Biotecnología Aplicada, Vol. 8, No. 1, 8-19 (1991)

Artículos originales completos

High Efficiency Integration of Human Growth Hormone Gene in Transgenic Mice

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Recibido en abril de 1990
Aprobado en junio de 1990

ABSTRACT

Transgenic mice carrying the mouse metallothionein-I promoter fused to the human growth hormone gene were produced by microinjection of fertilized 1 cell embryos. A high efficiency of transformation was achieved. Eighteen out of forty newborn mice were transgenic (45%). Transgenic founder mice (F0) and (F1) derived progeny that expressed human growth hormone gene grew significantly larger (up to 2.03 times) than control non transgenic littermates.

RESUMEN

Se obtuvieron ratones transgénicos que incorporaron a su genoma la construcción híbrida conformado por el gen de la hormona de crecimiento humana bajo el control del promotor de la metalotioneina-I de ratón. Una alta eficiencia en la generación de los ratones transgénicos (45 %) fue alcanzada. Tanto los transgénicos fundadores (F0) como una línea de primera generación (F1) obtenida, expresan en el suero la hormona de crecimiento humana y presentan un notable incremento de peso corporal (hasta 2,03 veces) en comparación con sus hermanos no transgénicos.

INTRODUCTION

In recent years the introduction of foreign DNA into mice has become commonplace, and the transgenic mouse assay system is being used to address many important biological problems (Palmiter and Brinster, 1986). Attention has been given to the transfer of heterologous growth hormone (GH) genes fused with mouse metallothionein promoter (mMT-I), to accelerate growth in animals (Palmiter *et al.*, 1982, 1983). A major effort is being made to express GH genes in farm animals (Hammer *et al.*, 1985, 1985a, 1986, Pursell *et al.*, 1987, Ebert *et al.*, 1988).

The overall efficiency of producing transgenic mice varies between different gene constructs, and it is thought to be affected by a variety of factors (Brinster *et al.*, 1985).

In this study we report a high efficiency generation of transgenic mice (45%) carrying the hybrid human growth hormone gene (hGH) fused to the mMT-I promoter/regulator region.

MATERIALS AND METHODS

Structure of the mMT-I/hGH fusion gene construct

Chromosomal human growth hormone gene was a gift of J. Martial from Liege University, Belgium. The hGH gene, a 2.6 kilobase (kb) fragment was released from the phGH plasmid by BamHI digestion

and cloned into the Bgl/II site of the pMT-I plasmid (figure 1a). The resulting plasmid (pMTI-hGH) was used for microinjection. The MT-I/hGH fusion gene was released from the pMTI-hGH plasmid by EcoRI digestion (figure 1b).

Southern Blot analysis

For Southern Blot analysis, 10 μg of DNA in a reaction volume of 300 μl were completely digested with 10-15 units of either EcoRI or PvuII per μg of

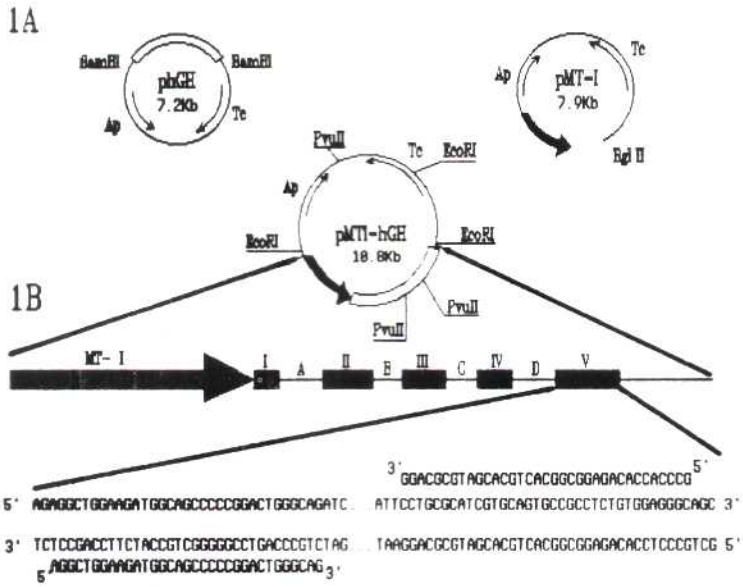

FIG. 1: (a) Outline of the plasmid pMTI-hGH used for the generation of transgenic mice. Relevant restriction sites are depicted; (b) A schematic representation of the mMT-I/hGH gene. Black boxes represent the exons of the hGH gene. The sequence of the primers used for PCR amplification of a fragment of the exon 5 in the hGH gene is showed below.

