2 Development of Genetic Tools for Transgenic Animals

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There is a chronic need to develop transgenic fish for aquaculture and genetically engineered farm animals for various agricultural and medical purposes. To meet this need we have developed new lines of vectors based on transposable elements and border elements for genetically engineering animals in an efficient, cost-effective manner. The principles of two newly developed tools for fish are described. First, a transposable element system, based on the *Tc1/mariner* family of transposons that is active in animals from fish to mammals, is described. Second, the effectiveness of border elements, derived from insects and birds, to insulate transgenes from position effects is presented. Fish are used as a model system and as an example of the type of needs that can be met because these species are especially convenient and inexpensive for use in the development of laboratory procedures.

Introduction

The aquatic resources of the world are being exhausted by over harvesting of finfish and other aquatic organisms. As the world's population grows, its fisheries are being depleted at increasing rates. The USA suffers from a staggering international trade imbalance in fisheries products, about US$3 billion per year, the third largest contributor to its annual imbalance of payments. The US consumption of about 20 kg/person requires the harvest of a total of about 6 Mt per year (Parfit, 1995). To meet this demand, the US fishing fleet is harvesting more fish and depleting the fish stocks required to maintain fish populations, which has led in the last 4 years to a decline in wild fisheries off the coasts of the USA. As a result, in future years we will not be able to produce sufficient quantities of fish for our national needs. The situation will get worse if we continue with a 'fishing as usual' policy.

 Transgenic Animals in Agriculture
(eds J.D. Murray, G.B. Anderson, A.M. Oberbauer and M.M. McGloughlin)

Placing long-term moratoria on fishing in some regions is an economically and politically difficult choice, and the strategy will not result in increased harvests in the long run. Another way to increase yields of fish is through aquaculture of genetically superior stocks of fish that can be farmed at a faster rate for lower cost.

There are two methods for achieving improved stocks of fish and other commercially important animals. The first is classical breeding, which has worked well for land animals, but which takes decades to bring about major changes. For example, by controlled breeding, growth rates in coho salmon (*Oncorhynchus kisutch*) increased by 60% over four generations (Herschberger *et al.*, 1990) and body weights of two strains of channel catfish (*Ictalurus punctatus*) were increased by 21–29% over three generations (Dunham *et al.*, 1994). The second method is genetic engineering, a selective process by which genes whose behaviours we think we understand are introduced into the chromosomes of animals or plants to give these organisms a new trait or characteristic, such as improved growth or greater resistance to disease. The results of genetic engineering have exceeded those of breeding in some cases. In a single generation, increases in body weight of 58% were obtained in common carp (*Cyprinus carpio*) with extra rainbow trout growth hormone I genes (Chen *et al.*, 1993), with increases of more than 1000% in salmon with extra salmon growth hormone genes, and less in trout (Agellon *et al.*, 1988; Du *et al.*, 1992; Devlin *et al.*, 1994).

The advantage of genetic engineering in fish is that an organism can be altered directly in a very short period of time if the appropriate gene has been identified (Fletcher and Davis, 1991; Hackett, 1993; Iyengar *et al.*, 1996). The disadvantage of genetic engineering in fish is that few of the many genes that are involved in growth and development have been identified and the interactions of their protein products is poorly understood. At present, we cannot produce certain changes that might be possible through breeding programmes; however, as we identify genes in fish genomes, our ability to introduce new characteristics into fish will improve and allow us to avoid the long process of breeding. Well-developed procedures that work well for genetic analysis in other organisms are lacking in fish and other economically important animals. These include methods for insertional mutagenesis (gene tagging) and efficient procedures for producing transgenic animals. Transgenic DNA is not efficiently incorporated into chromosomes. Only about one in a million of the foreign DNA molecules integrates into the cellular genome, generally several cleavage cycles into development. Consequently, most transgenic fish are mosaic (Hackett, 1993). As a result, fish raised from embryos into which transgenic DNA has been delivered must be cultured until gametes can be assayed for the presence of integrated foreign DNA. The screening is extremely tedious, time-consuming and therefore relatively expensive. Many transgenic fish fail to express the transgene due to position effects. Consequently, transgenic fish are not routinely made as frequently as they

are needed. A simple, reliable procedure that directs early integration of exogenous DNA into the chromosomes of animals at the one-cell stage is needed. Moreover, we need a method for getting the genes to express reliably and continuously once they are in animal chromosomes.

