Murine spermatogonial stem cells: targeted transgene expression and purification in an active state

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A 400 bp fragment of the spermatogonia-specific *Stra8* locus was sufficient to direct gene expression to the germinal stem cells in transgenic mice. A fractionation procedure was devised, based on immunomagnetic sorting of cells in which the promoter drives the expression of a surface functionally neutral protein tag. The purified cells expressed the known molecular markers of spermatogonia Rbm, cyclin A2 and EP-Cam, and the β_1 - and α_6 -integrins characteristic of the stem cell fraction. A 700-fold enrichment in stem cells was determined by the ability of the purified fractions to re-establish spermatogenesis in germ cell-depleted recipient testes.

INTRODUCTION

A number of adult tissues are in a dynamic state, with new cells continually differentiating from a pool of stem cells, self-renewed by asymmetric cellular divisions. In the case of the male germ line, the spermatogonial stem cells constitute a small fraction of the testicular germ cells, in the range of 30 000 per adult testis, located in the most peripheral region of the seminiferous tubule (reviewed by de Rooij and Grootegoed, 1998). Mitotic divisions generate with a strict periodicity the first differentiated cell type, the A₁ spermatogonia, from which differentiation follows a complex series of steps (reviewed by Bellve *et al.*, 1977). Better knowledge of the properties of the stem cell fraction would be useful in investigating important questions in both basic and applied research, including the molecular controls of germinal differentiation and their pathological alterations (male sterility syndromes), new technologies for transgenesis (Nagano *et al.*, 2001) and the possibility of trans-differentiation (Tersikh *et al.*, 2001 and references therein). The demonstration that spermatogonial stem cells can proliferate and differentiate in

the seminiferous tubules of infertile recipients has provided a functional assay (Brinster and Zimmermann, 1994), and enriched preparations were obtained by selection based on the expression of cell surface molecules (Shinohara *et al.*, 2000). Our initial aim was to identify a promoter that directs transgene expression to the earliest stages of male germ line differentiation. The *Stra8* gene, expressed in spermatogonia (Oulad-Abdelghani *et al.*, 1996), appeared to be a hopeful candidate. We determined that a 400 bp *Stra8* promoter fragment was sufficient for testicular expression of a reporter gene. Unlike the endogenous gene, however, this truncated promoter was only expressed in a minor fraction of premeiotic germ cells, which were purified by a procedure based on the expression of a neutral heterologous surface marker. These cells exhibited a high efficiency (~700-fold increased) in the establishment of stable spermatogenesis upon transplantation in germ cell-depleted testes.

RESULTS AND DISCUSSION

Promoter activity of an upstream fragment of the *Stra8* locus

A mouse genomic library was screened using as a probe the complete *Stra8* cDNA. A single 16 kb clone was obtained, which contains five exons corresponding to the 5'-region of *Stra8* cDNA (Figure 1). A fragment spanning nucleotide positions -371 to +29 relative to the transcriptional start site, including a TATA box-like sequence and two putative binding sequences for retinoic acid receptors, was amplified and cloned into the luciferase reporter vector pXp1. Three lines of transgenic mice carrying the resulting construct (pStra8Luc) showed identical

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Fig. 1. Genomic structure and upstream region of the *Stra8* gene. (A) Partial genomic organization of the *Stra8* gene, showing the first five exons (E1–E5). The numbers below the cDNA refer to the positions of exons along the cDNA sequence. (B) DNA sequence of the 5'-flanking region of *Stra8*. +1 indicates the start of transcription, and the ATG in bold letters (+102) the translation initiation site. The TATA-box like sequence (boxed) and putative binding sites for retinoic acid receptors (RARE1 and RARE2) are indicated.

expression patterns, with high luciferase activity in testis extracts, and in no other tissue except brain (Figure 2A). Not expected from the initial report (Oulad-Abdelghani *et al.*, 1996), the latter may be unique to the truncated form of the promoter. It was not further documented, and the following studies addressed the question of expression site(s) in the testis.