Transgenic mice

Transgenic mice were generated by direct microinjection of the 4.1 kb EcoRI fragment (Fig. 1a) into fertilized 1 cell mouse eggs, followed by embryo transfer into the oviducts of day 1 pseudopregnant foster mothers (Gordon,1980, Hogan *et al.*, 1986). DNA from small tail pieces was extracted by phenol-chloroform as described (Hogan *et al.*, 1986). Pups carrying the foreign DNA were identified by Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1985). For this purpose 2 primers were designed so that they extend a fragment of 183 base pairs (bp) in the exon 5 of the hGH gene (figure 1b). To the reaction volume of 100 μl (100 mM Tris Hcl pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin (w/v), 200 μM dNTP, 0.2 μM of each oligonucleotide primer) 2.5 units of T.aquaticus DNA polymerase (Enzibiot, CIGB, Cuba) were added. The reaction was subjected to 30 cycles of 1 min 93 °C, 10 sec 55 °C and 15 sec 70 °C. The products of the PCR were analyzed in a 1.2% agarose gel.

DNA for 10 hours. After electrophoresis in a 0.8% agarose gel,the DNA was transferred to nitrocellulose filters (BA 85, Schleicher & Schuell) and hybridized with [alpha^{32}-P]dATP labeled pM13-hGH according to Southern (1975).

The pM13-hGH contains the BamHI hGH fragment (Seeburg *et al.*, 1982) inserted into the BamHI site of the M13mp19. The probe for hybridization was prepared by primer extension. Two hundred ng of single stranded pM13-hGH DNA were mixed with 3 pmoles of pentadecamer (15 mer) sequencing primer (# 1200, New England Biolabs) in 5 mM $MgCl_2$, 50 mM Tris-HCl pH 8 and 5 mM 2-mercaptoethanol. After an incubation at 65 °C, 25 °C, and 0 °C for 15 min each, a mixture containing 1 μl of 10 μM dATP, 1 μl of 1 mM (dTTP, dCTP, dGTP), 1 μl of 100 mg/ml BSA, 50 μCi of [alpha^{32}-P]dATP (Amersham) and 10 units of DNA polymerase I Klenow fragment was added to a final volume of 10 μl. The reaction was carried out at 25 °C for 2 hrs. Finally, the reaction was stopped by adding 90 μl of 25 mM EDTA, 0.5% SDS, and

100 mM NaCl, and the DNA was ethanol precipitated.

After hybridization, the filters were washed under stringent conditions (twice at room temperature for 15 min. with 2xSSC, 0.1% SDS, twice at room temperature with 0.2xSSC, 1 % SDS, once at 45 °C for 15 min. with 0.2xSSC, 0.1% SDS, once at 55 °C for 15 min. with 0.2xSSC, 0.1% SDS). The nitrocellulose filters were then exposed to RX films (Fuji) at -70 °C for 24 hours and 1 week.

Gene copy number of transgenic mice was calculated by Tail blot as described by Palmiter *et al.* (1982). Briefly, hybridized pMTI-hGH DNA was quantified from excised small pieces of nitrocellulose that were placed in a liquid scintillation cocktail, and associated radioactivity measured in a scintillation counter (LKB Wallac, Sweden). As control, known amounts of the plasmid pMTI-hGH were used.

Growth measurement and hormone assays

Transgenic and control (non transgenic) littermates were fed *ad libitum*, and the drinking water was supplemented with 25 mM zinc sulphate ($ZnSO_4$) starting from the third week of life for a period of 10 weeks, to induce the mMT-I promoter.

Animals were weekly weighed during the above-mentioned period of time, and individual record was kept. Blood samples were collected by retroorbital puncture at the 3rd and 6th weeks of life. The concentration of hGH in serum was measured using a solid phase radioimmunoassay kit (Allegro, S.J. Capistrano, CA, USA).

X-ray radiography

For X-ray study, mice were anesthetized with ketamine (100 mg/kg) and xilazine (10 mg/kg), taped out as described (Hughes and Tarner, 1970) and X-rayed at 3 kilovolt potential for 1.5 seconds with a target to film distance of 115 centimeters.

Statistical analyses

All statistical comparisons were performed using one way Anova test.

RESULTS

Integration of the mMT-I/hGH gene

The 4.1 kb EcoRI restriction fragment of the pMTI-hGH plasmid was isolated from vector sequences and injected into the pronuclei of fertilized B6D2F1 x OF-1 murine eggs to generate transgenic mice. In overall, 323 mouse eggs were microinjected in two sets of experiments. Survival of microinjected eggs was 51 %. Survived embryos were transferred to the oviduct of pseudopregnant B6D2F1 females, and 40 pups obtained. Eighteen (45%) mice produced in this fashion were identified to be transgenic as determined by PCR (figure 2).