Here, we present the results of work in our laboratory over the past 5 years to improve transgenesis in animals. The experimental animal we use is the fish because: (i) they are easy to raise; (ii) embryos can be obtained daily in large numbers; (iii) embryos develop outside the mother, which greatly reduces the expense and effort of raising transgenic individuals; (iv) development is rapid, about 2 days to hatching in zebrafish; (v) development takes place in an optically clear chorion, allowing visual inspection of development as it occurs following experimental genetic manipulation of the embryos; and (vi) the results appear to be applicable to all other vertebrates which we have tested. The initial drive for transgenic fish came from attempts to enhance production of fish in Minnesota. Our goal was to use genetic engineering to improve the characteristics of several species of fish so as to increase their commercial value. To do this, several genetic tools were developed which work as well in land vertebrates as in fish.

Results

Early results of genetic engineering in fish

The first goal of the Minnesota Transgenic Fish Group (MTFG, formed by Drs Kevin Guise, Anne Kapuscinski, Anthony Faras and Perry Hackett) was growth enhancement of commercially valuable fish, with the understanding that if we succeeded in this area, we could continue improvement of fish species for Minnesota aquaculture. More than 60,000 embryos of walleye, northern pike and rainbow trout were microinjected with DNA constructs that contained a growth hormone gene and the required genetic switches to ensure expression of the transgene. From many thousands of embryos that were microinjected, only about 1000 fish survived to adulthood. The low survival rate was due to many factors including constant stress of moving from one fish facility to another in various regions of the state, the lack of knowledge of indoor rearing of wild game fish, the natural cannibalism practiced by the fish at early stages, and possible lowered fitness of growth-enhanced, transgenic fish.

Although the number of surviving founder fish was low, many were transgenic. Only one or two actively expressing fish are required to serve as broodstock for future generations. Initially we used a construct composed of the Rous sarcoma virus long terminal repeat sequence (which harbours enhancers and a semi-constitutive promoter) juxtaposed with the bovine growth hormone gene (RSV/bGH) for test purposes in the northern pike. In later studies we switched to an ‘all-fish’ construct composed of the carp

β-actin enhancers plus promoter driving a chinook salmon growth hormone gene (β-act/csGH) (Liu *et al*., 1990) for northern pike, walleye, rainbow trout and Atlantic salmon (Gross *et al*., 1992). About 10,000 northern pike embryos were injected with either of the two transgenic constructs. Of several thousand embryos injected with the RSV/bGH construct 1218 were examined by radioimmunoassay and 36 (3%) had elevated levels of bGH in their blood. Of the several thousand embryos that were injected with β-act/csGH, 1398 were screened to yield 88 (6%) with elevated csGH in the blood (MTFG, unpublished observations). This was in the range we expected.

The initial results were encouraging. There was an almost 40% increase in size of the fish, similar to what has been seen with other experiments of this type. However, these results did not take into consideration the sex of the fish, females are larger than males, or problems that could occur due to fish crowding. At later times, when several of the confounding parameters were considered, we found that only microinjected males showed a low but consistent increase (about 25%) in size over a 16 month time interval (Gross *et al*., 1992). Upon further consideration of our results compared with those of others who obtained larger fish (Agellon *et al*., 1988; Zhang *et al*., 1990; Du *et al*., 1992; Chen *et al*., 1993; Devlin *et al*., 1994; Dunham *et al*., 1994), we realized that we had not removed the prokaryotic vector sequences from our transgenic DNA. This was a serious omission because prokaryotes do not have a prejudice against CpG dinucleotide base pairs in their DNAs as do animal cells, where these base pairs are sites for DNA methylation (an activity associated with inhibition of gene expression). Thus, it is very possible that although the transgenes were present in a mosaic fashion in our fish, they were not active.

Our studies and those of others indicated that mosaicism was common, i.e. that the transgenic DNA integrated into the fish chromosomes after the initial cleavages, yielding fish that had the transgene in some tissues but not others. This determination came from Southern blotting of various tissues including muscle, fin, blood, kidney, spleen, heart and brain (Hallerman *et al*., 1990). Thus, by using PCR techniques on small samples of fin from the experimental fish, we could determine whether or not the transgenic construct was present in the fin but not necessarily in the most important tissue, the gonads. Presence in the gonads is important for passage of the trait to offspring. To determine further our abilities to get the transgenic DNA into fish chromosomes, we sacrificed a sample of the fish and carried out Southern blotting analysis of tissues. Thirty per cent of the fish had the transgene in one or more of the tissues analysed, but only about 12% had the gene in fin tissue. Thus, a positive signal in the fin samples represented only about 40% of the transgenic fish.

