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CHARACTERIZATION OF A CELL LINE DERIVED FROM ZEBRAFISH (*BRACHYDANIO RERIO*) EMBRYOS

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SUMMARY

During the last decade, zebrafish (*Brachydanio rerio*) have emerged as a novel and attractive system to study embryogenesis and organogenesis in vertebrates. The main reason is that both extensive genetic studies and detailed embryologic analysis are possible using this small tropical fresh water teleost. However, in vitro analysis using cell culture or molecular genetics are still far less advanced than in other vertebrate systems. Here we report the generation and characterization of a fibroblast like cell line, ZF4, derived from 1-day-old zebrafish embryos. The hyperploid cell line has been stable in multiple passages for more than 2 yr now and is the first zebrafish cell line that can be maintained in conventional medium containing mammalian serum. Using a series of plasmids for expression of a marker gene, we evaluate in ZF4 cells the relative strength of expression from several different viral, fish, and mammalian promoters. Stable integration can be obtained by using G418 selection. We hope that our cell line will be a useful tool for the analysis of gene regulation in zebrafish.

Key words: zebrafish; cell line; chromosomes; transfection; promoter strength.

INTRODUCTION

The study of development and differentiation is most advanced in those organisms of the animal kingdom that allow the application of a broad spectrum of experimental tools. Classical genetic approaches in combination with an advanced molecular genetics, and experimentally well accessible embryogenesis have enhanced our understanding of invertebrate development (*Drosophila melanogaster*: St. Johnston and Nüsslein-Volhard, 1992; *Caenorhabditis elegans*: Wilkins, 1993). Few vertebrates allow the extensive application of classical genetic tools, like large scale chemical mutagenesis, to identify novel regulatory genes. The zebrafish (*Brachydanio rerio*) has emerged as a novel genetic system with some extraordinary features that allow the fast and efficient generation of new mutations affecting defined aspects of development (Streisinger et al., 1981). Analysis of mutations affecting embryonic pattern formation in zebrafish has led to novel and exciting insights into vertebrate development (Kimmel et al., 1989; Ho and Kane, 1990; Hatta et al., 1991; Krauss et al., 1992).

In contrast to the advanced genetics and embryology, in vitro approaches have not been well developed in zebrafish. For several reasons the establishment of stable zebrafish cell lines seems desirable: cell lines can be used as tools to study transcriptional regulation of genes; to test whether zebrafish cells are susceptible to the infection of viruses (e.g., to develop schemes for insertional mutagenesis in zebrafish); or as feeder cells to establish the culture of more demanding cell types.

Although a variety of cell lines established from a number of fish species have been described (Wolf and Quimby, 1962; reviewed by: Hightower and Renfro, 1988), there is only one report of stable cell lines from zebrafish (Collodi et al., 1992a,b). These cell lines are dependent on a complex medium that includes trout embryo extract (Collodi and Barnes, 1990).

Here we report the generation and initial characterization of a fibroblastlike cell line obtained by continuous culture from zebrafish embryos. This cell line (ZF4) is fully adapted to grow in standard medium supplemented with mammalian serum. We demonstrate that standard transfection procedures can be used to introduce foreign DNA into these cells, and that Neomycin resistance allows selection for cell lines that have stably integrated plasmid DNA. In addition, we compare the strength of a variety of heterologous promoters in ZF4 cells to obtain efficient trans-gene expression in these cells.

MATERIALS AND METHODS

Zebrafish strains. We used a zebrafish (*B. rerio*) strain which was genetically wild type except for an allele at the *fr* pigment pattern locus which results in interrupted stripes of melanocytes (Kirschbaum, 1975). *fr* Segregates as a recessive mendelian allele in crosses with other zebrafish. The fish were originally obtained from a pet shop in Tübingen in 1988 and maintained by inbreeding in the laboratory. We used these fish because of their excellent breeding condition. Fish were maintained on a 14 h/10 h cycle of light/dark according to standard conditions (Westerfield, 1989). Onset of light in the morning triggers the fish to spawn, and fertilized embryos were collected from breeding traps 2 h after the light went on.

