PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Gene Transfer Into the Germ Line of Mice and Into Embryonal Carcinoma Stem Cells

E. F. Wagner

Phil. Trans. R. Soc. Lond. B 1984 **307**, 313-317
doi: 10.1098/rstb.1984.0133

Phil. Trans. R. Soc. Lond. B **307**, 313–317 (1984) [313]
Printed in Great Britain

Gene transfer into the germ line of mice and into embryonal carcinoma stem cells

By E. F. Wagner

European Molecular Biology Laboratory, Postfach 10.2209, *D*-6900 *Heidelberg, F.R.G.*

Recombinant genes can be introduced into the germ line of mice by microinjection into the fertilized egg or via embryonal carcinoma stem cells. A regulated, cell-type specific expression of the newly introduced gene following microinjection is usually not found. However, in one series of experiments the insertion of human growth hormone genes in the germ line led to prenatal recessive lethal mutations. Alternatively, embryonal carcinoma stem cells can be used to introduce and express genes in mice, for example after infection with selectable retroviral vectors. In addition, these cells are useful for differentiation studies *in vitro* following DNA-mediated gene transfer.

It is unclear how genes are regulated during the development of a multicellular organism. An understanding of developmentally regulated gene expression should facilitate the formation of new concepts concerning complex biological phenomena such as cellular differentiation, determination and regeneration as well as control of cell growth and neoplasia. To study some of these problems, an experimental approach must be sought within the context of a developing organism. Recent findings in two experimental systems will be reviewed: the introduction, expression and fate of recombinant DNA following microinjection into the fertilized mouse egg; and gene transfer studies with embryonal carcinoma (e.c.) cells.

Gene transfer into the germ line of mice

Parallel with the development of various gene transfer systems for cultured cells (Pellicer *et al.* 1980*a*, *b*), attempts were made to use the fertilized mouse egg as the target cell for microinjection of cloned DNA. The ultimate goal of this approach is that after reintroduction of a developmentally regulated recombinant gene, the correct expression of that gene in the appropriate cell type can be analysed at the level of the whole organism.

To date, a number of cloned genes have been stably introduced into mice by microinjection into one of the two pronuclei of the fertilized egg (see table 1). The genes in various copy numbers were usually transmitted through the germ line, and new strains of mice homozygous for the acquired genotype were obtained. The drawback of this approach has been the failure to find regulated tissue or cell specific expression of the foreign gene. In table 1, some of the characteristics concerning the mode of expression of various genes together with the references are listed. Expression of a newly introduced gene is often found in inappropriate tissues with great variations in the level of expression. If the gene is linked before injection to an inducable promoter such as the mouse metallothionein (MT), expression has usually, but not always occurred. However, first indications of a possible cell type specific expression, have come from mice carrying the chicken transferrin gene or mouse immunoglobulin genes. In the former example, a strong preference for expression of the transferrin gene was found in mouse liver, where transferrin is normally synthesized. The introduced immunoglobulin κ gene is expressed in the spleen, but not in the liver of mice carrying the injected gene.

Table 1. Modes of expression of genes microinjected into fertilized mouse eggs

gene injected	expression: specific	expression: unspecific	expression: no	reference
HSV-TK	·	+	·	Wagner *et al.* 1981
MT-TK	·	+	·	Brinster *et al.* 1981
	·	+	·	Palmiter *et al.* 1982
β-globin:				
human	·	·	+	T. A. Stewart *et al.* 1982
rabbit	·	+	+	Costantini & Lacy 1981
	·	+	+	Lacy *et al.* 1983
M-MuLV	·	+	·	Harbers *et al.* 1981
	·	+	·	C. Stewart *et al.* 1983
growth hormone:				
MT-GH rat	·	+	·	Palmiter *et al.* 1982
MT-GH human	·	+	·	Palmiter *et al.* 1983
GH human	·	·	+	Wagner *et al.* 1983
transferrin chicken	+	+	·	McKnight *et al.* 1983
immunoglobulin kappa	+	·	·	Brinster *et al.* 1983

