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REVIEWS

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Xenopus, the Next Generation: *X. tropicalis* Genetics and Genomics

NICOLAS HIRSCH,[1] LYLE B. ZIMMERMAN,[2] AND ROBERT M. GRAINGER[1]*
[1]*Department of Biology, University of Virginia, Charlottesville, Virginia*
[2]*Developmental Biology Division, National Institute for Medical Research, The Ridgeway, Mill Hill, London, United Kingdom*

ABSTRACT **A small, fast-breeding, diploid relative of the frog *Xenopus laevis*, *Xenopus tropicalis*, has recently been adopted for research in developmental genetics and functional genomics. *X. tropicalis* shares advantages of *X. laevis* as a classic embryologic system, but its simpler genome and shorter generation time make it more convenient for multigenerational genetic, genomic, and transgenic approaches. Its embryos closely resemble those of *X. laevis*, except for their smaller size, and assays and molecular probes developed in *X. laevis* can be readily adapted for use in *X. tropicalis*. Genomic manipulation techniques such as gynogenesis facilitate genetic screens, because they permit the identification of recessive phenotypes after only one generation. Stable transgenic lines can be used both as in vivo reporters to streamline a variety of embryologic and molecular assays, or to experimentally manipulate gene expression through the use of binary constructs such as the GAL4/UAS system. Several mutations have been identified in wild-caught animals and during the course of generating inbred lines. A variety of strategies are discussed for conducting and managing genetic screens, obtaining mutations in specific sequences, achieving homologous recombination, and in developing and taking advantage of the genomic resources for *Xenopus tropicalis*.**

Key words: embryology; development; transgenesis; genetic screens; mutagenesis; GAL4; UAS; *Xenopus*; genomics

INTRODUCTION

Xenopus has been an invaluable model for studying fundamental cellular and developmental problems. Cell biological studies in this system have greatly clarified chromosome replication, chromatin and nuclear assembly, control of the cell cycle, in vitro reconstruction of cytoskeletal dynamics, and signaling pathways. Embryologic work has untangled mechanisms of induction, early fate decisions and morphogenesis, patterning of the vertebrate body plan, and early organogenesis. The vitality of research on *Xenopus* is due to several factors, including the ability to stimulate production of eggs year round, the external development of embryos, and the large number of eggs per spawning. The size of the eggs and embryos is large enough to readily permit microsurgical manipulation and injection, and *Xenopus* embryos show remarkable powers of healing subsequent to these procedures.

Important insights into gene function have been obtained by injecting *Xenopus* embryos for misexpression or overexpression of gene constructs. This approach is limited to the delivery of experimental gene products early in development to broad regions of the embryo. Later stages of development are difficult to affect due to the short half-life of many injected gene products, including synthetic mRNAs, and diffuse application of injected constructs can obscure the subtleties of temporal and spatial cellular interactions. These problems can be addressed by using the transgenic technology developed for *X. laevis* (Kroll and Amaya, 1996; Amaya and Kroll, 1999) to express constructs tissue-specifically at later developmental stages, but variations in expression level and developmental abnormalities can complicate analysis of the primary products of transgenesis. Alternative approaches making use of stable transgenic lines, discussed here, are relatively difficult to implement in *X. laevis* due to its long generation time.

Loss-of-function experimental designs would also complement misexpression/overexpression studies of

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Drs. Hirsch and Zimmerman contributed equally to this manuscript.

*Correspondence to: Robert M. Grainger, Department of Biology, P.O. Box 400328, University of Virginia, Charlottesville, VA 22904-4328. E-mail: rmg9p@virginia.edu

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gene function. These approaches typically include analysis of null mutations as well as antisense "knockdown" strategies, but have not been readily applicable to *X. laevis*. Both are complicated by the allotetraploid origin of *X. laevis*, reflected in four copies of many genes. Knockdown strategies can require the application of multiple antisense sequences, and visualization of recessive embryonic phenotypes may require mutagenesis of four separate alleles.

With the advent of genomic resources for *Xenopus* research, there is a strong impetus to develop such multigenerational genetic and transgenic approaches in the close relative *Xenopus tropicalis*, where there are far better prospects for performing these experiments than in *X. laevis* (Amaya et al., 1998). *X. tropicalis* has a much shorter generation time (just under 3 months for males, compared with over twice that for *X. laevis*) and a true diploid genome. A set of tools has been developed for rapidly uncovering mutations in amphibia (Krotoski et al., 1985) that has led to the characterization of several interesting mutants in *X. laevis* (Krotoski et al., 1985; Dudek et al., 1987), despite its drawbacks as a genetic system.

One might fairly ask why develop a new vertebrate genetic system when these methods are already so advanced in organisms like the mouse and zebrafish? With regard to the mouse, internal development, requirements for culturing embryos, and the small number of embryos per litter severely hamper the utility of this system for experimental embryology. The small number of embryos per litter makes it a less attractive system for some kinds of genetic screening as well. Nonetheless, the striking insights generated by mouse genetics, particularly in recent years by gene targeting technology, emphasize the value of genetic approaches. The zebrafish system offers the advantages of large, externally developing, transparent embryos and a relatively short generation time that overcomes many of the limitations of the mouse system for studying developmental phenomena. However, although zebrafish embryos are excellent for some manipulations, particularly blastomere injections and transplants, the embryos are relatively fragile, and one cannot perform many of the tissue transplant and explant experiments that have made *Xenopus* such a powerful embryologic system. Although *Xenopus* is not transparent at early stages, embryos become transparent at tadpole stages and, therefore, are extremely useful for scoring mutant phenotypes and fluorescent reporter gene expression. Some have also argued that the ancestral genome duplication in teleosts (Woods et al., 2000; McClintock et al., 2001) makes zebrafish a less than ideal system for genetics. Although this duplication has not hampered the isolation of many valuable mutations, it is apparent that there is a pool of redundant genes, whose functions are unlikely to be revealed in conventional recessive screens.

Another reason that developing genetics in *X. tropicalis* is particularly timely is the recent development of a reliable amphibian transgenesis protocol. Discussed in detail below, this system is arguably the most efficient available for making transgenic vertebrates. One person, at modest cost, can prepare several hundred transgenic embryos per day. Procedures for transgenesis in the mouse are costly, and it is difficult to obtain large numbers of embryos. There are currently limitations in zebrafish as well; first-generation animals are generally mosaic and, therefore, only F1 (first filial generation) animals can be used for experiments. Because the *Xenopus* procedure involves insertion of the transgene in sperm nuclei, mosaicism is rare and first generation animals can be used for many kinds of experiments, for example in promoter mapping by using fluorescent reporter constructs in living embryos (Offield et al., 2000), or in identifying putative gene trap embryos (see below). This finding makes transgenesis extremely useful because it dramatically shortens the time required for many experiments. Although the transgenic procedure works well in both *X. laevis* and *X. tropicalis*, the latter species is advantageous because of the shorter time needed to generate and expand lines. Also, evaluating promoter constructs from the diploid *X. tropicalis* greatly simplifies what has been a severe limitation of *X. laevis*, because it can be challenging to ascertain which of the duplicated genes in *X. laevis* is active, or whether both contribute to different elements of gene expression.

