Advances in Functional Analysis in Urodele Amphibians

Sukla Ghosh, Stéphane Roy*, Alex Pfeifer[¶], Mark Schmitt[¶], Inder M. Verma[¶], Susan V. Bryant and David M. Gardiner

Developmental Biology Center and Department of Developmental Cell Biology,
University of California at Irvine, Irvine, California 92697;
[¶]Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California 92037
*Current address: Université de Montréal, Faculty of Dentistry, Dept. of Stomatology, P.O. Box 6128 Station Centre-ville, Montreal (Qc) H3C 3J7, Canada
Correspondence to: Dr. David M. Gardiner, Department of Developmental and Cell Biology, 4238 BioSci II, University of California Irvine, Irvine, CA 92697-2275,
phone: 949-824-2792; fax: 949-824-5385, email: dmgardin@uci.edu

Introduction

Urodele amphibians have long been an important model organism for studies of fertilization, embryology, regeneration and evolutionary biology. Our particular research efforts have focused on the fact that urodeles are unique among vertebrates in their ability to regenerate lost body parts as adults. Although urodeles, and axolotls in particular, have historically offered many attractive and important features for experimental work, research progress in recent years has been significantly impeded by a lack of techniques for functional analysis. In contrast, we have witnessed remarkable advances in studies of a select group of organisms, identified as model organisms largely based on the ability to conduct studies involving functional analysis in one form or another.

The challenge of being able to achieve efficient and controlled somatic cell transgenesis is not limited to research on organisms such as the axolotl, and in reality is the ultimate goal of applied technologies involving gene therapy. The motivation to develop appropriate technologies is intense, and is leading to rapid improvements in viral vector technologies (Somia and Verma 2000; Pfeifer and Verma 2001). As a consequence, there are new opportunities to utilize the power of molecular genetics to investigate the many issues of development, regeneration and evolution that experimental work with axolotls can contribute to.

Although the expression of many genes involved in regeneration has been described and studied (see Gardiner, et al. 1999), techniques for somatic gene transfer have so far been of limited utility. Most studies have involved introduction of reporter gene constructs into blastema cells by either biolistics, direct injection of plasmid DNA, or infection with replication incompetent retrovirus. Though such studies have contributed valuable data, until recently it has not been possible to alter expression in sufficiently large populations of cells in order to generate the dramatic phenotypic changes seen in comparable studies in the chick or mouse. Other variably successful techniques that we have tested in our own laboratory include injection of DNA into blastemas, in vivo electroporation, adenovirus and bacculovirus viral vectors, and *in vitro* lipofection. Typically, there is some level of transfection and expression of the transgene, but the efficiency is low (<<10%) and expression is transient. We have determined that lipofection and subsequent selection of cells expressing a selectable marker (neomycin-resistance) would be feasible for *in vitro* studies and for subsequently grafting cells into tissues *in vivo* (unpublished observations).

In contrast to previous techniques for introducing transgenes into axoltol cells, the use of vaccinia viral vectors has been successful (see fig. 1A-E, (Roy, et al. 2000). The techniques for generating the recombinant vaccinia vectors, and the utility of vaccinia for functional analysis are among the subjects of this report and are discussed below. Although vaccinia virus has been successfully used to over-express *Shh* gene, and can be used for other secretory proteins, there are limitations to the use of this vector. The transcription and translation of endogenous genes of the host cell are impaired after infection, and expression of the transgene is transient. Thus vaccinia vectors are appropriate for functional analysis of secreted molecules, such as intercellular signals, but would not be appropriate as a vector to study the function of intracellular molecules such as transcription factors.

In order to have a more appropriate gene delivery system that would be capable of over-expressing transcription factors, we have been working to optimize the conditions for use of a lentiviral vector (Naldini and Verma 1999; Trono 2000) to transduce genes in axolotl cells both *in vitro* and *in vivo*. This vector offers several advantages including the ability to transduce genes into both dividing and non-dividing cells. In addition, the transgene is stably integrated into the host genome, which allows for prolonged expression without inhibiting the endogenous response of the host cell. In this report, we summarize progress to date with our studies utilizing both the vaccinia viral vector and the lentiviral vector.