There are probably many reasons why most genes introduced into mice are not regulated, whereas correct tissue-specific expression after P-element mediated gene transfer in *Drosophila* seems to be the rule (Goldberg *et al.* 1983; Hazelrigg *et al.* 1984; Scholnick *et al.* 1983; Spradling & Rubin 1983). The regulation of stage- and tissue-specific gene expression in mammals might be quite different from *Drosophila* (e.g. the presence of methylated sequences in mammals; Jaenisch & Jähner 1984), and various classes of genes might be regulated in very different ways. Some genes may be regulated exclusively by structural sequences within or around the gene and be expressed independent of the position on a particular chromosome. Others might require the correct position, the right chromatin structure and additional *cis*- or *trans*-acting elements which we are just beginning to understand. However, the latest findings on the preferential expression of newly introduced genes in mice are encouraging and should lead to the dissection of events governing tissue-specific gene expression during differentiation and development.

What are the other possible consequences when changing the genetic make-up of a mammal? The integration of newly introduced DNA may disrupt important chromosomal regions, which could lead to developmental defects, sterility, neoplasia or to defects in metabolism. There have been numerous reports on insertional mutagenesis in non-mammalian systems (McClintock 1956; Roeder & Fink 1980; Kidwell *et al.* 1977) and more recently in mice (Jenkins *et al.* 1981; Copeland *et al.* 1983; Jaenisch *et al.* 1983). In one case, a recessive embryonic lethal mutation was experimentally induced in mice by insertion of a Moloney murine leukaemia virus copy into the α1(I) collagen gene (Schnieke *et al.* 1983). We have tested the mutagenic potential of recombinant DNA in the germ line of mice as a possible alternative tool for a molecular identification of gene functions essential for normal development (Wagner *et al.* 1983).

Six individual strains of mice (HUGH strains) were obtained from eggs injected with the cloned human growth-hormone gene in a pBR322 vector. All were heterozygotes containing between 1 and 20 intact copies of the foreign sequences in unique and often complex integration patterns. The six HUGH strains stably transmitted the newly inserted sequences through the germ line to their progeny and no indication of human growth hormone expression was found (see table 1). When the individual heterozygotes were mated with each other, only four of the

HUGH strains yielded viable homozygotes. In HUGH/3 and HUGH/4, no postnatal homozygotes were found, when a total of 31 and 52 offspring, respectively, were analysed. In addition, litter sizes at birth in these two strains were markedly reduced compared with the other HUGH strains, suggesting that homozygous animals die prenatally. In Mintz's laboratory, experiments are now in progress to establish the developmental stage at which these animals die. The molecular cloning of the integration site in HUGH/3 and HUGH/4 may reveal genomic coding sequences which are disrupted. Identification of these sequences may indicate whether a normal cellular house-keeping function is affected or if, more optimistically speaking, a novel gene could be identified, which is essential for a given developmental process. Additional experiments are needed to establish if this high incidence (33%) of obtaining homozygous recessive prenatal lethals reflects a mutagenic potential of recombinant DNA introduced into the germ line of mice by egg-injection.

Gene transfer into mouse embryonal carcinoma stem cells

Almost ten years ago it was found that e.c. cells lose the malignant phenotype and participate normally in development (to form all somatic as well as germ cells) after reintroduction into early embryos (for review see Mintz & Fleischman 1981; Stewart 1984). Therefore, these cells can be used as vectors for introducing new genetic information into the germ line of mice. The major advantage of using e.c. cells as opposed to direct DNA injection into fertilized eggs as an experimental system to study regulation of gene expression during differentiation and development is the fact that cells carrying the desired genetic change can first be selected and characterized before reintroduction into the animal. In addition, these cells serve as a powerful tool for *in vitro* differentiation studies of early mouse development. The current state of the field is summarized in *Embryonic and germ cell tumours in man and animals* (Gardner 1983) and in *Teratocarcinoma stem cells* (Silver *et al.* 1983).