The 400 bp *Stra8* promoter directs transgene expression to a minor fraction of spermatogonia

Analysis of luciferase expression in the prepubertal testis revealed expression at an early spermatogonial stage in the newborn testis, with identical results again in two independently established transgenic lines. Between birth and puberty, differentiation of germ cells progresses in a synchronous manner (Bellve *et al.*, 1977). Luciferase activity during that period reached high levels prior to the entry into meiosis at day 10 post-partum (p.p.), a critical period illustrated in Figure 2B by measurements performed on *Sycp1-luc* transgenic males, in which the reporter is expressed at the very beginning of meiosis (leptotene to zygotene stages; Sage *et al.*, 1999). Expression appeared to be restricted to the germinal fraction since extracts of pure cultures of Sertoli cells were inactive. In the two transgenic families analyzed, a limited number of positive cells were detected (Figure 3) in the periphery of the tubules (average 0.1 cell per section). A minor fraction contained ≥ 4 cells (up to 20), often located close to each other, a pattern evocative of sister cells generated by successive cell divisions. The profile of expression of the truncated promoter appeared, therefore, more restricted than that of

Fig. 2. Organ-specific expression of the 400 bp *Stra8* promoter in the mouse. (A) Luciferase activity in organ extracts from transgenic *Stra8-Luc* animals. Control testis, testis extract from a B6D2 control male. (B) Expression during post-natal development in testis, adult liver and Sertoli cells of *Stra8-luc* animals and parallel measurements performed on a transgenic *Sycp1-luc* animal (transgene expression at the early pachytene stage; Sage *et al.*, 1999). The SEM is indicated ($n = 2-3$).

the endogenous gene, which is expressed in all classes of spermatogonia. The observed increase in luciferase activity between days 1 and 20 p.p. suggests that the number of expressing cells increases after birth, and a detailed analysis is currently in progress.

Expression of a foreign neutral surface marker as a means to purify cells that express a transgene

A method was devised for purification of the cells in which the 400 bp *Stra8* promoter is active. It is based on the expression on the cell surface of a protein that is not present in the normal mouse, does not interfere with cellular functions and is efficiently recognized by monoclonal antibodies. A recombinant gene designated *CD4HAgl* (Figure 4) was engineered for this purpose. This gene encodes a protein whose N-terminal domain is made of the first two extracellular domains of the human CD4

Fig. 3. Expression of the *Stra8-Luc* transgene and of the endogenous gene in the adult and newborn testis. (A) Immunocytochemical analysis of luciferase expression in a tubule section of adult *Stra8-Luc* testis. (B) A group of four cells (circled) and two cells connected by a cytoplasmic bridge (arrow). (C) Cells positive by immunocytochemical analysis with anti-luciferase antibodies were counted on three testis sections from distinct animals (240, 260 and 290 tubule sections, respectively). Bars show the fraction (percent) of cells in each class (average and SEM). (D) Immunocytochemical analysis with anti-luciferase antibodies of a testis section at day 1 p.p. (E) Immunofluorescence detection with anti-*Stra8* antibody on an adult testis section. Bars: 50 μ m.

molecule [insufficient for ligand recognition and recognized by a variety of monoclonal antibodies (Bedinger *et al.*, 1988)], linked to the transmembrane and cytoplasmic regions of the influenza hemagglutinin (HA).

Two independent transgenic families were generated in which the *CD4Haglo* construct is expressed by the 400 bp *Stra8* promoter. Expression of the transgene in testicular cells was verified by immunological detection (not shown). All the males of the two families showed fully normal spermatogenesis and complete fertility.

Magnetic cell sorting of CD4-positive cells

Suspensions of well-isolated testicular cells ($20\text{--}30 \times 10^6$ cells per testis) were prepared from adult *Stra8-CD4Haglo* males. Paramagnetic beads coated with anti-CD4 monoclonal antibodies were used to sort the positive cells. A small fraction of the input (1 in 10^3 cells) attached to the beads. Of homogeneous

Fig. 4. Schematic representation of the *CD4Haglo* construct.

Table I. Haploid contaminants in CD4-positive germ cell preparations

	No. of experiments	Fractionation procedure	Total No. of cells	Round spermatids (%)	Elongated spermatids (%)
Adult (4–5 months)	4	1 $\times$ immuno magnetic sorting	1730	0.9 (± 0.1)	3.5 (± 0.2)
Prepubertal (3 weeks)	1	1 $\times$ immuno magnetic sorting	580	<0.2	<0.2
Adult (4 months)	1	2 $\times$ immuno magnetic sorting	500	<0.2	0.2

The indicated numbers of cells were examined after Hoechst 33258 staining for the presence of cells with the characteristic morphology of either round spermatid or elongated spermatid nuclei (SEM in parentheses).