Cell culture. All procedures involving embryos were performed at 28° to 29° C. After 24 h of development, embryos were harvested in batches of 50, washed, and incubated for 2 min in a 0.2% solution of bleach (commercial grade, Clorox) in water. Embryos were washed 3 times in Dulbecco's modified Eagle's medium (DMEM)/F12 (GIBCO, Grand Island, NY), with HEPES) supplemented with 100 Iu of penicillin per ml, 100 μg streptomycin per ml, and 0.25 Fungizone per ml. In the same medium, the chorions were removed manually with forceps. Embryos were transferred into fresh DMEM/F12 medium supplemented with 10% heat inactivated fetal bovine

serum (GIBCO) and antibiotics as above. Embryos were mechanically dissociated into small clumps of cells and plated at two to six embryos per well in 48-well plates. Plates were incubated at 28.5° C in a 4% CO_2 atmosphere. The media was changed every 4 to 7 days during the first 3 wk. Wells with colonies of fish cells appeared after 2 to 3 wk. Colonies were transferred into fresh wells, and after 2 to 3 additional wk into six-well plates. Cells were expanded into 25-cm^2 flasks, and the medium changed every 3 to 4 days. All plasticware was from Corning.

During initial passages, cells were very sensitive to EDTA. Hence cells were passaged by washing first with Hanks' balanced salt solution (HBSS; minus calcium, minus magnesium) and dissociation using trypsin only at 0.025% in HBSS (minus calcium, minus magnesium). During passage numbers greater than 80, we no longer observed such a strong lethal effect of EDTA (we continue not to use EDTA).

All further culture was performed using DMEM/F12 with carbonate or HEPES, 10% heat inactivated fetal bovine serum (GIBCO) at 4% CO_2, 28° C. Cells can be frozen using standard procedures [10% dimethylsulfoxide (DMSO) as cryoprotectant].

Growth assays were performed by plating cells in 60-mm tissue culture plates at different densities and the cells trypsinized and counted after 1, 2, 3, 4, and 7 days.

Karyotype analysis. Karyotype analysis was performed according to a procedure modified from Lindl and Bauer, 1989. Cells were plated at 0.5 $\times$ 10^5 per ml on 60-mm plates, allowed to grow for 2 days, the media changed, 24 h later media exchanged for media with 50 ng/ml colchicine, and incubated for 2 h. Cells were trypsinized and resuspended in 0.5 ml media; 9.5 ml of hypotonic solution (6.5 m*M* NaCl, 3.35 m*M* KCl; at 37° C) were added to the cells, which were then incubated at 37° C for 30 min. Cells were centrifuged and resuspended in 0.5 ml hypotonic solution; 5 ml of ice-cold fixative (3:1 methanol:glacial acetic acid) were slowly added. After 30 min the cells were washed once with fixative and resuspended in 1 ml fixative. Chromosome spreads were prepared and treated with 0.25% trypsin for 1 min. Chromosome spreads were stained with 0.8% Giemsa prepared in glycerol:methanol 1:1. Karyotypes were analyzed on photographic prints.

Genotype analysis. Genomic DNA was isolated from ZF4 cells as well as from adult zebrafish according to standard procedures (Maniatis et al., 1989). The purified DNA was digested by restriction enzymes and subjected to Southern analysis. It was probed at high stringency with the BamHI Hind III fragment from exon 4 of the zebrafish *wnt-1* gene (Molven et al., 1991).

Cell transfection. ZF4 cells were plated at 2.5 $\times$ 10^5 per 60-mm dish, and the medium changed after 1 day. Calcium-phosphate DNA coprecipitates were formed according to the standard procedure described in Maniatis et al. (1989), using 5 pmol of the supercoiled plasmid DNA. Precipitates were added to the cells and incubated 6 h to overnight. The cells were washed with HBSS and shocked for 4 min with 15% glycerol in HBSS (Friedenreich and Schartl, 1990). Cells were allowed to grow in standard medium for 48 to 72 h, and harvested.

