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PUBLISHER'S NOTE

Expression of Concern: Gene trap analysis of germ cell signaling to Sertoli cells: NGF-TrkA mediated induction of Fra1 and Fos by post-meiotic germ cells

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Journal of Cell Science was alerted to duplicated HPRT blots in Fig. 4A and Fig. 7C of this paper. The authors state that the conclusions of the paper are not affected by the duplicated control blots, but were unable to locate the original data from almost 20 years ago. Without the original full blots, the journal is unable to determine whether the results and conclusions reported in the paper are compromised.

The journal is publishing this Expression of Concern to make readers aware of these issues. The authors offer cell lines used in the paper for replication by any interested investigators and apologise to readers for any inconvenience caused.

Gene trap analysis of germ cell signaling to Sertoli cells: NGF-TrkA mediated induction of *Fra1* and *Fos* by post-meiotic germ cells

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SUMMARY

Analysis of complex signalisation networks involving distinct cell types is required to understand most developmental processes. Differentiation of male germ cells in adult mammals involves such a cross-talk between Sertoli cells, the somatic component which supports and controls germinal differentiation, and germ cells at their successive maturation stages. We developed a gene trapping strategy to identify genes, which, in Sertoli cells, are either up- or down-regulated by signals emitted by the germinal component. A library of ~2,000 clones was constituted from colonies independently selected from the Sertoli line 15P-1 by growth in drug-containing medium after random integration of a promoter-less *βgeo* transgene (*neo^r-lacZ* fusion), which will be expressed as a fusion transcript from a 'trapped' cellular promoter, different in each clone. A first screen conducted on 700 events identified six clones in which β-galactosidase activity was increased and one in which it was repressed upon addition of germ cells. The targeted loci were identified by cloning and sequencing the genomic region 5′ of the insert. One of them was identified as the gene encoding Fra1, a component of the AP1 transcription regulatory complex. Accumulation of *Fra1* mRNA was induced, both in 15P-1 and in freshly explanted Sertoli cells, by addition of either round spermatids or nerve growth factor (NGF). The effect of NGF was mediated by the TrkA receptor and the ERK1-ERK2 kinase kinase pathway. *Fos* and *Fra1* transcription were induced within the first hour after addition of the neurotrophin, but, unlike what is observed after serum induction in the same cells, a second wave of transcription of *Fra1*, but not of *Fos*, started 16 hours later and peaked at higher levels at about 20 hours. These results suggest that AP1 activation may be an important relay in the Sertoli-germ cell cross-talk, and validate the gene trapping approach as a tool for the identification of target genes in cell culture systems.

Key words: Spermatogenesis, Nerve growth factor, Sertoli cell, Signal transduction

INTRODUCTION

Germinal differentiation in the adult mammalian testis is a highly ordered process. Throughout its progression from spermatogonia to meiosis and spermiogenesis, a germ cell remains in intimate contact with a Sertoli cell. There is general agreement that the many morphological and functional changes that germ cells undergo during spermatogenesis are controlled by Sertoli cells (reviewed by Griswold, 1995). Several biochemical properties of Sertoli cells have a cyclic variation suggesting a critical role of the associated spermatogenic cells. A new cycle is initiated when germ cells have reached a defined stage of differentiation, before the completion of the ongoing cycle, thus generating the classical pattern of stages, each with a defined assortment of differentiation steps (Leblond and Clermont, 1952). Microdissection of isolated tubule segments has demonstrated the stage-specific expression of a series of proteins by the Sertoli cell, including growth factors, cytokines and proteases (reviewed by Parvinen, 1993). These notions imply a continuous cross-talk between the Sertoli cell and the associated germ cells, in which, at each maturation stage, specific signaling systems trigger the synthesis and/or the release of the required Sertoli factors.

Our knowledge of the mediators of Sertoli-germ cell interactions remains, however, imperfect. Owing to its complex architecture, the seminiferous epithelium has been a difficult area for biological studies, and many of the modes of signaling between the germ cells and their somatic partner remain to be identified at the molecular level. In vitro analysis conducted on simplified culture systems may offer useful alternatives. We previously described one such system, based on the properties of a Sertoli differentiated cell line, 15P-1. These cells express a series of Sertoli-specific genes, and form with male germ cells in cocultures multicellular complexes which support the progression of pachytene spermatocytes to the haploid state (Rassoulzadegan et al., 1993; Vincent et al., 1998).