The recent efforts to establish genomic resources for *Xenopus*, particularly a large expressed sequence tag (EST) database, and the decision to sequence the *X. tropicalis* genome, are already becoming an extraordinary resource for the *Xenopus* community in general and provide another strong impetus for genetic studies in *X. tropicalis* in particular. Genomic projects now under way are discussed further below and, in more detail, in the article by Steve Klein in this issue.

In this review, we will discuss the basic features of the *X. tropicalis* system, how it might be especially useful for embryologic studies, and ways in which transgenesis in this organism can be used to study gene function. We will then discuss several different strategies for genetic analysis and ways in which genomics will bring powerful new tools to this system.

EVALUATION OF *X. TROPICALIS* PLOIDY

In addition to the introduction to *X. tropicalis* presented in this and subsequent sections, more information can be found at the Grainger lab *X. tropicalis* Web site (http://faculty.virginia.edu/xtropicalis/) and in the accompanying article in this issue (Hirsch et al., 2002). Some important parameters are listed in Table 1, which compares features in *X. tropicalis* and *X. laevis*. One important issue is the evaluation of ploidy of *X. tropicalis*, because a conventional diploid vertebrate genome is important for genetic and genomic studies. All of the available data support the conclusion that *X. laevis* is of allotetraploid origin (meaning that the species arose through a hybridization in which both parental genomes were retained) and *X. tropicalis* diploid (Thiebaud and Fischberg, 1977; Jeffreys et al., 1980;

TABLE 1. Comparison of Properties Between *Xenopus tropicalis* and *Xenopus laevis*

Property	Species	
	X. laevis	*X. tropicalis*
Ploidy	Allotetraploid	Diploid
Haploid chromosome number	18 chromosomes	10 chromosomes
Genome size	3.1×10^9 bp	1.7×10^9 bp
Temperature range	16–22°C	22–32°C[a]
Adult size	10 cm	4–5 cm
Egg size	1–1.3 mm	0.7–0.8 mm
Eggs per spawning	300–1000	1,000–9,000
Generation time[b]	8–12 months	3–4½ months

[a]Optimal temperature range is 25–28°C.
[b]Rate of development for both *X. tropicalis* and *X. laevis* depends significantly on husbandry conditions. The numbers shown here for *X. tropicalis* would be achieved using currently optimized conditions for raising a population to sexual maturity: 3 months for males, 4½ months for females. The time needed to reach sexual maturity for *X. laevis* is often reported to be well over a year. Under the same rapid growth conditions used for *X. tropicalis,* the *X. laevis* would be less than this reported figure, but would typically still require 8 months or more to reach sexual maturity; the fastest males may reach sexual maturity in 6 months.

Fig. 1. Adult *Xenopus laevis* and *Xenopus tropicalis* appear similar, except for size. Female *X. laevis* (left) and female *X. tropicalis* (right).

Burki and Fischberg, 1985; Graf and Fischberg, 1986; Schmid and Steinlein, 1991; Tymowska, 1991). Many arguments for a diploid *X. tropicalis* genome are based on its relationship with *X. laevis*; in *X. laevis*, large numbers of genes have been cloned and shown to be present in duplicate (but not higher copy numbers), consistent with tetraploidy and not higher-order polyploidy (Graf and Kobel, 1991). Approximately 15–20 *X. tropicalis* and *X. laevis* gene structures have been compared, by genomic Southern analysis, with *X. laevis* yielding approximately twice the number of hybridizing fragments in most cases (Jeffreys et al., 1980; Mohun et al., 1988; L. Zimmerman and R. Grainger, unpublished results). Two gene clusters have been examined at the molecular and genetic level with regard to ploidy. The globin locus has been shown to be diploid in *X. tropicalis* and duplicated in *X. laevis* (Jeffreys et al., 1980). The major histocompatibility complex (MHC) is a much larger cluster, encompassing many genes and approximately 4 megabases. It has been mapped genetically in a variety of *Xenopus* species, including *X. tropicalis*, as well as in zebrafish, and in nearly all cases individual genes have resolved to a diploid state (presumably evolution prefers these genes as single copies; Salter-Cid et al., 1996; Nonaka et al., 1997). The MHC is highly structurally conserved, in a single linkage group, with the chromosomal order of genes maintained from human through mouse to *X. tropicalis*. Elements of the structure are also conserved in higher ploidy *Xenopus* as well as zebrafish, but in these polyploid species, they map to linkage groups on several chromosomes, consistent with a genome duplication event followed by random gene loss from the duplicated clusters to restore diploidy of individual genes. In *X. tropicalis*, this large and complex single genetic structure is conserved intact, strong evidence that this species contains a typical diploid tetrapod genome.

The *X. tropicalis* genome has also been evaluated cytologically and its DNA content determined. Cytologically, *X. laevis* has been described as a functional diploid of allotetraploid origin, with 36 chromosomes and 18 bivalents (reviewed in Tymowska, 1991). In addition, most *X. laevis* chromosomes can be arranged into quartets by their similar specific replication pattern, also consistent with tetraploidy (Schmid and Steinlein, 1991). *X. tropicalis* is cytologically the sole diploid species of the genus *Xenopus* (Tymowska and Fischberg, 1973; Tymowska, 1991) with 20 chromosomes (n = 10), approximately half that of *X. laevis*. The genome size of *X. tropicalis* is 1.7×10^9 bp (*X. laevis* is 3.1×10^9 bp) (Thiebaud and Fischberg, 1977).