lacZ expression vectors and β-galactosidase assay. The following expression plasmids were used to assess the strength of different promoters to drive expression in ZF4 cells. pSVβ (SV40 early promoter/enhancer), pCMVβ (CMV immediate early promoter/enhancer), pTKβ (HSV TK promoter) and pAdβ (adenovirus 2 major late promoter) all contain an intron (SV40 late splice) 5′ to the lacZ reading frame and an SV40 late polyadenylation signal (MacGregor and Caskey, 1989). The carp actin lacZ expression vector was constructed after insertion, by blunt end ligation, of the Hind III-BamHI lacZ fragment from pRSVlacZ (Edlund et al., 1985) into the KpnI digested FV-2–1 carp actin expression vector (Liu et al., 1990; Moav et al., 1992). This expression vector contains 1.2 kb of carp actin upstream sequences, the first intron and non-coding part of the first exon as well as the salmon growth hormone polyadenylation signal. pRSVlacZ expresses lacZ from the RSV LTR (Edlund et al., 1985). SV40lacZ is pCH110 (Pharmacia) with the SV40 early promoter and SV40 poly A site. The mouse HSP68lacZ plasmid (phspPTlacZpA), which contains 800 bp of the mouse HSP68 promoter, the lacZ gene, and the SV40 polyA site, was obtained from S. Darling, University College London.

Cells were transfected as described with 5 pmol of expression plasmid, supplemented with pUC18 to a total of 30 μg DNA (Maniatis et al., 1989). Experiments were performed in triplicates. Cells were harvested 72 h after the glycerol shock, and a lacZ assay was performed according to Miller (1972). Cells from each 60-mm dish were harvested, washed, resuspended in 0.2 ml Tris-HCl, pH 7.8, 2 m*M* phenylmethylsulfonylfluoride, and sonicated 5 times for 1 s. The cleared supernatant was taken as extract. Protein in the extract was determined by the Biorad protein assay (Biorad Laboratories). Twenty microliters of extract were incubated with 480 μl of buffer (100 m*M* sodium phosphate, pH 7, 10 m*M* KCl, 1 m*M* $MgSO_4$, 50 m*M* mercaptoethanol) and 100 μl of substrate (4 mg/ml *O*-nitrophenyl-β-D-galactoside in 0.1 *M* sodiumphosphate pH 7) at 29° C until a yellow color developed (30 min to 2 h). The incubation time was recorded and the reaction was stopped by the addition of 250 μl of 1 *M* sodium carbonate. Optical density (OD) was measured at 420 nm. Activity was determined as OD_{420} units per hour of incubation and milligrams of extract protein. Background of β-galactosidase activity in ZF4 cells was lower than 0.2 OD_{420} $U \cdot mg^{-1}$ protein $\cdot h^{-1}$. Values determined for the expression constructs are corrected for background as measured using extracts from non-transfected cells.

G418 selection. ZF4 cells were transfected with pRSVgeo [containing the Hind III-Sal I gal-neo fusion gene from pSAβgeo (Friedrich and Soriano, 1991) in Hind III-BamHI vector fragment from pRSVlacZ] and selection started 2 days later with G418 (GIBCO) at 600 μg/ml. Mixed lines of ZF4RSVgeo cells were selected for 3 wk and expanded. Percentage of survival after 10 days of selection at various G418 concentrations was determined for both ZF4 and ZF4RSVgeo cells.

Results

Establishment of cell lines. In an attempt to establish cell lines from zebrafish, we cultivated dissociated zebrafish embryos at various stages of development. The earliest stages we used were blastoderm embryos (3 to 4 h after fertilization), hoping for the establishment of stem cell-like, undifferentiated cell lines. In our hands, most of the blastoderm cells died within the following 1 or 2 days, and in a few cases cells differentiated to phenotypes of several kinds. Continuous culture of blastoderm-derived cells was not successful.

In contrast, we were able to establish several cell lines from 1-day-old embryos. Embryos were mechanically dissociated and transferred to 48-well plates. After 24 to 48 h, cells started to migrate from the embryo fragments, and after about 1 wk, most wells were confluent with various cell types (fibroblastlike, epithelial, neuronlike, melanocytes or spheroid, non-adherent cells). Cells went through a crisis after 2 to 3 wk of culture, and few wells with colonies of cells survived. Colonies were passaged to fresh wells and expanded. Only colonies of cells of fibroblastlike phenotypes could be established. The rare frequency at which colonies were obtained argues for clonal origin of each cell line. Attempts to subclone by limited dilution failed during early passages of the cell lines. Seven independent lines of similar phenotypes (ZF1 to ZF7) were established, but only one (ZF4) was further characterized due to its good growth characteristics. Frozen stocks were established for the other six cell lines.

