Transfer of a foreign gene into the brain using adenovirus vectors

Saïd Akli[1], Catherine Caillaud[1], Emmanuelle Vigne[2], Leslie D. Stratford-Perricaudet[2], Livia Poenaru[1], Michel Perricaudet[2], Axel Kahn[1] & Marc R. Peschanski[3]

The ability of a replication-deficient adenovirus vector to transfer a foreign gene into neural cells of adult rats *in vivo* has been analysed. A large number of neural cells (including neurons, astrocytes and ependymal cells) expressed an *E. coli* lacZ transgene for at least 45 days after inoculation of various brain areas. Injecting up to 3×10^5 pfu in 10 µl did not result in any detectable cytopathic effects — these were only observed for very high titres of infection (>10^7 pfu $10\mu l^{-1}$). Adenovirus vectors therefore appear to be a promising means for *in vivo* transfer of therapeutic genes into the central nervous system.

[1]*U 129 INSERM, Institut Cochin de Génétique Moléculaire, 24 rue du Faubourg Saint Jacques, 75014 Paris, France*
[2]*URA 1301 CNRS, Institut Gustave Roussy, PR2, 39 rue Camille Desmoulins, 94805 Villejuif, France*
[3]*CJF 91-02 INSERM, Faculté de Médecine, 8 rue du Général Sarrail, 94010 Créteil, France.*

Correspondence should be addressed to M.R.P.

To transfer therapeutic DNA safely and efficiently into the central nervous system (CNS) is a formidable challenge in the development of active therapies for brain diseases. In most cases, retroviral vectors are not useful because they are unable to infect postmitotic cells, including most neural cells[1]. Herpes *simplex*-derived vectors infect neural cells but there remain problems of pathogenicity and stability of gene expression[2–4]. Replication-deficient adenoviruses appear to offer a workable alternative as they have been successfully used to transfer foreign DNA into a variety of cells and organs, including postmitotic cells such as hepatocytes and myotubes[5–8]. Previous use of live adenovirus vaccines in human populations emphasizes the safety of this type of vector[9,10]. We have therefore analysed the ability of an adenovirus vector to transfer *in vivo* the *E. coli lacZ* gene in neural cells of adult rats. The *lacZ*-containing, replication-deficient adenovirus vector (Ad.RSVβgal) has been described previously[11]. This vector contains the gene for β-galactosidase targeted to the nucleus by the SV40 nuclear localization signal (nls)[12].

Gene transfer to neural cells

Preliminary studies showed that Ad.RSVβgal infected non-dividing neural cells (both neurons and astrocytes) in primary culture (C.C. *et al.*, manuscript submitted). In order to analyse neural infection *in vivo*, we injected virus suspensions into specific areas of the brain in anaesthetized adult rats using stereotactic methods. No gross adverse effect of this inoculation on the animals' health and behaviour was observed up to 60 days after inoculation (the longest time studied). Animals were perfused at various times after inoculation and their CNS was sectioned. Sections were treated histochemically with the X-gal stain in order to reveal β-galactosidase activity, which was visualized as a blue reaction product in infected cells. In all cases, a large number of cells were histochemically stained. Neural cells were infected, including neurons (Fig. 1*a*,*b*) and glia identified as astrocytes (Fig. 1*c*) or microglia (data not shown) in double-staining experiments using specific immunocytochemical markers. When injections were aimed at the ventricular system, a wealth of ependymal cells expressed the gene (Fig. 1*d*).

In the brain parenchyma, labelled cells were mostly found within 500 to 1,000 µm from the needle track. Borders of the area containing infected cells tended to respect anatomical boundaries such as large fibre tracts or lamellae. For example, injections aimed at the nucleus of the XIIth nerve (Fig. 1*a*,*b*) produced a virtually complete and intense staining of most, if not all, neurons within the nucleus while surrounding areas contained only scattered infected cells. Similarly, infected ependymal cells were observed over several millimeters after intracerebroventricular injections, while adjoining neural tissue did not contain any stained cells (Fig. 1*d*). It is likely, therefore, that the topography of β-galactosidase expressing cells at the injection site corresponds to a limited spread of viral particles within the tissue and that anatomical boundaries such as large fibre tracts or the *glia limitans* prevent their diffusion. Spread of viral particles within the ventricular system, as determined from infection of ependymal cells, is apparently much wider.

