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Transplantation of the human insulin gene into fertilized mouse eggs

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A circular recombinant plasmid composed of a 12.5 kb fragment of human DNA including the entire insulin gene and the 4.3 kb bacterial plasmid pBR322 was microinjected into fertilized C57BL/6 mouse eggs. 753 eggs were injected with 30 000 gene copies in a volume of 1–2 pl; 379 eggs survived micromanipulation and were subsequently cultured to the blastocyst stage. From 282 embryos that were transferred into the uteri of pseudopregnant ICR/Swiss foster females, 60 fetuses and corresponding placentas could be recovered at day 16–19 of pregnancy. High molecular weight DNA was extracted from these tissues and was screened with radioactively labelled hybridization probes for the presence of the injected DNA sequences. By restriction endonuclease analysis in conjunction with Southern blot hybridization, we found that in two normally developed fetuses at day 18, the fetal and placental tissues contained the human insulin gene including the flanking regions and bacterial plasmid sequences. Our results indicate that the injected DNA integrated into the mouse genome within its pBR322 region and properly replicated with the host DNA during development. The intensities of the hybridization bands suggest that at least one copy of foreign plasmid DNA was present per cell in the two fetuses and their placentas.

Key words: recombinant DNA/pBR322 plasmid/microinjection/gene transfer/mouse fetus

Introduction

Recombinant DNA technology has provided the means to select and clone distinct portions of prokaryotic and eukaryotic genomes. Genes of interest can be characterized in detail and used either unaltered, or experimentally modified, for DNA-mediated transformation. Various techniques have been used to transfer purified genes into mammalian cells, including the use of viral vectors, calcium precipitation (Mantei, *et al.*, 1979; Mulligan *et al.*, 1979; Wigler *et al.*, 1979; Wold *et al.*, 1979), and liposomes (Fraley *et al.*, 1979; Fraley *et al.*, 1980). Because of the low efficiencies of these methods, detection of transformed cells usually requires employment of a selection system such as thymidine kinase. Successful gene transfer into a recipient cell can then be demonstrated by molecular hybridization with the appropriate probes.

One approach to the study of the regulation of mammalian genes in a developing organism involves transfer of specific genes of interest into eggs or early embryos, and monitoring of their structure and possible expression during development. Microinjection with its high transformation frequency (Capecchi, 1980; Anderson *et al.*, 1980) provides a powerful method for transferring cloned genes for which selection systems are not available into early embryos. In mammals, preimplantation embryos of the mouse provide suitable recipients for gene transfer because of their well characterized genetic background and the relative ease with which they can be collected from pregnant females, manipulated and cultured *in vitro*, and then retransferred into foster mothers for further development. Thus, the transfer, integration and possible expression of foreign genetic material in the developing animal can be conveniently studied at various stages of embryonic, fetal and adult life and, eventually, transmitted to the next generation. This approach was first used by injecting purified simian virus 40 (SV40) DNA into the cavity of mouse blastocysts (Jaenisch and Mintz, 1974). Several of the surviving animals carried copies of the injected DNA in some of their organs.

More recently cloned viral, bacterial and eukaryotic DNA was injected directly into the fertilized mouse egg, in order to increase the probability of integration of donor DNA into the recipient genome and its propagation to all cells of the developing embryo, including germ line and extraembryonic tissues. In this way, recombinant DNA molecules containing segments of herpes simplex virus (HSV), SV40, and pBR322 (Bolivar *et al.*, 1977) were introduced into the pronuclei of fertilized mouse eggs (Gordon *et al.*, 1980). Two of the newborn mice derived from plasmid-injected eggs were found to contain donor DNA-specific sequences, though in rearranged form (Gordon *et al.*, 1980).

In the present report, we describe the successful transfer of recombinant DNA plasmids containing a defined eukaryotic gene, the human insulin gene, into fertilized mouse eggs. The plasmid we employed comprises a 12.5 kb region of the human genome including the insulin gene (Ullrich *et al.*, 1980) and the 4.3 kb bacterial plasmid pBR322 (Bolivar *et al.*, 1977).

