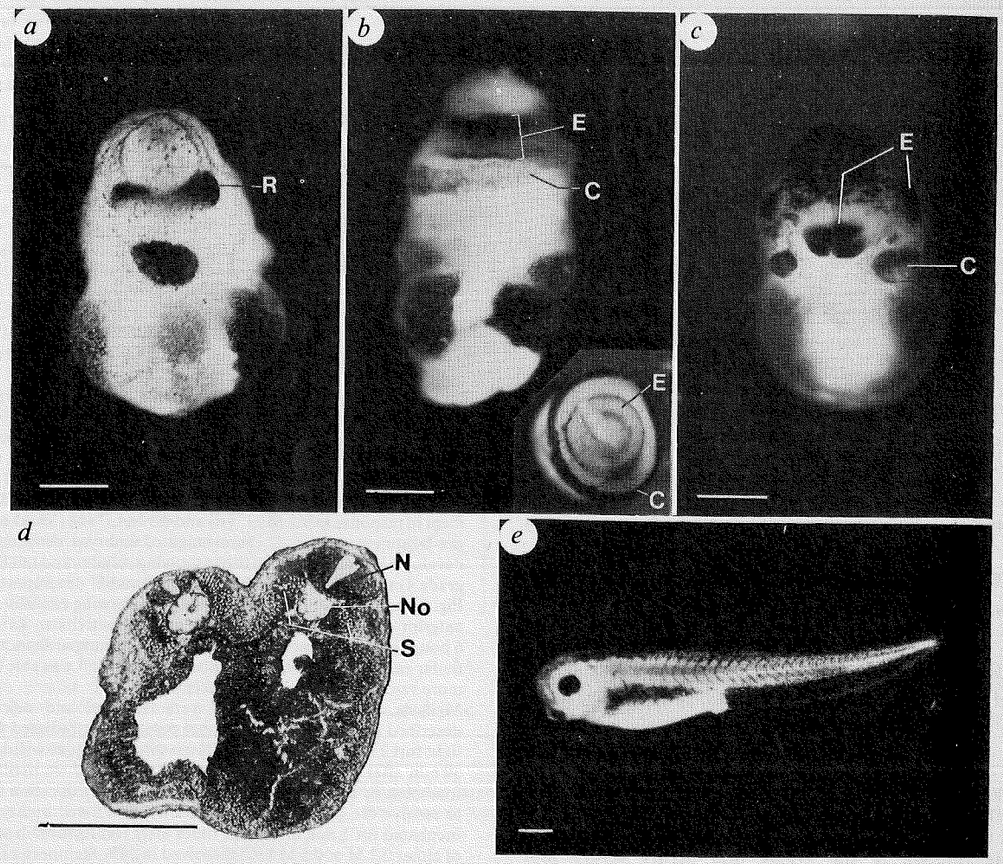

Fig. 2 *a*, *X. laevis* embryo exposed to UV light prior to first cleavage and then treated with 0.3 M LiCl for 5 min at the 32-cell stage. Note the presence of retinal pigment (R) which would not develop without LiCl exposure. *b*, An embryo with dorso-anterior radial symmetry. This embryo, like that in *a*, was UV-irradiated. Exposure to 0.3 M LiCl for 5 min 4.5 h following fertilization (16–32 cells) restored dorso-anterior development, causing rescue of eye (E) and cement gland (C) in a radial manner (inset: top view of radial, anterior embryo). *c*, A 'Janus twin' embryo exhibiting duplication of dorso-anterior structures. This embryo was injected with 4 nl of 0.3 M Li^+ into a ventral, vegetal cell at the 32-cell stage. The eyes (E) and cement gland (C) of the head on the right side are labelled. *d*, A section just posterior to the head of an embryo displaying duplication of the head with one posterior axis. This embryo was microinjected similarly to the embryo in *c*. Embryos were fixed, sectioned in paraplast and stained as described previously[12]. The duplicate axis is unlabelled. No, notochord; N, neural tube; S, somites. See Fig. 4 for experimental details. *e*, A control, untreated embryo at stage 40. Scale bars, 0.5 mm.

