

Transfer of the Gene for Neomycin Resistance into Goldfish, *Carassius auratus*

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ABSTRACT

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A recombinant DNA construct containing the neo gene controlled by a promoter from the Rous sarcoma virus (RSV) was microinjected into newly fertilized, dechorionated goldfish (*Carassius auratus*) eggs. The neo gene confers resistance to the neomycin analog drug G-418. Results of Southern blot analyses were consistent with incorporation of single or multiple copies of the gene into the genomic DNA of one examined fish. Evidence of neo mRNA in RNA dot-blot analysis indicated that the RSV promoter had initiated transcription within a piscine genome. The utility of the neo gene as a selectable marker for transgenic fish was evaluated by G-418 selection on newly hatched and juvenile fish but proved inconclusive. Reasons for the discrepancy between neo expression and G-418 selection results are discussed.

INTRODUCTION

Gene transfer in vertebrates was carried out first in mice (Gordon et al., 1980), but the technology did not receive widespread attention until Palmiter et al. (1982) transferred a rat growth hormone gene into mouse embryos, giving rise to transgenic mice that grew significantly faster and larger than control mice. These experiments inspired a wide variety of researchers to seek to improve economic traits in domesticated and semi-domesticated animals through gene transfer. A number of such gene transfer experiments have been carried out in fish (reviewed by Maclean et al., 1987).

Most gene transfer experiments in fish to date have followed the design of the early mouse work, utilizing in recombinant constructs the mouse metallo-

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thionein promoter to regulate the expression of a protein coding sequence (Zhu et al., 1985). Activities of other promoter sequences have not been demonstrated in transgenic fishes. Retroviral long terminal repeat elements would seem likely to promote the expression of introduced DNA coding sequences because their expression is independent of the location of genomic integration, and in many cases is not tissue or species specific. While piscine retroviruses have not been well characterized, the key functional domains of the promoter region of the avian Rous sarcoma virus (RSV) have been identified (Luciw et al., 1983) and subcloned (Petersen et al., 1984), enabling their use in the construction of expression vectors.

To date, all gene transfers in fish have been achieved through microinjection of individual eggs. In mammalian cells, however, mass gene transfer has been carried out through calcium phosphate precipitation (Graham and Van der Eb, 1973), electroporation (Neumann et al., 1982), or lipofection (Felgner et al., 1987). Such mass transfer schemes generally include a means of selecting transformed cells on the basis of some phenotype conferred through the integration and expression of the introduced DNA construct – often novel drug resistance. No such selection scheme has been demonstrated to date in fish. Demonstration of a protocol for selection of transformants would be a practical contribution toward the development of effective mass gene transfer protocols in fish. A selection regime has been demonstrated (Southern and Berg, 1982) for transformation of mammalian cells with a bacterial gene, *neo*, conferring resistance to the neomycin analog G-418. The possibility of whole animal selection on the basis of introduced G-418 resistance has been demonstrated (Steller and Pirrotta, 1985) in *Drosophila* sp.

Our group reports the successful transfer into goldfish of a construct bearing the structural gene for neomycin resistance, *neo*, controlled by the RSV promoter. The objectives of this study were: to evaluate the ability of the RSV promoter to regulate expression of a foreign gene in transgenic fish, and to evaluate the usefulness of neomycin resistance as a selectable marker for identification of transgenic individuals expressing novel gene products.

MATERIALS AND METHODS

Embryo production

Spontaneous ovulation of goldfish was carried out using the methods of Stacey et al. (1979). Sexually mature fish were maintained under a long photoperiod regime (16 h light: 8 h dark). On day 1, breeding fish were transferred from holding tanks to breeding aquaria at $15 \pm 1^\circ\text{C}$. Aquaria were supplied with floating artificial plants, and the water temperature was increased overnight to $21 \pm 1^\circ\text{C}$. Spontaneous ovulation usually occurred during the last half of the dark phase on day 3. If no ovulation occurred on day 3, the fish were injected intraperitoneally with 2 mg/kg body weight of carp pituitary gland extract.