While this manuscript was in preparation several other groups reported the successful introduction of eukaryotic genes into the mouse genome (Wagner *et al.*, 1981; Wagner *et al.*, 1981a; Constantini and Lacy, 1981; Gordon and Ruddle, 1981). In two of these cases, expression of HSV thymidine kinase (Wagner *et al.*, 1981) at late fetal stages and rabbit globin genes (Wagner *et al.*, 1981a) in adult mice have been observed.

The introduction of the insulin gene into the genome of mouse embryos differs from these other gene transfer experiments in one major respect. Uncontrolled constitutive expression of a metabolic regulatory protein such as insulin will undoubtedly have profound if not lethal effects on the developing fetus. Our experimental system probably excludes those random integration events which result in uncontrolled insulin gene expression. The maintenance of the human insulin gene in term fetuses most likely reflects cellular control of this gene's expression, be it regulated or silenced. Preliminary data of this work have already been presented at the ICN-UCLA Symposium on Molecular and Cellular Biology, March 1981 (Illmensee *et al.*, 1981).

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Table I. Microinjection of recombinant DNA plasmid containing human insulin gene into fertilized C57BL/6 eggs.

Series	Eggs[a]		Development stage reached				
			Preimplantation		Postimplantation		
	Injected	Cultured (%)	Cleavage to Compaction	Morula and Blastocyst (%)	Decidua	Fetus[b] (%)	
						Dead	Alive
1	40	14 (35.0)	6	8 (57.1)	1		2 (25.0)
2	99	59 (59.6)	8	51 (86.4)	5		10 (19.6)
3	36	61 (44.9)	18	43 (70.5)	8	3	10[c] (30.2)
4	82	45 (54.9)	15	30 (66.7)	10		6 (20.0)
5	69	40 (60.0)	13	27 (67.5)	9		5 (18.5)
6	120	56 (46.7)	11	45 (80.4)	8	1	7 (17.8)
7	131	61 (46.5)	13	48 (78.7)	2	2	5[c] (14.6)
8	76	43 (56.6)	13	30 (69.8)	7	2	7 (30.0)
Total	753	379 (50.3)	97	282 (74.4)	50	8	52 (21.3)

[a]As controls 75 noninjected C57BL/6 eggs were cultured under the same conditions and developed into 69 (92%) morulae and blastocysts.
[b]Fetuses derived from DNA-injected eggs were recovered together with their corresponding placenta between day 16 and 19 of pregnancy, and further processed for DNA analysis (see **Materials and methods**).
[c]Two normally-developed fetuses both autopsied at day 18 of pregnancy, Nr. 26 of series 3 and Nr. 46 of series 7, as well as their corresponding placenta were found to contain the injected DNA plasmid (see **Results**).

Results

Gene transplantation

753 fertilized C57BL/6 eggs were injected with 1–2 pl of DNA solution; 379 (~50%) eggs survived micromanipulation and were subsequently cultured *in vitro*. Normal development to the morula and blastocyst stage was observed in 282 (~74%) embryos which then were transferred into the uteri of 19 pseudopregnant ICR/Swiss foster females. Upon autopsy of these females at days 16–19 of pregnancy, 60 (~21%) fetuses with their corresponding placenta were recovered and prepared for DNA extraction (Table I).

DNA analysis

We employed a variety of hybridization probes representing various specific regions of the injected DNA molecule to investigate the presence and state of integration of the human insulin gene and its flanking regions in the mouse genome. High molecular weight (mol. wt.) DNA from injected fetal and placental tissues was digested with restriction endonucleases and then screened using the Southern blot hybridization procedure. In initial experiments, the use of a probe consisting of human insulin cDNA sequences excised from a plasmid (Sures *et al.*, 1980) resulted in a high level of background hybridization (data not shown), probably due to the $(C:G)_n$ sequences at both ends of the cDNA which originated from the cloning procedure. Improved results were obtained with a hybridization probe composed of a subcloned 310 bp PstI fragment (see Figure 1) containing part of the first intervening sequence, the prepeptide, the B-chain, and part of the C-peptide regions of the human preproinsulin gene. The initial screening with the 310-bp probe revealed that two (fetuses 26 and 46, see Table I) out of the 60 fetuses and their respective placentas contained the injected DNA sequences; these were characterized in more detail using several restriction enzymes. Here we present data obtained with individuals 46 and 26, most of which is identical; some differences were, however, observed.