morphology, these cells were determined to be diploid by cytometric analysis (not shown). Preparations from adult testis were initially contaminated by a small amount of elongated and round spermatids (Table I), a specific problem in the fractionation of adult testicular cells with their largely predominant haploid cell population. Near-homogeneous preparations (<0.1% contaminants) could be generated by two modifications of the procedure. One was to use testicular cells from 3-week-old males, in which the first wave of meiosis has just been completed (Bellve *et al.*, 1977). The second possibility was to perform two successive fractionation steps with anti-CD4-coupled beads on adult testis cells. The counterpart of a high degree of purity (close to 100%) was, however, a lower yield, a problem that would clearly not be encountered in large-scale preparations. The estimated yields (30 000 cells per testis) were those expected for the stem cell fraction (de Rooij and Grootegoed, 1998), with a very minor fraction corresponding to their immediate progeny (Figure 3).

Spermatogonia marker genes

Only a few proteins characteristic of the premeiotic murine germ cells have been identified that can be employed as purification markers. Immunocytochemical analysis (Table II) detected in 80–90% of the CD4-positive purified cells the RBM protein (Elliott *et al.*, 2000), which in the mouse is exclusively present in spermatogonia (H. Cooke, personal communication), Ep-Cam (Anderson *et al.*, 1999), and the β_1 - and α_6 -integrins, known markers of the stem cell population (Shinohara *et al.*, 1999). These values are minimal estimates because, in order to count

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Table II. *In situ* detection of spermatogonia-specific gene expression

	Immunofluorescence staining of CD4-HA Δ glo-positive cells (1 $\times$ immunomagnetic sorting) by antibodies against				
	RBM	Ep-Cam	β_1 -integrin	α_6 -integrin	SCP1
Positive	168	101	130	88	0
Weak to negative	21	15	20	21	110
% positive	89	87	87	81	<1

Cells obtained by immunomagnetic sorting were analyzed by immunofluorescence for the presence of RBM and Ep-Cam proteins, expressed in spermatogonia (Anderson *et al.*, 1999; Elliott *et al.*, 2000), of the synaptonemal complex SCP1 protein, expressed in pachytene cells, and of the β_1 - and α_6 -integrins characteristic of the stem cell fraction (Shinohara *et al.*, 1999).

sufficient numbers of cells, the analysis was performed after only one round of immunomagnetic sorting. Labeling with antibodies directed against synaptonemal complex protein 1 (SCP1), a pachytene spermatocyte marker (Sage *et al.*, 1999), did not detect any measurable contamination. RNA of cyclin A2 (spermatogonia) was readily detected by RT-PCR assays (Ravnik and Wolgemuth, 1999), but not that of cyclin A1 (spermatocyte stage) (Figure 5). RNA from the *W* locus (Kit receptor) could not be detected at significant levels, in agreement with a recent report concluding that the protein is absent in stem cells (Shinohara *et al.*, 2000).

Restoring spermatogenesis in a recipient testis

The stem cell quality of the purified cells was definitely established by assaying their ability to restore complete spermatogenesis cycles in cell-depleted seminiferous tubules (Brinster and Zimmermann, 1994). CD4-positive cells were purified by magnetic sorting from double-transgenic *Stra8-CD4HA Δ glo;ROSA26* males (Zambrowicz *et al.*, 1997) and injected into the seminiferous tubules of males previously irradiated to the point at which spermatogenesis was arrested. A total suspension of germ cells was injected in one testis (20 000 cells per injection) and, in the contralateral organ, the same suspension mixed with 2000 CD4-positive purified cells (one-round sorting from adult testis). Ten injections were performed in each testis, on two males per experiment, in three independent experiments conducted with fresh preparations of purified cells. The males were killed after 12 weeks and examined for the appearance of *ROSA26* β -galactosidase-positive germ cells (Figure 6; Table III). Staining of tubules was clearly stronger and more extensive upon injection of purified cells compared with injection of the total germ cell preparation. Moreover, the number of injection sites at which staining was detected was systematically close to 10 out of 10 in the testes that had received purified cells, and much lower in the controls. Staining of histological sections confirmed the presence of X-Gal-positive germ cells at every differentiation stage. When results were quantitated and reported relative to the number of injected cells, the values obtained with purified cells were in the range of 700-fold greater than those recorded with the initial cell suspension (Table III). Two series of injections were performed in which cells that had

Fig. 5. RT-PCR assays for expression of the c-kit receptor, cyclins A1 and A2, and GAPDH as a control, performed on RNA prepared from total germ cells and from CD4-positive cells.