Why did only 3–6% of the fish show elevated levels of transgenic GH when 30% of the fish had the transgene in one or more tissues? The answer is not known. Our best speculation, which is fairly well founded on other

systems, is that expression of the transgenes is dependent not only on the accompanying genetic control elements, but also on regulatory sequences in the fish chromatin near the site of integration of the construct. There are about 2,000,000,000 potential sites of entry of the transgenic material, and the regulatory units around these sites play a role in transgene expression. Likewise, the problem of mosaicism is widespread in every laboratory that is attempting to make transgenic fish (Hackett, 1993). Screening transgenic fish would be much easier, and the results of the procedure far more predictable, if mosaicism were reduced. Accordingly, we initiated studies to improve the rate of early integration of transgenes into fish and to reduce the effects of neighbouring chromatin sequences on expression of integrated transgenes.

Efficacy of co-delivery of transgenes plus integrase to produce transgenic animals

Our first goal was to enhance integration by using a recombinase protein, an enzyme that is associated with restructuring DNA sequences. There are many types of recombinase proteins found in nature. We elected to examine recombinase proteins that were known to mediate integration of DNA sequences into chromosomal DNA. For this, specific recombinase-binding DNA sequences had to be added to both sides of the transgenic DNA construct (Fig. 2.1). However, some recombinases require host-specific cofactors for efficient activity; the key was to find a recombinase that either had no such requirement or required a factor(s) that was ubiquitous in a wide range of animal cells. We first used murine retroviral integrase synthesized from a baculovirus expression vector and from extracts of retrovirus-packaging cells (Ivics *et al*., 1993). We achieved a 10- to 40-fold enhancement of transgene expression, although we had hoped to get a much higher level of activity. There may have been several factors contributing to the low activity: (i) the integrase preparations made in the baculovirus-infected cells may have had low specific activities as a result of denaturation or poor solubility; (ii) the chromatin may have needed to be 'activated' for integration (a site in the chromosome may have needed to be 'opened' or cleaved), possibly by an endonuclease that produces ends complementary to those of the transgene; (iii) integrase may have to be packaged together with target DNA (as happens with reverse transcriptase and viral RNA during virus assembly in retrovirus-infected cells) to form an 'integration kit'; moreover, (iv) host factors probably are necessary for efficient function of integrase, and therefore mixing purified integrase with substrate DNA may not have mimicked the natural retroviral process faithfully. Nevertheless, the most important conclusion of this early work was that recombinases could be employed to enhance integration of transgenic DNA. To avoid the problem of host factors and find an alternative to using

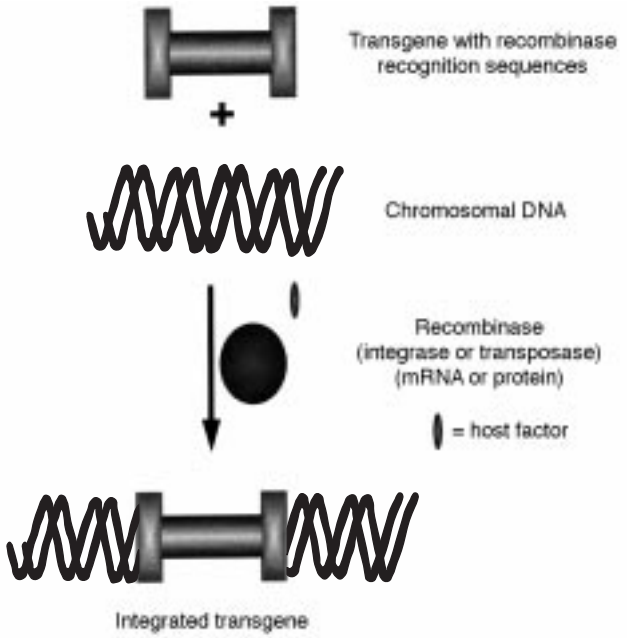

Fig. 2.1. Recombinase-directed integration of transgenes into chromatin. The schematic illustrates the principle of using either a transposase or integrase protein (large oval), with or without the assistance of cofactors (small, narrow oval) to mediate the integration of transgenic DNA (horizontal bar) into chromatin (double helix). The transgene is shown with recombinase recognition sequences (vertical rectangles flanking the transgene).

oncoviruses (or their products), we investigated repetitive elements in fish genomes (Izsvák *et al*., 1995; Izsvák *et al*., 1997). This was done in order to find other types of sequences that might harbour recombinases to catalyse efficient integration of DNA sequences into animal chromosomes. We especially scrutinized Tc1-type transposable elements, hereafter referred to as TcEs.