Few plasmid-sized molecules should be present in our mouse tissue DNA preparations, because we used a high mol. wt. DNA purification scheme (Wigler *et al.*, 1979). However, to confirm that our hybridizations were not due to unintegrated plasmid DNA, we electrophoresed undigested DNA from the mouse tissues containing human DNA sequences. Unintegrated plasmids should migrate faster than undigested high mol. wt. chromosomal DNA and be detectable by hybridization; none were observed (data not shown). Furthermore, attempts to transform *Escherichia coli* with various amounts of fetal and placental DNA at transforma-

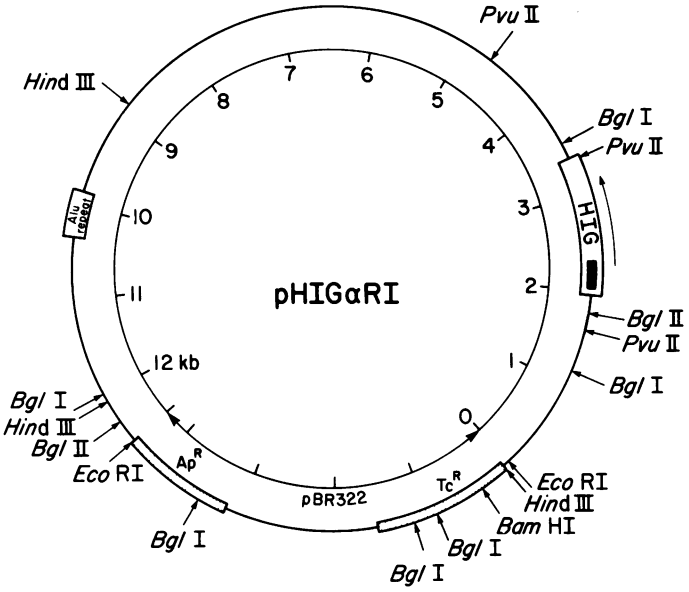

Fig. 1. Restriction endonuclease map of pHIGαRI recombinant DNA plasmid. Not all PvuII and BglI restriction endonuclease cleavage sites are shown. The human insulin gene is shown (HIG) with an arrow designating the direction of transcription (5′ to 3′). The solid bar in the human insulin gene region represents that portion of the gene used as hybridization probe, referred to in the text as the 310 bp probe. Tc^R, tetracycline resistance gene; Ap^R, ampicillin resistance gene. Sequences numbered 0–12 kb flank the insulin gene within the human genome (Ullrich *et al.*, 1980); pBR322 designates a bacterial plasmid (Bolivar *et al.*, 1977).

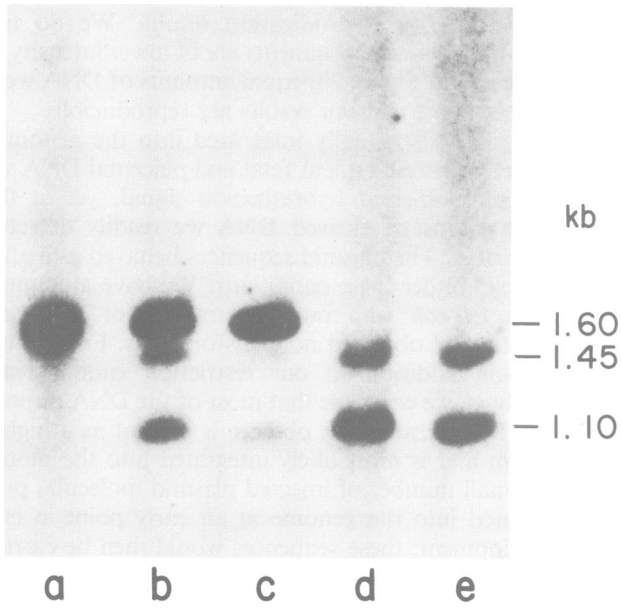

Fig. 2. PvuII restriction endonuclease cleavage in conjunction with Southern hybridization. DNA from **(a)** human placenta, **(b)** mouse fetus 46, **(c)** mouse placenta 46, **(d)** control mouse fetus, and **(e)** control mouse placenta, electrophoresed on a 1% agarose gel and hybridized with the 310 bp human insulin gene probe.