ated embryos to try to restore dorso-anterior development to one side of the embryo and thereby rescue normal development. Embryos at the 32-cell stage were used because they could be easily injected without significant leakage, and were most sensitive to rescue by Li^+. Microinjection of Li^+ into a cell in the vegetal-most tier caused significant rescue of normal development in a dose-dependent manner (Fig. 3). We used the IAD to quantify axis development, because the embryos developed morphologies identical to those defined by the IAD scale. For example, in Fig. 3, vegetal cell injections of 0.3 M Li^+ reduced the average IAD to 2 in embryos which would otherwise develop into grade 4.9 or 5 embryos. Out of 81 embryos, 30% were scored as grade 0 (normal), 13% as grade 1 (slightly microcephalic), 16% as grade 2 (cyclopic), 17% as grade 3 (microcephalic), 16% as grade 4 (acephalic), and 8% as grade 5 (radial ventral). We could not assign an IAD score to embryos injected with solutions of Li^+ greater than 0.3 M. These concentrations approach lethal levels and in the surviving embryos, there is an over-enhancement of dorso-anterior structures so that the embryos tend towards radial dorso-anteriorization. Injection of Li^+ into an animal cell gave a lower frequency of rescue than with vegetal cell injections. It remains to be determined whether the injected vegetal cell is altered by Li^+ or whether it acts as a local source of Li^+.

The restoration of bilateral symmetry in UV-irradiated embryos by Li^+ microinjection prompted us to determine whether dorsal structures are duplicated in normal embryos when they are microinjected with Li^+. We microinjected into vegetal lower-tier cells as this injection gave the best rescue of UV-irradiated embryos. From 112 embryos microinjected with Li^+ into a ventral, vegetal cell, we obtained 97 embryos that had duplication of dorso-anterior structures (Fig. 4). Most of these embryos developed into 'Janus' twins (Fig. 2*c*) that lack posterior development but consist of two heads which have normally formed eyes and cement glands. A small number (~5%) developed into twins which have a single posterior axis but with twinned heads, central nervous systems, notochords and somites (Fig. 2*d*). Injection into a dorsal cell failed to duplicate dorsal structures. Out of 97 embryos injected into a dorsal, vegetal cell, 89 survived to form a single body axis. Most (~80%) developed normally, but the remainder developed enhanced dorso-anterior structures, similar in external appearance to the 'imbalanced' embryos produced by Cooke[5].

To see whether dorsal rescue is specific for Li^+, we microinjected 4 nl of 0.4 M solutions of NaCl, KCl, CsCl, RbCl or NH_4Cl, 50 mM solutions of $CaCl_2$ or $MgCl_2$, or a 10 mM solution of $ZnCl_3$ (higher concentrations of the latter three salts usually killed the embryos). All solutions were made up in 200% Steinberg's solution which, when injected alone into UV-irradiated embryos, did not cause rescue. Among the cations tested, only Li^+ was able to cause significant rescue of dorsal structures.

Based on cell transplant experiments Gimlich and Gerhart[11] showed that a vegetal, dorsal cell at the 32-cell stage carried sufficient information to promote complete dorsal development. This information is translated by inductive cell interactions during cleavage to form dorsal elements. Our experiments show that Li^+ is able to cause expression of dorsalizing information in cells that otherwise lack or do not express this information. Thus, the potential for cells to undergo dorsal development exists radially around the embryo even after axis specification, and such development will occur when stimulated by Li^+ at the appropriate time.

The ability to produce radial dorsal embryos with L^+ should facilitate investigations on the molecular and cellular differences between dorsal and ventral pattern formation in *X. laevis*.