Adaptation of transposable elements as genetic tools in animal genetic engineering

The wide distribution of TcEs suggested that these elements require few, if any, species-specific host factors. In contrast, other mobile DNAs such as *P* elements, which appear to have requirements for specific host cofactors, do not function in fish (our unpublished observations and Gibbs *et al*., 1994). Transposase-deficient TcEs carrying marker genes can be mobilized by transposase provided in *trans*. These features suggested that TcEs would be

suitable for many genetic applications. However, because species-specific constraints of TcE transposition never had been evaluated rigorously, it was important to transfer elements from a species that was relatively close in evolution to the species in which they were to be applied. Indeed, we were unsuccessful in mobilizing the *Tc1* element from *Caenorhabditis elegans*, in fish.

Fish transposable elements belonging to the Tc1 family were discovered 3 years ago (Radice *et al*., 1994). Since then, we have identified TcEs in a dozen species of fish, indicating that TcEs are prevalent components of many fish genomes (Izsvák *et al*., 1995). Due to a variety of mutations in their transposase genes (Radice *et al*., 1994; Izsvák *et al*., 1995; Ivics *et al*., 1996), none of the identified fish TcEs encoded an active transposase. We found that the majority of fish TcEs can be classified into two major subfamilies, zebrafish- and salmonid-type elements. Although the two subfamilies of fish TcEs appear to have a common ancestor, they are characteristically different in their encoded transposases and their flanking sequences (Ivics *et al*., 1996). These findings suggested that a heterologous, *salmonid* transposon could be revived for use in developmental genetic studies in fish and other animals.

Structural and functional features of TcEs

TcEs contain a single gene encoding a transposase flanked by inverted repeats (IRs). Transposons spread when the transposase is expressed and their flanking IR sequences are exposed. The transposase catalyses the excision of the transposon from its original location and promotes its reintegration elsewhere in the genome (a 'cut-and-paste' mechanism). In leaving a site, the transposon leaves behind a gap in chromosomal DNA, which is often repaired by a mechanism that can regenerate a portion (a 'footprint') of the transposon at its original site of insertion. The prototype *Tc1* transposon has short, 54-bp IRs flanking its transposase gene. In contrast, most of the fish TcEs have long, 210–250 bp, IRs at their termini and directly repeated (DR) DNA sequence motifs at the ends of each IR. In this respect, fish TcEs are similar to other transposable elements from flies. We proposed that these IR/DR elements form a group of TcEs on the basis of the organization of their IRs, and that they transpose by a similar mechanism (Izsvák *et al*., 1995; Ivics *et al*., 1996). The direct repeats in the IR/DR flanks are the cores of the binding sites for transposase. These observations suggested that the number of putative transposase binding sites in the IR/DR-transposons is twice that of most known TcEs; therefore, these may be sites for regulation of mobility. An important observation is that *Minos*, a TcE from the fly *Drosophila hydei*, is active in *Ceratitis capita*, a non-drosophilid species of fly (Loukeris *et al*., 1995). This suggested to us that, unlike *P*-elements, TcEs of the IR/DR class could be active beyond the species in which they were found. Multiple sequence alignment of TcE

transposases allowed us to identify highly conserved amino acid domains of functional importance (Fig. 2.2) (Ivics *et al.*, 1996). Phylogenetic sequence comparisons show that the C-terminal halves of TcE transposase proteins which make up the catalytic centre and contain the DDE box, are highly conserved and shared by other recombinase proteins, suggesting similar mechanisms for DNA cleavage and joining. A nuclear localization signal (NLS), and casein kinase II (CK-II) phosphorylation sites which mediated nuclear translocation were identified. Tc1 transposase has a bipartite DNA-binding domain overlapping the NLS motif at a cluster of basic amino acids. The N-terminal region (marked as a string of leucines) of the fish transposases has a striking sequence similarity to the bipartite *paired* domain. Our identification of functional domains in TcE transposases was important because it allowed us to improve the efficiency of the system.