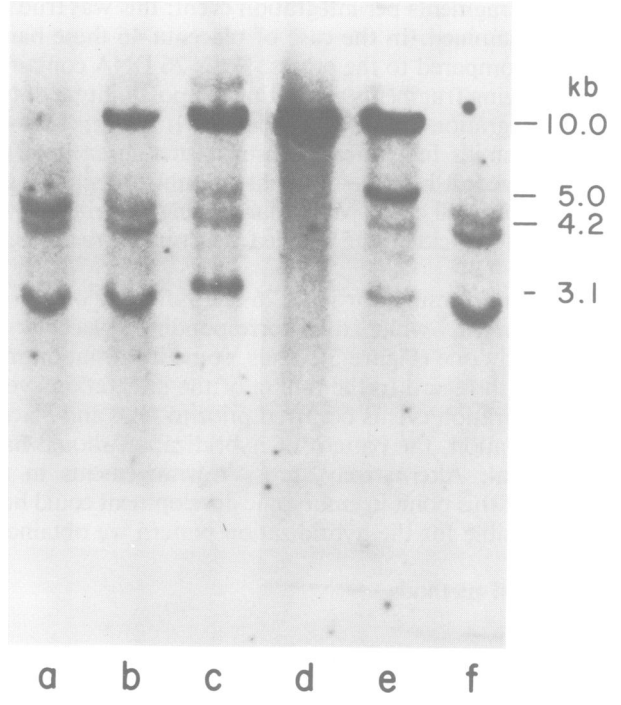

Fig. 3. BglII restriction endonuclease cleavage in conjunction with Southern hybridization. DNA from **(a)** control mouse fetus, **(c)** fetus 46, **(b)** a mixture of equal amounts of samples a and c, **(d)** human placenta, **(e)** placenta 46, and **(f)** control mouse placenta, was analyzed as described in Figure 2. To verify identity of hybridizing bands, samples were mixed **(lane b)**, necessitated by altered migration of the control mouse DNA samples.

tion efficiencies of 5 x 10^6 transformants per microgram of pBR322 plasmids were unsuccessful (data not shown).

The restriction enzyme PvuII cleaves the injected plasmid close to either side of the human insulin gene sequences, and leaves most of the 1.43-kb gene intact (see Figure 1). This enzyme was employed to examine whether the size of the insulin gene region was maintained after integration. PvuII endonuclease digestion of total human placental DNA and injected plasmid pHIGαRI yielded a 1.6 kb band, detectable by hybridization with the 310-bp probe (Figure 2). This fragment has been completely sequenced and is known to contain most of the human preproinsulin gene (Ullrich *et al.*, 1980). The placentas and fetuses 26 and 46 each displayed this 1.6-kb hybridizing fragment, in addition to two bands of 1.1 and 1.45 kb, which resulted from cross-hybridization of the endogenous mouse insulin genes (Figure 2).