MATERIALS AND METHODS

Animal Procedures

Axolotls (*Ambystoma mexicanum*) were spawned either at the Indiana University Axolotl Colony or at UC Irvine. Larvae were maintained at 20-22°C in 40% Holtfreter's solution. Animals (5-7 cm) were anesthetized in 0.1% MS222 solution for amputations to induce regeneration and for virus injections. Animals were euthanized at the end of the experiment. For Victoria blue staining, samples were fixed overnight in Bouin's fixative and then processed as in Bryant and Iten (1974). For X-gal staining (Miller 1972), samples were fixed for 30 min in phosphate-buffered saline

containing 1% formaldehye, 0.2% glutaraldehyde, 0.5 mM EDTA, 0.02% NP-40 and 2 mM MgCl2 for 30 min.

Axolotl cells *in vitro* were derived from a cell line established previously from limb connective tissue cells (Gardiner, unpublished). Explants of limb mesenchymal tissues were cultured as described previously (Groell, et al. 1993), and cells that migrated from the explants were cultured and passaged serially to establish the cell line. The axolotl limb connective tissues cells (ACT1) were cultured as described previously (Groell, et al. 1993).

Generation of Recombinant Vaccinia Virus

Vaccinia virus expressing genes of interest are generated through homologous recombination between a schuttle vector [pSC65, (Chakrabarti, et al. 1997)] and wild-type vaccinia virus as described previously (Mackett, et al. 1985). The pSC65 shuttle vector contains a synthetic early/late promotor that is active during the early and late phases of viral replication, the *Lac-Z* gene which allows for positive selection of recombinant virus. Homologous recombination disrupts the wild-type vaccinia virus thymidine kinase gene, which allows for negative selection of wild-type virus grown in the presence of BrdU. These two criteria (Bgal expression and lack of BrdU incorporation) allow for double selection and purification of high titer recombinant vaccinia virus (Chakrabarti, et al. 1985).

To generate *Shh*-vaccinia, we cloned chicken *Shh* (gift from Juan Carlos Ispisua-Belmonte) between the SalI and SmaI sites of the pSC65. The resulting plasmid, pSVShh was used to drive homologous recombination after being transfected into RK13 cells (ATCC CCL-37) infected with the wild-type vaccinia virus. Recombinant viruses were then selected by plaque assay and purified as describe previously (Mackett, et al. 1985).

Generation of Lentiviral Vector

A third-generation, Tat-free packaging system (Dull, et al. 1998) was used to produce recombinant lentivirus. A *Lac-Z* plasmid together with the two packaging plasmids (encoding human immunodeficiency virus (HIV) gag, pol, and rev) and the plasmid coding for VSV-G envelope were transfected into 293T cells using the calcium phosphate method. The virus was harvested by collecting the cell culture medium, which was filtered through 0.45 mm filters and concentrated by ultracentrifugation (Naldini, et al. 1996; Pfeifer, et al. 2000). The resulting pellet was resuspended in 200 ml Hanks' buffer for addition to cells *in vitro* or injection *in vivo*.

Viral Transduction

White or albino axolotls were used for microinjection of virus to allow for detection of the X-gal reaction product. Viral injections were made into mature limbs or into the regenerating blastemas. Vaccinia-*LacZ* or recombinant *Shh* virus of high titer was injected at single (300-500 nl) or multiple (1-2 (l total volume) sites. The titer of vaccinia-*LacZ* and vaccinia-*Shh* was $>10^9$ pfu/ml, as assayed by X-gal staining of RK 13 cells 72h after infection with a serial dilution of the virus preparation. To determine the earliest time of transgene gene expression after infection, virus was added to the culture medium of the ACT1 cells. *LacZ*-lenti virus was injected in multiple injections (1-2(l total volume of virus containing polybrene to a final concentration of 100 (g/ml). ACL1 cells were plated at $1.6x10^5$ cells per well in a six well plate two days prior to infection, and were infected with 5(l of virus/ well in the presence of polybrene (4(l/ml). The titer of *LacZ*-lenti was $3x10^9$ I.U./ml, as assessed by X-gal staining of 293T cells 72h after infection with a serial dilution of the virus preparation.