X. TROPICALIS HUSBANDRY AND DEVELOPMENT

X. tropicalis closely resembles *X. laevis*, although adults are approximately one quarter the size of *X. laevis* (see Fig. 1) and, therefore, can be housed at significantly higher densities. Adults will survive at temperatures from 22–32°C, although animals should not be kept at or near the lower limit for more than a few days. The optimal temperature for raising adults is 25–28°C. Egg production in females appears to be more consistent when animals are maintained at 25°C. Matings can be induced by human chorionic gonadotropin (HCG) treatment, as with *X. laevis*, but at lower doses (typically a 15 U prime followed a day later with a 75-U boost), and with a faster response time, 3 to 4 hr from boost injection to egg deposition. Matings are effectively done at temperatures from 22–25°C. In vitro fertilization can be done by procedures similar to *X. laevis*. Embryos can be kept at 22–25°C in simple salt solutions (those typically used for *X. laevis*). A system for raising tadpoles at high efficiency to metamorphosis can be quite simple: success is achieved by feeding tadpoles the powdered mix Sera Micron (Sera Part-

ners), accompanied by partial water changes (to avoid shock) every day or two. Metamorphosed animals can be fed the standard pellet foods used for *X. laevis* (small sizes for smaller animals), supplemented with blackworms (*Lumbriculus variegatus*) to achieve more rapid growth. Postmetamorphic animals can be raised in the standing water, flow through, or recirculating systems used for *X. laevis*. Recirculating systems (e.g., from Marine Biotech or Aquatic Habitats) have been very successful in promoting rapid growth of healthy animals. The generation time for males raised under currently optimal conditions is just under 3 months, whereas females take as little as 4.5 months to reach sexual maturity. The current Grainger lab facility-wide average is approximately 4.5 months for males and 6 months for females.

Although the generation times achieved with current husbandry conditions are compatible with performing multigenerational studies, improvements in husbandry regimens are likely to further shorten these times. Improvements in food sources, housing conditions, and development of strains that develop more rapidly (there is evidence showing growth differences between strains; R. Grainger, unpublished) are all likely to be beneficial in this regard. Hormone treatments may be useful for accelerating the generation time, e.g., by treatment of froglets with androgens and gonadotropin-releasing hormone (GnRH; McCreery and Licht, 1984; Stamper and Licht, 1993) to accelerate gonadal development. Although males can be remated at biweekly intervals, females are usually given a 2- to 3-month rest period between matings to achieve high success rates. Improved husbandry and hormone treatments of females to keep egg production at high levels (Wallace and Jared, 1968; Wallace et al., 1970) may shorten this refractory period significantly.

The relatively long lives of *Xenopus* species (*X. tropicalis* live 8 years or more; Biegler, 1966), the ability to tag individuals with permanent identifying chips, and their compatibility with large groups of conspecifics simplifies many aspects of stock management, although a means for long-term storage of genetic stocks is very desirable. Successful matings after sperm freezing have been reported (R. Tompkins and M. Sargent, unpublished observations). Sperm nuclei can be frozen for at least several years and used to generate lines by nuclear transplantation (R. Grainger and E. Amaya, unpublished observations).

X. tropicalis development closely resembles that of *X. laevis*, but embryos are slightly smaller and develop more rapidly. Egg diameter is 0.7–0.8 mm (*X. laevis* is 1.0–1.3 mm). The number of eggs per spawning is typically over a thousand and can be upward of 5,000 (*X. laevis* spawnings are generally 300–1,000). Other than size differences, the general features of embryologic development are identical between *X. tropicalis* and *X. laevis* (Fig. 2). The rate of development depends on temperature (embryos develop normally between 20°C and 28°C, and possibly at higher temperatures). At 23°C, where *X. tropicalis* and *X. laevis* can both be raised, *X. laevis* takes an average of approximtately 40% longer to reach the neural tube stage than *X. tropicalis* (D. Shook, unpublished observations).

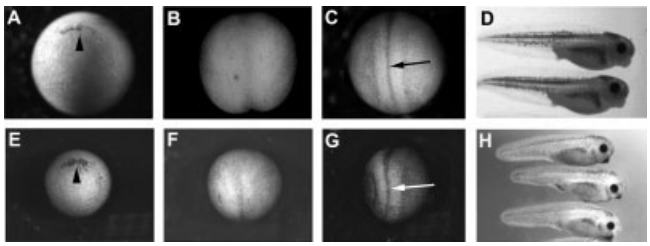

Fig. 2. A comparison of embryonic development between *X. laevis* (A–D) and *X. tropicalis* (E–H). **A,E:** Vegetal view of early gastrula stage embryos, future dorsal is at the top. Arrowheads mark dorsal lip of the blastopore. The anterior of the embryo is toward the top in the next four panels. **B,F:** Dorsal view of neural plate stage embryos. The darkened stripe in the center of the embryo is the presumptive floorplate. **C,G:** Dorsal view of neural tube stage embryos. Arrows mark the region of neural-fold fusion. **D,H:** A lateral view of tadpole stage embryos; anterior is to the right. Magnification is the same at each stage.

EMBRYOLOGIC PROCEDURES AND ASSAYS IN *X. TROPICALIS*

All of the embryologic procedures typical of the *X. laevis* system that have been tried with *X. tropicalis* (isolation of animal caps, transplants, explants, and recombination of tissues) work well (Offield et al., 2000; Hirsch et al., 2002, this volume), as do injection into embryos (e.g., of mRNA). The slightly smaller size of *X. tropicalis* embryos is not a significant impediment to these procedures. It is also quite helpful that RNA probes and antibodies from *X. laevis* cross-react with *X. tropicalis* very well (N. Hirsch and R. Grainger, unpublished observations; Khoka et al., 2002, this volume), so that new reagents do not have to be generated to move between the two species. There also appears to be a conservation of the biological activity of *X. laevis* mRNA when injected into *X. tropicalis*, although sequence differences (e.g., in untranslated regions) can be used to our advantage to distinguish injected mRNA from endogenous mRNA. In the transgenic procedure, promoters from *X. laevis,* in general, preserve the same tissue-specificity in *X. tropicalis* (J. Gray and R. Grainger, unpublished observations).

The ability to use transgenic reporter lines for embryologic experiments has revolutionized the kind of experiments possible in the *Xenopus* system. Although such lines can be prepared in both *X. laevis* and *X. tropicalis*, the more rapid development of *X. tropicalis* makes it far easier to generate populations of transgenic animals for such experiments. Reporter lines, in which a specific gene promoter construct drives expression of a fluorescent marker like green fluorescent protein (e.g., *Pax-6*/GFP or *Rx*/GFP transgenic lines), provide extraordinary spatial and temporal profiles of gene expression in vivo, permitting assays that would otherwise be highly impractical. Conventional assays for molecular markers require tissue fixation, followed

by considerable labor, to obtain information about differentiation at a specific point. One can now isolate tissues according to their gene expression pattern, for example, identifying an area in the young embryo expressing a gene in a particular presumptive tissue that could not be morphologically recognized. As an example of the utility of such reporter lines in studying temporal control, a lens-specific GFP reporter line has been used to provide an in vivo assay for gene activation to examine the events controlling the onset of lens differentiation in *X. tropicalis* (Offield et al., 2000). By using conventional "snapshot" assays for gene expression, this experiment would have required a daunting number of embryologically manipulated samples at different points. Finally, induction assays, e.g., expression of markers in isolated tissues, in response to an applied inducing signal, can be accomplished by simply counting positively expressing tissues (Hirsch et al., 2002).