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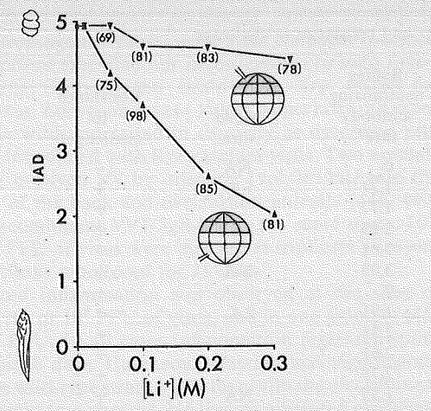

Fig. 3 Rescue of normal development by Li^+ microinjection into radially symmetric ventral, embryos. Embryos were fertilized and irradiated with UV light as described in Fig. 1, and then microinjected with 3–5 nl of LiCl dissolved in distilled H_2O or 200% Steinberg's solution at varying concentrations (horizontal axis) into either a top mostanimal tier cell (▼) or lowest vegetal tier cell (▲). The embryos for each experimental group were scored for axis development (vertical axis) using the index of axis deficiency (IAD)[7]. An IAD score of 0 indicates normal development whereas a score of 5 indicates complete lack of dorsal structures with only radial ventral development. The average IAD is plotted here for each group of injected embryos. Note that the average IAD is reduced in both animal and vegetal cell injections, but more significantly in the latter. Microinjection of >0.3 M Li^+ increased the frequency of dead embryos and the formation of exclusively dorso-anterior embryos that are not scorable with the IAD. The numbers in brackets refer to numbers of embryos injected with LiCl at the indicated concentration. (For 0.01 M LiCl, 78 embryos were microinjected in the animal cell and 86 embryos were injected into the vegetal cell. At 0 M, 269 control embryos were scored.)

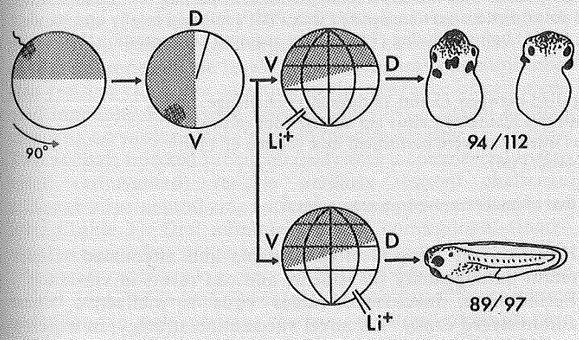

Fig. 4 Summary of twinning experiments. Fertilized eggs were placed in a 5% solution of Ficoll (Pharmacia) in 20% Steinberg's solution and rotated within 30 min after fertilization with the sperm entry point (SEP) towards gravity. The rotation ensures the position of the dorsal side of the embryo (D) as 180° opposite the SEP (ventral side, V) in the meridian passing through the SEP and the animal–vegetal axis. Eggs were kept in this orientation for 20 to 30 min and the dorsal (upper) side was marked with Nile blue sulphate[14]. After the rotation period, eggs were allowed to orient normally in 20% Steinberg's solution with the vegetal pole downwards. At the 32-cell stage, embryos were microinjected either in a ventral, vegetal-most cell or a dorsal, vegetal-most cell with 3–5 nl of 0.3 M LiCl dissolved in 200% Steinberg's solution. At stage 40, the embryos were scored for development either as having a single body axis or with duplicated dorso-anterior development. The twins resulting from ventral cell injection are usually Janus twins with two diametrically opposed anterior ends, although some show closely approximated heads as in Fig. 2*c*.