In addition to cells labelled around the needle track, for which endocytosis of viral particles at the perikaryal level was likely, remote groups of neurons that send axonal projections to the area of injection were labeled, such as

nigral neurons after injection into the striatum (Fig. 2*b*). The nuclear and perinuclear localization of the staining in nigral cells, as well as the lack of a profuse staining in the nigro-striatal axonal pathway, indicates that the viral particles rather than the enzyme was taken up by axonal terminals and transported retrogradely from the striatum to the substantia nigra. In contrast, no histochemical staining was observed in neurons that have axons crossing (but not ending in) the injection site, suggesting that viral particles cannot be endocytosed by axons of passage. No labelled neural cells were observed in the control animals that received only saline injections.

Efficiency of gene transfer

Considerable numbers of cells were infected after inoculation with high titre virus suspensions, but many cells were also infected when much lower titres were used. In rats injected with a 10 µl suspension titering 3.3×107 pfu ml^{-1} (a maximum of 3.3×10^5 infectious particles) several thousand neural cells still expressed the *lacZ* gene 8 to 30 days after inoculation (Fig. 3*b*). Quantitative estimations of infected ependymal cells made in two animals injected intracerebroventricularly gave comparable results at 8 (n = 3,240) and 30 days (n = 3,468), that is, a mean of 1 labelled cell per 100 injected pfu. It is important for the interpretation of this result to recall that "pfu number" was determined on human 293 cells competent for defective adenovirus replication. Consequently, the actual number of adenovirus vector particles intrinsically able to infect rat cells in which replication of the defective virus is impossible is likely to be much lower[6,7]. Injections as low as 3,300 pfu resulted in labelling of close to a hundred cells (Fig. 3*c*). Intensity of labelling also varied, most probably depending upon the concentration of viral particles to which the cells were exposed. When high titre virus suspensions were used (over 10^9 pfu ml^{-1}) staining intensity was usually very strong in the central area of the injection site where cells were exposed to the highest viral particle concentration. In these cases, labeling was not limited to nuclei despite the nuclear localization signal sequence added to β-galactosidase, but diffused to cytoplasm and processes (dendrites and axons of neurons) producing a complete "Golgi-like" staining of the cells (Fig. 1*b*, 4). In contrast, in most cells located at the periphery of the injection site and in retrogradely infected neurons (that is, in cells that were not exposed to the most concentrated viral suspension), only the nuclei were labelled (Fig. 2*a*,*b*). Similarly, nuclear localization of the enzyme was the rule when lower titre virus suspensions were used (Fig. 3*b*,*c*).

The apparent relationship between the intensity of X-gal staining and the concentration of viral particles to which the cell was exposed suggests that a single neural cell, in particular a neuron, can take up a large amount of viral particles by endocytosis. This high infectability would explain the cytopathogenicity (characterized by neuronal death, gliosis, vascular inflammatory response and tissue loss) observed at the site of injection of high titre suspensions (Fig. 3*a*). Adenovirus infection is clearly not systematically cytopathic: well preserved neural cells expressing *lacZ* were still observed 45 days after inoculation of high titre virus suspensions (Fig. 5) and there was no evidence of cytopathogenicity after injections of lower titre virus suspensions (10^7 pfu ml^{-1})(Fig. 3*b*,*c*). Cytopathogenicity may relate, therefore, to the endocytosis of large numbers of viral particles by cells located close to the injection site.

Discussion

Our results demonstrate that cells in the CNS, including neurons, can be successfully infected in large numbers by a replication-deficient adenovirus and consequently express a transferred foreign gene. Numerous potential applications of this possibility can be envisioned for both scientific and clinical goals.

The Ad.RSVβgal appears as an appealing means to analyze neuronal- and glial- morphology in specific areas of the brain by providing Golgi-like staining of cells at the injection site. Filling up of the axons by the enzyme may additionally provide ways to analyse projections of discrete neuronal populations. One significant advantage of this technique for neuroanatomy is the easy combination of the X-gal stain with all sorts of other tracing techniques, in particular immunocytochemistry. The use of vectors in which the nuclear localization sequence would be omitted might improve the efficacy of such a technique.