A large number of established e.c. cell lines, mostly derived from tumours of grafted embryos are available, and recently stem cell lines derived directly from embryos grown *in vitro* have been obtained (Evans & Kaufman 1981; Martin 1981; Axelrod 1984). By using the calcium phosphate method, selectable genes along with unselectable ones, were introduced into established e.c. cells (Pellicer *et al.* 1980*b*; Linnenbach *et al.* 1980; Wagner & Mintz 1982; Bucchini *et al.* 1983; Nicolas & Berg 1983). Problems were encountered with respect to the relative low transformation efficiency (about 100-fold less than in L-cell transformations), the integration of multiple and rearranged gene copies, the low or non-expression of co-transferred genes and the instability of expression under non-selectable conditions. With the development of novel transducing retroviral vectors new possibilities exist without the above mentioned disadvantages (Gilboa 1983; Mann *et al.* 1983). Foreign genes can be introduced into e.c. cells and mouse embryos by virus infection. In collaboration with E. Gilboa, several viral vectors, containing Moloney murine leukaemia virus regulatory regions as well as selectable (neo) and non-selectable (human β-interferon cDNA) genes were used to infect various established e.c. cell lines. We have been able to obtain a high frequency of clones under selectable conditions (efficiency *ca.* 10^{-3}). The clones contain a single intact copy of the recombinant gene and seem to express stably the gene in the absence of selection (unpublished results). These viruses should not only be useful for efficient transfer of genes into totipotent e.c. cells and embryos, but they are currently also used for studying the block to retrovirus replication in e.c. cells and in early mouse embryos (Stewart *et al.* 1982; Gautsch & Wilson 1983).

In a different set of experiments we asked whether a cellular oncogene (c-*onc*) might exert a biological effect on transfection into F9 teratocarcinoma cells (unpublished experiments in collaboration with R. Müller, E.M.B.L.). For this approach we chose the c-*fos* gene, which is expressed at high levels specifically in placenta and late-gestation foetal membranes and which has therefore been suggested to have a function during prenatal development (for review see Müller & Verma, 1984). It was found that introduction of either the normal mouse or human c-*fos* gene along with a selectable marker gene (*neo*), induces colonies of cells that are morphologically markedly different from F9 stem cells. The transformed cells are greatly enlarged, very flat, grow in an epitheloid fashion and stop proliferating after reaching a maximum clone size of about 400 cells. The phenotype of these cells is being analysed currently with respect to expression of various differentiation markers (Müller & Wagner 1984). The preliminary data suggest that the normal c-*fos* gene can induce differentiation *in vitro* and lends support to the hypothesis that c-*onc* genes may have a vital function in cell differentiation and embryonic development.

The work on the introduction of human growth hormone genes into the germ line of mice was done in Beatrice Mintz's laboratory at the Institute for Cancer Research in Philadelphia. I wish to thank Colin Stewart and Ulrich Rüther from the E.M.B.L. in Heidelberg for stimulating discussions and Ines Benner for preparation of the manuscript.

References

Axelrod, H. R. 1984 Embryonic stem cell lines derived from blastocysts by a simplified technique. *Devl Biol.* **101**, 225–228.

Brinster, R. L., Chen, H. Y., Trumbauer, M., Senear, A. W., Warren, R. & Palmiter, R. D. 1981 Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* **27**, 223–231.

Brinster, R. L., Ritchie, K. A., Hammer, R. E., O'Brien, R. L., Arp, B. & Storb, U. 1983 Expression of a microinjected immunoglobulin gene in the spleen of transgenic mice. *Nature, Lond.* **306**, 332–336.

Bucchini, D., Lasserre, C., Kunst, F., Lovell-Badge, R., Pictet, R. & Jami, J. 1983 Stable transformation of mouse teratocarcinoma stem cells with the dominant selective marker *Eco.gpt* and retention of their developmental potentialities. *EMBO J.* **2**, 229–232.