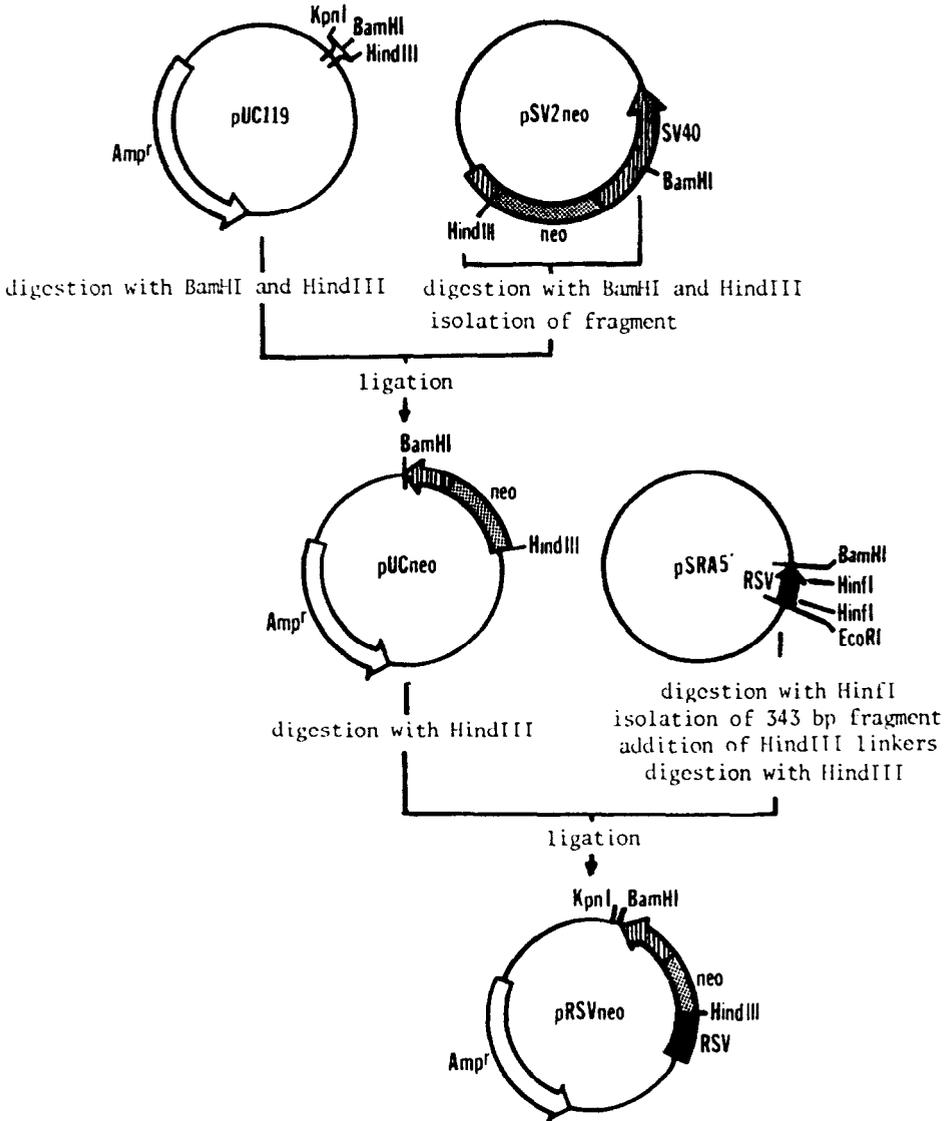


Fig. 1. Construction of the plasmid pRSVneo. A BamHI-HindIII restriction fragment (stippled) containing the neo gene and an adjacent intron from the vector (SV40, vertical lines) was isolated from plasmid pSV2neo (Southern and Berg, 1982) and ligated into the polylinker of pUC119. HindIII linkers were ligated onto a 343 bp fragment (black) removed from pRSA5' (Petersen et al., 1984) containing the RSV promoter/enhancer region. This fragment was ligated in turn into the HindIII site of pUCneo to produce the pRSVneo construct utilized in the gene transfer experiment. Correct orientation of the RSV promoter element was confirmed by restriction site mapping.

Approximately 10 h later, eggs and sperm were collected separately by manual stripping of the fish.