As there is an important degree of homology between hGH gene and endogenous murine (mGH) gene (figure 2a), we expected some false positive among the tested animals. To ascertain which mice were really transgenic for the hGH gene, a Taq 1 endonuclease restriction analysis was performed. There are no sites for this restriction enzyme in the amplified fragment of the mGH gene (figure 2b) while two sites for Taq 1 enzyme were found in the transgenic animals bearing the hGH gene. This finding is in line with the fact that two sites are reported for Taq 1 endonuclease in the amplified fragment (exon 5 of the hGH gene, Seeburg *et al.*, 1982). The results of the restriction analysis are shown in figure 2d. PCR analysis of tail DNA from F0 mice revealed the presence of the microinjected transgene in eighteen animals (figure 2c).

The integrated genes were characterized with respect to copy number by Tail dot hybridization (Palmiter *et al.*, 1983) and it ranged from 40 to 380 copies per cell (figure 3). These results are in line with the high copy number of the integrated genes reported for this (Palmiter *et al.*, 1983) and for other gene constructs (Brinster *et al.*, 1985, Castro *et al.*, 1989).

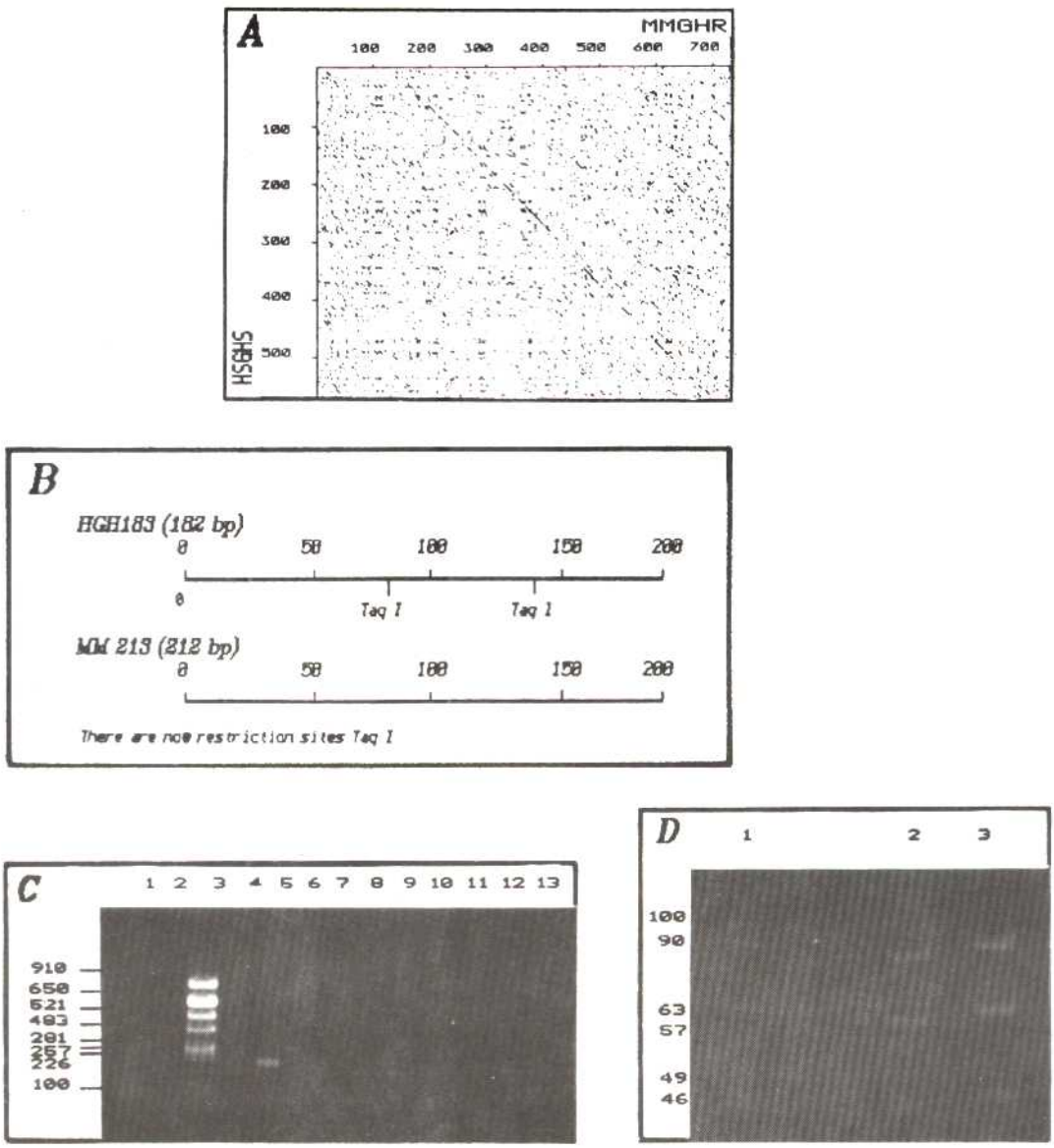

FIG. 2. (a) Dot matrix of homology between cDNAs from the hGH gene (HSGHS, 586 bp) and murine GH gene (MMGHR, 772 bp) as processed by BIOSOS (CIGB, Havana, Cuba); (b) Restriction map for Taq 1 endonuclease of the exon 5 of the hGH gene (hGH 183), and the amplified fragment of the mGH (MM 