Development of scientific and potentially clinical applications will make use of other recombinant replication-deficient adenovirus vectors specifically designed to address issues of interest. Due to deletion of

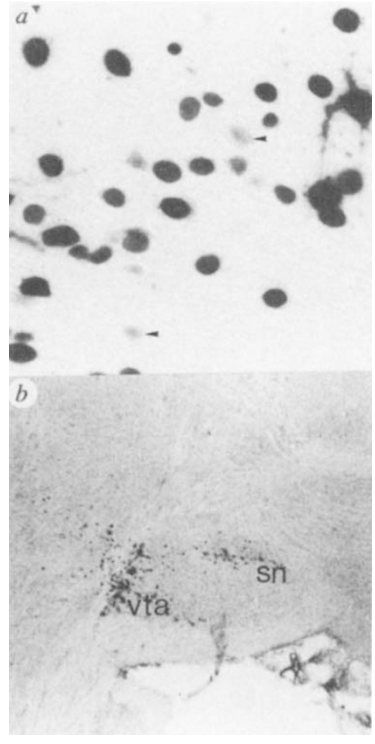

Fig. 2 Photomicrographs showing β-galactosidase histochemical staining four days after adenovirus injection into the striatum. *a*, Reaction product in cells at the periphery of the injection site. This is mostly in the nuclear-perinuclear region and intensity ranges from very faint (arrowheads) to strong, extending into processes (arrow); ×400. *b*, Histochemical staining of neurons in the substantia nigra (SN) and in the ventral tegmental area (VTA) that have transported the viral vectors retrogradely from the striatum; ×20.

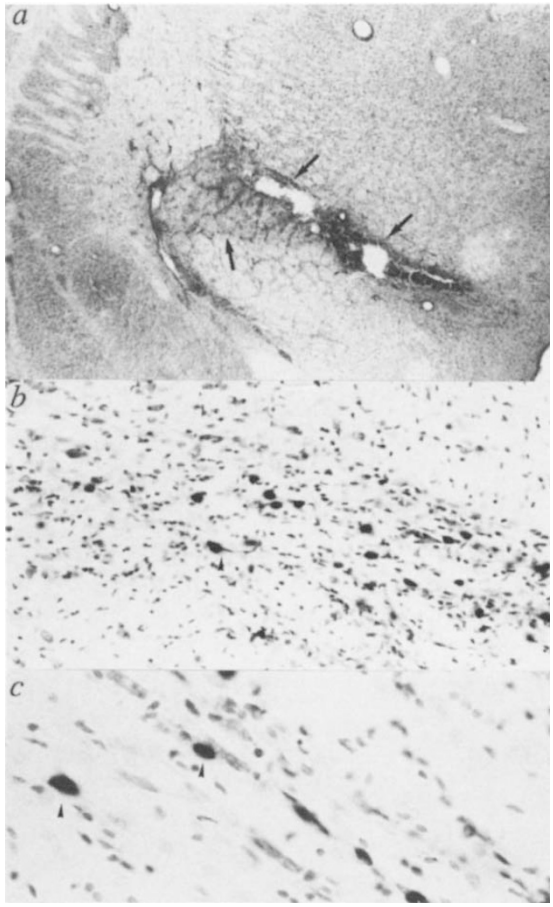

Fig. 3 Photomicrographs one week after injection of adenovirus suspension into the thalamus at different dilutions (sections counterstained with cresyl violet). *a*, Cytolytic effect observed after injection of 10 µl of a 3.3×10^{10} pfu ml^{-1} suspension (total: 3.3×10^8 pfu). Tissue loss and gliotic areas (arrows) reveal cell death induced by the viral injection; ×20. (section not treated with X-gal). *b*, Staining after injection of a total of 3.3×10^5 pfu. Several thousand cells still expressed the gene, exhibiting mostly nuclear-perinuclear staining except for a few short neurites (arrowhead). There was no conspicuous cytolytic effect; ×200. *c*, Staining after injection of 3.3×10^3 pfu. A few cells still expressed the gene (arrowheads) in a small area corresponding to the tip of the needle track as determined from the vertically-oriented mechanical lesion. There was no apparent cytolytic effect; ×400.

the E1 and part of the E3 region, it is possible to substitute up to 7.5 kilobases (kb) of exogenous DNA into the genome of the adenovirus vector currently used, which permits accommodation of many mammalian cDNAs. For instance, this type of vector has been used to transfer a 7 kb cDNA encoding a minidystrophin gene to skeletal muscle[13] and a 4.5 kb cDNA encoding CFTR to airway epithelium[14].