Fig. 6. The CD4-positive fraction differentiates efficiently after re-implantation in a recipient testis. X-Gal detection of β -galactosidase activity 12 weeks after injection of a total germ cell suspension from a double-transgenic *ROSA26;Stra8-HA Δ glo* male (20 000 cells), either supplemented (A and B) or not (C and D) with 2000 purified CD4-positive cells. (A and C) Intact seminiferous tubules (bar: 500 μ m). (B and D) Sections of tubules showing β -galactosidase-positive cells at every differentiation stage (bar: 100 μ m).

not been retained on the beads in the fractionation procedure were compared with the purified population. In this case (data not shown), the efficiency of colonization was too low to allow an accurate determination. The increase in colonization efficiency (700-fold as a minimal estimate) was comparable to the yield in purified cells, thus indicating a high degree of purity. The fact that after 12 weeks, corresponding to multiple initiations from the stem population with the 10 day period characteristic of the adult mouse (Bellve *et al.*, 1977), the tubule sections still showed a full complement of 'blue cells' demonstrates the establishment of stem colonies of β -galactosidase-positive cells. Experiments are in progress to check that fertility will indeed be efficiently restored in the case of sterile *W* mutants (Ogawa *et al.*, 2000).

Table III. Increased efficiency of the CD4-positive fraction in the stem cell repopulation assay

Cell fraction	Cells injected	Positive injection sites (out of 10) (a)	Length of X-Gal staining (mm) (b)	Staining intensity (arbitrary units) (c)	Repopulation efficiency (a × b × c)	Efficiency per injected cell
Total germ cells	20 000	2.3 ± 0.99	0.55 ± 0.4	75 ± 17.5	95	4.7 × 10 ⁻³
CD4-positive cells	2000	9.5 ± 0.69	4.80 ± 1.59	146 ± 9.9	6660	3.33

Same experiment as in Figure 6, performed on a total of six recipient males. A total of 20 000 total germ cells prepared from ROSA26;CD4-HA α adult testes were injected at 10 distinct sites in one testis of each animal, and the same number of cells supplemented with 2000 purified CD4-positive cells in the contralateral testis. X-Gal staining of the tubules was performed 12 weeks after injection, the length of the stained region was measured on the extended positive tubules and average stain intensities were estimated by image analysis with MacBAS v2.2.

The availability of homogeneous preparations of active germinal stem cells opens the way to a variety of research avenues, including gene transfer and re-implantation as a new approach in transgenesis (Nagano *et al.*, 2001), transcriptome studies, and possible transdifferentiation into other cell types depending on the local environment (Tersikh *et al.*, 2001 and references therein).

METHODS

Transgenic mice. Transgenic lines were generated by micro-injection in C57BL/6 × DBA/2 (B6D2) F₁ fertilized eggs according to standard procedures (Hogan *et al.*, 1994).

Plasmid construction. (i) *pStra8Luc*. The -371/+29 fragment amplified from *Stra8* genomic DNA (primers Stra8-5' and -3') was inserted in the *Sma*I site of pXp1 (Li *et al.*, 1994). (ii) *pStra8 HA α* . The CD4HA α marker has been assembled from PCR fragments amplified using primers CD4-1 and -2 (first two domains of the human CD4), HA-1 and -2 for the HA coding region with a Gly-Gly-Ser-Ser spacer (Bodmer *et al.*, 1994), and glo-1 and -2 for the 3'-UTR of the β -globin gene.

Reverse transcription and PCR amplification. RNA analysis was performed as described previously (Vidal *et al.*, 2001), using primers gapdh-1 and -2 for Gapdh expression; cycA1-s and -r for Cyclin A1, confirmed by hybridization with cycA1-h; cycA2-s and -r for Cyclin A2, confirmed by hybridization with cycA2-h; and ckit-s and -r for *W* (Kit receptor) expression (Table IV).

Luciferase activity in cell extracts. Activity was determined using the Dual-Luciferase Reporter (DLRTM) Assay System according to the manufacturer's instructions (Promega). Protein concentrations were determined according to Bradford (1976).

Immunocytochemical localization of luciferase in testis sections. *In situ* detection of the protein was performed on 12- μ m-thick cryo-sections, using rabbit anti-luciferase IgGs (Sigma) and FITC-conjugated goat anti-rabbit IgGs (Sigma).