An active TcE vector system for vertebrate animals

The two components of any transposon system are an active transposase and the DNA sequences that are mobilized. We searched for a transposase-producing IR/DR element in a number of fish species; however, *all* the elements that we examined appeared to be inactive due to deletions and other mutations. Therefore, we used the accumulated sequence data to reconstruct a salmonid transposase gene from sequence alignments of TcEs found in 11 fish species. Since parsimony analysis could not resolve the phylogenetic relationships among salmonid-type TcEs (Ivics *et al.*, 1996), we engineered a consensus transposon with an intact transposase gene from salmonid elements which we call *Sleeping Beauty* (SB). A series of ten

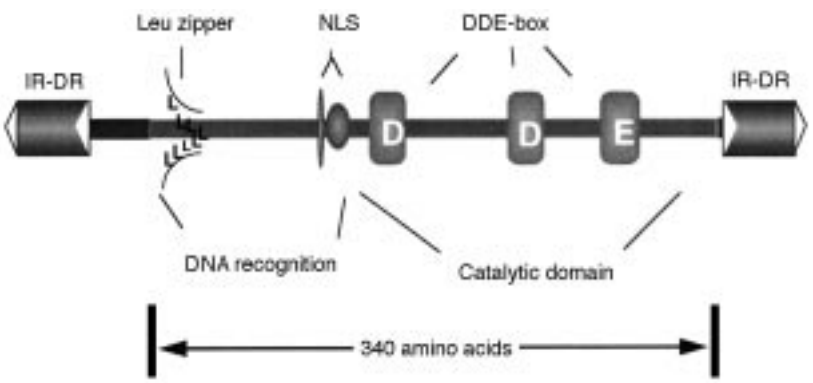

Fig. 2.2. Schematic map of a salmonid TcE. The conserved domains in the transposase and IR/DR flanking sequences are shown. The domains are defined in the text.

constructs (Fig. 2.3) were made by a PCR-mutagenesis strategy to step-by-step produce a synthetic gene encoding a putative salmonid transposase protein of 340 amino acids that is nearly identical to the consensus, and that possesses all the most conserved domains (Ivics *et al*., 1997). By this method, selected nucleotides were changed in codons to restore the amino acids that were in the putatively active transposase gene many millions of years ago. We did not blindly use a 'majority-rule' consensus sequence; e.g. at some loci it appeared that C→T mutations had been fixed where deamination of $5^{m}C$ residues had occurred (which leads to C being converted to T which in turn can lead to the 'repair' of the mismatched G residue to an A). We could test for various expected activities of the resurrected transposase, and thus the accuracy of our engineering, by examining several

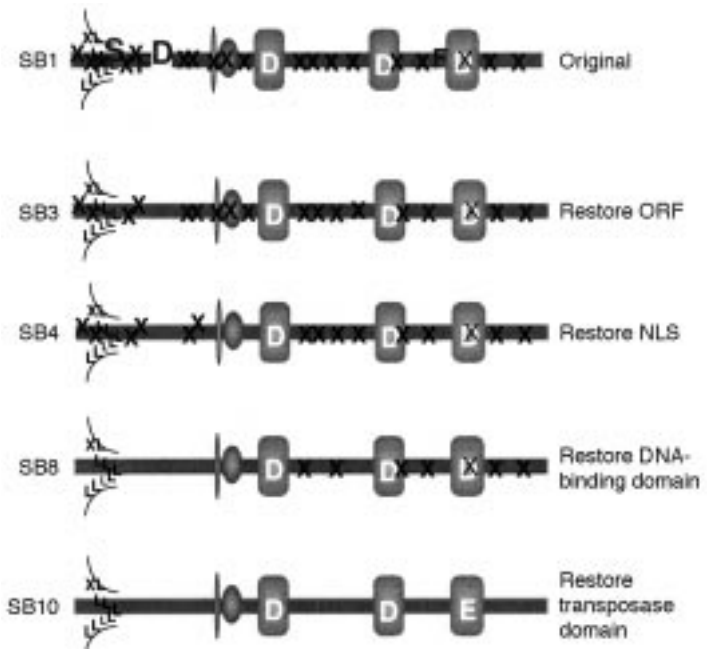

Fig. 2.3. Molecular reconstruction of the SB transposase gene. Several types of site-specific changes were made: black-D (a major deletion was filled in) and S (translational termination codons) and F (frameshift mutations) were replaced. Residues marked by Xs were changed to the consensus. In the right margin, the net results, based on various functional tests, are indicated. SB1 is the initial salmonid TcE transposase gene with the conserved domains indicated. The first two stages resulted in a complete open reading frame for the transposase (SB3). Systematic replacement of specific amino acids restored the bipartite nuclear localization signal (SB4), the DNA-binding domain specific for salmonid but not zebrafish TcEs (SB8) and catalytic domain (SB10). The methods used for the site-specific mutagenesis are described in detail in Ivics *et al*. (1997).

specific functions which are found in active transposase proteins (see right margin of Fig. 2.3).