Above all, adenovirus vectors represent clearly a promising means to transfer foreign genes into the brain with a therapeutic goal. Relatively low titres of adenovirus can transfer foreign genes into a significant number of brain cells without triggerring pathological effects. It should be mentioned, however, that spread of viral infection after intraparenchymal injections was relatively limited. This characteristic could be very useful to deliver a therapeutic gene into a precise, limited brain structure (such as, tyrosine hydroxylase into the substantia nigra of Parkinson's disease patients). In contrast, it may restrict therapeutic applications in cases where a large and widespread population of neurons needs reprogramming (for example, spinal motorneurons in motorneuronal diseases or striatal neurons in Huntington's disease). Infection of ependymal cells several millimeters away from an intra-cerebroventricular injection, however, suggests that delivery of a substance encoded by the transgene to large brain areas could be obtained through this route, provided this substance can cross the brain-ventricular border. Although cytopathic effects were only observed after injection of high titre virus suspensions and, therefore, were tentatively attributed to multiple infections of single cells, further optimization of the delivery process is clearly needed. Very precise pathogenicity analysis in animals, including non-human primates, is required before application of this technique can be proposed as the basis of treatment in the human CNS. Inocuity of adenovirus vectors for brain cells could be improved by the construction of a new generation of vectors with larger deletions of viral genes (M. Perricaudet

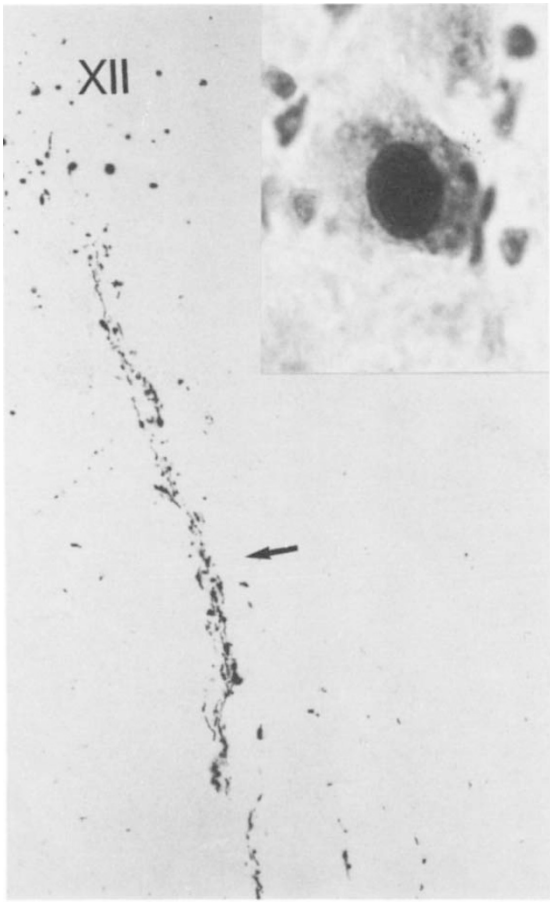

Fig. 5 Photomicrographs showing β-galactosidase histochemical reaction product in a rat that was allowed to survive for 45 days after adenovirus injection into the nucleus of the XIIth nerve (XII). A few cells still expressed the gene as demonstrated by nuclear-perinuclear histochemical staining. Among them were motorneurons as exemplified in the inset (counterstained with cresyl violet). A few axons also contained the enzyme (arrow); ×40, inset ×1,000.