RESULTS AND DISCUSSION

ß-*gal vaccinia virus can transduce axolotl cells in vitro and in vivo*

Vaccinia virus is a member of the orthopoxvirus genus of the Poxviridae family (Moss 1991). It is a DNA virus that has been used as a replication-competent vector for the transient expression of transgenes. It can be grown to high titers and can accommodate foreign genes up to 25 kb.

Vaccinia virus with the *Lac-Z* gene inserted into its genome was able to infect axolotl limb cells *in vitro* and *in vivo*. When cultured axolotl limb cells were infected, no expression was observed at 3 h, but at 6 h and 12 h scattered expressing cells were observed (data not shown). Injection of mature axolotl limbs resulted in staining by 4 days (not examined earlier) that was still intense at least 12 days post-injection (Table 1A, Fig. 1A). Tissues in the mature limb that expressed the transgene included muscle and connective tissue fibroblasts (Fig. 1B).

TABLE 1
ß-Gal Activity after Ectopic Expression of *lacZ*

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**10**	**12**	**14**	**20**
Mature Limbs (subcutaneous injections)												
Vaccinia				++				++		+		
HIV		++	++		++			++	++		+	-
Regenerating Limbs (medium/late bud blastema)												
Vaccinia	+	++	++	++	++	+	+	-				
HIV	+	++	++		++			++			+	-