We have four sources of synthetic SB transposase protein:

1. Extracts of tissue-cultured zebrafish or carp EPC cells transfected with a eukaryotic expression plasmid carrying the SB transposase gene driven by the human cytomegalovirus (CMV) promoter.
2. Purified SB transposase protein, or portions thereof, obtained from extracts of *Escherichia coli* transformed with the SB transposase gene in a pET (Novagen) bacterial expression vector; this vector directs the addition of a histidine-tag for purification and an epitope-tag for detection of the recombinant protein.
3. Extracts of 5-h old zebrafish embryos microinjected at the one-cell stage with SB transposase mRNA synthesized *in vitro*.
4. SB transposase expressed in bacculovirus-infected insect cells.

We examined some of the biochemical activities of the SB protein. One such test is shown in Fig. 2.4, the mobility-shift assay. In this assay, if a protein can bind to a specific DNA sequence, which is crucial for a transposase protein, then it will convert a small DNA molecule into a larger complex that will migrate slower during electrophoresis through a gel. We tested the DNA-binding activity of the amino-terminal fragment (123 amino acids, called N123) of SB transposase, expressed in *E. coli* and isolated via a histidine tag, that contains the putative DNA-recognition motif (Ivics *et al.*, 1997). The target for the N123 was a 320-bp IR/DR DNA sequence from either a salmonid TcE or the zebrafish Tdr1 transposon. The salmonid IR/DR sequence could be shifted by N123 to two positions, complex 1 with one bound N123 polypeptide and complex 2 with two N123 molecules. The SB IR complexed with N123 could be completed by SB IR/DR fragments, but not with closely related IR/DR DNA sequences from zebrafish. We conclude that our synthetic transposase protein has DNA-binding activity, and this binding is specific for salmonid IR/DR sequences. These data suggest that the SB transposase will have the ability to mobilize specific vectors based on our SB transposon without disturbing endogenous TcEs of the host.

Two crucial tests for a transposase are its ability to cut DNA precisely out of one DNA molecule and then to insert it into another DNA sequence. We tested excision and integration activities of our SB transposase by an inter-plasmid marker-transfer assay. The indicator (donor) plasmids for monitoring transposon excision and/or integration had two features: (i) a marker gene that, when recovered in *E. coli* or in fish cells, can be screened by virtue of either the loss or the gain of a function, and (ii) transposase-recognition sequences in the IRs flanking the marker gene. Care was taken during these constructions to keep the total size of the marked transposons around 1.6 kb, the natural size of TcEs found in teleost genomes. Using the assay system, we examined the integration activity of SB transposase. Table 2.1 shows that the number of recombinant plasmids (doubly resistant to the antibiotics

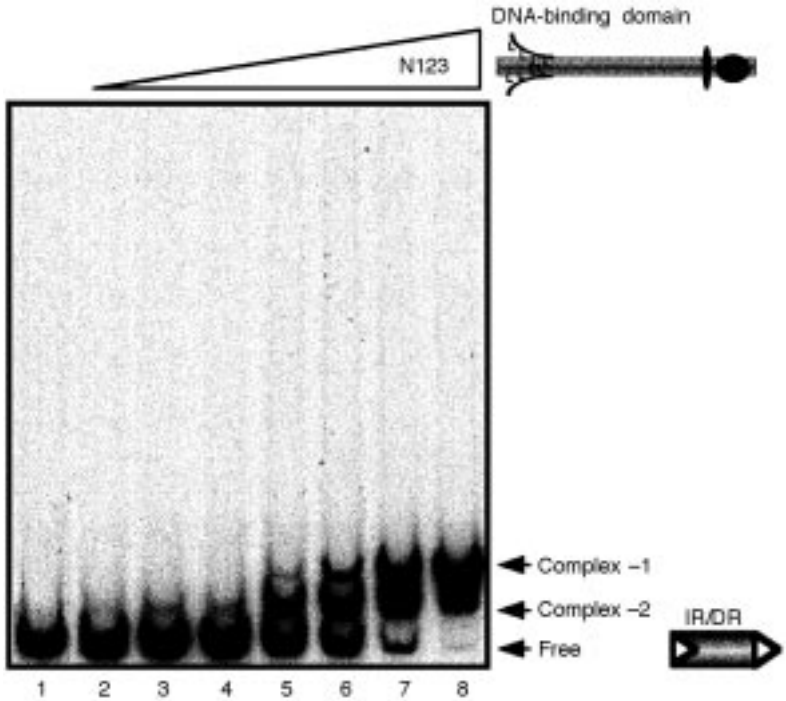

Fig. 2.4. DNA-binding activities of SB transposase. Mobility-shift analysis of the ability of the amino-terminal third (first 123 amino acids; N123) of SB transposase to bind to the inverted repeats of fish transposons. The N123 peptide was purified from *E. coli* in which it was cloned and expressed. ^{32}P-labelled IR sequences were combined with the N123 peptide in varying amounts plus unlabelled poly(dI:dC) non-specific competitor DNA. Lane 1: probe only; lanes 2–8: 100,000-, 50,000-, 20,000-, 10,000-, 5000-, 2500- and 1000-fold dilutions of the N123 preparation. The binding of two N123 peptides to each IR/DR DNA segment did not display cooperative binding kinetics.