Embryologic procedures possible with the *Xenopus* system afford potentially powerful opportunities in conjunction with the genetic strategies described below. That one can perform such extensive transplants in *Xenopus* means that it will be feasible to make genetic chimeras. To illustrate an extreme example, it is possible to transplant the entire animal cap (giving rise to the entire ectodermal layer) from one embryo to another, thereby enabling one to examine properties of genetically modified ectoderm in an embryo with wild-type mesoderm and endoderm (or vice versa). Another method that will be highly useful is the ability to transplant germ cells from one embryo to another during neurula stages in *Xenopus* species, including *X. tropicalis* (Blackler and Fishberg, 1961). Such transplants would allow one to use wild-type hosts as carriers of germ cells homozygous for mutations that are lethal at later stages, thereby providing an unlimited source of homozygous mutant embryos when two such chimeric animals are mated. Although the method requires a set of transplantations to set up "carrier" animals, because frogs are fertile for several years, the procedure can be viewed as quite efficient. The operation entails transplanting gut tissue from one neurula to another (the site of germ cells at this stage). Survival rates to adulthood are upward of 80% (Blackler and Fishberg, 1961).

The higher temperatures tolerated by *X. tropicalis* also open the possibility of making chimeras between frog and mammalian tissues, a potentially very powerful combination for cellular and developmental assays (e.g., for detecting products secreted from mammalian cell lines). The conservation of developmental properties between mammals and amphibians can also be examined this way, for example, as described by Hirsch et al. (2002) regarding the ability of mouse tissue to induce lenses in *X. tropicalis*.

TRANSGENESIS IN *X. TROPICALIS* AND ITS APPLICATIONS

As noted earlier, the *Xenopus* transgenesis procedure, developed by Kroll and Amaya (1996), has been a major impetus in developing the genetic potential of this system. The procedure, which involves injection of eggs with sperm nuclei preincubated with transgene DNA, was developed for *X. laevis* and was modified to work in *X. tropicalis* (Offield et al., 2000). Recent improvements (Hirsch et al., 2002) have further enhanced the efficiency.

The transgenic technique has been used in several ways but most frequently to make reporter lines (Hirsch et al., 2002). The first point that should be emphasized is that constructs are readily incorporated into the genome with this technique and can be used to score gene expression in primary transgenic embryos. It is feasible and useful to generate lines from these embryos, which, if they survive to sexual maturity, generally transmit the transgene through the germline, maintaining the appropriate tissue-specific expression pattern in offspring. Second filial generation (F2) tadpoles from a *Pax-6*/GFP transgenic line show a consistent pattern of GFP expression (Fig. 3A), which is identical to that of a primary transgenic tadpole expressing the same transgene (Fig. 3B). Offspring from transgenic lines simplify traditional mRNA or antisense injection experiments by providing hardy embryos, consistently expressing the reporter gene, in which changes of expression can be assayed in vivo. Injection of such reagents into primary transgenic embryos often results in nonspecific developmental abnormalities, because these embryos have already been stressed by the transgenesis procedure. We are aware of over 20 such reporter lines at present (Hirsch et al., 2002). Most of the lines have promoters from brain or sensory tissue genes driving GFP, but there are also lines driving red fluorescent protein (RFP) variants (Matz et al., 1999) and ones that ubiquitously express GFP. Several transgenes have been inserted simultaneously in some animals. In one case, germline transmission has been obtained with a *Pax-6* promoter driving GFP, a muscle-specific promoter driving RFP, and a crystallin promoter driving RFP. These very striking embryos have green brain tissue, red muscle, and yellow lenses, the latter resulting from the mix of red and green reporter proteins expressed in eye tissues (Fig. 4). Such multireporter lines will be extremely useful in genetic screens, as outlined in a later section.

Transgenic inserts commonly occur as tandem repeats at a small number of loci (Kroll and Amaya, 1996; Hirsch et al., 2002), presumably in the paternal genome, and are inherited in standard Mendelian manner. Founder animals will be hemizygous for a given insertion and transmit it to 50% of their outcrossed progeny. Lines bearing crystallin and *Pax-6* promoters driving GFP have now been inbred to homozygosity for these loci (Hirsch et al., 2002).

The transgenic technique can also be used for tissue- or stage-specific expression of designer constructs to perturb gene function. As noted earlier, the *Xenopus* system has been very useful for misexpression studies using injected RNAs, but this approach is often hampered by nonspecific effects resulting from broad ex-

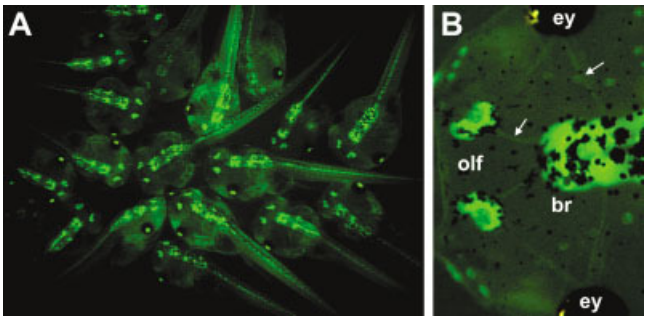

Fig. 3. *Pax-6*/green fluorescent protein (GFP) transgenics show complex patterns of gene expression in later development, demarcating numerous subdomains within the brain, spinal cord, and sensory tissues. **A:** A group of *Pax-6*/GFP transgenic F2 siblings at late swimming tadpole stages (Nieuwkoop and Faber stage 43–45; Nieuwkoop and Faber, 1956). Note the consistency of GFP pattern and intensity between tadpoles. **B:** A high-magnification view of the head of a *Pax-6*/GFP primary transgenic tadpole at stage 46. *Pax-6*/GFP is expressed in the brain (br), eye (ey; GFP fluorescence is blocked in this image by the pigmented retina), and olfactory placodes (olf). Arrows point to the olfactory nerve (between olfactory placodes and brain) and optic nerve (between eye and brain), which show low, but detectable, levels of GFP fluorescence. Small clusters of cells on the skin, which may be part of the lateral line (Winklbauer, 1989), also express GFP.