Fertilization of eggs was carried out by the addition of milt followed by the addition of well water. Five min after fertilization, goldfish eggs were dechorionated by a 4–6-min incubation in 0.25% trypsin in Holtfreter's solution (Grand et al., 1941). Dechoronation was stopped by addition of 5% fetal bovine serum in Holtfreter's solution. Dechorionated eggs were washed several times in Holtfreter's solution and transferred to Holtfreter's solution on an agar petri dish.

Plasmid construction

The plasmid pRSVneo contains the neo structural gene, whose product (aminoglycoside 3'-phosphotransferase II) confers resistance to the neomycin analog G-418, under the control of the RSV promoter. Construction of the plasmid is shown in Fig. 1.

Microinjection

Plasmids were linearized with KpnI (which cuts in the polylinker of the plasmid), extracted with phenol/chloroform, precipitated in ethanol, and redissolved in 88 mM NaCl, 10 mM Tris·HCl, pH 7.6 to a final concentration of 25 ng/ μ l. Borosilicate glass microinjection needles with an inner tip diameter of approximately 2 μ m were prepared by use of a vertical pipette puller (David Kopf Instruments, Model 700-C). The needles were filled with plasmid and blue dye (FD&C Blue #1, McCormick and Co.) solution and microinjection was carried out using a Brinkman MM33 micromanipulator. Approximately 2 nl of plasmid solution were injected, with volume controlled by timing the insertion/withdrawal of the injection needle at constant fluid flow rate. DNA was injected into the center of the germinal disc of the fish eggs prior to first cleavage. Microinjected eggs were allowed to develop in Holtfreter's solution until the blastula stage and in well water post-blastula.

Probe labelling

To derive a probe for Southern and DNA dot-blot analyses, BamHI–HindIII plasmid restriction fragments were separated by 1% agarose gel electrophoresis in TAE buffer (0.04 M Tris acetate, 2 mM EDTA, pH 8.0). The excised 2.3 kb band containing the neo gene was phenol–chloroform extracted, the DNA precipitated by ethanol and sodium acetate, and redissolved in TE (10 mM Tris·HCl (pH 7.6), 1 mM EDTA). This fragment was labelled by the procedure of Feinberg and Vogelstein (1983) to 0.5–2 $\times 10^9$ cpm/ μ g. DNA probes were used without purification from unincorporated nucleotides.

The 2.3 kb BamHI–HindIII fragment including the neo gene was subcloned into pTZ18R (Pharmacia) under the control of the phage T7 promoter to produce a transcript complementary to neo mRNA. One μ g of linear DNA was

transcribed using ^{32}P -UTP following the method of Schenborn and Mierendorf (1985) to produce probes used in RNA dot-blot analyses of goldfish.

DNA analysis

Genomic DNA was isolated from 1–2-month-old fish which had been bisected and stored at -90°C . Posterior halves of fish were individually homogenized in three volumes of 0.2 M Tris·HCl (pH 8.0), 0.1 M EDTA, 0.5% (*w/v*) sodium dodecyl sulfate (SDS) buffer with 100 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer–Mannheim) using a Dounce homogenizer (Wheaton). High molecular weight DNA was extracted following the method of Maniatis et al. (1982).

DNA dot-blot analysis (Kafatos et al., 1979) was used to detect the presence of the neo gene within the genome of goldfish. Five μg of genomic DNA were denatured in 0.4 M NaOH and, following addition of an equal volume of 2 M ammonium acetate, were spotted onto a nitrocellulose filter using a Hybri-dot system (Bethesda Research Laboratories). The filter was baked in vacuo for 2 h at 80°C . The filters were prehybridized in 50% deionized formamide, $5\times\text{SSC}$ ($1\times\text{SSC}=0.15\text{ M NaCl}$, 15 mM Na citrate, pH 7.2), $5\times\text{Denhardt's}$ solution ($1\times\text{Denhardt's}=0.02\%$ Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 50 mM pyrophosphate (pH 6.5), 0.1% SDS, 0.1 mg/ml denatured calf thymus DNA (Sigma), and 0.1 mg/ml yeast tRNA (Boehringer–Mannheim) at 42°C for at least 4 h. The filter was hybridized with 1×10^6 cpm/ml of ^{32}P -labelled probe in the same solution used for prehybridization for 20 h at 42°C . The filter was washed three times for 10 min in $2\times\text{SSC}$, 0.1% SDS at room temperature, and three times for 30 min in $0.2\times\text{SSC}$, 0.1% SDS at 60°C , air dried, and exposed to Kodak XAR5 X-ray film at -80°C (Maniatis et al., 1982).