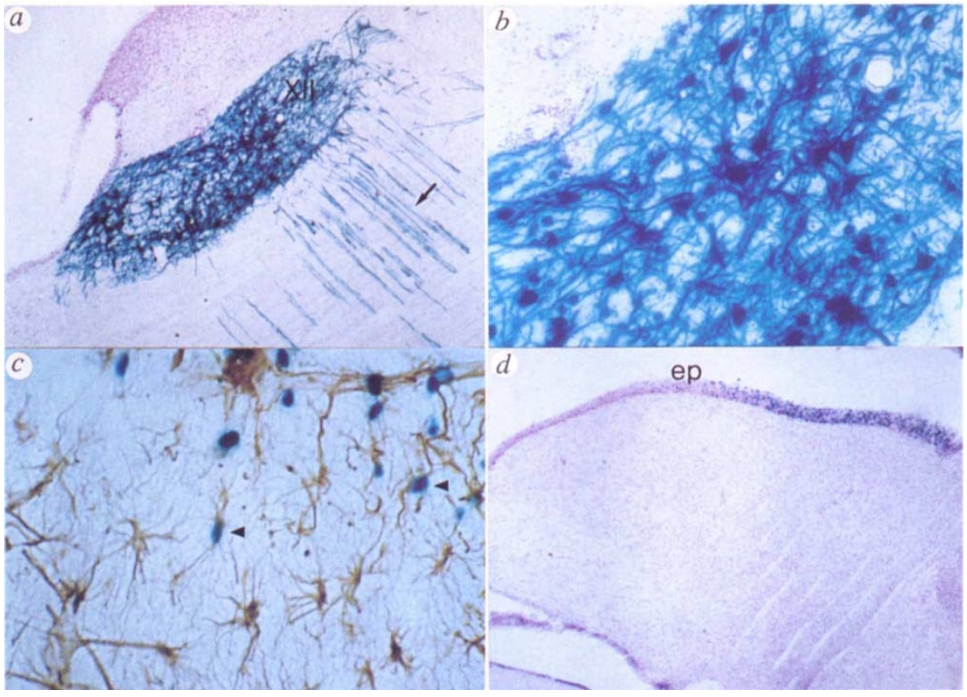

Fig. 1 Photomicrographs showing β-galactosidase histochemical staining (in blue) in various cell populations in the rat brain four days after adenovirus injection. *a*,*b*, Labelling observed after injection into the nucleus of the twelfth nerve (XII). A large number of neurons in the XII nucleus have been infected, staining extending in all neuronal processes including axons (arrow in *a*), providing a "Golgi-like" appearance of the cells; *a*, ×100; *b*, ×400. *c*, Staining of cells at the periphery of the injection site after injection into the striatum. Some of the infected cells can be identified as astrocytes (arrowheads) by the brown reaction product indicating immunoreactivity for GFAP (glial fibrillary acidic protein); ×400. *d*, Staining in ependymal cells (ep) following an intra-cerebroventricular injection; ×100. Sections in *a*, *b* and *d* have been counterstained with cresyl violet.

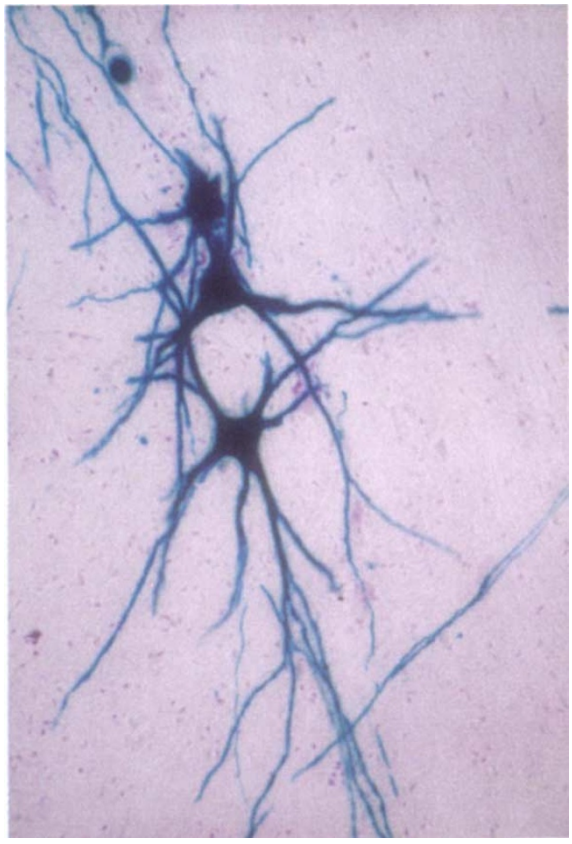

Fig. 4 Photomicrograph showing "Golgi-like" β-galactosidase histochemical staining in neurons of the medullary reticular formation four days after injection of 10 μl of an adenovirus suspension containing 10^{11} pfu ml^{-1}.

et al., in preparation). If efficiency and safety of foreign gene transfer into brain cells by adenovirus vectors is confirmed, it will open new avenues in the treatment of many genetic and acquired neurological diseases for which this method may work as an alternative to drug treatment or brain transplantation of fetal tissues.