Oral vaccination of the fox against rabies using a live recombinant vaccinia virus

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Rabies, a viral disease affecting all warm-blooded animals, is prevalent in most parts of the world[1], where it propagates amongst wild animals, particularly the fox and dog. The public health and economic consequences of infection in man and livestock are well known. Attempts to control the disease by vaccinating wild carnivores with inactivated or attenuated rabies virus remain controversial, and we have instead evaluated here the potential of a recombinant vaccinia virus to protect foxes against the disease. We have found that the administration of vaccinia virus (VV) or a recombinant harbouring the rabies surface antigen gene (VVTGgRAB) is innocuous to foxes. The recombinant virus can elicit the production of titres of rabies-neutralizing antibodies equal or superior to those obtained with conventional vaccine, and 10^8 plaque-forming units (PFU) of VVTGgRAB administered subcutaneously, intradermally or orally confers complete protection to severe challenge infection with street rabies virus.

Table 1 Rabies-neutralizing antibodies and resistance to challenge

Vaccine and route	Dose (PFU)	Animals	Rabies neutralizing antibody titre*	Mean titre	Resistance to challenge†	Fraction surviving
No vaccine		#	0	NA	−	0/6
Conventional vaccine subcutaneous‡		440	1.21	1.49	+	2/2
		441	1.77		+	
VVTGgRAB, intradermal§	10^8	446	3.03	2.82	+	2/2
		449	2.61		+	
VVTGgRAB, subcutaneous§	10^8	439	3.03	NA	+	2/2
		442	0		+	
VVTGgRAB, oral scarified‖	10^8	437	1.91	2.4	+	4/4
		438	2.33		+	
		447	2.61		+	
		452	2.75		+	
VVTGgRAB, oral‖	10^4	408	1.35	NA	+	1/4
		427	0		−	
		428	0		−	
		429	0		−	
	10^6	416	0.8	0.4	+	2/4
		425	0.8		+	
		426	0		−	
		430	0		−	
	10^8	414	2.33	2.57	+	4/4
		431	2.61		+	
		433	2.61		+	
		451	2.75		+	
VVTGgRAB in bait	10^8	411	1.07	1.8	−¶	4/5
		412	1.63		+	
		420	2.61		+	
		423	2.19		+	
		424	1.49		+¶	

Rabies neutralizing antibody titres were determined in accordance with recommendations laid down by the World Health Organisation[27]. Titres are expressed as the $\log_{10}$ of the final neutralizing dilution (FND). For conversion to international units (IU) IU = 59/[antilog (3.5 − log FND)]. Foxes were monitored for 50 days following challenge and the presence of rabies virus in the brain of the animals succumbing to challenge was verified by immunoflorescence (not shown). Challenge virus GS6 (street virus comprising salivary glands from rabid foxes dispersed in phosphate-buffered saline (PBS) (20% w/v) as described previously[28]) was injected in a volume of 1 ml containing 5,700 mouse LD_{50} units (intracerebral), ~17,000 fox LD_{50} units (intramuscular). Vaccinia Copenhagen strain and VVgRAB were grown on tissue culture cells as described elsewhere[29]. Supernatants were recovered, the cell pellets homogenized, recentrifuged and pooled with the supernatant. Virus was purified by sedimentation through a sucrose cushion (36% w/v) for 2 h, 14,000 r.p.m., Beckman SW28 rotor. Pellets were suspended into PBS, sonicated briefly and banded by centrifugation on a sucrose gradient (20–40% w/v; 12,000 r.p.m., 45 min, SW28) before diluting (PBS) to the required concentration. Virus was titred on BHK21 cells. NA, not applicable.

* Only the 28-day titre is given.

† +, Resisted challenge, −, succumbed to rabies. All animals not resisting died between 15 and 25 days after challenge.

‡ 'Rabisin ND' vaccine, lot 5532; neutralizing antibody titres obtained with live attenuated virus are similar (not shown).

§ Virus was inoculated intradermally as described (see text); subcutaneous injections were performed in a volume of 1 ml.

‖ Virus was administered by direct application into the mouth by syringe (vol. 1 ml).

¶ Two animals were observed to have ingested only a part of the vaccine.

Four animals were vaccinated with vaccinia virus Copenhagen strain, two animals received no treatment.