Southern blot analysis was carried out by complete digestion of 10 μg of goldfish genomic DNA with KpnI, BamHI, or SstI (each of which cuts the plasmid only once), followed by electrophoresis through a 0.8% agarose gel, and transfer to a nitrocellulose filter (Southern, 1975). The Southern blot was probed using the same hybridization conditions as the dot-blot analysis.

RNA analysis

Anterior halves of fish stored at -90°C were homogenized using a polytron (Kinematica) in a solution containing 4 M guanidinium isothiocyanate, 0.1 M β -mercaptoethanol, 0.5% sarkosyl, 5 mM sodium citrate, pH 7.0. Total RNA was isolated following the methods of Maniatis et al. (1982) or Davis et al. (1986). RNA dot-blot analysis (White and Bancroft, 1982) was used to test for neo gene expression. Total RNA was dissolved in $6\times\text{SSC}$, and 7.4% (*v/v*) formaldehyde, and heat-denatured at 65°C for 15 min. Serial amounts of RNA (0.25, 0.5, 1, 2, or 4 μg) were dotted directly onto nitrocellulose equilibrated with $6\times\text{SSC}$. The RNA was bound to the filter by baking under vacuum at 80°C for 2 h. The prehybridization was carried out for more than 4 h at 60°C

in 50% deionized formamide, $5\times$ SSC, $5\times$ Denhardt's solution, 50 mM sodium phosphate pH 6.5, 0.1% SDS, 100 μ g/ml denatured calf thymus DNA, and 100 μ g/ml yeast tRNA. The filter then was hybridized with 2×10^6 cpm/ml of 32 P-labelled probe in the same buffer as in prehybridization for 20 h at 60°C. After hybridization, the filter was washed three times for 10 min with $2\times$ SSC, 0.1% SDS at room temperature, three times for 30 min with $0.2\times$ SSC, 0.2% SDS at 68°C, air dried, and exposed to Kodak XAR5 X-ray film at -80°C .

G-418 selection

For the drug selection screenings, solutions of G-418 in static water aquaria were aerated to achieve initial saturation levels of dissolved oxygen. Dissolved oxygen was maintained above 60% saturation by periodic aeration. Temperature was maintained at $20\pm 1^\circ\text{C}$. The pRSVneo-injected or non-injected control goldfish at a population density of 12 g/l were exposed separately to 4 mg/ml or 8 mg/ml solutions of G-418. Mortality of fish was monitored every 4 h for the first 24 h and every 12 h afterward. Dead individuals were removed upon observation and stored in liquid nitrogen. Drug selection was continued until 50% of the control fish in 4 mg/ml G-418, or until 90% of those in 8 mg/ml, had died. Surviving fish were transferred into fresh well water upon termination of the test.

RESULTS

Molecular analysis of injected fish

The RSVneo construct was successfully introduced into goldfish using standard microinjection techniques. The survival rate for groups of microinjected fish has ranged from 10% in early experiments to close to 50% currently, compared with survival rates of above 90% in uninjected control groups. The results reported here are from early injection studies, where despite high mortality rates among injected fish, we were still able to effect transfer and apparent expression of the injected construct.

To determine what percentage of the surviving fish were transgenic, i.e., bearing the RSVneo construct integrated within their genome, DNA isolated from the fish was analyzed by DNA dot-blot. Fig. 2 shows the results of a representative DNA dot-blot analysis of control and pRSVneo-injected goldfish. Neither of the control fish (individuals 1E and 1F, spotted again at 1K and 1L) showed positive hybridization to the neo probe. On the blot shown in Fig. 2, two out of 48 pRSVneo-injected fish (fish # 30, spotted at 2L; and # 47, spotted at 5K) showed hybridizations of intensities equivalent to approximately one copy of the gene per genome. Two other putative transgenic individuals were observed among 11 additional DNA samples probed on a similar dot-blot (results not shown). All dot-blot analyses were repeated, and yielded