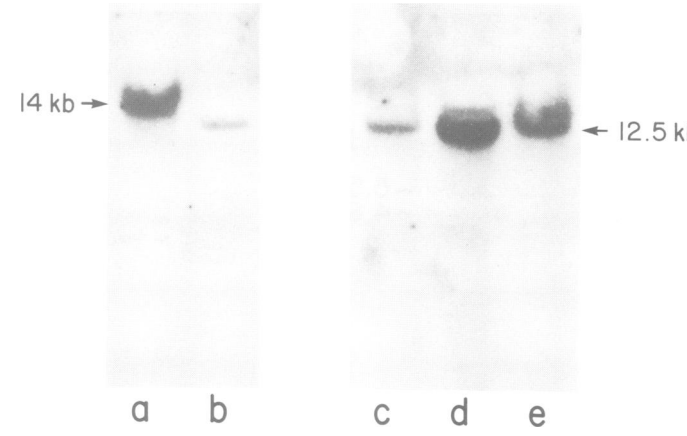

Fig. 4. EcoRI endonuclease cleavage of genomic DNA in conjunction with Southern hybridization: **(a)** human placenta DNA; **(b, c)** 50 pg plasmid DNA (pHIGαRI) mixed with mouse liver DNA; **(d)** mouse fetus derived from injection 46; and **(e)** mouse placenta 46. EcoRI fragments containing mouse insulin gene sequences electrophoresed off the gel. A radiolabelled 310 bp human insulin gene fragment was used as hybridization probe. It should be noted that the 12.5 kb EcoRI fragment represents only part of the native 14 kb human genomic EcoRI fragment containing the human insulin gene. The gene was originally isolated from an AluI-HaeIII partially digested human gene library kindly provided by T.Maniatis, as described (Ullrich *et al.*, 1980).

Bg1II restriction endonuclease cleaves the injected plasmid close to the 5′ end of the human insulin gene, and yields a 10-kb fragment containing the insulin gene and ~8.7 kb of 3′ region flanking human DNA sequences (Figure 1). Bg1II digestion of DNA from fetuses and placentas 26 and 46 as well as human placenta and spleen generated a 10-kb fragment which hybridized with the 310 bp probe in both the positive mouse tissues and human controls (Figure 3). The endogenous mouse Bg1II bands for fetal and placental DNAs were identical in size, i.e., 3.1, 4.2, and 5.0 kb, respectively. Although small differences in size would not be detected, this digestion indicated that this 10 kb portion of the plasmid maintained its size after integration in both individuals.

Cleavage of the original plasmid with EcoRI yields a 12.5-kb fragment containing the human insulin gene (see Figure 1). EcoRI endonuclease digestion of DNA from fetuses and placentas 26 and 46 gave rise to a 12.5-kb fragment hybridizing to the human insulin gene probe (Figure 4), equal in size to the original human DNA region within the injected plasmid.

To determine whether chromosomal integration had occurred within the pBR322 region of the injected plasmid, we hybridized EcoRI blots of the fetal and placental DNA with a pBR322 probe (Figure 5). An integration event should result in two or more hybridizing fragments, depending on the location and mode of recombination between plasmid and mouse DNA. This experiment revealed a different pattern for each DNA examined: fetus 26 contained four bands of 2.0, 4.4, 5.1, and ~25 kb, and its placenta, two bands of 4.6 and 5.5 kb; fetus 46 showed two hybridizing bands of 3.8 and 4.3 kb,

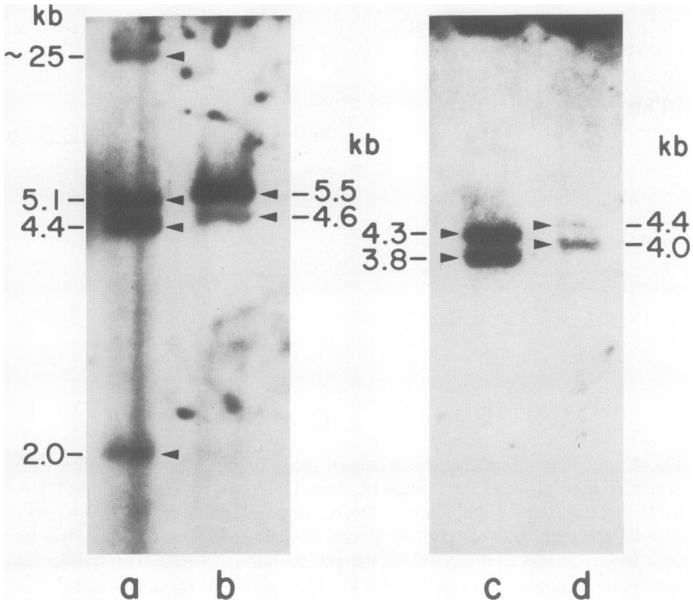

Fig. 5. EcoRI restriction endonuclease cleavage in conjuntion with Southern hybridization, using radiolabelled pBR322 as probe. **(a)** fetus 26, **(b)** placenta 26, **(c)** fetus 46, and **(d)** placenta 46.

