hypothesis is that these genes are required for procession through mitosis. Consistent with this idea, each of these mutants have nuclear morphologies that resemble the nuclei of *Drosophila* cell cycle mutants. For example, the nuclei of *spb* resemble those of *aurora*, a mutant with monopolar spindles (Glover et al., 1995); the nuclei of *zom* resemble those of *fizzy*, a mutant with a metaphase arrest (Dawson et al., 1993); the nuclei of *ogr* resemble those of *pebbles*, a mutant with defective cytokinesis (Jürgens et al., 1984).

Consistent with the idea that the phenotypes of *spb*, *zom* and *ogr* are related to the cell cycle is the relationship of the time of onset of the phenotypes with the progress of cell death. Embryos mutant for *spb* first show their phenotype at about 8 hours in the late epiboly embryo, and then their phenotype rapidly progresses, within 1 to 2 hours, to necrosis. At about this stage the cell cycle is relatively rapid, about 2 to 3 hours long (Kimmel et al., 1994). In contrast, the phenotype of *zom* is evident during early segmentation but then the phenotype slowly progresses, over about 6 to 10 hours, to a less than complete necrosis. By this stage the cell cycle has slowed considerably, about 4-6 hours long, and furthermore, many cells of the embryo have withdrawn from the cell cycle.

The function of *spr* and *trl* is less clear. In the case of both of these genes, mutant cells lysed after transplantation into the

nervous system anlagen of wild-type hosts; the survival of mutant cells transplanted into other tissues, such as muscle, was better in the case of *spr* and almost complete in the case of *trl*. As in the case of *spb*, *zom* and *ogr*, the *spr* and *trl* mutations could be in genes required for the cell cycle. Abnormal nuclei have not been seen in these mutants. However, if the arrest was in the interphase portion of the cell cycle, as in the case of the mutant *string* in *Drosophila* (Edgar, 1990), such abnormalities may not have been detected in our experiments. Nevertheless, other possibilities have not been eliminated for either gene, such as the idea that the mutations are in genes required for the survival of cells of a particular tissue, e.g., tissues of nervous system.

The function of *plt* is more enigmatic. Mutants of *plt* are difficult to identify at 15-18 hours but quite consistently and dramatically die at 22 hours. Yet cells of mutant *plt* donors when transplanted into wild-type hosts survive for as long as we can follow the cells, up to 7 days postfertilization. Thus, *plt* seems to not be a general cell lethal. One possibility is that *plt* is required for the extracellular matrix, if so, then single mutant cells or small groups of mixed mutant cells could be rescued by the wild-type host environment, a cell non-autonomous mutation. Another possibility is that the function of *plt* is autonomously required in the periderm, the fish's first skin, which is clonally derived from the enveloping layer; due to its epithelial morphology, transplanted cells usually do not contribute to the enveloping layer, and thus our experiments inadequately test this possibility.

The relationship of the maternal-zygotic transition with the Early Arrest Group loci

During the maternal-zygotic transition, gene products transcribed from the zygotic genome supplant products supplied maternally. In zebrafish, this transition begins at cycle 10, the beginning of the midblastula transition, with the activation of zygotic transcription (Kane and Kimmel, 1993). Which genes are required to be transcribed for normal early development to occur? Reported in this volume (Kane et al., 1996), the inhibition of RNA polymerase II by injection of alpha amanitin into early embryos causes an arrest in morphogenesis at the beginning of epiboly, 4.5 hours postfertilization, 1.5 hours after the beginning of the midblastula transition. However the cell cycle continues in the alpha amanitin injected embryos, so that at 8 hours postfertilization, about the time that the experimentally injected embryos die, the cells of experimentally injected embryos are approximately the same size as cell in control embryos, approximately in cycle 15.

In *Xenopus* (Newport and Kirschner, 1982) and *Drosophila* (Edgar and Schubiger, 1986) the major transcripts present at midblastula transition are predominantly products which are generally required in all cells of the embryo, e.g., ribosomal RNA and tRNA. Mutations in the genes for these transcripts would be expected to affect most or all cells in the embryo. Of the mutants isolated in the Tübingen screen, those of the Early Arrest Group have phenotypes that affect most of all cells of the embryo, making the genes of this group the most likely candidates for such generally required products. If so the comparison of the time of onset of the phenotypes of the Early Arrest Group could give some approximation of the extent of the maternal-zygotic transition.

The phenotypes of mutants of the Early Arrest Group first

become evident at 7-8 hours in the case of *spb* mutants; the latest phenotypes are expressed at about 22 hours in mutants of most of the Class II mutants. Given that the end of midblastula transition occurs at late cycle 13, about 4.5 hours post-fertilization (Kane and Kimmel, 1993), less than 4 hours separates the end of midblastula transition from the earliest morphological phenotype. The phenotypes of *zom* and *ogr*, both evident at 12-14 hours, extends further the possible length of the zygotic-maternal transition: if these genes are necessary for progression through mitosis then maternal products must be sufficient for *zom* and *ogr* function at least until early segmentation. The relevance of the later phenotypes of the Early Arrest Group will remain unclear until a better idea of their function is proposed; nevertheless, the late timing of these Class II phenotypes compare more closely to time of onset of phenotypes of *Drosophila* mutants at *Minute* loci, which become evident as the imaginal disks begin to develop. Thus it seems reasonable that the zygotic-maternal transition continues in zebrafish from the midblastula transition until the end of the first day of development or further.

The mutants of the Early Arrest Group are likely to be useful in the analysis of the cell cycle of the zebrafish. Furthermore, many of these mutants will be useful for the study of the effects of ectopic death of cells during development. Currently the chromosomal locations of the Early Arrest Group mutants are being determined; these mapped locations will be helpful in screening genes cloned from other species as candidates for the Early Arrest Group genes.

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