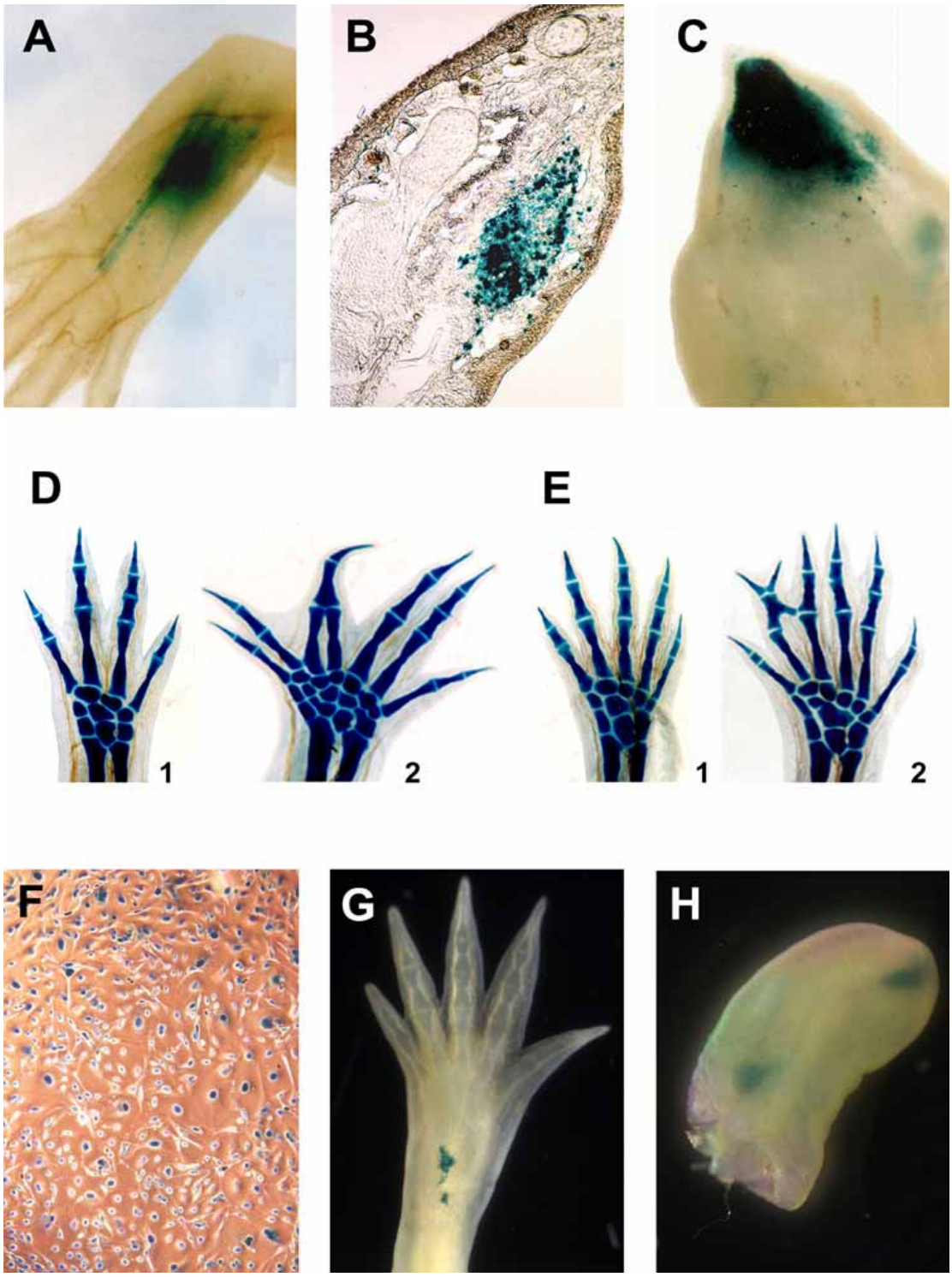

FIG. 1. X-gal staining of axolotl cells and tissues transduced with ß-gal vaccinia virus or ß-gal lenti virus, and skeletal preparations of axolotl forelimbs and hind limbs injected with *Shh* vaccinia virus. (A) X-gal staining in a mature limb 12 days after ß-gal vaccinia injection. (B) Section through a region of X-gal staining in mature limb tissues 4 days after ß-gal vaccinia injection. (C) X-gal staining in a medium bud blastema 4 days after ß-gal vaccinia injection. (D) Control forelimb (1) and *Shh* vaccinia virus-injected forelimb displaying extra digits and carpals (2). (E) Control hindlimb (1) and *Shh* vaccinia virus-injected hindlimb displaying extra digits and tarsals (2). (F) X-gal staining of axolotl limb cells 7 days after ß-gal lenti transduction *in vitro*. (G) X-gal staining in a mature limb 6 days after ß-gal lenti transduction *in vivo*. (H) X-gal staining in a medium bud blastema (upper right) and in adjacent stump tissues (lower left) 6 days after ß-gal lenti transduction *in vivo*. Images in A-E reprinted with permission from Roy, et al. (2000).

ß-gal activity could be detected in regenerating blastemas as early as 18 h after injection, but did not appear to persist as long as in mature limb tissues. The most intense staining was detected between 2-5 days after injection (Table 1 and Fig. 1C), after which staining declined and was not detected by 8 days (Table 1). When injected at several sites in a blastema, virtually all cells were positive for ß-gal activity (Fig. 1C). Although the staining was localized to the site of injection for single injections, the area of infected cells was larger than observed with injections of lentivirus (Fig. 1G,H). Since the vaccinia vector is replication competent, we presume there is secondary transduction of cells adjacent to the site of infection. In spite of the likelihood of lateral transmission of viral infection, we did not observe transgene expression in the overlying epidermis. Transmission between tissue layers may not occur because this strain of virus binds tightly to cell membranes of secreting cells (Blasco and Moss 1992). Injection of *Lac-Z* expressing virus into the blastema at different stages of regeneration did not affect growth, differentiation or pattern formation of the regenerated limb.

Ectopic expression of shh-vaccinia induces digit duplications in regenerating axolotl limbs.