213). No restriction sites are present for Taq 1 in the murine gene; (c) PCR amplification of a 183 base pair fragment of the hGH gene in transgenic mice. Line 1: human genomic DNA, line 2: DNA from wild mouse, line 3: molecular weight markers (PBR 322-Alu I fragments), lines 4 to 13: DNA from tested mice. The size of the restriction fragments (in bp) is shown (left); (d) Polyacrylamide gel electrophoresis (ethidium bromide staining) of Taq 1 restricted DNA. Line 1: PBR plasmid, line 2: human DNA, line 3: DNA from mouse FO CB-5. Molecular weight markers are shown (left).

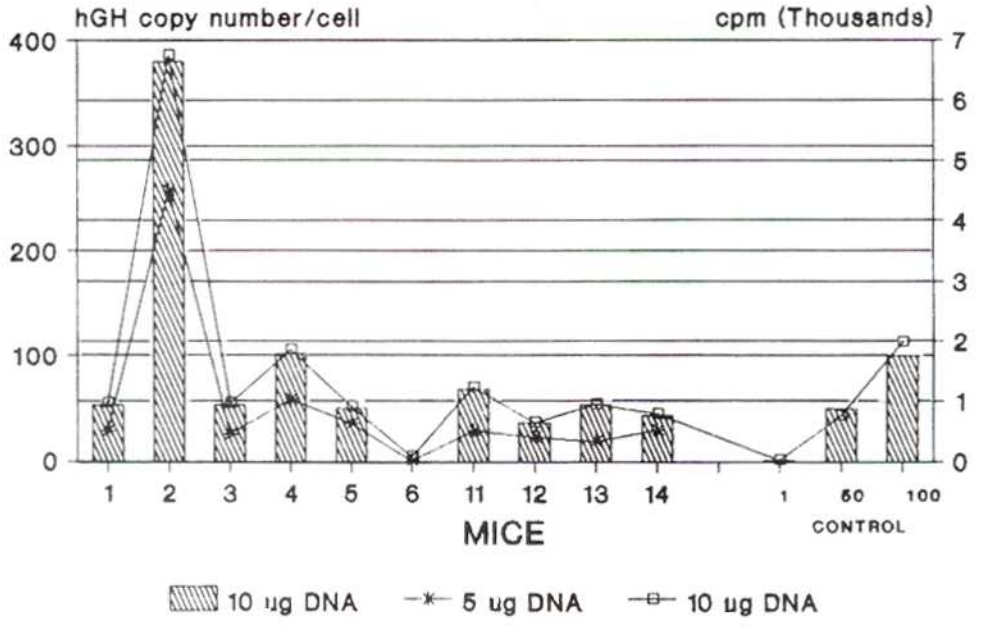

FIG. 3. "Tail blot" (Palmiter *et al.*, 1982) of DNA from FO mice. Five and ten micrograms of total DNA from FO mice CB-1 to CB-14 were used. Known amounts of pMTI-hGH plasmid which correspond to 1, 50 and 100 copies per cell were mixed with 10 μg of wild mouse DNA and were blotted as control.

Southern blot analyses from tail DNA of some transgenic mice were performed (figure 4). All 18 DNAs from F0 founder mice were initially screened by digestion with EcoRI,and hybridization with [alpha^{32}-P]dATP labeled pMT13-hGH as described in Materials and Methods. Hybridization resulted in a signal at 4.1 kb. In figure 4 (top panel) we show the results of Southern Blot analysis of founder mice F[0hGH] CB-1 through F[0hGH] CB-6 (hereafter to be named CB-1 to CB-6). The presence of the 4.1 kb signal can be expected when concatamers of the microinjected transgene are integrated in the host genome in tandem arrays.