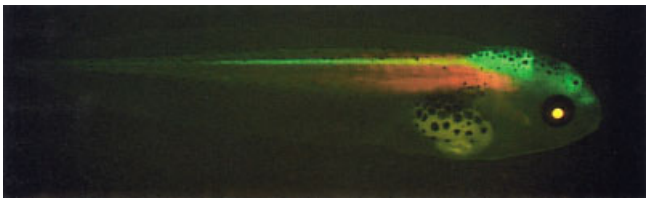

Fig. 4. A triple transgenic *X. tropicalis* tadpole. Red fluorescence in the muscle is produced by expression of a transgene coupling the cardiac actin promoter with red fluorescent protein (RFP). Green fluorescence in the brain is produced by the *Pax-6* promoter coupled to green fluorescent protein (GFP). The yellow color of the lens of the eye is produced by the combination of *Pax-6*/GFP expression in the neural retina and gamma-crystallin/RFP expression in the lens.

pression of a construct or because injected mRNAs are short-lived. The *Xenopus* transgenic method was first used to inhibit FGF signaling during neural induction with the intent of avoiding effects on earlier FGF signaling by using this targeted approach (Kroll and Amaya, 1996) in primary transgenic animals. A similar approach has been used to examine muscle gene expression (Polli and Amaya, 2002) and BMP signaling (Hartley et al., 2001).

Because targeted gene constructs may be designed to interfere with key developmental events, clearly it might be impossible to expect them to be propagated in transgenic lines without some way to effect conditional expression. One method of this kind that has been extremely valuable in the *Drosophila* system is the use of GAL4/UAS lines. This approach uses a tissue-specific promoter driving the yeast transcription factor GAL4, which is inserted into one line, and a UAS target sequence of GAL4, linked to the test construct, in another (Phelps and Brand, 1998). Either line alone is inactive, but when crossed, the result is targeted expression of the construct. The generation of permanent lines affords ready opportunity to repeatedly prepare embryos expressing a construct at a consistent level (which would not be expected to occur in primary transgenic embryos). The prospect of crossing lines with different promoters and target constructs, as these become available, should also be of great value, as it has been in the *Drosophila* system. A similar strategy has been demonstrated both in *X. laevis* (Hartley et al., 2002) and in *X. tropicalis* (Chae et al., 2002). In the latter study, a hormone-inducible version of GAL4 was used to accomplish temporal regulation of transgene activity, complementing the spatial control afforded by choice of tissue-specific promoters. The fragment length polymorphism recombinase system (Broach et al., 1982; Golic and Lindquist, 1989) provides another way of generating conditional gene expression and this system has now been shown to be usable in *Xenopus* as well (Werdien et al., 2001; E. Amaya, unpublished observations).

UNCOVERING MUTATIONS IN *X. TROPICALIS*: GYNOGENESIS AND INBREEDING

Several different strategies are being applied to the *X. tropicalis* system to identify mutations. The first approach used in this system has been to uncover naturally occurring mutations, either in wild-caught animals or during inbreeding.

A strategy to rapidly uncover recessive mutations in wild-caught animals was used successfully in *X. laevis* (Krotoski et al., 1985; Tompkins and Reinschmidt, 1991) and has now been used to reveal developmental mutants in *X. tropicalis* as well. The approach involves the technique of gynogenesis: generation of embryos in which only the maternal genome is present. This technique allows one to immediately reveal a recessive mutation, circumventing the laborious and time-consuming inbreeding process. Sperm, treated with ultraviolet (UV) light to inactivate their DNA, are used to fertilize eggs, thereby initiating haploid development. Haploid embryos develop abnormally, potentially obscuring detection of recessive phenotypes. However, if haploid embryos are subjected to several thousand pounds of hydrostatic pressure within a few minutes of fertilization, the extrusion of the second meiotic polar body is suppressed, thereby generating diploid embryos with only a maternal gene complement (Tompkins, 1978). One can then score these embryos for developmental phenotypes. The frequency of phenotypes in gynogenetic diploid embryos is not exactly Mendelian, because crossing-over during meiosis reduces the fraction of homozygous loci uncovered with increasing distance from the centromere (Reinschmidt et al., 1979), but the fraction of mutant embryos is generally still high enough to identify many phenotypes.

Screening of gynogenetic diploids from wild-caught animals has now been used to identify several muta-

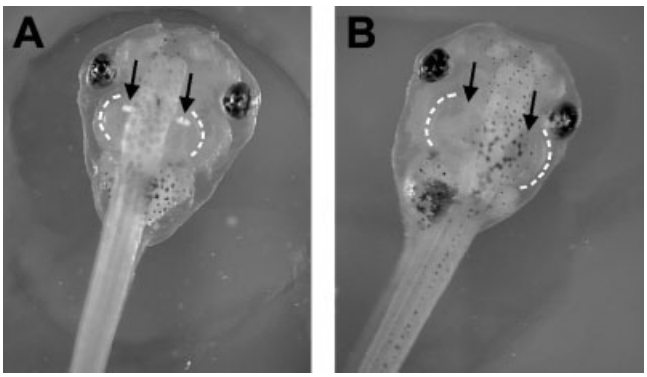

Fig. 5. A putative *X. tropicalis* ear mutant uncovered by gynogenesis. A normal tadpole, stage 42, is on the left (**A**) and a homozygous mutant tadpole on the right (**B**). Note the enlarged otic vesicles (compare white outlines) and lack of otoconia (white calcifications within the inner ear, marked by black arrows) in the mutant.

tions in *X. tropicalis*. From one screen of 160 wild-caught animals, 42 potential developmental mutations were identified (S. Noramly and R. Grainger, unpublished observations). Mutations in many of these animals have been confirmed in offspring generated from founders by conventional two-generation sib-crosses. These confirmed mutants exhibit several very interesting developmental defects, including alterations in left–right asymmetry and effects on the ear and brain, that are being characterized at present. One putative ear mutant identified by gynogenesis is shown in Figure 5. Here one can see that the otic vesicle, the precursor of the inner ear, is enlarged and undifferentiated in the abnormal animal; it has not formed otoconia (arrows in Fig. 5) that are one of the first signs of differentiation within the amphibian inner ear. One can also see from this image that gynogenetic diploids are indistinguishable from wild-type embryos (except when mutant phenotypes are present).

Phenotypic screens are facilitated when performed on a uniform genetic background. Toward that end, two *X. tropicalis* strains have now been extensively inbred, one originating from Nigeria and one from the Ivory Coast (R. Grainger, unpublished observations). The Nigerian line is now in its seventh generation of inbreeding and the Ivory Coast line in its sixth generation. It is widely held that animals inbred for seven generations or more generally provide a usable genetic background for screens. The use of these inbred lines for genetic screens and genomic projects is discussed in later sections. One might expect that developmental mutations would be revealed during the process of inbreeding. Indeed, a very interesting lethal mutation, resulting in reduced heart development and severe tail defects, was identified this way (N. Hirsch, L. Zimmerman, and R. Grainger, unpublished). These strains have been compared by several molecular criteria and are sufficiently polymorphic to be useful in genetic mapping studies (D. Wright and D. Stemple, unpublished observations).