respectively, and the corresponding placenta, bands of 4.0 and 4.4 kb (Figure 5). Our hybridization results indicate that the human DNA insert of plasmid pHIGαRI remained intact in each of the tissues examined (Figure 4), yet Figure 5 shows that some change occurred in the pBR322 region of the injected plasmid. One simple interpretation of this hybridization result is that the pBR322 region of the injected plasmid integrated into the genome, yielding two bands per integration event: one from each end of the plasmid, connected to mouse DNA.

Discussion

In this paper we have reported the injection and stable integration of the human insulin gene into mouse embryos. Two out of 60 fetuses which survived the injection and reimplantation procedures contained human insulin gene sequences as detected by Southern hybridization. This transformation frequency is comparable to that obtained after DNA injection into cultured cells (Capecchi, 1980; Anderson *et al.*, 1980). Restriction cleavage with PvuII, BglII, and EcoRI showed that the entire human DNA insert region had maintained its size in the two mouse fetuses and their corresponding placentas from the time of embryo injection up to 18 days of prenatal development.

Although large numbers (~30 000) of plasmids were injected into each fertilized egg, simple hybridization patterns were observed in both cases. By comparing the intensity of the human insulin gene fragment in control human placental DNA (Figure 2, lane a) with the injected mouse fetal and placental tissues (Figure 2, lanes b and c), one can estimate that the number of hybridizing DNA molecules in these samples is roughly equal. This is consistent with each cell of the transformed fetus and placenta containing ~1–2 copies of the human insulin gene. Alternatively, a fraction of the cells in the fetuses and placentas could contain many copies while other cells contain none. We favor the former possibility because we obtain extremely simple hybridization patterns; the second model would require that each of the many plasmids contained within a cell be integrated in tandem, to be consistent with our hybridization results. We do not understand why the mouse fragments are of lower intensity in Figure 2, lane c than Figure 2b; equal amounts of DNA were analyzed in each lane and our results are reproducible.

Are these plasmids actually integrated into the genome? When we electrophoresed uncut fetal and placental DNA we detected no plasmid-sized hybridization signal, yet at the same concentrations of cleaved DNA we readily detected plasmid sequences. The plasmid sequences behaved as higher mol. wt. species under these conditions. We have attempted to transform *E. coli* with our preparations of fetal and placental DNA and obtained no transformants. From these experiments, in addition to our restriction endonuclease cleavage analysis, we conclude that most of the DNA responsible for the hybridization we observe is present as a higher mol. wt. form and is most likely integrated into the mouse genome. A small number of injected plasmid molecules probably integrated into the genome at an early point in embryonic development; these sequences would then be carried to progeny cells in conjuntion with genomic DNA replication.

Hybridization of EcoRI-digested DNA from both the positive fetuses and placentas with radiolabelled pBR322 strongly suggested that integration into the mouse genome occurred within the pBR322 region of the plasmid pHIGαRI. Opening the DNA circle in the pBR322 region for integration should, after EcoRI digestion, result in two pBR322-hybridizing fragments per integration event; this was true for all tissues examined. In the case of placenta 46 these bands were weak compared to the others. Fetus 26 DNA contained four hybridizing fragments suggesting the possibility that two separate integration events had occurred. It appears that the detected plasmids integrated at distinct sites in each of the tissues analyzed; it is interesting that a doublet of similar size was generated in all cases. Molecular cloning experiments will be required to elucidate the detailed structure of the integration sites.

Hybridization results for fetus 26 are indicative of two integration events, while the corresponding placenta 26 displayed only one (Figure 5). Thus, some information may be gained with regard to the timing of the integration event. Had all integration events occurred prior to fetal and placental differentiation, the pattern of hybridization should have been identical. Alternatively, gross rearrangements in the genome after this point in embryonic development could have been responsible for the hybridization pattern we obtained.