Founder male CB-5 and some of his F1 progeny were analyzed in a more detailed way by Southern Blot (figure 4, bottom). In the founder mice CB-5, we found an additional signal of about 6 kb after EcoRI digestion, which does not appear in the DNA of analyzed F1 mice. This fact suggested that the microinjected DNA was integrated in the founder mice CB-5 in more than one chromosomal site.

In order to detect specific internal fragments of the injected transgene, the DNA was digested with Pvu II (figure 4, bottom, CB-35 and CB-45). As shown in figure 1, Pvu II cuts twice the hGH gene, generating an internal fragment of 1.05 kb. Digestion of tail DNA with Pvu II results in signals of the expected size in all tested animals. We concluded that no rearrangements occurred in the microinjected gene during integration. However, different patterns of hybridization are found in the F0 CB-5 and his F1 progeny CB-45.

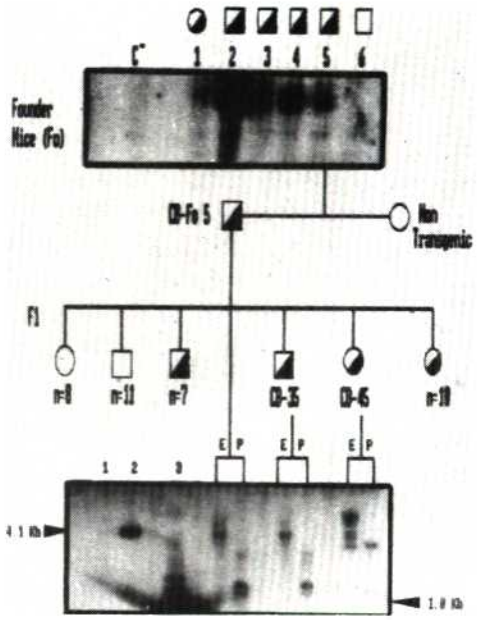

FIG. 4. **Top**. Southern Blot analysis of the integration of the hGH gene in transgenic founder mice. Ten micrograms of EcoRI digested DNA were used. C⁻ = DNA from wild mouse; lines 1 to 6 DNA from FO mice. **Center**. Schematic representation of the generation of the F1 line obtained from transgenic CB-FO-5 male. Shaded squares and circles represent transgenic males and females respectively, open squares and boxes represent non-transgenic males and females, respectively. **Bottom**. Southern Blot of DNAs from F1 transgenic mice. Line 1, one copy of hGH gene per cell; line 2, fifty copies of the hGH gene; line 3, molecular weight markers (pBR 322-Alu I fragments); DNA from CB-FO-5 and his progeny CB-35 and CB-45, E = EcoRI digestion, P = Pvu II digestion. Arrows indicate the size in kb of the mMT-I/hGH fusion gene (left) and the internal Pvu II fragment (right).

Expression of hGH in transgenic mice

The injected fusion gene spans all the necessary elements for the secretion of the hGH. Using a human specific radioimmunoassay, hGH was detected in 2 out of 9 tested transgenic founder mice (males CB-2 and CB-5) and in the F1 progeny derived from the F0- CB-5 (table 1). Table 1 also summarizes the growth ratio of these transgenic mice compared to sex matched littermates.

We found no correlation between growth rate and mMT-I/hGH gene copy number, as the biggest founder mice (CB/5) had less copies than some of their littermates.

In order to assess the transmission of the hGH transgene to the F1 progenies, expressing transgenic founders were matted to nontransgenic counterparts. One transgenic line derived from the CB/5 founder was established. Seventeen (17) transgenic F1 (9 males and 8 females) were detected by increased body weight after supplementing the drinking water with 25 mM $ZnSO_4$. This finding was confirmed by the detection of elevated levels of hGH in the serum of transgenic mice (table 1).

Three different levels of expression of the hGH gene could be found, namely < 10 ng/ml (animals F0-CB-2, F1-CB-43 and F1-CB-56, data for animal F1-CB-56 not shown); > 25 ng/ml (< 40 ng/ml

(F0-CB-5, F1-CB-35, 44, 48, 49, 53 and 54) and in the case of the female F1-CB-45, the value of the hGH in the serum was out of the upper range of detection of the employed RIA system; we estimated it at the levels of hundred nanograms per ml.