Copeland, N. G., Jenkins, N. A. & Lee, B. K. 1983 Association of the lethal yellow (A^y) coat color mutation with an ecotropic murine leukemia virus genome. *Proc. natn. Acad. Sci. U.S.A.* **80**, 247–249.

Costantini, F. & Lacy, E. 1981 Introduction of a rabbit β-globin gene into the mouse germ line. *Nature, Lond.* **294**, 92–94.

Evans, M. J. & Kaufman, M. H. 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature, Lond.* **292**, 154–156.

Gardner, R. L. (ed.) 1983 Embryonic and germ cell tumours in man and animals. *Cancer Surveys* **2** (1).

Gautsch, J. W. & Wilson, M. C. 1983 Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. *Nature, Lond.* **301**, 32–37.

Gilboa, E. 1983 Use of retrovirus derived vectors to introduce and express genes in mammalian cells. In *Experimental manipulation of gene expression* (ed. M. Inouye), pp. 379–394. London and New York: Academic Press.

Goldberg, D. A., Posakony, J. W. & Maniatis, T. 1983 Correct development expression of a cloned alcohol dehydrogenase gene transduced into the *Drosophila* germ line. *Cell* **34**, 59–73.

Harbers, K., Jähner, D. & Jaenisch, R. 1981 Microinjection of cloned retroviral genomes into mouse zygotes: integration and expression in the animal. *Nature, Lond.* **293**, 540–542.

Hazelrigg, T., Levis, R. & Rubin, G. M. 1984 Transformation of *white* locus DNA in *Drosophila*: dosage compensation, *zeste* interaction, and position effects. *Cell* **36**, 469–481.

Jaenisch, R., Harbers, K., Schnieke, A., Chumakov, I., Jähner, D., Löhler, J., Grotkopp, D. & Hoffmann, E. 1983 Germline integration of Moloney murine leukemia virus at the *Mov*13 locus leads to recessive lethal mutation and early embryonic death. *Cell* **32**, 209–216.

Jaenisch, R. & Jähner, D. 1984 Methylation, expression and chromosomal position of genes in mammals. *Biochem. biophys. Acta* (In the press.)

Jenkins, N. A., Copeland, N. G., Taylor, B. A. & Lee, B. K. 1981 Dilute (*d*) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. *Nature, Lond.* **293**, 370–374.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Kidwell, M. G., Kidwell, J. F. & Sred, J. A. 1977 Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility and male recombination. *Genetics*, *Princeton* **86**, 813–833.

Lacy, E., Roberts, S., Evans, E. P., Burtenshaw, M. D. & Costantini, F. 1983 A foreign β-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. *Cell* **34**, 343–358.

Linnenbach, A., Huebner, K. & Croce, C. M. 1980 DNA-transformed murine teratocarcinoma cells: Regulation of expression of simian virus 40 tumor antigen in stem versus differentiated cells. *Proc. natn. Acad. Sci. U.S.A.* **77** (8), 4875–4879.

Mann, R., Mulligan, R. C. & Baltimore, D. 1983 Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**, 153–159.

Martin, G. 1981 Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. natn. Acad. Sci. U.S.A.* **78** (12), 7634–7638.

McClintok, B. 1956 Controlling elements and the gene. *Cold Spring Harb. Symp. quant. Biol.* **21**, 197–216.

McKnight, G. S., Hammer, R. E., Kuenzel, E. A., & Brinster, R. L. 1983 Expression of the chicken transferrin gene in transgenic mice. *Cell* **34**, 335–341.

Mintz, B. & Fleischman, R. A. 1981 Teratocarcinomas and other neoplasms as developmental defects in gene expression. In *Adv. Cancer Res.* **34**, 211–278.

Müller, R. & Verma, I. M. 1984 Expression of cellular oncogenes. In *Curr. Topics Microbiol Immunol.* **112**, 73–115.