Methodology

Adenovirus vectors containing *LacZ* gene were prepared according to standard procedures[7]. In the first set of experiments, thirteen adult Sprague-Dawley rats (Charles River France) were anaesthetized using chloral hydrate (400 mg kg^{-1}, intraperitoneally) and placed in a stereotactic apparatus. A viral suspension containing 3.3×10^{10} to 10^{11} pfu per ml was injected over 10 min using a Hamilton syringe (10 μl, total injection: 3.3×10^{8} to 10^{9} pfu) into the XIIth nucleus ($n = 6$) or into various regions of the forebrain ($n = 7$). After four days' survival time, rats were reanesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.1 M; pH 7.4). The CNS was removed, post-fixed for 4 h then cryoprotected overnight in 30% sucrose. Parasagittal sections (48 μm-thickness) were cut on a cryostat. Every fourth section was incubated for 4–12 h at 28–30 °C with the X-gal stain as described[15]. In three rats, X-gal treated sections were rinsed in phosphate buffer then further treated using classical immunohistochemical techniques with Vectastain kits (Vector labs) using a rabbit polyclonal antibody raised against GFAP (1/500) to identify astroglial cells. A few sections were treated alternatively with the monoclonal antibody OX42 that stains microglial cells. Sections were then mounted on gelatinized slides and every other section was counterstained with cresyl violet before final coverslipping from toluene. Two additional "control" animals were treated following the same protocol except that they received injections of 10 μl saline at the level of the nucleus of the XIIth nerve.

In a second set of experiments, the 3.3×10^{10} pfu ml^{-1} suspension was either used directly or diluted using sterile saline before injection into the brain of ten rats. Six rats were allowed to survive for one week and the others were sacrificed every week thereafter. Other methods were as described above except that sections were cut in the coronal plane.

In a third set of experiments, nine rats were injected with the highest viral titre and sacrificed ($n = 3$) every fifteen days after injection. Surgical and histological methods were as described above (sections cut in the coronal plane).

Acknowledgements

S.A. & C.C. contributed equally to this work

The authors wish to thank S.L. Juliano for help with the manuscript. This work has been supported by INSERM, CNRS, University Paris V and by grants from l'Association Française contre les Myopathies and the Ministère de la Recherche et de l'Espace.

Received 9 November; accepted 9 December 1992.

1. Culver, W.C. *et al. In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 256, 1550–1552 (1992).
2. Johnson, P.A., Miyanohara, A., Levine, F., Cahill, T. & Friedman, T. Cytotoxicity of a replication defective mutant of herpes *simplex* virus 1. *J. Virol.* **66**, 2952–2965 (1992).
3. Fink, J.D. *et al. In vivo* expression of β-galactosidase in hippocampal neurons by HSV-mediated gene transfer. *Hum. Gene Ther.* **3**, 11–19 (1992).
4. Wolfe, J.H., Deshmane, S.L. & Fraser, N.W. Herpes virus vector gene transfer and expression of β-glucuronidase in the central nervous system of MPS VII mice. *Nature Genet.* **1**, 379–384 (1992).
5. Quantin, B., Perricaudet, L.D., Tajbakhsh, S. & Mandel, J.L. Adenovirus as an expression vector in muscle cells *in vivo*. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2581–2584 (1992).
6. Jaffe, H.A. *et al.* Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. *Nature Genet.* **1**, 372–378 (1992).
7. Stratford-Perricaudet, L.D., Levrero, M., Chase, J.F., Perricaudet, M. & Briand, P. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum. Gene Ther.* **1**, 241–256 (1990).
8. Rosenfeld, M.A. *et al.* Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium *in vivo*. *Science* **252**, 431–434 (1991).
9. Straus, S. E. Adenovirus infections in Humans. in *The Adenoviruses* (ed. Ginsberg, H.S.) 451–496 (Plenum Press, New York, 1984)
10. Chanock, R.M., Ludwig, W., Heubner, R.J., Cate, T.R. & Chu, L.-W. Immunization by selective infection with type 4 adenovirus grown in human diploid tissue culture. I. Safety and lack of oncogenicity and tests for potency in volunteers. *JAMA* **195**, 445–452 (1966).
11. Stratford-Perricaudet, L.D., Makeh, I., Perricaudet, M. & Briand, P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. clin. Invest.* **90**, 626–630 (1992).
12. Kalderon, D., Roberts, B.L., Richardson, W.D. & Smith, A.E. A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499–509 (1984).
13. Ragot, T. *et al.* Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice. *Nature* (in the press).
14. Rosenfeld, M.A. *et al. In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* **68**, 143–155 (1992).
15. Sanes, J.R., Rubenstein, J.L.R. & Nicolas, J.F. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133–3142 (1986).