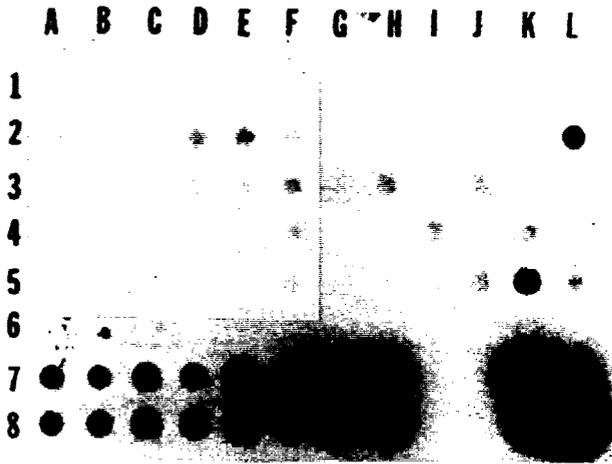


Fig. 2. DNA dot-blot analysis of goldfish microinjected with pRSVneo. Total genomic DNA was probed with the 2.3 kb BamHI-HindIII fragment of pRSVneo. Dots at positions 1 E, F, K, and L contain DNA from non-injected control goldfish. Dots in rows 2-5 contain DNA from 48 different pRSVneo-injected goldfish. Two fish showed positive hybridization signals, fish # 30 (at position 2L) and # 47 (at position 5K). Rows 7 and 8 represent dilutions of pRSVneo with (row 8) or without (row 7) added goldfish genomic DNA. Dilutions shown are: A-B, 0.5 copies per genome equivalent; C-D, 1 copy; E-F, 5 copies; G-H, 25 copies; and K-L, 100 copies per genome.

identical results. Thus, roughly 7% of the goldfish surviving through 1 month of age carry the transferred construct in at least some of their tissues.

To examine the mode of genomic integration, the DNA of putative transgenic fish was further examined in Southern blots. Fig. 3 shows the results of Southern blot analysis of genomic DNA from one control and two putative transgenic fish (# 30 and # 47), cleaved with the restriction enzyme BamHI and probed with the 2.3 kb neo probe. The neo probe detected a 5.3 kb band with mobility equal to that of the linearized plasmid in all four test individuals (two of which are shown in Fig. 3), which may be evidence of circularization and persistence of the introduced genetic construct. Higher and lower molecular weight bands observed upon the DNA of fish # 47 were interpreted as being junction fragments indicative of genomic integration of the introduced construct. Results from Southern blots of SstI- and KpnI-digested DNA agreed with the results with BamHI-digested DNA.

To determine to what extent the transferred neo gene was expressed, dot-blots of total RNA prepared from the fish were probed for evidence of the neo-specific mRNA. Expression of the neo gene was evident on the RNA dot-blot of two of the four putative transgenic fish. On the blot of one of these individuals (Fig. 4A), this was apparent as a stronger hybridization signal than that observed upon RNA of an uninjected control fish. To rule out the possibility of pRSVneo DNA contamination of the RNA samples, in a separate experi-

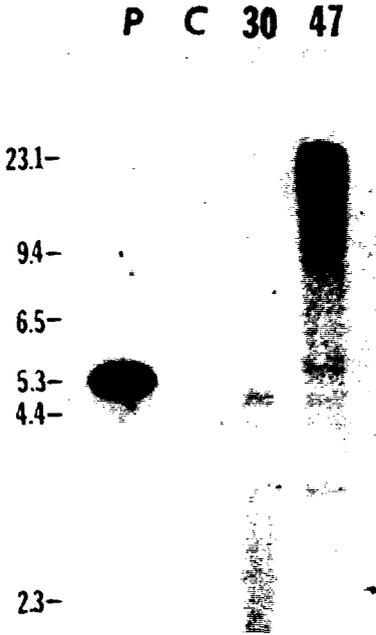


Fig. 3. Southern blot analysis of BamHI-digested genomic DNA from three goldfish, probed with the 2.3 kb BamHI-HindIII fragment of pRSVneo. Lane C contains DNA from one control, non-injected fish. Lanes labelled 30 and 47 contain DNA from the fish which showed positive hybridization signals in DNA dot-blot. Lane P contains linearized pRSVneo DNA. Locations of bands for size markers (λ DNA cut with HindIII) are indicated on the left.

ment, RNA was treated with DNase and RNase. DNase treatment had a negligible effect on the results of RNA dot-blot hybridization, while RNase treatment destroyed hybridization, indicating that hybridization had been RNA-specific (results not shown). Screening of dot-blot of lower concentrations of RNA from a second series of individuals (Fig. 4B) revealed evidence of neo gene expression in another individual without problems of high background hybridization.