ZF4 cells were grown continuously for more than 2 yr and the highest passage numbers at present exceed 150. During this time, no change in phenotype was observed. ZF4 cells attach and express fibroblastlike phenotypes when grown at low density (Fig. 1 *A*). At high density, ZF4 cells do not show any signs of contact inhibition, but grow in multiple layers and form foci (Fig. 1 *B*). During continued culture without passage, cells within the foci become vacuolated, and dying cells can be observed.

The growth rate of ZF4 cells slowed down with increasing passage number. At low passage numbers (10 to 15) we measured cell doubling times of about 23 h (data not shown), whereas we now measure cell doubling times in the range of 2 days (Fig. 2).

Karyotype of the ZF4 cells. Karyotype analysis at passage numbers above 100 reveals a hyperploid set of chromosomes, with counts in the range of 110 to 120 being most frequent (mean:

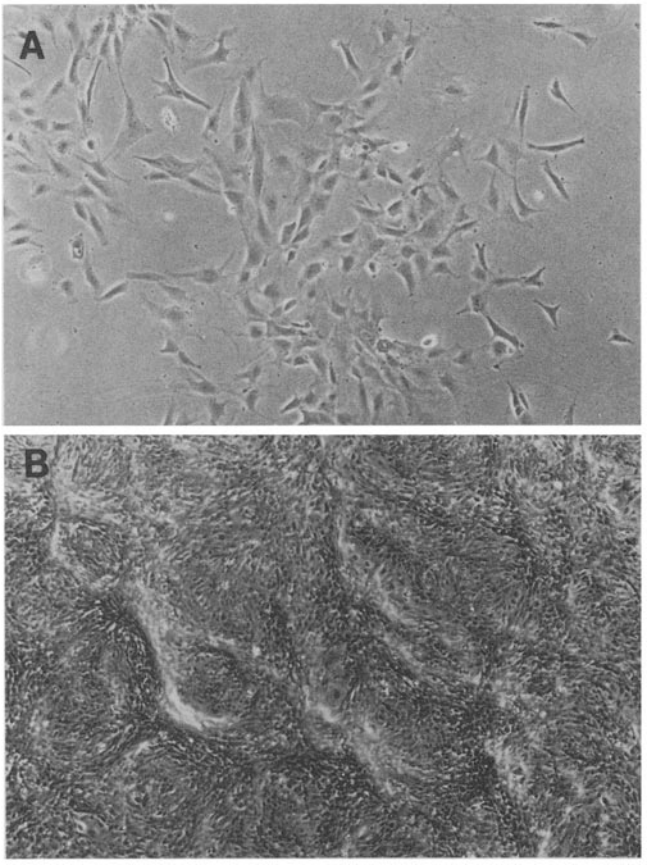

FIG. 1. Phase contrast micrograph of zebrafish embryo derived cell line ZF4. *A*, appearance of cells growing at low density (×100); *B*, cells growing at high density do not show contact inhibition and form multiple foci (×40).

120.7; minimum: 65; maximum 230) (Fig. 3). The diploid chromosome number for zebrafish has been determined as 50 (Endo and Ingalls, 1968). We find most chromosomes to be metacentric, like the karyotype published for zebrafish (Endo and Ingalls, 1968). An assignment of individual chromosomes is not possible due to the high similarity in morphology among the haploid set of metacentric chromosomes.

Verification of the origin of ZF4 cells. We used Southern blot analysis to verify the origin of the ZF4 cells (Fig. 4). A probe for exon 4 of the zebrafish *wnt-1* gene was made available to us by Anders Fjose (Molven et al., 1991). We used genomic digests of DNA from adult *fr* zebrafish as well as DNA isolated from ZF4 cells. The BamHI HindIII digest recognizes the fragment used as probe, whereas the PstI or Hind III digests cut intron sequences. Introns of the zebrafish *wnt-1* are larger than those of the mammalian counterparts (Molven et al., 1991). The presence of the tested restriction sites and the identity of the fragments' length proves the zebrafish origin of the ZF4 cells.