Transmission of rabies occurs predominantly through biting. Large quantities of virus are shed in the saliva and the aggressive behaviour of the rabid animal facilitates transmission. Infection by this route is invariably fatal and an immune population is therefore never established. Prophylactic measures have aimed at eliminating or reducing the population of the principal reservoir through poisoning or gassing[2], though a decrease in the carrier population density rarely reduces the rate of advance of the disease front[3]. In Europe, culling of foxes has reduced the number of rabies outbreaks but has not contained the disease. Vaccination, perhaps supported by culling in low-density fox populations, seems more likely to be effective than mere destruction of foxes[4]. An immune population is better able to limit the spread of the disease than one which is sparse but susceptible[4,5].

Oral administration could facilitate the vaccination of large numbers of wild foxes and this was first attempted in North America and Europe[6,7]. Live attenuated rabies virus introduced into chicken heads, sausages or dog biscuits and distributed in the wild has been successfully used to vaccinate wild foxes[8], and field trials are in progress in Switzerland, West Germany[9] and Canada (C. D. MacInnes, personal communication). However, the virus is often unstable and attenuated viruses retain pathogenicity for rodents and can revert to virulence[10]. Furthermore, inactivated rabies virus is ineffective when administered orally[11]. A novel vaccination strategy, in which a recombinant vaccinia virus bearing a foreign antigen coding sequence is used as the immunizing agent[12–14], seemed to hold some promise for the vaccination of foxes against rabies.

The causative agent of rabies is a rhabdovirus, and the glycoprotein (G) which traverses the envelope surrounding the virus, is the sole viral protein capable of inducing and reacting with virus-neutralizing antibodies or of conferring protection against rabies[15,16]. The relative innocuity of vaccinia virus, which has been used extensively to control and eradicate smallpox in man[17], has stimulated its development as a cloning and expression vector, and derivatives expressing surface antigens from influenza, hepatitis B and herpes simplex have been used to confer protection against these diseases[14]. We recently developed a recombinant VV (VVTGgRAB) expressing the rabies G coding sequence[18] and found that mice scarified with the live recombinant virus VVTGgRAB resisted severe challenge with street rabies virus[18–20]. VV itself is an enveloped virus and

the recombinant VVTGgRAB presenting rabies G protein elicited protection against rabies even after chemical inactivation[19]. We therefore extended our investigations to wild foxes.

Vaccinia virus (Copenhagen strain) was first tested for innocuity to foxes. European foxes (*Vulpes vulpes*) of both sexes captured in the wild and raised in captivity as described previously[21] were inoculated with live vaccinia virus. Two animals (nos 445, 448) received VV by injection (vol. 0.1 ml) into the depilated skin of the back and two further animals (446, 449) received the recombinant VVTGgRAB by the same route. 10^2, 10^4, 10^6 or 10^8 PFU of virus were injected at triplicate sites and cutaneous reactions monitored for 15 days.

Mild localized inflammation was observed at the sites of injection with 10^6 or 10^8 PFU of virus, and in one animal (445) at 10^2 and 10^4 PFU. In all cases inflammation regressed spontaneously within 8 days. Cutaneous reaction was significantly greater with the wild-type strain of VV than with the recombinant VVTGgRAB strain (not shown). No lesions appeared elsewhere than at the site of injection and there was no evidence here of contact transmission of vaccinia virus between animals. In test animals receiving live recombinant vaccinia virus by direct application into the mouth (Table 1) no impairment of digestive or alimentary function was observed.

We subsequently examined the ability of the recombinant virus to elicit the production of antibodies directed against rabies virus. Animals inoculated intradermally, subcutaneously or orally with VVTGgRAB were bled on days 0, 8, 14 and 28; serum was separated and titred for the presence of rabies-neutralizing antibodies. High titres of neutralizing antibodies were present in sera from all but one animal treated with 10^8 PFU of VVTGgRAB (Table 1). Further, scarification of oral mucous membranes before oral administration (to facilitate penetration of the virus) did not yield improved titres of neutralizing antibodies. All animals presented only low levels of antibody capable of neutralizing VV (not shown), in agreement with other reports[22].