G-418 selection

Forty pRSVneo-injected fish were exposed to 4 mg/ml G418. After 20 had died, the remaining fish were transferred to fresh well water. All fish died within 7 days after the test, presumably due to stress attributable to the drug exposure. Fig. 5 shows a series of DNA dot-blot of pRSVneo-injected fish that were subjected to drug selection with G-418. The fish were grouped by time of death during the drug selection trial. One fish, which survived the drug selection trial but later died of fungal infection, showed a hybridization signal with an inten-

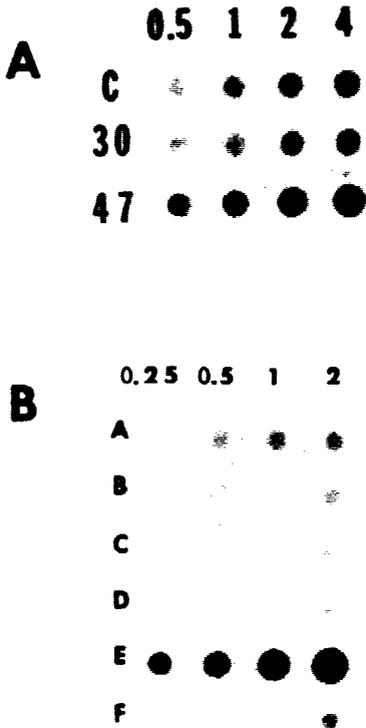


Fig. 4. A, An RNA dot-blot analysis of total RNA from two pRSVneo-injected fish, spotted at 0.5, 1, 2, and 4 µg RNA per dot for each fish. Row C contains total RNA from a non-injected control fish. Rows 30 and 47 contain total RNA from fish which showed positive DNA dot-blot hybridization signals. B, RNA dot-blot analysis of total RNA from six other pRSVneo-injected fish spotted at 0.25, 0.5, 1, and 2 µg RNA per dot for each fish.

sity of roughly one gene copy per genomic equivalent. DNA from groups of fish sensitive to G-418 showed no significant hybridization signal.

In a second G-418 trial, 63 pRSVneo-injected fish were exposed to 8 mg/ml G-418. After 90% of the control fish exposed to G-418 in a separate vessel had died, the nine surviving test fish were transferred to fresh well water. The test individuals died within 3 days after the test. DNA was extracted from these fish and analyzed by dot-blot. None of the DNA from these test fish exhibited strong hybridizations (results not shown). DNA from three of these individuals, however, showed hybridizations with an intensity of less than one gene copy per genomic equivalent. This may be evidence of chimerism in these individuals with respect to the introduced pRSVneo construct. Chimerism, or mosaicism, results from integration of the introduced DNA construct only into some cell lineages in a given individual.

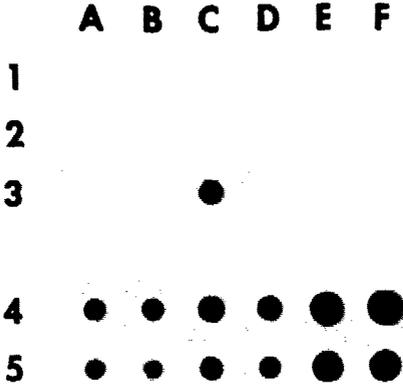


Fig. 5. DNA dot-blot analysis of fish exposed to 4 mg/ml G-418. Dots are arranged in order of increasing survival time from 20 h to 8 days. Dot 1F represents pooled DNA from four individuals which died after 20 h of exposure; dot 2A, three fish which died after 23 h; 2B, seven fish, 28 h; and 2C, six fish, 28 h. After 28 h, all surviving fish were transferred to fresh water. All these fish later died. Dot 2D represents five fish which died 40 h after the beginning of the test; 2E, five fish, 40 h; 2F, three fish, 64 h; 3A, three fish, 64 h; 3B, three fish, 3 days; and 3C, one fish, 8 days. Rows 4 and 5 are positive controls, including pRSVneo plasmid DNA spotted at concentrations equivalent to 0.5 gene copies per genome (4A, 4B, 5A, and 5B), one copy per genome (4C, 4D, 5C, and 5D), and five copies per genome (4E, 4F, 5E, and 5F). Negative controls, DNA from uninjected goldfish were present on the same blot but are not shown.