Materials and methods

Mice

Groups of two females and one male of the inbred C57BL/6J strain were continously housed in small cages. Every morning those females with a vaginal plug were removed to harvest their fertilized eggs. Females of the outbred ICR/Swiss strain were selected in proestrus and mated with sterile, vasectomized males of the same strain. Females showing a plug the next morning were stored to be used as pseudopregnant foster mothers.

Recipient eggs

Approximately 10–12 h after mating both oviducts were removed from the C57BL/6 females and transferred to a depression slide containing 1 ml of modified Whitten's medium (Hoppe and Pitts, 1973) supplemented with bovine testes hyaluronidase (Sigma) at 0.2 mg/ml. The ampullae were punctured to release the eggs. After removal of the cumulus cells, fertilized eggs were washed twice and pooled in a drop of medium covered with paraffin oil (Fisher). The eggs were stored at 37°C in an atmosphere of 5% CO_2/5% O_2/90% N_2 (Carbagas). For microinjection, about five eggs were transferred in a small drop of medium onto a special microscopic slide and covered with halofluorocarbon oil (Voltalef 10S). After manipulation, the surviving eggs were cultured in glass tubes containing 1 ml of gassed medium.

Donor DNA

The pHIGαRI recombinant plasmid comprises a 12.5 kb EcoRI fragment of fetal human DNA derived from λCh4A-HIGα (Ullrich *et al.*, 1980), which was subcloned into the EcoRI site of pBR322. The overall size of this circular DNA molecule is 16.9 kb (Figure 1). The 12.5 kb human DNA fragment contains the 1.43 kb long preproinsulin gene, ~2.5 kb of flanking sequences upstream from the gene (including the potential RNA polymerase binding region), in addition to 8.7 kb of nucleotide sequences downstream from the insulin gene (Ullrich *et al.*, 1980). A 500 bp sequence which is highly repeated (~100 000 copies) in mammalian genomes and a member of the Alu family (Bell *et al.*, 1980; Rubin *et al.*, 1980; Houck *et al.*, 1979), is located 5 kb downstream from the insulin gene, and is also present in the injected plasmid. The remainder of the 12.5 kb DNA fragment has not been characterized in detail. This work was carried out under P2 containment in accordance with National Institutes of Health guidelines for recombinant DNA research.

Microinjection

The preparation of micropipettes was carried out as previously described (Hoppe and Illmensee, 1977). For each injection series, ~10 pl of DNA solution (0.5 μg/μl) was sucked from a microdrop stored under halofluorocarbon oil (Voltalef 10S) into the injection pipette (1–2 μm diameter). After having attached a fertilized egg to the holding pipette in the appropriate position, the injection capillary was gently pushed through the zona pellucida into the egg, and ~1–2 pl (~30 000 molecules) of DNA solution was injected into either the cytoplasm or the male pronucleus. The injected volume was estimated by measuring the distance covered by the meniscus within the pipette and by determining the inner diameter of the capillary.

Embryo transfer

Pseudopregnant ICR/Swiss females (day 3 postcoitus) were anesthetized with 0.5 ml Avertin (25 mg/ml Tribromethanol, Fluka) and opened dorsally by a small incision in order to prepare their uteri for embryo transfer. Approximately five injected embryos that had developed to the morula and blastocyst stage (day 4 postfertilization) were sucked into a small glass pipette and subsequently transferred into each uterine horn. Following these operations and proper wound closure, the females were caged individually. At day 16–19 of pregnancy, they were killed by cervical dislocation and the fetuses with their corresponding placenta collected and frozen for DNA extraction.

DNA isolation and analysis

Mouse and human high mol. wt. DNA was isolated by the procedure of Wigler *et al.* (1979) and was stored under ethanol at −20°C until use; plastic tubes and containers were routinely employed. Supercoiled bacterial plasmids were isolated by acid phenol extraction (Zasloff *et al.*, 1978). Cloned human DNA sequences were excised from plasmids by restriction endonuclease cleavage, and were purified using preparative acrylamide gels for use as hybridization probes. Restriction endonucleases were obtained from Bethesda Research Laboratories (Rockville, MD) or New England Biolabs (Beverly, MA). DNA fragment mol. wt. markers (wild-type phage λ cleaved by HindIII and EcoRI) were purchased from Boehringer Mannheim.