GENERATION OF MUTATIONS IN *X. TROPICALIS*: STRATEGIES FOR INDUCED MUTAGENESIS

Although the isolation of mutations carried by wild-caught animals has been successful, large-scale screens will require more efficient mutagenesis on a more-consistent genetic background. Several strategies, including chemical mutagenesis, induction of deletions, and insertional mutagenesis, are under way in a number of laboratories.

Chemical mutagenesis has been an extremely valuable method for generating developmental mutations in many systems. Indeed the recent successes in the zebrafish system (Driever et al., 1996; Haffter et al., 1996) have been an inspiration to investigators interested in developing genetics in *Xenopus*. Chemical mutagenesis, although not done on *Xenopus* before, has been performed in other amphibians. In axolotls, ethyl methane sulfonate was used to generate dominant lethal mutations (Armstrong and Gillespie, 1980). The efficiency of the mutagen N-ethyl-N-nitrosourea (ENU) in zebrafish (Grunwald and Streisinger, 1992; Riley and Grunwald, 1995), and the likelihood that a similar strategy will be feasible in *X. tropicalis*, have led several labs to initiate studies using ENU to induce mutations. As in the zebrafish procedure, males are being treated with ENU to mutagenize sperm, and phenotypes will be evaluated in subsequent generations.

Although results from ENU screening are not yet available, there are several features of the *Xenopus* system that should enhance the prospects for these screens. First, the efficient procedure for preparing gynogenetic diploid embryos provides a rapid way to screen phenotypes of F2 embryos. The time and space reduction involved in eliminating the screening of F3 families, used in some zebrafish screens (Driever et al., 1994), will be extremely valuable in facilitating *X. tropicalis* screens. Second, the use of transgenic reporter lines will be very useful in focusing screens on particular regions of the embryo. The multireporter transgenic line described earlier (Fig. 4) provides an illustration of how several tissues can be highlighted at once with this method and would be expected to enable investigators to discern more subtle phenotypes than those assessed by morphology alone. Another example shown previously, the *Pax-6*/GFP line (Fig. 3), illustrates the subtlety of the gene expression patterns that can be observed in *Xenopus* tadpoles with this method. This image also highlights another important point: *Xenopus* embryos become transparent at tadpole stages, facilitating visual screening of both morphology and gene expression.

The primary disadvantage of ENU screens is that one cannot readily clone the point mutations induced by this mutagen. There are several reasons, however, to be hopeful about the prospects for cloning genes responsible for ENU mutations in the future. First, the *X. tropicalis* genome project now under way, in conjunction with polymorphism identification and map-

ping (discussed below), greatly simplifies strategies for positional cloning. In addition, the availability of genomic sequence also makes it possible to apply strategies that are not dependent on a high-density genetic map. The method of amplified fragment length polymorphism (AFLP) mapping can be used to examine a large number of molecular markers simultaneously. Polymerase chain reaction (PCR) amplification of fragments of genomic DNA can identify single-nucleotide polymorphisms between different strains of *X. tropicalis*. The offspring from a cross of polymorphic individuals carrying a given ENU-induced mutation could be screened to identify amplified polymorphic fragments linked to the mutation. These polymorphic AFLP sequences could then be cloned, located on *X. tropicalis* genomic sequence, and used to suggest nearby candidate genes for further tests. Second, highly refined strategies for using microarrays, also discussed later in this review, are likely to aid in identifying target genes, or at least pathways, affected in a particular mutation and to accelerate candidate gene approaches.

A strategy that will become increasingly more useful as the *Xenopus* genome project advances will be the generation of a deletion panel. Large deletions generated by gamma-irradiation are being collected and mapped as part of the zebrafish genomics effort to systematically eliminate gene function throughout the genome. Although a panel of this sort is a valuable long-term goal, more modest screens using gamma-irradiation would quickly provide a source of developmental mutants (M. Khoka, T. Grammer, and R. Harland, personal communication). Large deletions might be mapped even before a bank of genetic markers is available by using cytologic techniques; the striking lampbrush chromosomes of amphibians (Gall, 1954) may be very useful in this regard. *X. tropicalis* lampbrush chromosome preparations allow one to readily identify chromosomes (J. Gall, personal communication) and should provide sufficient resolution for mapping purposes.

Because the resources required for cloning mutations generated by chemical or gamma-ray mutagenesis remain formidable, there is a great deal of interest in techniques for insertional mutagenesis, because this methodology provides a way of readily cloning the mutated gene. One insertional strategy that has received some attention in *Xenopus* is "gene-trapping," a method used with great success in several developmental systems (Wurst et al., 1995; Chen et al., 2002). In a study using *X. laevis* transgenesis, a DNA construct containing a splice-acceptor fused to GFP was randomly inserted into the genome (Bronchain et al., 1999). Theoretically, when this transgene inserts within an intron, the cell's own splicing machinery creates a chimeric mRNA containing the GFP message, which is transcribed under the control of the endogenous gene's promoter. This process results in GFP expression only in cells where the endogenous gene's own promoter is active and possible disruption of gene function by the inserted transgene. The interrupted gene may be cloned by using PCR techniques to identify sequence adjacent to the GFP insert. Striking, localized expression of gene trap constructs was seen when this method was used on *X. laevis* embryos and the sequence adjacent to at least one insert cloned by 5′ rapid amplification of cDNA ends technology. Similar technology has been applied to *X. tropicalis*, where 15 embryos with tissue-specific GFP expression were isolated in a preliminary screen (O. Bronchain, L. Zimmerman, N. Hirsch, E. Amaya, and R. Grainger, unpublished observations). Due to primitive husbandry techniques in these early experiments, all the embryos died before reaching metamorphosis. In a more recent screen, several putative gene traps have reached sexual maturity and in one case have shown a mutant phenotype that is now being further characterized (T. Nakayama and R. Grainger, unpublished observations).

Although this approach shows promise, gene trap insertions generated by nuclear transfer-based transgenesis are relatively laborious if raised to the level of performing large genetic screens. An alternative approach uses transposable elements. One example is the Sleeping Beauty transposable element system (Ivics et al., 1997) that has been shown to be active in cells from several vertebrates, including *Xenopus* (Lam et al., 1996; Izsvak et al., 2000). In preliminary experiments, mobilization of Sleeping Beauty system transposons has been shown to be very efficient in *Xenopus* embryos (E. Amaya, unpublished observations) and shows promise as a system for generating enhancer traps and gene traps (P. Mead, unpublished observations).