DISCUSSION

The results reported here showed successful transfer of the pRSVneo construct into goldfish, *Carassius auratus*. Integration of the transferred construct was strongly suggested. Three of the transgenic individuals exhibited hybridization phenotypes consistent with single genomic integration sites. The observation of junction fragments exhibited by goldfish # 47 indicated genomic integration of the microinjected construct. The multi-banded hybridization pattern of function fragments exhibited by this individual was indicative of multiple genomic integration sites, presumably due to delayed genomic integration with subsequent mosaicism for the neo gene in this transgenic individual. Although the frequency of mosaicism in transgenic animals is, for technical reasons, not firmly established, 30% has been suggested (Wilkie et al., 1986) as a low estimate for transgenic mice. Our identification of one putative chimera among four transgenic individuals would be in agreement with this estimate.

Expression of the neo gene was shown through detection of neo mRNA in two individuals. High levels of background hybridization in an RNA dot-blot (Fig. 4A) were not observed among DNA dot-blots. This may be attributed to: amplification of cross-hybridizing sequences relative to their genomic repre-

sentation in a population of mRNA transcripts, strong binding of RNA-DNA hybrids relative to DNA-DNA hybrids, or less stringent washing in preparation of the RNA dot-blot for autoradiography. Subsequent screening of smaller concentrations of RNA at higher stringency (Fig. 4B) showed greater contrast between positive and negative results.

Identification of promoters active in fish is an important practical aspect of developing gene transfer as a means of genetic improvement. Adding to previous reports of metallothionein promoter activity in fish (Maclean et al., 1987), our data indicated the activity of the RSV promoter in the genome of goldfish. Definitive demonstration of correct function of the RSV promoter in goldfish would require evidence of proper initiation of mRNA transcription (through S1 nuclease mapping or primer extension), production of full-length mRNA transcripts (northern blot), and translation of mRNA into its protein product (radioimmunoassay). Activity of the RSV promoter also has been inferred in northern pike (manuscript in preparation) and in zebrafish (G.W. Stuart, University of Oregon at Eugene, personal communication, 1988).

Our group has been exploring the use of mass transfer techniques to overcome the tedium of injecting the large numbers of eggs needed to generate a phenotypically improved transgenic fish. While mass transfer techniques might prove less efficient than microinjection, the ability to manipulate large numbers of eggs at one time might compensate for the loss in transfer efficiency. A key element necessary to make mass transfer feasible is the development of a selection system to differentiate transgenic from non-transgenic fish without sacrifice of the transgenic fish. One approach to such a selection system would be to distinguish transgenic from non-transgenic individuals on the basis of transferred antibiotic resistance. The neo gene, conferring resistance to G-418, was utilized in this gene transfer study because of its known potential as a selectable marker. A wide range of eukaryotic organisms has proven sensitive to G-418 (Davies and Jimenez, 1980).

A gene conferring a selectable phenotype would ultimately be utilized as one component of a construct which also bears a gene of economic importance. Thus, selection would identify individuals likely to exhibit economically improved phenotypes. Individuals exhibiting high levels of marker-gene expression would be likely to exhibit high levels of expression of the economically important gene.

Trials based upon neo expression as a selectable marker were inconclusive. Two observations related to these trials must be explained.

Some putatively chimeric individuals did not survive exposure to G-418. Mosaicism for the introduced neo construct could complicate the response of an individual to G-418 selection. If genomic integration of the construct had been delayed beyond the stage of differentiation of cell lineages in the embryo, then only certain tissues of the adult would be transgenic. Thus, the sensitivity or resistance to a given concentration of G-418 of a chimeric individual might be

a function of how many tissues and which particular tissues were expressing the introduced gene. The problem of response of chimeric individuals to drug selection may ultimately have to be circumvented by testing the progeny derived from the original transgenic individuals. Mosaic parents with some transgenic germ cells would pass the novel gene to a portion of their progeny. These transgenic progeny would not be chimeric.