Restriction endonuclease digests of high mol. wt. DNA were carried out at a 5- to 10-fold excess of enzyme and the reactions were terminated by adding EDTA (20 mM final concentration). Digested DNA (15 μg) was analyzed on 1% agarose (Seakem HP) gels in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA (pH 7.9) buffer at 75 V for 8–10 h. DNA was transferred from agarose gels to nitrocellulose filters according to the procedure of Southern (1975). These filters were then baked, preannealed, incubated with ^{32}P-labelled DNA probes, and washed as previously described (Cordell *et al.*, 1979). Autoradiography was carried out by exposure of Kodak X-Omat film to dried filters at −70°C, in conjunction with a Dupont Lightening Plus intensifying screen, for 1–5 days (Swanstrom and Shank, 1978).

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References

Anderson, W.F., Killos, L., Sanders-Haigh, L., Kretschmer, P.J., and Diacumakos, E.G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5399-5403.

Bell, G.I., Pictet, R., and Rutter, W.J. (1980) *Nucleic Acids Res.*, **8**, 4091-4109.

Bolivar, F., Rodriquez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977) *Gene*, **2**, 95-113.

Capecchi, M.R. (1980) *Cell*, **22**, 479-488.

Constantini, F., and Lacy, E. (1981), *Nature*, **294**, 92-94.

Cordell, B., Bell, G., Tischer, E., De Noto, F.M., Ullrich, A., Pictet, R., Rutter, W.J., and Goodman, H.M., (1979) *Cell*, **18**, 533-543.

Fraley, R., Fornari, C. and Kaplan, S. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3348-3352.

Fraley, R., Subramani, S., Berg, P. and Paphadjopoulos, D. (1980) *J. Biol. Chem.*, **255**, 10431-10435.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7380-7384.

Gordon, J.W. and Ruddle, F.H. (1981) *Science (Wash.)*, **214**, 1244-1246.

Hoppe, P.C., and Pitts, S. (1973) *Biol. Reprod.*, **8**, 420-426.

Hoppe, P.C., and Illmensee, K. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5657-5661.

Houck, C.M., Rinehart, F.P., and Schmid, C.W. (1979) *J. Mol. Biol.*, **132**, 289-306.

Illmensee,K., Bürki,K., Hoppe,P.C., and Ullrich,A. (1981) in Brown,D.D. (ed.), *Developmental Biology using Purificed Genes*, ICN-UCLA Symposium on Molecular and Cellular Biology, **23**, Academic Press, NY, pp. 607-619.

Jaenisch, R., and Mintz, B. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1250-1254.

Mantei, N., Boll, W., and Weissmann, C. (1979) *Nature*, **281**, 40-46.

Mulligan, R.C., Howard, B.H., and Berg, P. (1979) *Nature*, **227**, 108-114.

Rubin, C.M., Houck, C.M., Deininger, P.L., Friedmann, T., and Schmid, C.W. (1980) *Nature*, **284**, 372-374.

Southern, E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.

Sures, I., Goeddel, D.V., Gray, A., and Ullrich, A. (1980) *Science (Wash.)*, **208**, 57-59.

Swanstrom, R., Shank, P.R. (1978) *Anal. Biochem.*, **86**, 184-192.

Ullrich, A., Dull, T.J., Gray, A., Brosius, J., and Sures, I. (1980) *Science (Wash.)*, **209**, 612-614.

Wagner, E.F., Stewart, T.A., and Mintz, B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 5016-5020.

Wagner, T.E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R.L., and Gault, J.B. (1981a) *Proc. Natl. Acad. Sci. USA*, **78**, 6376-6380.

Wigler, M., Sweet, R., Sim, G.K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) *Cell*, **16**, 777-785.

Wold, B., Wigler, M., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5684-5688.

Zasloff, M., Ginder, G.D., and Felsenfeld, G. (1978) *Nucleic Acids Res.*, **5**, 1139-1152.