Table 1
EXPRESSION OF mMT-I/hGH FUSION GENE IN TRANSGENIC FOUNDER (FO) MICE AND THEIR F1 PROGENY

Mouse	Sex	Gene copy #/cell	Serum hGH* (ng/ml)	Relative growth ratio**
F0-CB-5	m	40-45	36.0	1.59
F0-CB-2	m	380	9.6	1.54
F1-CB-35	m	40-45	28.0	1.79
F1-CB-38	f	40-45	26.0	ND
F1-CB-43	m	ND	3.3	1.21
F1-CB-44	m	ND	36.0	1.64
F1-CB-45	f	40-45	> 100.0	1.54
F1-CB-49	m	ND	6.5	1.34
F1-CB-53	f	ND	32.3	1.53
F1-CB-54	f	ND	30.0	1.59
F1-CB-64	f	ND	ND	1.87
F1-CB-65	f	ND	ND	1.66
F1-CB-67	f	ND	ND	2.03
F1-CB-68	f	ND	ND	1.89
F1-CB-70	m	ND	ND	1.28
F1-CB-71	m	ND	ND	1.12

* hGH levels were measured three weeks after supplementation of the water with $ZnSO_4$ (sixth week of life).
** Relative weights of transgenic mice compared with sex matched littermates at 13 weeks of life.
ND = not determined.

The progeny of the founder male CB/5 was used for comparison of growth rate (figures 5a and b), and weight gain (table 2). Transgenic and non transgenic sex-matched littermates were weighed weekly, and absolute and daily weight gain were calculated.

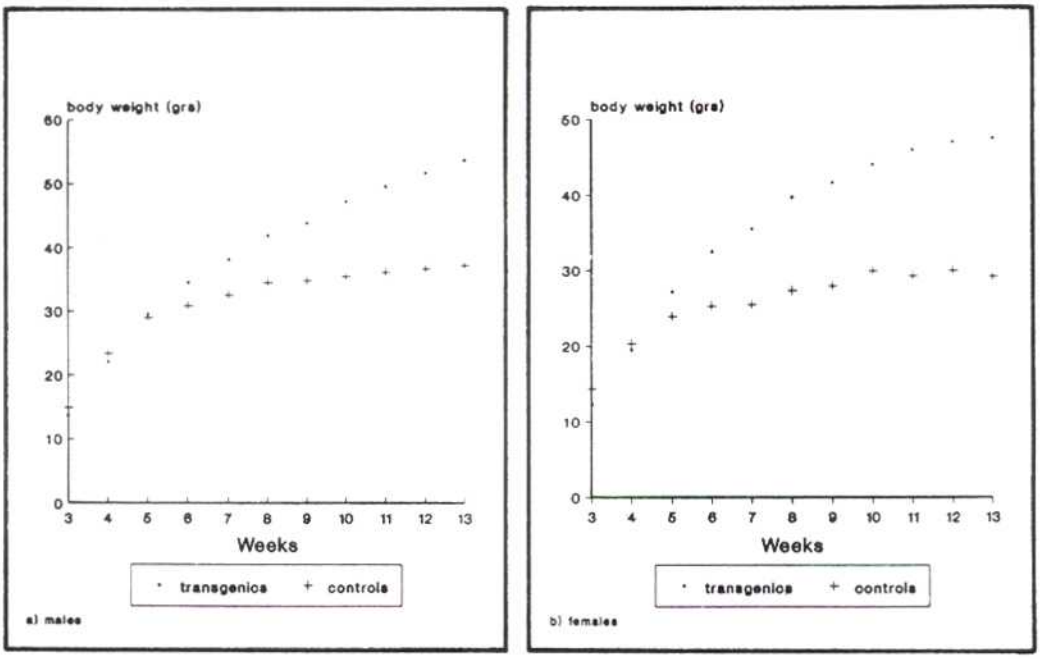

FIG. 5. Growth of transgenic F1 mice. (a) Males, (b) females. Each point represents the mean of 8 mice at least (see text for statistics).

Table 2
BODY WEIGHT GAIN IN TRANSGENIC MICE EXPRESSING hGH GENE

Absolute weight gain * at 13 weeks, (g ± SD)				**Daily weight gain ** (g/day ± SD)**			
males		**females**		**males**		**females**	
trans.	**controls**	**trans.**	**controls**	**trans.**	**controls**	**trans.**	**controls**
39.91	22.07	35.08	14.99	0.57	0.31	0.50	0.21
±8.3	±3.31	±7.63	±2.39	±0.13	±0.05	±0.12	±0.04
P <0.001		P <0.001		P <0.001		P <0.001	

* Absolute weight gain was calculated as: N = AW - WW
where:
AW = adult weight at 13 weeks
WW = weight at weaning (3 weeks)
** Daily weight gain = N/70 days

We found no differences in body weight between transgenic and control mice at weaning (data not shown, $P > 0.1$). Starting from the 7th week of life, a statistically significant difference ($P < 0.05$) in the body weight of both transgenic males and females was observed. The adult body weight at the age of 13 weeks was also statistically different for both transgenic males (53.59 g vs. 37.1 g, $P < 0.001$) and transgenic females (47.39 g vs. 29.17 g, $P < 0.001$) (figure 6).

DISCUSSION

The overall efficiency of generating transgenic mice is commonly measured as: (1) percent of newborn pups that carry the foreign gene (range from 10 to 33%, Brinster *et al.*, 1985, De Pamphillis *et al.*, 1988) and (2) percent of transgenic pups out of the total number of injected embryos (range 1 to 4%, Hogan *et al.*, 1986, De Pamphillis *et al.*, 1988).

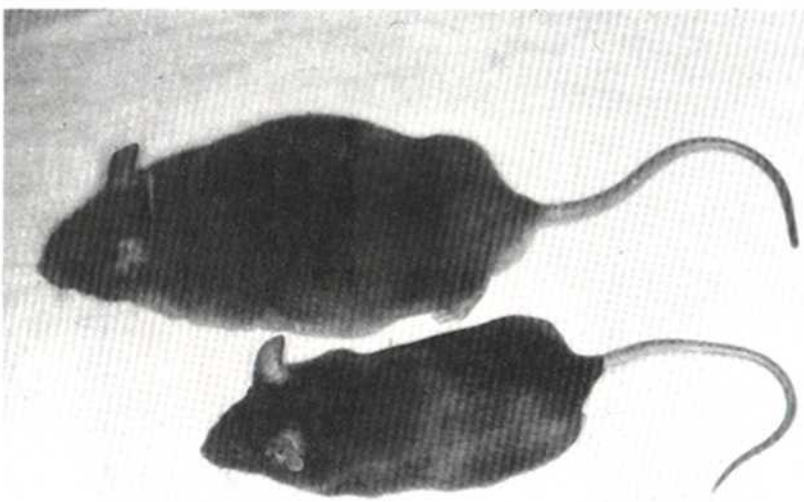

FIG. 6. Transgenic F1 mouse CB-35 (top) and non transgenic littermate CB-36 at 13 weeks of life.

The ability of transgenic mice to grow heavier and faster than control littermates should be measured not only in terms of body weight at the desired time, but also as the absolute and daily gain of weight. As expected,transgenic mice showed increased absolute and daily weight gain ($P < 0.05$) despite sex when compared to sex-matched littermates (table 2).

In this study we report a highly efficient transformation of mice with the mMT-I/hGH fusion gene using standard microinjection techniques (Hogan *et al.*, 1986).

Forty-five (45) percent of newborn mice were identified to carry the fusion gene and 5.6% of the microinjected embryos resulted in transgenic mice. Both parameters of integration are the highest reported to our

knowledge for this transgene, and also for any gene constructs introduced into transgenic mice. The possibility of cross hybridization with endogenous mGH was ruled out with the Taq I endonuclease assay (figures 2c and 2d).

Southern Blot analysis revealed that the microinjected mMT-I\hGH gene was integrated in the genome of the mice. In the founder mouse CB-5, there are two integration sites of the mMT-I\hGH gene. This can be concluded from the different patterns of hybridization found in tested animals after digestion with either EcoRI or PvuII (figure 4). Multiple integration sites have been reported for the mMT-I\hGH transgene (Brenig and Brem, 1988). We found no hybridization signals in control (non injected) mice. Thus, it can be concluded that no cross hybridization of the hGH gene with endogenous mGH occurred under stringent conditions.

The microinjected DNA was integrated without rearrangements, as it can be concluded from Southern Blot analysis of PvuII restricted DNA (figure 4, bottom). Signals of the predicted size (1.05 and 1.3 kb, respectively) appeared in all tested animals after digestion with PvuII. Differences in the intensity of the upper bands can be explained as a result of replication cycles of the injected DNA in the nucleus of the embryos that are independent of cell division; such a cycle could generate different amounts of the specific fragments. Similar events have been reported for transgenic mice (Palmiter *et al.*, 1982, Brinster *et al.*, 1985) and also for transgenic fish (Brem *et al.*, 1988).

The expression of the hGH in transgenic mice might be regulated by the promoter/regulator region of the mouse metallothionein-I gene. It has been proposed that mouse MT, a small cysteine rich metal binding protein, is involved in heavy metal detoxification in the organism (Durnam and Palmiter, 1981). MT has a high affinity for Cd, Zn, Hg, and Cu, thus the use of the promoter/regulator region of the MT gene enables the on-and-off switch of the transcription of the fused gene.

Our data showed that high levels of induction are obtained when transgenic mice are supplemented with 25 mM $ZnSO_4$ in water, the shunt of hGH is more likely to take place between the second and fifth weeks after the induction of the promoter (i.e., 4-8 weeks of life) and increased growth is achieved at least for another 15 weeks (data not shown).

Although Palmiter *et al.* (1983) reported expression of microgram quantities of hGH/ml in the serum of transgenic mice, we were able to detect no more than a few hundred nanograms (table 1) using a very sensitive commercial RIA system. However, the adult body weight of our transgenic mice (average 53.59 g for males and 47.39 g for females) is not different from that reported by others (Palmiter *et al.*, 1983, Brem and Brenig, 1987). These results confirm the suggestion by Palmiter *et al.* (1983) that less than 100 ng of hGH per milliliter are sufficient to stimulate maximal skeletal and muscle growth in transgenic mice.

We found no correlation between the patterns of hybridization of the hGH gene in the Southerns and the different levels of expression that occurred in transgenic F0 and F1 mice (table 1).

Radiographic studies carried out by us (figure 7) and others (Mathews *et al.*, 1988) confirm the fact that the growth increment of transgenic mice is not only due to increased muscle growth or obesity, but also to increased skeletal growth.

No significant difference was found in the adult weight of transgenic males compared to transgenic females ($P = 0.1261$) while non transgenic control male mice are significantly heavier ($P < 0.05$) than female non transgenic counterparts. The absolute and daily weight gain also differs significantly only for control mice, but not for transgenic mice (data not shown). This finding suggests that the expression of exogenous hGH acts strongly enough to promote nearly the same adult body weight of transgenic mice despite the sex.

Increased growth of transgenic mice is heritable in Mendelian fashion, but it has been reported that transgenic females expressing the hGH gene are sterile (Palmiter *et al.*, 1983, Bartke *et al.*, 1988). We have failed so far to obtain F1 progeny from expressing transgenic females.

To sum up, the present results clearly demonstrate that high efficiency generation of transgenic mice can be achieved for the hGH gene by direct microinjection of one cell mouse eggs.

ACKNOWLEDGMENTS

The authors thank Arturo Alfaro for skilled technical assistance and Dr. Jorge Gavilondo for his critical reading of the manuscript.

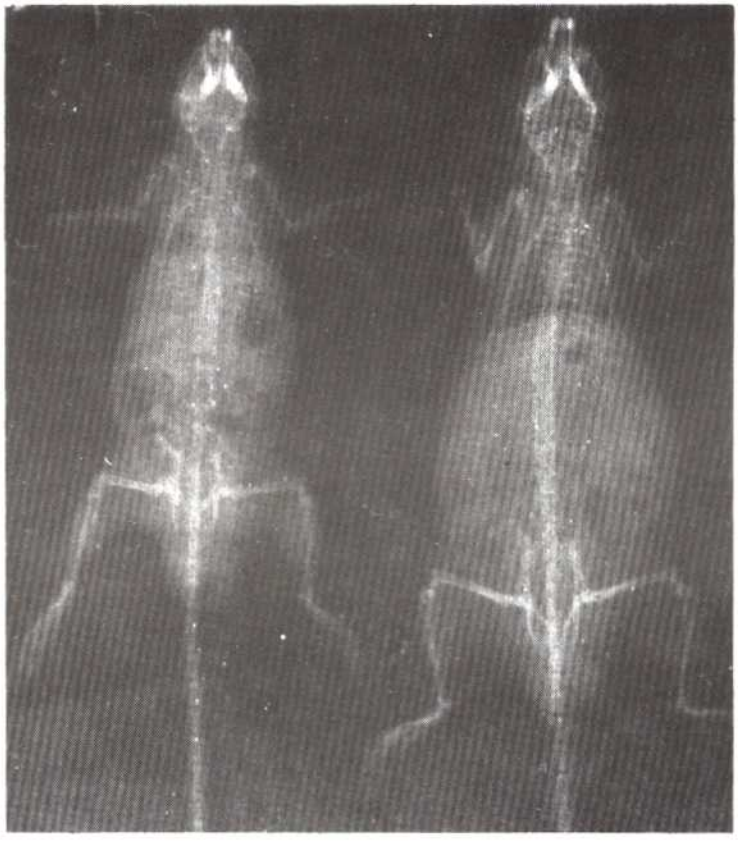

FIG. 7. X-ray radiography of transgenic (right) and non transgenic (left) F1 mice.

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