Immunocytochemical analysis of isolated cells. Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 10 min. Primary antibodies were a polyclonal rabbit anti-mouse RBM protein (kindly provided by Dr H. Cooke, MRC, Edinburgh, UK), rat anti-mouse CD29 (β_1 integrin) (BD Biosciences, Heidelberg, Germany) and rat G8.8 monoclonal antibody against the mouse Ep-CAM protein (Anderson *et al.*, 1999), obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA. Depending on the

primary antibody, the secondary antibody was either FITC-conjugated goat anti-rabbit IgG (Sigma) or anti-rat R-phycoerythrin-conjugated antibodies (Sigma). FITC-conjugated rat anti-human CD49f (α_6 -integrin) monoclonal antibody was from BD Biosciences.

Immunomagnetic sorting. Total germ cells were prepared as described previously (Vidal *et al.*, 2001). Immunomagnetic isolation of CD4-positive cells from total testicular cells of *Stra8*-CD4HA α mice was performed using the CD4 Positive Isolation Kit (DynaI, Oslo, Norway) according to the manufacturer's instructions.

Germ cell transplantation. Recipient 3-week-old B6D2 males were γ -irradiated [⁶⁰Co, 3Gy, 1 Gy/min, 1.17–1.33 MeV on a Theratron 780 (Theratronics)], a dose that had been determined to be sufficient to arrest germinal differentiation. Three to four weeks later, after examination of control animals for the absence of spermatogenesis, purified *Stra8*-CD4HA α ;ROSA26 cells (1× sorting) were injected in the tubules. CD4-positive purified cells (10⁴) from one male were resuspended in 100 μ l of buffer together with 10⁵ total germ cells from the same animal. Total germ cells only (10⁶/ml, same preparation used for immunomagnetic sorting) were injected in the contralateral testis. Ten microlitres of cell suspension were injected at 10 distinct sites per testis. Ten to twelve weeks later, the transplanted males were killed and seminiferous tubules were stained with X-Gal. Three measurements were found to be important to evaluate the extent of colonization of the recipient testis: the number of injection sites from which β -galactosidase-positive cells had developed; the length of stained tubule extending from these sites; and the intensity of X-Gal staining determined using MacBAS v2.2 (FujiphotoFilm Co. Ltd) and Adobe Photoshop 5.5 (Adobe Systems Inc.) applications. The product of the three values was taken as an estimate of the colonization potential of the injected cells.

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Table IV. Oligonucleotide primers and probes

Oligonucleotide	Amplified sequence and internal probes
Stra8-5' 5'-CTATTCCTCTCACATCTTC-3' and Stra8-3' 5'-GCCCTGACGAGTCAGTCACT-3'	<i>Stra8</i> genomic sequence -371 to +29
CD4-1 5'-GGACGGAATTCCTCGATCAAGGCCACAATGAACCG-3' and CD4-2 5'-GGAGCTACCTCCGCTGGAGGCCTTCTGGA-3'	Human CD4 cDNA: first two domains
HA-1 5'-TCCAGCGGAGGTAGCTCCGGGATCTATCAG-3' and HA-2 5'-GCAGAGGGAAAAAGATCTCATCAGATGCATATTCTGC-3'	Influenza hemagglutinin (nucleotides 669–794)
Glo-1 5'-TGAGATCTTTTCCCTCTGC-3' and glo-2 5'-GGACGGAATTCCTCGATGGCATATGTTGCCAAAC-3'	Rabbit β -globin gene 3'-UTR
gapdh-1 5'-CACCACCAACTGCTTAGCC-3' and gapdh-2 5'-CGGATACATTGGGGTAGG-3'	<i>Gapdh</i> mRNA
cycA1-s 5'-CAGAGCTCCAAGAGTGGAG-3' and cycA1-r 5'-AGTGGAGATCTGACTTGAGC-3'	Cyclin A1 mRNA
cycA1-h 5'-CCTGTGGCCAGGAGATCACAG-3'	Cyclin A1 internal probe
cycA2-s 5'-CACCTCGAGGCATTCGGG-3' and cycA2-r 5'-CGGGTAAAGAGACAGCTGC-3'	Cyclin A2 mRNA
cycA2-h 5'-CAGCCTGCCTTACCATTTCATG-3'	Cyclin A2 internal probe
ckit-s 5'-CAACAGCAATGGCCTCACGAGT-3' and ckit-r 5'-GTGGTCACCTTTGCTCTGCTC-3'	<i>W</i> (Kit receptor) mRNA

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