Transfection and selection. We tried to optimize conditions for transfection of ZF4 cells with supercoiled plasmid DNA. For comparison, we used conditions described by Friedenreich and Schartl (1990) for *Xiphophorus* and carp cell lines. The conditions we find are very similar to those described by Friedenreich and Schartl. Comparison of calcium phosphate coprecipitation with the DEAE dextran method revealed higher toxicity of the DEAE method. A glycerol shock 6 to 18 h after addition of the precipitate increased the uptake of DNA significantly.

We used the RSV lacZ expression vector for these experiments, because it also allowed us to evaluate the fraction of cells expressing the transfected DNA using histochemical staining for lacZ (data not shown).

G418 selection can be used as a tool to obtain stable transfection of ZF4 cell lines (Fig. 5). Use of G418 at a concentration of 800 to 1000 μg/ml kills all ZF4 control cells within 10 days. G418 at 600 to 800 μg/ml kills all control cells within 15 to 20 days, whereas resistant colonies grow at a normal rate. Higher concentrations significantly slow down the growth of resistant colonies.

Relative strength of various eukaryotic promoters in ZF4 cells. Little is known about the activity of heterologous promoters in zebrafish cells. By transfection, we tested eight different vectors for β-galactosidase expression in ZF4 cells (Fig. 6). We choose lacZ as the marker gene because there are easy assays for histochemical detection in fixed tissue. We plan to extend the study of the promoter/enhancer combinations in zebrafish embryos to reveal any potential tissue specificity of the expression pattern.

We tested six promoter/enhancer combinations from mammalian viruses [simian virus (SV40) early/promoter/enhancer; human cytomegalovirus (CMV) immediate/early promoter enhancer; herpes simplex virus thymidine kinase promoter (HSV TK); adenovirus 2 major late promoter; Rous sarcoma virus (RSV) long terminal repeat (LTR)] as well as one mouse promoter (for heat shock pro-

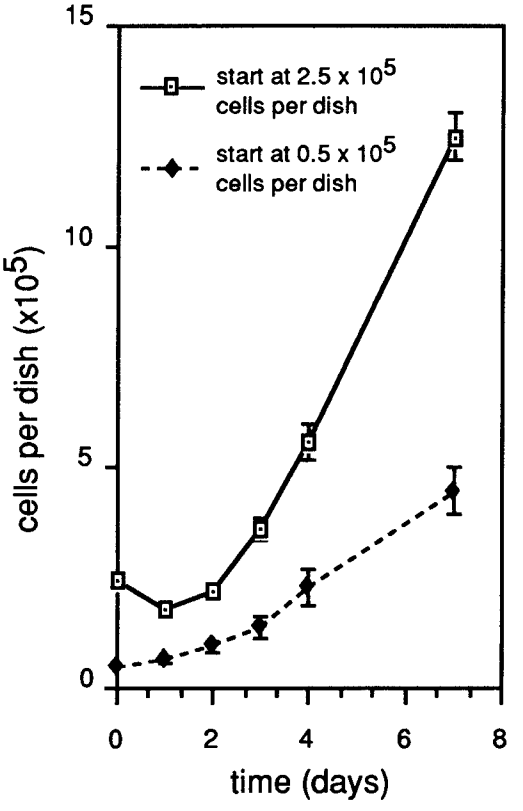

FIG. 2. Growth rate of zebrafish ZF4 cells cultured in vitro. Assays were performed in 60-mm tissue culture dishes. Shown are the mean of three determinations and the standard deviation.