STRATEGIES FOR INHIBITING PARTICULAR GENE ACTIVITIES AND TARGETED GENE INACTIVATION IN *X. TROPICALIS*

As the number of cloned or sequenced genes increases, so is there an increasing interest in making mutations in known genes. During the past decade, targeted gene inactivation experiments in the mouse have revolutionized our understanding of gene function in development. Yet this remains a very expensive technique that has limitations: even with newer methods for targeting gene inactivation to defined stages or tissues, the function of a gene in a particular cellular or developmental process can be elusive. It would be of great value to be able to control the extent of a mutant tissue in the way that one could in *Xenopus*, where tissue transplantation techniques permit endless possibilities for the formation of chimeric embryos to untangle the particular functions of genes in specific tissues or processes.

Even without targeted gene inactivation techniques for *Xenopus* analogous to those in the mouse, two knockdown strategies can be used effectively at present. Morpholino antisense oligonucleotides have provided a powerful means for significantly reducing the level of gene activities in embryos from organisms like the zebrafish and *Xenopus*, where injection into fertilized eggs is relatively straightforward (Heasman et al., 2000; Nasevicius and Ekker, 2000). The dupli-

cated genome of *X. laevis* may complicate the effectiveness of this strategy when using a single oligonucleotide, but highlights the utility of a diploid species like *X. tropicalis* for such experiments. The utility of this morpholino strategy for studying events in young embryos through tadpole stages is illustrated by Nutt et al. (2001).

Although this technology brings an extraordinary resource to the *Xenopus* system, morpholino treatments may not reduce levels of gene expression sufficiently well, or for long enough during embryogenesis, to replace the need for more definitive strategies for inhibiting gene activities. Another great step forward will entail use of the GAL4/UAS system, described earlier, for targeting gene expression (Chae et al., 2002; Hartley et al., 2002). This method will allow investigators to very specifically target the expression of dominant inhibitory constructs to study the effects of a given gene at a particular stage and region of the embryo. The effectiveness of this method is limited by the potency of a given construct, but the concern with morpholino technology about targeting to any stage or region of the embryo (or at later stages) is eliminated.

Turning to future prospects, the *X. tropicalis* system offers several possibilities for developing a knockout strategy based on homologous recombination, however these remain largely untested. Although creating a totipotent cell line analogous to murine embryonic stem cells is daunting, the totipotency of nuclei is well documented in *Xenopus* (Gurdon, 1962; Gurdon et al., 1975). It may be feasible to make cell lines from young *X. tropicalis* embryos in which genes could be modified and then nuclei transplanted back into enucleated eggs to test for function. Cell lines from *X. laevis* and *X. tropicalis* have been made by several groups (Smith and Tata, 1991), and although many of these are aneuploid, some are not (D. DeSimone and D. Stemple, unpublished observations). In addition, it may be feasible to make diploid cell populations from early passages of cultured embryonic cells that would be suitable for selection experiments. Drugs that could be used to select for recombination events in cells transfected with a mutated gene construct are effective in *Xenopus* cells (Kroll and Gerhart, 1994). It may even be possible to inactivate genes in haploid embryonic cells, as long as the gene is not required for growth in vitro. Transplantation of nuclei from such cells into an irradiated egg would generate haploid embryos in which phenotypes might be directly scored, if the abnormalities associated with haploidy itself did not interfere with scoring. Alternatively, using unirradiated host eggs might allow the possibility of generating an embryonic line that could be subsequently inbred to generate homozygous mutant embryos. Haploid cell lines have been created from amphibians (where one can use UV-inactivated sperm to readily create haploid embryos, as noted earlier) for early studies on somatic cell genetics (Freed and Mezger-Freed, 1970). Although classic nuclear transplantation techniques result in few normal embryos, the more recent modifications of these methods (e.g., using cells in the G_o phase of the cell cycle; Campbell et al., 1996) have not been reported in amphibians.

Another strategy that may be promising for *X. tropicalis* is the homologous recombination strategy developed by Golic and collaborators for *Drosophila* (Rong and Golic, 2000, 2001; Rong et al., 2002). In this approach, in vivo excision of an introduced targeting construct by using a rare-cutting restriction enzyme enhances homologous integration by presentation of recombinogenic DNA ends. The frequency of recombination for some genes was at a level (approximately 1 event per 5,000 animals) that might allow transfer of this technology to *X. tropicalis*, where the transgenic methods in place could allow one to generate the required constructs.

Two other very different approaches developed recently are likely to be of great value for identifying mutations in known genes of *X. tropicalis*. These both entail screening DNA from a panel of chemically mutagenized individuals to identify those with mutations in a given gene. In zebrafish ENU screens, the mutation frequency for a given gene is approximately 1 per 650 mutagenized genomes, with an average loss-of-function frequency per locus of 1 in 1,600 mutagenized genomes (Driever et al., 1996; Haffter et al., 1996). One would expect, therefore, to see mutations in a given gene relatively frequently. In a study in which the *rag1* gene was sequenced in 2,600 F1 mutagenized fish (Wienholds et al., 2002), an allelic series of mutations (a total of 15 mutations) in the *rag1* gene were identified and subsequently characterized. A second, related strategy, known as "TILLING" has been developed for *Arabidopsis* (Colbert et al., 2001). In this strategy, dye-labeled primers are used to amplify loci of interest from candidate heterozygous DNA samples, followed by heat denaturation and annealing to form mutant–wild-type heteroduplex DNA with unpaired "bubbles" representing mutagenized sequences. These can be cleaved by treatment with the endonuclease CEL I, a member of the S1 nuclease family, giving a pair of shorter dye-labeled fragments that can be resolved on sequencing gels. This approach is compatible with sib-selection approaches, in which pools of different DNA samples or primer sets can be assayed in a single gel lane. Both procedures could in principle be applied to a population of *X. tropicalis* generated from ENU treatment.

GENOMIC INITIATIVES FOR *X. TROPICALIS*

In parallel with the development of the *X. tropicalis* system, there has been an intense effort to develop genetic and genomic resources for *Xenopus* researchers by the National Institutes of Health, a plan for which was crafted at two meetings sponsored by NIH, in 1999 and 2000 (summarized at the NIH Web site; http://www.nih.gov:80/science/models/xenopus/). A primary conclusion from the meetings was the importance of developing an EST resource for *Xenopus* researchers, a set of priorities for other genomics initiatives and a

Xenopus database, and support for genetics projects using *X. tropicalis*. A recent decision by the Joint Genome Institute of the Department of Energy to sequence the *X. tropicalis* genome adds another powerful dimension to the genomics initiative. These projects are summarized in an article by S. Klein in this issue and so will only be discussed here as they relate to particular issues concerning the *X. tropicalis* system.