ampicillin and kanamycin) was about 20-fold higher than control transformations lacking SB transposase mRNA. The physical translocation of the *Ap*-marked TcE was confirmed by Southern hybridization; we did not find any evidence that double antibiotic resistance was due to co-transformation by two original plasmids. Based on the estimated average number of plasmids injected per embryo, the activity shown in Table 2.1 corresponds to an average of 20–50 insertions per zebrafish embryo. Approximately 5×10^5 3-kb plasmids were injected per embryo. This corresponds to about 1.5×10^9 bp of total injected DNA, nearly the same as the zebrafish genome (about 1.6×10^9 bp). Assuming that the plasmid DNA formed nucleosomes, this assay is an excellent test of the system *in vivo*. In the course of these

Table 2.1. SB transposase-dependent transposition in zebrafish embryos. We evaluated the transposition activity of SB transposase by co-microinjecting 200 ng ml^{-1} of SB transposase mRNA, made *in vitro* by T7 RNA polymerase from a Bluescript expression vector, plus about 250 ng ml^{-1} each of target and donor plasmids into one-cell stage zebrafish embryos. Low molecular weight DNA was prepared from the embryos at about 5 h post-injection, transformed into *E. coli* cells, and colonies selected by replica plating on agar containing 50 mg ml^{-1} kanamycin and/or ampicillin. The data show results from three independent experiments and the numbers of putative transposition events is given per experiment.

Reagent	− SB txpase	+SB txpase[a]
Indicator (*Ap*) plasmid	+	+
Target (*Km*) plasmid (total)	43,858	46,492
Transposition events[b]	1	19
Transposition frequency	0.002%	0.041%

[a] SB txpase, SB transposase mRNA.

[b] Number of true *Km*/*Ap* plasmids found in 5 h zebrafish embryos.

experiments, we found that a high percentage of the embryos did not survive beyond 4 days. Insertional mutagenesis studies in the mouse have suggested that the rate of recessive lethality is about 0.05. Assuming that this rate is applicable to zebrafish, the approximate level of mortality suggests that with the microinjection conditions we used there were, coincidentally, about 20 insertions per genome, which is in the range of that suggested by the inter-plasmid assays.

Using an assay to monitor transfer of a gene from a plasmid to a chromosome (rather than an inter-plasmid assay as with the zebrafish embryos) in transformed human cells and cultured fish EPC cells, we found a comparable 12-fold stimulation of integration of a transposon-like substrate DNA by SB transposase (Table 2.2). HeLa cells were transfected with SB transposase and an SV40–neomycin–phosphotransferase-II expression vector flanked by IR/DR repeats. The transfected cells were selected with G418 and stained with methylene blue to indicate survivors. The staining patterns clearly demonstrated that the integration of an SV40-*neo* construct flanked by IR/DR repeats into HeLa cells was significantly increased when an active transposase construct was coinjected when compared with control constructs with an inverted SB coding region or a defective transposase gene lacking the DDE-catalytic domain but containing the DNA-binding and NLS functions. These data suggested that the SB system is active in vertebrates other than fish and that the activity of the SB transposase was due to its catalytic ability rather than merely binding to the vector DNA and facilitating its movement into the nucleus. Thus, the data in Tables 2.1 and 2.2 demonstrate that the SB transposon system is about as active for inserting DNA into chromatin in humans as in fish.

Table 2.2. SB transposase-dependent transposition in vertebrate chromosomes. Two plasmids were constructed, one encoding the SB transposase behind a cytomegalovirus promoter and the other with a transposon carrying an SV40-*neo* construct flanked by the SB IR sequences. The plasmid with the *neo* transposon was delivered to cultured cells by lipofection; the results show the relative levels of transformation when the plasmid with the SB transposase gene was co-transfected or not.

Cell line	− SB txpase	+ SB txpase	Increase
Carp EPC	362	868	2.4X
Mouse LMTK$^-$	66	308	4.7X
Human HeLa	34	417	12.3X

SB txpase, plasmid encoding SB transposase.