Müller, R. & Wagner, E. F. 1984 Induction of differentiation after transfer of c-*fos* genes into F9 teratocarcinoma stem cells. *Nature*, *Lond.* (In the press.)

Nicolas, J. F. & Berg, P. 1983 Regulation of expression of genes transduced into embryonal carcinoma cells. In *Teratocarcinoma stem cells* (ed. L. M. Silver, G. R. Martin & S. Strickland), *Cold Spring Harb. Conf. on Cell Proliferation* **10**, pp. 469–485.

Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. & Evans, R. M. 1982*b* Dramatic growth of mice that develop from eggs microinjected with metallotionein-growth hormone fusion genes. *Nature*, *Lond.* **300**, 611–615.

Palmiter, R. D., Chen, H. Y. & Brinster, R. L. 1982*a* Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* **29**, 701–710.

Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E. & Brinster, R. L. 1983 Metallothionein-human GH fusion genes stimulate growth of mice. *Science*, *Wash.* **222**, 809–814.

Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J. M., Sim, G. K., Silverstein, S. & Axel, R. 1980*a* Altering genotype and phenotype by DNA-mediated gene transfer. *Science*, *Wash.* **209**, 1414–1422.

Pellicer, A., Wagner, E. F., El Kareh, A., Dewey, M. J., Reuser, A. J., Silverstein, S., Axel, R. & Mintz, B. 1980*b* Introduction of a viral thymidine kinase gene and the human β-globin gene into developmentally multipotential mouse teratocarcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **77** (4), 2098–2102.

Roeder, G. S. & Fink, G. R. 1980 DNA rearrangements associated with a transposable element in yeast. *Cell* **21**, 239–249.

Schnieke, A., Harbers, K. & Jaenisch, R. 1983 Embryonic lethal mutation in mice induced by retrovirus insertion into the α1(I) collagen gene. *Nature*, *Lond.* **304**, 315–320.

Scholnick, S. B., Morgan, B. A. & Hirsh, J. 1983 The cloned dopa decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell* **34**, 37–45.

Silver, L. M., Martin, G. R. & Strickland, S. (eds) 1983 *Teratocarcinoma stem cells. Cold Spring Harb. Conf. on Cell Proliferation* **10**.

Spradling, A. C. & Rubin, G. M. 1983 The effect of chromosomal position on the expression of the *Drosophila* xanthine dehydrogenase gene. *Cell* **34**, 47–57.

Stewart, C. L. 1984 Teratocarcinoma chimaeras and gene expression. In *Chimaeras in developmental biology* (ed. N. Le Douarin & A. McLaren). New York: Academic Press. (In the press.)

Stewart, C. L., Harbers, K., Jähner, D. & Jaenisch, R. 1983 X chromosome-linked transmission and expression of retroviral genomes microinjected into mouse zygotes. *Science*, *Wash.* **221**, 760–762.

Stewart, C. L., Stuhlmann, H., Jähner, D. & Jaenisch, R. 1982 De novo methylation, expression and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4098–4102.

Stewart, T. A., Wagner, E. F. & Mintz, B. 1982 Human β-globin gene sequences injected into mouse eggs, retained in adults, and transmitted to progeny. *Science*, *Wash.* **217**, 1046–1048.

Wagner, E. F., Covarrubias, L., Stewart, T. A. & Mintz, B. 1983 Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line. *Cell* **35**, 647–655.

Wagner, E. F. & Mintz, B. 1982 Transfer of nonselectable genes into mouse teratocarcinoma cells and transcription of the transferred human β-globin gene. *Molec. Cell Biol.* **2** (2) 190–198.

Wagner, E. F., Stewart, T. A. & Mintz, B. 1981 The human β-globin gene and a functional viral thymidine kinase gene in developing mice. *Proc. natn. Acad. Sci. U.S.A.* **78** (8), 5016–5020.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES