Mammalian Transgenesis by Intracytoplasmic Sperm Injection

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Coinjection of unfertilized mouse oocytes with sperm heads and exogenous DNA encoding either a green fluorescent protein (GFP) or β-galactosidase reporter produced 64 to 94 percent transgene-expressing embryos, reflecting DNA–sperm head association before coinjection. Nonselective transfer to surrogate mothers of embryos in the GFP series generated about 20 percent offspring expressing the integrated transgene. These data indicate that exogenous DNA can reproducibly be delivered into an oocyte by microinjected spermatozoa and suggest an adaptable method of transgenesis.

There now exist several methods of modifying mammalian genomes to carry recombinant DNA sequences transmissible through the germ line. The first of these methods to become widely used (pronuclear microinjection) was developed in the mouse in the early 1980s and entails injection of transgene (tg) DNA into a pronucleus of a one-cell embryo (*1, 2*). This method does not yet permit the outcome of tg insertion to be controlled or predicted because of the quasi-random nature of integration site and number of copies integrated into the host genome (*2*).

Greater control over the outcome of integration can be achieved by using (mouse) embryonic stem (ES) cell lines transfected with constructs capable of genome-targeted, homologous recombination (*3*). Transfected ES cell lines can be selected and characterized in vitro to confirm the construct integration site. Reconstitution of embryos with such gene-targeted ES cells may then be used to produce chimeric offspring. This method of genome modification is currently restricted to the one species for which established, germ line-contributing ES cells exist—the mouse.

Limitations in the available strategies for modifying mammalian germ lines have fueled a search for alternative methods, including the use of recombinant retroviruses to infect oocytes or preimplantation embryos (*4*), replication-deficient adenovirus-mediated delivery systems (*5*), and spermatozoa as vehicles for DNA delivery during in vitro fertilization (IVF) (*6*). In this last approach, live spermatozoa are used as a vector for introducing recombinant DNA into the oocyte in vitro; this system has triggered considerable controversy about its efficacy in promoting transgenesis (*6–8*). The biology of the phenomenon is poorly characterized and it is of limited use because of its unreliability (*7, 9*).

We have demonstrated that after intracytoplasmic sperm injection (ICSI) into metaphase II mouse oocytes, sperm heads, even though considered "dead" in that they are membrane disrupted at the time of injection, are able to support full development (*10, 11*). We therefore evaluated the ability of spermatozoa to transfer into an oocyte a replication-deficient fragment of plasmid pCX-EGFP containing a ubiquitously expressed GFP reporter (*12, 13*). We recorded expression of GFP monitored by epifluorescence microscopy for embryos that had been cultured in vitro for 3.5 days after sperm and DNA were preincubated for 1 min and then coinjected (Table 1). The proportion of embryos containing fluorescing blastomeres was lowest (26%) when pCX-EGFP DNA was coinjected with fresh spermatozoa, but it increased to higher values when the DNA was coinjected with spermatozoa that had been subjected to membrane disruption (confirmed by electron microscopy) by Triton X-100 (64%), freeze-thawing (82%), or freeze-drying (87%) (Fig. 1) (*14*). Coinjection of unfertilized oocytes with linearized pxCANLacZ DNA fragments and either freeze-thawed or freeze-dried sperm also generated a high proportion (92 to 94%) of embryos expressing the *lacZ* tg product β-galactosidase (Table 1) (*15*). Furthermore, coinjection of a sperm head with a mixture of two different tg DNAs (respectively encoding GFP and LacZ) produced embryos expressing both tg's from a single microinjection (Fig. 2) (*16*). These data indicate that coinjection of membrane-disrupted sperm heads and exogenous DNA into unfertilized oocytes can efficiently produce transgenic embryos. Because exogenous DNA can be demonstrated to decorate intact spermatozoa in a reversible fashion (*17*), membrane structures may act as a barrier to the stable association of sperm heads with extraneous, recombinogenic DNA.

Spermatozoa that had been washed with fresh medium after being mixed with pCX-EGFP DNA retained the ability to produce fluorescent blastocysts, albeit with a slightly reduced efficiency (63 versus 80%) compared with their nonwashed counterparts (Table 1) (*18*). This suggests a rapid association between exogenous DNA and spermatozoa during mixing (before injection).

To probe whether a similar interaction could occur inside the oocyte (after injection), we injected sperm heads and pCX-EGFP DNA serially, with no mixing before injection (Table 1). We consistently failed to observe exogenous (*GFP*) DNA expression, even though 75% positive control embryos (freeze-thaw sperm head–pCX-EGFP coinjection as for Table 1) were fluorescent. Freeze-thawed sperm heads coinjected with pCX-EGFP at 500 pg/μl (but not at 50 pg/μl) produced blastocysts expressing observable GFP. This threshold of GFP detection (corresponding to 50 to 500 pg of pCX-EGFP DNA per microliter) represents an average of 15 to 150 molecules per picoliter injected.

In contrast to coinjection with a sperm head, injection of a similar quantity of *GFP* tg DNA alone (*19*) did not preclude good parthenogenetic development (98% of oocytes surviving injection developed to the morula-blastocyst stage). Moreover, none of the resulting embryos exhibited observable tg

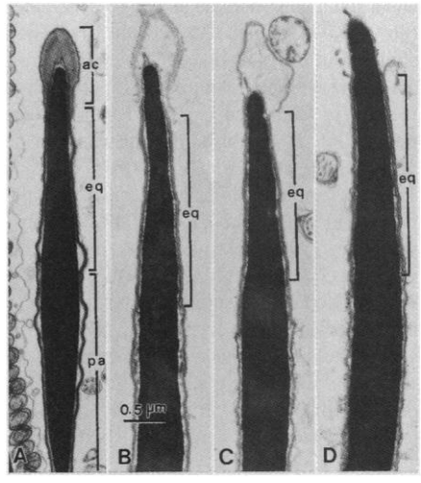

Fig. 1. Representative sagittal sections through the heads of mouse spermatozoa that were either intact (fresh) (**A**) or whose membranes had been disrupted by Triton X-100 (**B**), freeze-thawing (**C**), or freeze-drying (**D**) (*14*). ac, Acrosomal cap; eq, equatorial segment; pa, postacrosomal region. Plasma and acrosomal membranes (except for those in the equatorial region) are absent or disrupted. Disruption is clearest in the membranes of the acrosomal cap.

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expression (Table 1). Hence, in the absence of sperm heads there could have been little tg expression or epichromosomal persistence of transcriptionally active tg DNA. Collectively, these data argue for a preinjection association between exogenous DNA and sperm head submembrane structures, conceivably involving predominantly basic proteins of the perinuclear matrix (*20*). Sperm nuclei contain at least one endonuclease and at 25°C demembranated spermatozoa quickly lose their ability to support full development (*21*). Sperm genomic DNA was thus unlikely to be damage-free, consistent with the presence of single-strand breaks that would facilitate oocyte-mediated tg integration. Curiously, we observed mosaic embryos containing both GFP-positive and -negative blastomeres (+/− morulae-blastocysts) after sperm head–pCX-EGFP coinjection but not after injection of pCX-EGFP DNA alone (Table 1). The frequency of such +/− mosaics implies that tg DNA integration was sometimes delayed until after the first S-phase post-ICSI. Such delayed integration apparently did not occur unless tg DNA had been coinjected with a sperm head. One interpretation of this is that sperm-derived material stabilizes exogenous DNA within the early embryo, thereby facilitating delayed integration; in the absence of such material (for example, in parthenotes) the exogenous DNA would be degraded before it could integrate.

Our findings have implications for previous claims that live (membrane intact) spermatozoa promote transgenesis by IVF by acting as a vehicle for the introduction of extrinsic, recombinant DNA into oocytes (*6*). Even if we assume that there is a high efficiency of DNA association with the membranes of live spermatozoa (*17*), the critical step may be whether the DNA subsequently becomes associated with the appropriate submembrane structure. The marked variation of apparent tg integration (0 to 100%) (*7*) in the IVF method may reflect the degree of genomic damage (*21*) or a chaotic influence of temperature-sensitive sperm lipid-phase transitions (*22*).

We noted that after sperm head–pCX-EGFP coinjection, the developmental potential of embryos decreased as the proportion that contained fluorescent blastomeres increased (Table 1). In contrast, tg expression after sperm head–pxCANLacZ coinjection did not inhibit embryonic development (Table 1). It is possible that this reflects a deleterious effect of GFP expression; early embryonic development may be exquisitely sensitive to the evolution of H_2O_2 that accompanies maturation of the GFP chromophore (*23*).

To determine whether genomic integration of tg DNA constructs could be demonstrated in live offspring, we coinjected sperm heads that had been subjected to one of the three membrane disruption procedures with pCX-EGFP DNA, cultured the resulting embryos in vitro for 3.5 to 4 days (to the morula-blastocyst stage), and then transferred embryos to surrogate mothers nonselectively (not on the basis of fluorescence). Phenotypic analysis of tg integration was by examination of offspring under long-wave ultraviolet (UV) light (Fig. 3A) (*24*). A high proportion (17 to 21%) of offspring were transgenic with respect to observable GFP expression in skin (Table 2); this efficiency did not depend on the membrane disruption method used to prepare spermatozoa. Rates of zygotic development to term were comparable for each of the three groups of membrane-disrupted sperm heads (12 to 14%) but relatively low compared with rates obtained after microinjection of similarly treated heads in the absence of exogenous DNA (*11*).

These data are consistent with the results in Table 1. They indicate that embryos that contain GFP-negative cells are more likely to develop to term than those with cells that are all positive. Additionally, some pups scored negative are likely to have arisen from mosaic embryos that contained both GFP-positive and -negative cells at day 3.5 of culture. This implies a deleterious effect of the coinjected pCX-EGFP DNA on both pre- and postimplantation embryonic development. Further experiments are needed to establish

Table 1. In vitro culture and tg expression of embryos produced after microinjection of metaphase II oocytes with exogenous reporter-encoding DNA or sperm heads or both.

Fragment*	Sperm treatment†	No. of oocytes	Total morulae-blastocysts (m-b) and fluorescence (GFP) or staining (LacZ) on day 3			
			m-b (%)‡	−§	+/−§	+‡§
pCX-EGFP	None (fresh)	162	134 (83)[a]	100	13	21[a]
pCX-EGFP	Triton X-100	270	212 (79)[a]	75	37	100[b]
pCX-EGFP	Freeze-thaw	313	155 (50)[b]	28	31	96[b]
pCX-EGFP	Freeze-dry	278	154 (55)[b]	20	23	111[b]
px-CANLacZ	Freeze-thaw	151	110 (73)[a]	7	45	58[b]
px-CANLacZ	Freeze-dry	136	106 (78)[a]	8	32	66[b]
pCX-EGFP	Washed	153	114 (75)[c]	43	4	67[c]
pCX-EGFP	Not washed	117	83 (71)[c]	17	3	63[c]
	① Freeze-thaw					
→ ② pCX-EGFP		71	56 (79)	56	0	0
① pCX-EGFP →						
	② Freeze-thaw	51	35 (69)	35	0	0
pCX-EGFP alone	−	49	48 (98)	48	0	0

*Exogenous DNA fragments were pCX-EGFP-Bam HI–Sal GI or pxCANLacZ-Sal GI, Sal GI–Xho I, or Xho I. Fragments were mixed with sperm heads at DNA concentrations of 5 to 10 ng/ml. With the exception of the last three rows [see text and (*19*)], exogenous DNA was injected after mixing with sperm samples as described in (*14*). †Sperm treatments are as described in (*14*). Preparation of washed and nonwashed samples is described in (*18*). As a negative control, all experiments included same-day injection of a fresh aliquot of the appropriate sperm preparation mixed with NIM or CZB alone; after culture, no false-positive expression was ever observed. Serial injections were separated by 30 to 90 min per pair, with heads prepared by the freeze-thaw method (same-day positive control coinjection of freeze-thaw sperm premixed with DNA yielded fluorescent blastomeres in 75% of embryos). Injection of tg DNA alone was followed by parthenogenetic activation as described in (*19*). ‡When values in the same column with superscripts a and b are compared, they differ significantly ($P < 0.05$). Values in the same column with superscript c do not differ significantly. §Tg expression: −, negative; +, positive; +/−, m-b containing both + and − cells (mosaics).

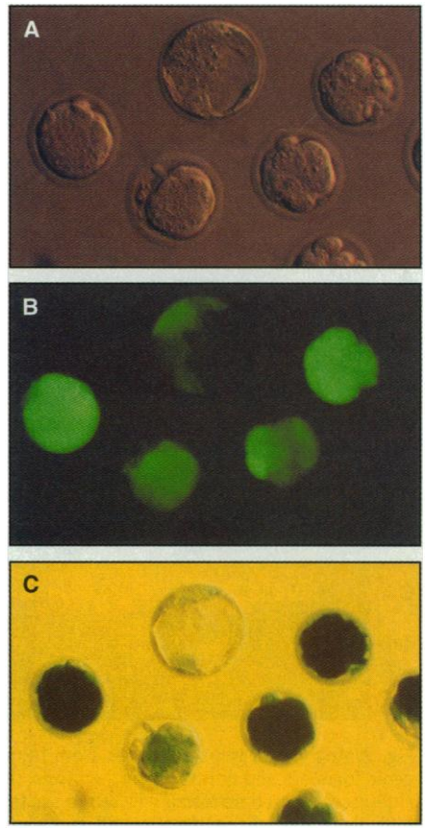

Fig. 2. Transgenic embryos produced by single-shot double transgenesis. Oocytes were microinjected with spermatozoa that had been preincubated with a mixture of pCX-LacZ and pCX-EGFP tg DNAs as described in (*16*). The same embryos are shown (×400) after 3.5 days viewed by Hoffman modulation contrast microscopy unstained (**A**), for GFP expression under long-wavelength (480 nm) UV light (**B**), and stained with X-gal for β-galactosidase expression (**C**).

whether the inhibition of postimplantation development is a consequence of tg expression or of the presence of exogenous DNA per se. Nevertheless, the fraction of pups expressing GFP ectopically here is comparable to the proportion obtained with the same construct by pronuclear microinjection (20/78 = 26%) in a laboratory experienced in pronuclear microinjection (*25*).

Physical analysis of tail-tip genomic DNA by Southern blotting or by polymerase chain reaction (PCR) (*26*) showed that all founder lines that exhibited green fluorescence possessed the tg, including one that was initially scored phenotypically negative but whose biopsied tail tip exhibited GFP expression (Fig. 3). In three cases, the tg was demonstrated by PCR in founders that lacked detectable green fluorescence, with nonexpression presumably being due to locally cis-active elements at the tg integration locus. Southern blot analysis indicated that tg copy numbers in founders ranged from ≤1 to >50 (Fig. 3B); this result resembles the pattern of tg integration after pronuclear microinjection. Both the physical characterization of genomic pCX-EGFP DNA and the efficiency of GFP expression suggest that tg DNA did not undergo gross rearrangements on integration.

A random selection of 12 GFP-expressing founders (8 females, 4 males; from Table 2 and analogous series) were crossed with nontransgenic animals and produced litters in all but one case (female). Of the 11 fertile founders, 8 produced pups expressing GFP ectopically in their skin with a frequency of 27 to 50% (average = 40%). The pattern of tg inheritance in most cases was consistent with Mendelian germ line transmission of a single locus *GFP* gene.

We have shown that membrane-disrupted mouse spermatozoa support the genomic integration and expression of exogenous tg DNA when they are microinjected together into unfertilized oocytes. An ICSI-based method has been described for the generation of *Xenopus* embryos in which sperm nuclei were first partially decondensed before DNA mixing (*27*). This supports the notion that sperm submembrane components can associate with exogenous DNA to promote transgenesis.

The method of mammalian transgenesis described here affords advantages over existing methods. Viral protocols imply extra steps in cloning, necessitating specialized containment facilities for the recombinant adenoviruses and retroviruses that must be engineered (*4, 5*). These methods still require either microinjection equipment to deliver the virus or removal of the zona pellucida. In addition, transgenesis by ICSI may circumvent certain drawbacks to pronuclear microinjection. Use of pipettes with an ~100-fold larger tip aperture (~0.78 μm^2 for a pronuclear microinjection tip of diameter 1 μm compared with ~78 μm^2 for an ICSI tip of diameter 10 μm) will facilitate the handling of large constructs such as yeast or mammalian artificial chromosomes. Association of tg DNA with sperm heads here suggests the further stabilization and protection of megabase and submegabase constructs. Whereas the generation of pronuclear zygotes is straightforward in the mouse, this is not true for species exemplified by the large commercial breeds; use of unfertilized metaphase II oocytes described here thus represents a major facilitatory simplification over methods that require zygotes. Moreover, zygotes are difficult substrates for pronuclear injection when their lipid richness renders them opaque, as in cattle and pigs; mouse zygotes are translucent. Conservation of certain features of spermatozoa (*28*) and their ability to support development after ICSI in different species (*29*) suggest that the technique described here will have widespread applicability.

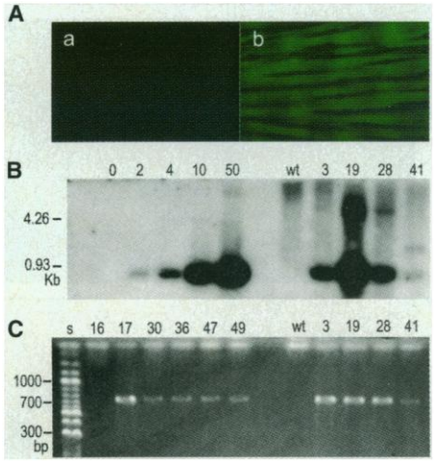

Fig. 3. Analysis of tail-tip biopsies from transgenic founders and nontransgenic controls. (**A**) Fluorescent stereomicroscopy (×40) of tail tips from nontransgenic (**a**) (mouse 16) and transgenic, green-fluorescent (**b**) (mouse 3) lines. Green fluorescent skin could be visualized through nongreen hairs. (**B**) Southern blot analysis of total DNA from control B6D2F$_1$ (wt) (0) and from mice 3 (5 to 9), 19 (>50), 28 (5 to 9), and 41 (2) using a pCX-EGFP fragment as probe. Estimated tg copy numbers per genome are shown in parentheses. (**C**) PCR analysis of total DNA from mice 16, 17, 30, 36, 47, 49, control B6D2F$_1$ (wt), 3, 19, 28, and 41.

Table 2. Development of phenotypically transgenic (green) pups and their siblings.

Sperm treatment*	No. of oocytes	m-b transferred†	Total pups	+ (green) pups‡
Freeze-dry	116	67 (4)	14	3§
Freeze-thaw	97	53 (3)	12	2§
Triton X-100	218	150 (9)	31	6§

*Each row records development of embryos and pups produced from oocytes coinjected with demembranated sperm heads and a fragment of plasmid pCX-EGFP (*13, 14*). †m-b, Morulae-blastocysts. Values in parentheses show the number of surrogate mothers used as recipients in embryo transfers. ‡Tg expression: +, positive pups are those expressing GFP ectopically in their skin. §Values do not differ significantly.

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12. The isolation and culture of B6D2F$_1$ mouse metaphase II oocytes for microinjection was essentially as described (*30*). Spermatozoa were collected from mature B6D2F$_1$ male mice in 400 μl of CZB medium [C. L. Chatot, J. L. Lewis, I. Torres, C. A. Ziomek, *Biol. Reprod.* **42**, 432 (1990)] as described (*11*). Spermatozoa for Triton X-100 extraction were isolated by finely chopping two caudae epididymides at 0° to 1°C in nuclear isolation medium (NIM) (*31*) and filtering the resulting sperm suspension to produce a final volume of 900 μl. Piezo-actuated microinjection of oocytes and culture of embryos in CZB medium under mineral oil equilibrated in 5% (v/v) CO_2 in air at 37°C has been detailed elsewhere (*30*). For microinjection, sperm heads were aspirated into a pipette attached to a piezoelectric pipette-driving unit and one injected per oocyte as described (*30*). Oocytes that lysed soon after injection were discarded. Where appropriate, dislocation of heads from tails was by the application of a single piezo pulse as described (*31*). This procedure disrupts membranes and thus represents a difference between the fresh spermatozoa used here and previous reports of live spermatozoa promoting transgenesis by IVF (*7*). We estimate that about 1 pl was displaced from the pipette interior per injection; 3 to 3.5 days after microinjection, we examined embryos for expression of GFP by epifluorescence microscopy with a UV light source (480 nm) with fluorescein isothiocyanate filters. This enabled the clear identification of nonfluorescent (non-GFP-expressing), weakly fluorescent, and strongly fluorescent embryos and mosaics, which were scored accordingly. Mouse methods strictly adhered to National Institutes of Health (Department of Health and Human Services) guidelines implemented by the University of Hawaii Animal Care and Use Committee.
13. The large (3.5 kb) Sal GI–Bam HI fragment of plasmid pCX-EGFP used here harbors a GFP gene expressed from a strong cytomegalovirus-IE–chicken β-actin enhancer-promoter combination [H. Niwa, K. Yamamura, J. Miyazaki, *Gene* **108**, 193 (1991)] but lacks a eukaryotic origin of replication [G. Zhang, G. Vanessa, S. R. Kain, *Biochem. Biophys. Res. Commun.* **227**, 707 (1996); T. Takada *et al.*, *Nature Biotechnol.* **15**, 458 (1997)].
14. We mixed spermatozoa with a pCX-EGFP fragment without further preparation (fresh) or after they had been subjected to one of three membrane-disruption protocols: freeze-thawing [T. Wakayama, D. G. Whittingham, R. Yanagimachi, *J. Fertil. Reprod.* **112**, 11 (1998)], freeze-drying (*11*), or Triton X-100 extraction. For Triton X-100 extraction, 100 μl of 0.5% (v/v in

NIM) Triton X-100 was added to 900 μl of sperm suspension in NIM [see (12)] and mixed by trituration for 30 s on ice. Cells were pelleted by centrifugation for 1 min at 20,000*g*, 2°C and thoroughly resuspended in 2 ml of ice-cold NIM before repelleting for 2 min at 20,000*g*, 2°C. We resuspended the final pellet in 400 μl of CZB or NIM. Before microinjection, we mixed 1 μl of the DNA fragment with 9 μl of sperm suspension (containing 2 to 5×10^5 spermatozoa in CZB or NIM) by pipetting, to give a final DNA fragment concentration of 7 ng/μl. We incubated the DNA-sperm mixture at room temperature (about 25°C) or on ice for 1 min and then mixed it with a polyvinylpyrrolidone (PVP; average M_r 360,000) solution to give a final concentration of about 10% (w/v) PVP; it was then placed on the microscope stage for microinjection. All injections were done in CZB-H at room temperature within 1 hour of sperm-DNA mixing or within 1 hour of sperm-Triton X-100 mixing.

15. Purified *lacZ*-harboring fragments of pxCANLacZ, linearized by digestion either with Sal GI or Xho I and Sal GI, were mixed with spermatozoa at concentrations of 4.5 and 9 ng/μl, respectively, and microinjected as described in (*14*); both fragments gave similar results; the pxCANLacZ Xho I–Sal GI fragment lacks a replication origin. The β-galactosidase encoded by pxCANLacZ contains a nuclear localization signal. We assessed pxCANLacZ β-galactosidase expression in day 3 embryos [T. Tsukui, Y. Kanegae, I. Saito, Y. Toyoda, *Nature Biotechnol.* **14**, 982 (1996)] after a 5-min fixation at room temperature in phosphate-buffered saline (PBS) (pH 7.6) containing 1% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde, and bovine serum albumin (BSA) (5 mg/ml). Fixed embryos were washed thoroughly in PBS containing BSA (5 mg/ml) and stained by incubation for 5 hours at 37°C in PBS containing BSA (5 mg/ml), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (1 mg/ml). We examined and scored embryos by light microscopy.
16. We used single-shot double transgenesis to generate embryos coexpressing two tg's after a single microinjection as described in (*14*). Sperm heads were coinjected with a DNA solution containing pCX-EGFP Sal GI–Bam HI fragment (2.5 ng/μl) and pCX-LacZ Sal GI–Pst I fragment (2.5 ng/μl). pCX-LacZ is a derivative of pCX-EGFP in which the *EGFP* gene is replaced by one that encodes β-galactosidase [M. Okabe, unpublished data]. After culture in vitro, we first scored embryos for GFP expression and then for β-galactosidase expression as described in (*12, 15*). For photography, we mounted embryos between a microscope slide and a coverslip and collected images to show development and GFP expression before fixation and staining to show LacZ expression.
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18. We divided the sperm suspension in each washing experiment into two 5-μl aliquots immediately after mixing and incubating with pCX-EGFP DNA for 1 min. One aliquot (washed sperm) was diluted and washed by mixing well with 50 μl of ice-cold, fresh CZB or NIM. We then pelleted both aliquots for 2 min at 20,000*g*, 2°C. The supernatant from the washed sperm aliquot was carefully removed and replaced with 5 μl of fresh CZB or NIM; we used the supernatant from the second aliquot to resuspend its own pellet (therefore, this sample was not washed).
19. We mixed a fresh dilution of the Sal GI–Bam HI fragment of plasmid pCX-EGFP (7 ng/μl in NIM) with an equal volume of PVP 20% (w/v) and injected about 1 pl per oocyte. After a recovery time of 5 to 10 min at room temperature, we transferred oocytes to Ca^{2+}-free CZB containing 10 mM $SrCl_2$ and the cytokinesis-blocking agent cytochalasin B at 5 μg/ml and incubated them for 6 hours at 37°C [A. Bos-Mikich, D. G. Whittingham, K. T. Jones, *Dev. Biol.* **182**, 172 (1997)]. We then transferred them to CZB and incubation continued under standard embryo culture conditions. We scored embryos for GFP expression after 3.5 days as described (*12*).
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24. Ectopic GFP expression in skin was clearly discernible as a green color when pups were examined 1 to 4 days after delivery by incidental illumination from a UV light source (480 nm).
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26. Tail-tip biopsies from 3- to 6-week-old, randomly selected green pups and their nongreen littermates were used for extraction of total, genomic DNA. Photography of tails was under a fluorescent stereomicroscope equipped with a 480/440-nm filter. In Southern blot analysis, 10 μg of genomic DNA per sample was digested with Eco RI and probed with the 733-base-pair Eco RI fragment of pCX-EGFP. We used forward (TTGAATTCGCCACCATGGTGAGC) and reverse (TTGAATTCTTACTTGTACAGCTCGTCC) oligonucleotide primers detection of the *GFP* gene by PCR of 1 μg of genomic DNA per reaction. Reaction parameters were 95°C for 9 min (1 cycle) and 94°C for 45 s, 60°C for 30 s, 72°C for 45 s (40 cycles). Electrophoretically separated products were visualized after staining with ethidium bromide.
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Control of Autoimmune Diabetes in NOD Mice by GAD Expression or Suppression in β Cells

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Glutamic acid decarboxylase (GAD) is a pancreatic β cell autoantigen in humans and nonobese diabetic (NOD) mice. β Cell–specific suppression of GAD expression in two lines of antisense GAD transgenic NOD mice prevented autoimmune diabetes, whereas persistent GAD expression in the β cells in the other four lines of antisense GAD transgenic NOD mice resulted in diabetes, similar to that seen in transgene-negative NOD mice. Complete suppression of β cell GAD expression blocked the generation of diabetogenic T cells and protected islet grafts from autoimmune injury. Thus, β cell–specific GAD expression is required for the development of autoimmune diabetes in NOD mice, and modulation of GAD might, therefore, have therapeutic value in type 1 diabetes.

Type 1 diabetes, or insulin-dependent diabetes mellitus, is the consequence of progressive T cell–mediated autoimmune destruction of pancreatic β cells (*1*, *2*). However, the initial events that trigger the destruction of β cells are incompletely understood. Several β cell autoantigens have been implicated in the triggering of β cell–specific autoimmunity (*1*, *3*). GAD is the strongest candidate in both humans and the NOD mouse, which is considered the best animal model of the human disease (*3*–*5*). In NOD mice, GAD, as compared with other β cell autoantigens examined, provokes the earliest T cell proliferative response (*4*, *5*). However, no unequivocal evidence exists to indicate that the β cell expression of GAD is required for the initiation of diabetes in NOD mice. To address this issue, we examined the effect of selectively suppressing GAD expression in the β cells of diabetes-prone NOD mice.

The suppression of β cell GAD expression was achieved by producing transgenic

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