Construction of expression vectors for use with the transposon system in fish

Parallel to our development of transposition vectors, we initiated development of expression vectors that can be used either to overexpress, ectopically express or block expression of genes. Optimally, expression vectors will have a predictable level of gene expression and not be regulated by nearby *cis*-acting chromosomal sequences (position effects). We have found that border elements from flies and chickens function in zebrafish and confer not only position-independent expression, but also maintain expression levels through at least the F3 generation (Caldovic and Hackett, 1995; Caldovic and Hackett, 1998). In many transgenic experiments, it is desirable to have expression of the transgene that is not subject to position–effect variegation (i.e. the expression is not modulated by chromatin sequences flanking the integration site). We tested chick lysozyme *A* elements and *Drosophila* heat shock *scs/scs′* elements in zebrafish and found that they confer position-independent expression of a CAT transgene in every case where we found transmission of the genes (Caldovic and Hackett, 1995; Caldovic and Hackett, 1998) (Table 2.3). The data in Table 2.4 show that overall, our efficiency of producing transgenic fish is low, about 2.4%. Rates of integration do not appear to be influenced by the presence of border elements.

Table 2.3 shows that whereas the FV3CAT expression vector has non-uniform expression without border elements, with either of the two border elements it confers position-independent expression that is maintained through three generations of fish. Moreover, within a factor of two in zebrafish, the expression per gene per cell at 1, 2 and 5 days of development is constant for transgenes flanked with either *scs/scs′* or *A* elements. These data suggest that the border elements can confer reliable levels of expression of a transgene regardless of cell type or stage of development.

Table 2.3. Frequency of transgenic fish in F1, F2, and F3 generations.

Transgenic line	Expression pattern in F1	Total F1 fish	F1 fish	F2 fish	F3 fish
FV3CAT[a]	Non-uniform	522	13 (2.5%)	None found	Not done
scs(FV3CAT)scs[b]	Uniform	1278	46 (3.6%)	44/96 (46%)	Not done
A(FV3CAT)A[c]	Uniform	190	16 (8.4%)	166/317 (52%)	138/191 (72%)[d]

[a] Four lines of FV3CAT without border elements are totalled.
[b] Seven lines of FV3CAT flanked by *scs/scs′* border elements are totalled.
[c] Three lines of FV3CAT flanked by *A* elements are totalled.
[d] These F3 generation were interbred, leading to the higher percentage transmission of transgenes.

Table 2.4. Frequency of transgenic fish in F0 generation. One- or two-cell zebrafish embryos were injected with approximately 10^6–10^7 copies of the constructs shown in the table. Transgenic fish were identified by taking a small sample of fin tissue (fin clip) and screening for the presence of the CAT transgene by PCR. Fish that gave positive results were further analysed by Southern blotting to ensure that integration of the transgenic DNA had occurred (Caldovic and Hackett, 1998).

Construct	Total of F0 fish	Transgenic fish
FV3CAT	180	4 (2.2%)
scs(FV3CAT)scs	204	8 (4%)
A(FV3CAT)A	286	4 (1.4%)

Discussion

The results reported here demonstrate that it is now possible to move exogenous DNA more efficiently into the chromosomes of fish and other vertebrates using the SB transposon system. The mobilization and integration of exogenous, transgenic DNA depends only on the presence of transposase and inverted repeats of the correct sequence. Thus, we expect that the SB transposon will be useful in mammals, as well as in fish and birds, for delivering transgenic constructs into the chromosomes of animals (and perhaps plants) for biotechnological exploitation. Moreover, we have found that border elements flanking transgenes can be used to stabilize the expression of the exogenous genes such that their expression is consistent from line to line and is maintained during vertical transmission through the germline.

The results will have applications to many areas of aquacultural biotechnology. Development of transposable elements for vectors in animals

will permit the following: (i) efficient insertion of genetic material into animal chromosomes; (ii) identification, isolation and characterization of genes involved with growth and development; (iii) identification, isolation and characterization of transcriptional regulatory sequences controlling growth and development; (iv) use of marker constructs for quantitative trait loci (QTL) analysis; (v) identification of genetic loci of economically important traits, besides those for growth and development, i.e. disease resistance; (vi) site-specific gene inactivation for obtaining stocks with specific inactivated genes. This could be useful for producing sterile transgenic fish so that broodstock with inactivated genes could be mated to produce sterile offspring for either biological containment or for maximizing growth rates in aquacultured fish. We have participated in the construction of a genetic vaccine for fish that protects them against a killer rhabdovirus, infectious haematopoietic necrosis virus (Anderson *et al*., 1996). Use of our vectors in this area should be especially important to worldwide aquaculture.

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