Direct protection testing was then performed. Twenty-eight days after vaccination, animals were challenged by injection of 5,700 mouse LD_{50} (50% lethal dose) units of rabies virus. All 12 animals recieving 10^8 PFU of VVTGgRAB either orally or parenterally resisted challenge (Table 1). One animal (442) exhibiting undetectable levels of rabies-neutralizing antibodies also resisted challenge, attesting to the relevance of cell-mediated immunity in defence against rabies[16,23]. Control animals receiving no vaccine succumbed to the disease after 15–25 days. Two animals injected subcutaneously with a commercial inactivated (adjuvanted) vaccine similarly resisted challenge, although virus-neutralizing antibodies were present at a reduced level (Table 1). Oral administration of conventional (inactivated) vaccine has previously been shown to be ineffective[11].

Animals receiving less than 10^8 PFU of VVTGgRAB showed a clear dose-dependent response, with one out of four and two out of four animals surviving challenge after oral administration of 10^4 and 10^6 PFU of VVTGgRAB, respectively (Table 1).

Oral administration is the only route appropriate to the vaccination of wild animals. Accordingly, the vaccine must be presented in a form suitable for ingestion. We thus prepared 'Plastipak' capsules (a gift from Dr Wandeler) containing 10^8 PFU of VVTGgRAB, inserted them into chicken heads (one capsule per head into the beak) and distributed them to test animals (one per fox). These animals similarly produced high titres of rabies-neutralizing antibodies and resisted severe challenge with rabies virus (Table 1).

Transmissibility is a major factor determining the impact of a live vaccine on a wild population. We therefore sought to establish whether animals vaccinated with VVTGgRAB might transmit the virus by contact with untreated control animals. Four test animals (two male, two female) were acclimatized (4 days) to sharing a pen (2 m^2) with an animal of the opposite sex. One animal from each pair received 10^8 PFU of live VVTGgRAB by direct application into the mouth (vol. 1 ml). Serum samples were collected from both vaccinated and control animals at 14 and 28 days, and intramuscular challenge with live rabies virus (see Table 1 legend) was performed at 28 days.

All vaccinated animals presented high titres of rabies-neutralizing antibodies (mean 2.72 at 28 days, units as in Table 1) and resisted challenge. In three out of four control animals no rabies-neutralizing antibodies were detectable and these animals succumbed to challenge (not shown). Surprisingly, the fourth control animal (523, female) presented significant levels of rabies-neutralizing antibodies (1.35 and 0.97 at 14 and 28 days, respectively) and resisted severe challenge infection.

Subsequent investigations into possible mechanisms of transmission revealed that both the relevant vaccinated male (517) and control female 523 consistently displayed aggressive behaviour, and reciprocal biting was observed within a few minutes of oral vaccination. Contamination of bite wounds with VVTGgRAB may, in this instance, have been sufficient to effect immunization. Such a mode of transmission requires a combination of rather exceptional circumstances, and we surmise that contact transmisson of VVTGgRAB in the field is likely to be rare.

We have shown that vaccinia virus and its recombinant VVTGgRAB bearing the rabies surface antigen are innocuous to healthy foxes; indeed, the thymidine kinase-negative recombinant may be more innocuous than wild-type strains of vaccinia virus[24]. Our recombinant virus can confer complete protection to severe challenge infection with rabies virus. Similar experiments are in progress with other animal vectors of rabies, notably the skunk and racoon[25]. Importantly, presentation to foxes of the live recombinant, encapsulated and introduced into chicken heads, also yields animals resisting severe challenge infection. Procedures for the production, stabilization and distribution of vaccinia virus are fully established[26]. Due to its efficacy, innocuity and stability, the recombinant virus may be a candidate for the large-scale vaccination of wild foxes and, possibly, other feral or domestic animals.

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