Shh is expressed in a small population of cells in the posterior-distal region of the developing axolotl limb bud, which is comparable to the spatially restricted expression in limb buds of the other vertebrates (Torok, et al. 1999). *Shh* is also re-expressed during relatively late stages of limb regeneration on the posterior of the blastema (Torok, et al. 1999). To test whether *Shh* has an equivalent function during both limb development and limb regeneration, we constructed a *Shh*-vaccinia expression vector that we injected into regenerating limb blastemas. As noted above, injection of control, *lac-Z* expressing virus did not alternate pattern formation. In contrast, injection of *Shh*-vaccinia virus into the anterior of regenerating limb blastemas induced the formation of ectopic distal limb elements (carpals/tarsals and digits; Fig. 1D,E). Ectopic *Shh* only altered pattern formation when expressed during the stages of regeneration when endogenous *Shh* is expressed. More than 50% of the limbs injected during medium-bud to palette stages of regeneration were induced to form supernumerary structures. In contrast, limbs injected at earlier stages (preamputation or early bud blastema) regenerated a normal limb pattern (Roy, et al. 2000).

Although vaccinia virus can be successfully used as a vector to test the function of secreted molecules, such as a signaling molecule, its utility is limited for two reasons. Infection by vaccinia severely impairs transcription and translation of the genome of the host cell (Ausubel 1987). As a consequence, it is not possible to test the function of any molecule, such as a transcription factor, that functions to trigger a response by the host cell. In addition, expression of the transgene is transient, presumably due in part to the impaired function and diminished survival of the host cell.

*ß-**gal** lentivirus can transduce axolotl cells in vitro and in vivo*

Lentiviruses belong to the retrovirus family of RNA virus, but differ from many retroviruses in that they can infect both dividing and non-dividing cells (Somia and Verma 2000; Pfeifer and Verma 2001). By far the best characterized lentivirus is the human immunodeficiency virus (HIV), which has been disabled (replication-incompetent) and developed as a vector for *in vivo* gene delivery. The use of the VSV-G envelope protein allows for vector concentration by ultracentrifugation, and expands the range of target tissues (Burns, et al. 1993; Naldini, et al. 1996).

We have determined that the HIV vector can transduce axolotl limb cells with high efficiency *in vitro*. Approximately 95% of the cells express the transgene (as evidenced by X-gal staining) after 7days of culture post-infection (Fig. 1F). We have also detected expression of the transgene in cells that have been cultured for 30 days. We presume that long-term expression occurs as a consequence of the stable integration of the transgene into the host cells' genome, which is a characteristic of HIV-based vectors (Somia and Verma 2000; Pfeifer and Verma 2001).

HIV-based vectors also transduce mature limb tissues (Fig. 1G) and blastema cells (Fig. 1H) *in vivo*. Expression of the transgene (*Lac-Z*) can be detected within 48 hours in mature limb tissues, and is readily detectable for about 2 weeks (Table 1). This time period is comparable to what we observed previously with injection of vaccinia virus into mature limb tissues. We have yet to section injected limbs to determine if the transgene is expressed at lower levels or in reduced density of cells over longer time periods. We presume that long term expression occurs since we have observed expression over at least 30 days *in vitro*. However, it is possible that the CMV promoter, which drives expression of *LacZ*, is shut-off in the axolotl cells *in vivo*.

Transgene expression in blastemas injected with HIV-based vectors persists at least twice as long as with injections of vaccinia vectors (Table 1). We readily detect expression for about 2 weeks, as compared to one week for vaccinia, and presume that expression persists beyond this period, albeit at lower levels or in reduced densities of transduced cells. Overall, the regions of expressing cells resulting from HIV injections are smaller and more localized as compared to vaccinia injections (compare Fig. 1A and G for mature limbs and Fig. 1C and H for blastemas). We presume this more limited region of expression is a consequence of the HIV vectors being replication incompetent, which would preclude lateral transmission of the virus.

Further analysis is underway to optimize the use of the HIV-based vector for functional analysis in the axolotl. This vector offers the valuable benefits of having a high rate of transduction both *in vitro* and *in vivo*, and being able to mediate the integration and long term expression of transgenes. Finally, and perhaps most significantly, HIV-based vectors will allow for the study of a wide range of molecules, including the many transcription factors that likely are involved in controlling the early events in the initiation of the regeneration cascade.

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