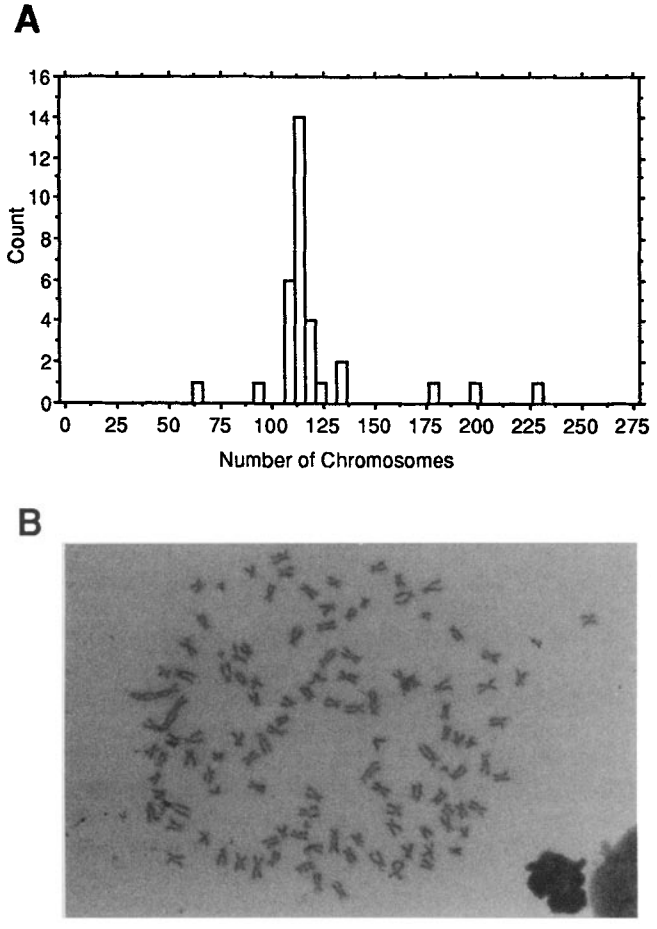

FIG. 3. Karyotype analysis of zebrafish ZF4 cells. *A*, chromosome number distribution according to counts from 32 metaphase preparations. *B*, Giemsa stain of ZF4 cell metaphase chromosome spread.

tein 68, HSP68lacZ) and one fish promoter from the carp actin gene. The vectors are described in detail in the methods section.

We find that among the viral promoter, the RSV-LTR, and the CMV immediate early promoter provide the highest expression levels, whereas others are only moderately active. When comparing the expression levels of pSVβ (containing an intron) to that of SV40 lacZ (no intron) expression, we observe about twofold higher expression in the presence of the intron. Therefore, similar to other vertebrates, introns may have a positive influence on RNA expression levels in fish (Gruss and Khoury, 1980). The mouse hsp68 and the carp actin promoter give intermediate levels of expression.

DISCUSSION

This paper describes the generation and characterization of a fibroblastlike cell line, ZF4, from zebrafish (*B. rerio*) embryos. This line is well adapted to grow in standard media supplemented with fetal bovine serum and has been maintained for more than 150 population doublings. This cell line has developed a hyperploid karyotype characteristic of transformed cell lines. Transient transfection of the cells is possible using the calcium phosphate coprecipitation method. Introduction of plasmids carrying the gene for neomycin phosphotransferase II allows the selection of stable transformed cell lines with G418.

A variety of promoter/enhancer combinations used for expression studies in mammalian systems are also active in ZF4 cells. We find that regulatory elements from mammalian viruses as well as a mammalian promoter are active at levels comparable to those of a fish promoter (carp actin gene; Liu et al., 1990). The carp β-actin promoter has also been extensively characterized in a variety of cell lines from other fish species (Moav et al., 1992). When comparing our results with viral promoter to those described from expression studies in other fish cell lines (Friedenreich and Schartl, 1990; Winkler et al., 1992; Inoue, 1992), the CMV and RSV promoter