The generation of a large number of ESTs (now well over 200,000 for *X. laevis* and 100,000 for *X. tropicalis*) has transformed the way the *Xenopus* community at large approaches analysis of gene structure. It is of particular value in some *X. tropicalis* projects, for example in identifying genes that one might want to target for morpholino antisense oligonucleotide treatment, and in determining which regions within the cDNA sequence to target.

The EST database has been extremely valuable for developing microarrays, which should be a useful resource for characterizing *X. tropicalis* mutations. For example, a gene chip that recognizes 15,000 unique embryonic *X. laevis* sequences and cross-reacts extremely well with *X. tropicalis* probes (K. Cho, personal communication) may be used to compare mutant and wild-type embryos to aid in identification of genes in ENU or gamma ray–induced mutations. One procedure that may provide a very powerful way to use gene chips to evaluate recessive phenotypes in a more systematic way entails use of the technique, mentioned earlier, for transplantation of presumptive germ cell tissue from mutant embryos into wild-type hosts. When two such hosts are mated, all of the embryos would have the recessive phenotype, providing an extraordinarily valuable resource to use in conjunction with screening microarrays for evaluating mutant gene expression before a phenotype was visible (when it might be too late to readily identify the causal agent leading to the mutation). Because there is no technology available in frogs, or in most vertebrate systems, for selecting the subset of recessive mutant embryos before they express a phenotype in a cross between two heterozygotes, the germ cell transplant technique is clearly advantageous.

Full-length *X. tropicalis* cDNA expression libraries, now under construction in the Blumberg laboratory, should provide an additional resource. These libraries will facilitate expression cloning strategies that have been so effective in identifying new genes in *Xenopus* (Smith and Harland, 1991, 1992). They will also be useful for trying to identify gene activities that might rescue an early mutant phenotype, for example, by injection of mRNAs from pools of clones in the library, followed by a sib-selection procedure to identify the defective gene. For later developmental mutations, it should also be feasible to use these libraries in conjunction with the *Xenopus* transgenic technique, which enables one to obtain approximately 20 inserts per transgenic animal (Kroll and Amaya, 1996). With an optimized transgenesis system one could potentially screen a library for rescue of a mutant phenotype by using it to make transgenic inserts in a population of embryos.

One of the most useful short-term resources will be the two *X. tropicalis* BAC libraries recently constructed, one under a program sponsored by the NIH Genome Institute and one by S. Qin (personal communication). These will enable investigators to clone gene promoter elements much more quickly and completely than was possible previously, further enhancing the utility of the *Xenopus* transgenic technology for many purposes. Recent work has also demonstrated that BAC clones can be directly used in the *Xenopus* transgenic system. A group of mouse BAC clones have been shown to integrate and transmit through the germline and, at least in the few cases examined, express appropriate tissue-specific patterns in F1 animals (N. Marsh-Armstrong, personal communication). BAC clones will also add a valuable resource to define genes responsible for a mutant phenotype. For example, BAC clones from a particular region of the genome might be used to attempt to rescue a developmental mutation generated by any one of several methods.

The construction of a genetic map for *X. tropicalis* would greatly facilitate identification of mutant genes generated in ENU or gamma ray screens and would serve as a vital link to genomic sequence as well. Efforts are under way to generate a radiation hybrid panel (A. Sater and D. Stemple, personal communication), and a polymorphism map is being developed (D. Wright and D. Morizot, personal communication). Polymorphisms are being mapped by using crosses from the two inbred strains, from Nigeria and Ivory Coast, described earlier. These lines are quite useful for this purpose, because they are polymorphic to each other. Animals with more extensive polymorphisms have been identified recently in populations obtained from Africa and should be very useful in the mapping effort as well.

Finally, the extraordinary prospect of a genome sequence for *X. tropicalis* greatly reinforces the utility of many of the other resources described in this section, and strengthens the rationale for developing genetic approaches in this system. Having a complete resource for purposes of gene discovery, analysis of gene organization and chromosome structure will transform, as it has in other systems, the way researchers can evaluate and clarify complex genetic networks controlling cellular and developmental processes. Connecting a physical map and genetic map will certainly be a very valuable link in the process of identifying point mutations by positional cloning.

PROSPECTS FOR THE *X. TROPICALIS* SYSTEM

Research using amphibian embryos in the past century experienced two major technical eras, each providing a series of questions for investigations that continue today. In the classic embryologic era, techniques for tissue transplantation, explantation, and recombination were used to demonstrate the existence and

location of inducing centers in developing embryos and to define important embryologic principles. In the molecular era, analysis of particular gene products, ranging from studies of cell cycle components in egg extracts to injection of mRNAs into embryos to study early developmental events, has added another dimension to our understanding of a multitude of cellular and developmental problems. This rich history, together with the many technical advantages of the *Xenopus* system, provides an extensive set of problems and tools to couple with genetic and genomic studies. With the prospect of performing genetic screens, identifying mutations in particular genes, and the development of reagents for genome-wide functional tests, *Xenopus* could arguably become the most comprehensive system for evaluating the challenging issues in cell and developmental biology.

Progress can be expected on long-standing questions, for example, the process of morphogenesis, with the likelihood that it will be possible to significantly clarify the cell biological behaviors and signaling mechanisms underlying complex tissue movements. Issues such as axial patterning, cell determination, and induction in the young embryo, defined in amphibian systems, have seen great insights generated as a result of the injection techniques developed in the past two decades, but precise mechanisms will be finally within reach with the refined tools afforded by the more subtle genetic manipulations now possible or anticipated in the *X. tropicalis* system. This new technology will make many fundamental problems approachable for the first time. Notable among these is the problem of organogenesis. The relatively late stages and localized areas in which definitive organs are formed have left this problem out of reach of techniques such as mRNA injection. One can expect that targeted expression of gene constructs, screens focused on identifying mutants in particular organs and the general facility of the *Xenopus* embryo for studies on tissue rudiments, will lead to the disclosure of new principles about how organs do form. Other complex developmental phenomena such as the later phases of tissue differentiation, metamorphosis, and limb formation will become more accessible as well, as one can look forward to strategies for understanding, and integrating, developmental phenomena at a level that would be inconceivable without the new genetic strategies and genomic resources that are so greatly expanding opportunities for *Xenopus* researchers.

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