Some individuals which were not demonstrably transgenic survived exposure to G-418. This can be explained in two ways. First, there could be a small amount of genetic variance for G-418 sensitivity in these fish. This is largely eliminated as a possibility due to the failure to detect such survivors in the control non-injected population. Alternatively, these individuals may have been chimeric but with transgenic cells at low frequencies. In such cases, dot-blot analysis can fail to identify chimeric individuals, because a single copy of introduced DNA in one-fifth of the cells would be undetectable (Wilkie et al., 1986). Cases have been noted in transgenic mice where DNA analysis did not reveal incorporation of introduced DNA, but biological activity of the product of the introduced gene was detected (Hammer et al., 1985).

Work to improve G-418 selection protocols is being continued. Because of the expense of G-418, development of other selection regimes is also under consideration.

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REFERENCES

- Davies, J. and Jimenez, A., 1980. A new selective agent for eukaryotic cloning vectors. *Am. J. Trop. Med. Hyg.*, 29: 1089-1092.
- Davis, L.G., Dibner, M.D. and Battey, J.E., 1986. *Basic Methods in Molecular Biology*. Elsevier, New York, NY, pp. 136-138.
- Feinberg, A.P. and Vogelstein, B., 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 132: 6-13.
- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrup, J.P., Ringold,

- G.M. and Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.*, 84: 7413-7417.
- Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H., 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. U.S.A.*, 77: 7380-7384.
- Graham, F.L. and Van der Eb, A., 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, 52: 456-467.
- Grand, C.G., Gordon, M. and Cameron, G., 1941. Neoplasma studies: cell types in tissue culture of fish melanotic tumors compared with mammalian melanomas. *Cancer Res.*, 1: 660-666.
- Hammer, R.E., Brinster, R.L. and Palmiter, R.D., 1985. Use of gene transfer to increase animal growth. *Cold Spring Harbor Symp. Quant. Biol.*, 50: 379-388.
- Kafatos, F.C., Jones, C.W. and Efstratiatis, A., 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res.*, 7: 1541-1552.
- Luciw, P.A., Bishop, J.M., Varmus, H.E. and Capecchi, M.R., 1983. Location and function of retroviral and SV-40 sequences that enhance biochemical transformation after microinjection of DNA. *Cell*, 33: 705-716.
- Maclean, N., Penman, D. and Zhu, Z., 1987. Introduction of novel genes into fish. *Bio/Technology*, 5: 257-261.
- Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 545 pp.
- Neumann, E., Schafer-Ridder, M., Wang, Y. and Hofschneider, P.H., 1982. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO J.*, 1: 841-845.
- Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C. and Evans, R.M., 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature (London)* 300: 611-615.
- Petersen, R.B., Hensel, C.H. and Hackett, P.B., 1984. Identification of a ribosome-binding site for a leader peptide encoded by Rous sarcoma virus RNA. *J. Virol.*, 51: 722-729.
- Schenborn, E.T. and Mierendorf, R.C., Jr., 1985. A novel transcription property of Sp6 and T7 RNA polymerases: dependence on template structure. *Nucleic Acids Res.*, 13: 6223-6236.
- Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98: 503-517.
- Southern, P.J. and Berg, P., 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.*, 1: 327-341.
- Stacey, N.E., Cook, A.F. and Peter, R.E., 1979. Spontaneous and gonadotrophin-induced ovulation in the goldfish, *Carassius auratus* L. Effects of external factors. *J. Fish Biol.*, 15: 349-361.
- Steller, H. and Pirrotta, V., 1985. A transposable P vector that confers selectable G-418 resistance to *Drosophila* larvae. *EMBO J.*, 4: 167-171.
- White, B.A. and Bancroft, F.C., 1982. Cytoplasmic dot hybridization. *J. Biol. Chem.*, 257: 8569-8572.
- Wilkie, T.M., Brinster, R.L. and Palmiter, R.D., 1986. Germline and somatic mosaicism in transgenic mice. *Dev. Biol.*, 118: 9-18.
- Zhu, Z., Li, G., He, L. and Chen, S., 1985. Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). *Z. Angew. Ichthyol.*, 1: 31-34.