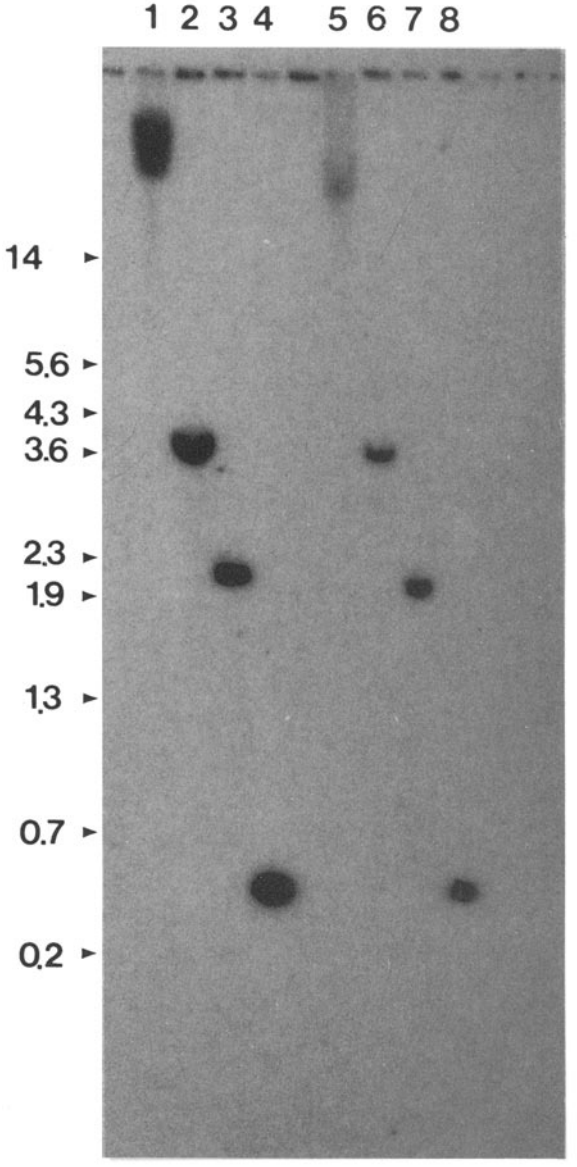

FIG. 4. Identification of ZF4 as a zebrafish-derived cell line. Genomic DNA was prepared from ZF4 cells (*lane 1–4*) and from one adult zebrafish (*lane 5–8*). DNA was digested with PstI (*lane 2* and *6*), Hind III (*lane 3* and *7*), or Hind III and BamHI (*lane 4* and *8*); DNA in *lanes 1* and *4* is not digested. DNA was separated on an agarose gel, and a Southern blot probed with the ^{32}P-labeled BamHI/HindIII fragment from the zebrafish *wnt-1* gene (Molven et al., 1991). Fragments that light up (*lane 2* and *6*: 3700 bp; *lane 3* and *7*: 2200 bp; *lane 4* and *8*: 550 bp) are the ones expected from the published genomic restriction map.

and enhancer seem useful for high-level expression in a variety of fish species. These studies provide some guidelines for promoter choice in future zebrafish gene expression studies. Further studies using transgenic zebrafish will be required to test whether these promoters exhibit any tissue specificity.

Zebrafish are rapidly becoming the system of choice to study the genetic control of embryogenesis in vertebrates (Roosen-Runge, 1936; Marcey and Nüsslein-Volhard, 1986). The unique features of this small tropical freshwater teleost (Streisinger et al., 1981) have already led to the identification and characterization of a number of developmental control genes (Kimmel et al., 1989; Ho and Kane, 1990; Hatta et al., 1991; Krauss et al., 1992; Ekker et al., 1992; Schulte-Merker et al., 1992). The fact that the fish are easy to breed and genetic methods allow experiments hardly possible in other vertebrates (Grunwald and Streisinger, 1992) has made zebrafish attractive to a variety of other disciplines from immunology (Ono et al., 1992) to toxicology (e.g., Bresch et al., 1990), neurobiology (e.g., Gatchalian and Eisen, 1992), and behavioral sciences (e.g., Gillis and Kramer, 1987).

The development of cell lines for zebrafish will broaden the usefulness of this vertebrate model system. Co-transfection assays in such cell lines will allow the convenient study of the influence of transcription factors and other regulatory proteins on the expression levels of genes important for the control of normal development of zebrafish. Transfection studies provide a powerful alternative to the generation of transgenic fish. Although the generation of germ line transgenic fish by microinjection into the cytoplasm of freshly fertilized eggs is possible at present (Stuart et al., 1988 and 1990; Culp et al., 1991; Bayer and Campos-Ortega, 1992), the process is still time consuming, a low percentage of the animals is transgenic, and expression is often inactivated after transmission through the germ line. Injection into the pronucleus, successfully applied in medaka (Ozato et al., 1992), unfortunately is not possible so far in zebrafish. Other methods for generating transgenic zebrafish have been tested, but germ line transmission has not been demonstrated so far (Buono and Linser, 1992; Powers et al., 1992). The ZF4 cell line may be useful to develop alternative methods for the introduction of DNA into the zebrafish germ line. The line can be used to test whether zebrafish are susceptible to any known viral vectors. Retroviruses are an excellent tool for insertional mutagenesis in mice (Friedrich and Soriano, 1991) and may prove to be of equal utility

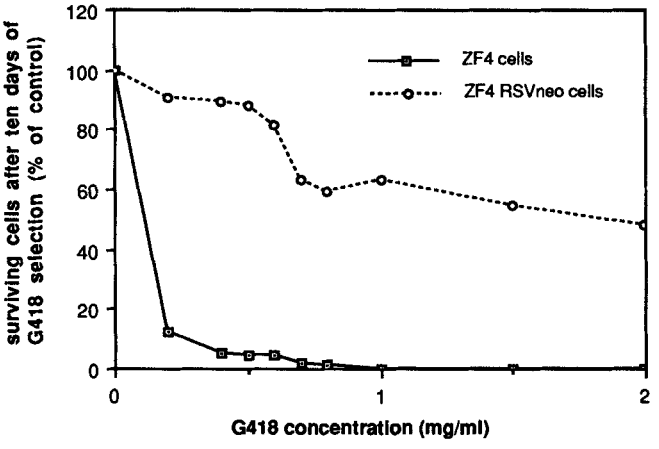

FIG. 5. Selection of stable transformed ZF4 cells with G418.

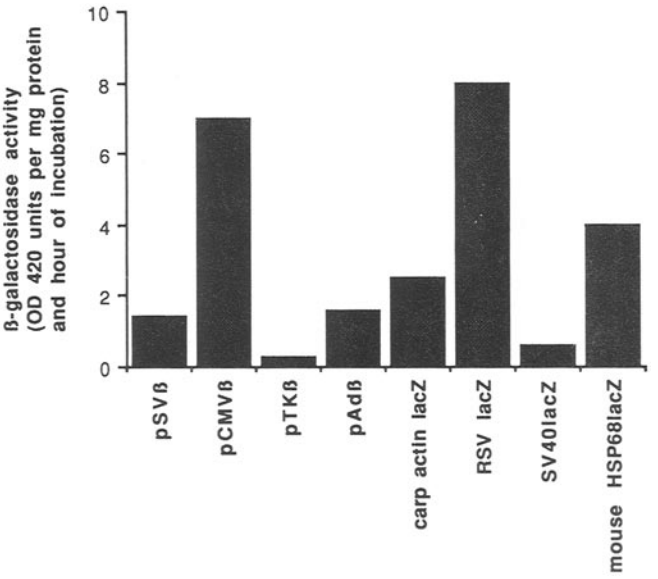

FIG. 6. Relative strength of several different eukaryotic enhancer/promoter combinations in transient expression assays in ZF4 cells. Expression vectors used in this study are described in the text.

for zebrafish mutagenesis. In addition, the cell line will also be useful as a system to test whether heterologous transposable elements can be mobilized in zebrafish cells and provide an other means for insertional mutagenesis.

Finally, it is desirable to develop embryonic stem cell lines (Evans and Kaufman, 1981) for zebrafish. During blastula stages it has been demonstrated that zebrafish cell fates are not yet specified (Kimmel and Warga, 1986), thus transplanted blastoderm stage cells can contribute to the germline of recipients (Lin et al., 1992). These cells, therefore, have the potential to serve as starting material for the development of pluripotent embryonic stem cell lines. Our hyperploid cell line may be used as feeder cells for the development of embryonic stem cell lines or other cells that may be more demanding than ZF4 cells.

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