We took advantage of the ability of 15P-1 cells to interact with male germ cells to devise a general strategy based on gene trapping (Friedrich and Soriano, 1991) for the identification of

genes whose expression in Sertoli cells is regulated by germ cells and/or defined effector molecules. The method, largely used in ES cell lines, involves the selection of drug-resistant clones after transfer of a promoter-less construct composed of a splice acceptor in front of a gene (*βgeo*), which encodes a protein with both β-galactosidase and neo^r activities. Integration into the intron of an expressed gene in the correct orientation is predicted to create a fusion transcript. If the coding sequences in both messengers are in frame, an active protein is produced. After transfer of *βgeo* into 15P-1 by a retroviral vector, 2,000 independent drug-resistant clones, each one of them corresponding to a distinct integration of the transgene, were selected in geneticin-containing medium. The resulting library could then be screened for clones in which β-galactosidase activity, reflecting that of the upstream cellular promoter, would be either up- or down-modulated upon application of the stimulus of interest (addition of germ cells, of cell fractions, of soluble factors). Isolation of the chromosomal sequences upstream of the integrated transgene could then identify the responsive cellular genes, and further characterization of their expression and regulation could be conducted.

As a test of the general usefulness of the system, we conducted a first screen on 700 clones by measuring β-galactosidase activity with and without an overnight preincubation with total germ cells. Seven clones were identified in which the activity of the trapped promoter is either up- or down-modulated. One of them, designated 11F7, exclusively responded to the addition of purified spermatids and to that of nerve growth factor (NGF). A member of the neurotrophin family, NGF is essential for the development and maintenance of sensory and sympathetic neurons of the peripheral nervous system (reviewed by Levi-Montalcini et al., 1996). In the established line PC12, it induces the expression of a series of characteristic neuronal properties (Greenberg et al., 1985; Kruijer et al., 1985). It is also present in the adult testis. Although still a somewhat controversial issue, its production has been assigned by several reports to the germinal component, predominantly the round spermatids, in the form of an unprocessed precursor (Ayer et al., 1988; Parvinen et al., 1992; Chen et al., 1997). It was shown to increase the viability of Sertoli cells in culture, to stimulate DNA synthesis and cell proliferation in isolated seminiferous tubules, and to increase the levels of androgen binding protein (Lonnerberg et al., 1992; Parvinen et al., 1992; Chen et al., 1997). The two receptors identified in nerve cells are also expressed in the testis, namely the tyrosine kinase TrkA and $p75^{NTR}$, a member of the TNF receptor family. Their precise localization, however, has been the subject of somewhat divergent reports (Ayer et al., 1988; Parvinen et al., 1992; Russo et al., 1994; Russo et al., 1996; Seidl et al., 1996; Chen et al., 1997). Our own results, in agreement with the conclusions of Seidl et al. (Seidl et al., 1996), confirmed the expression in Sertoli cells of TrkA, but not of $p75^{NTR}$.

We found that, in the 11F7 gene trap clone, the βgeo cassette was inserted in a 5′ intron of the 'Fos-related' gene *Fra1*. With Fos, Jun and a series of other proteins, Fra1 is a constituent of the AP1 complex (Cohen and Curran, 1988; Cohen et al., 1989). Fra1 and Fos thus appear as candidates for central regulatory functions, that will in turn modulate the expression of other genes. One example, which is not likely to remain unique, is provided by a previous study concluding that induction of the transcription of *Fos* by Follicle Stimulating Hormone leads to the secondary activation of the transferrin promoter (Chaudhary et al., 1996). To access this central regulatory node, and in a more general way, to validate the gene trap approach as a means to the identification of regulated genes in complex paracrine networks, we further analyzed *Fra1* and *Fos* induction by NGF in Sertoli cells.

MATERIALS AND METHODS

Cell culture

15P-1 and the gene trap derivatives were grown at 32°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO-BRL Life Technologies, Paisley, UK). For NGF treatment, 15P-1 cells were cultured for 24 hours before experimentation in the absence of serum, with 0.2% bovine serum albumin (Fatty acid free, Sigma A-6003, St Louis, MO, USA). NGF was obtained from Promega, Madison, WI, USA (mNGF 2.5S G5142), and the phosphorylation inhibitor PD 098,059 from Sigma.

Fractionation of germ cells

Total germ cells

After removal of the tunica albuginea of testes of C57BL/6 × DBA/2 F1 adult mice, the seminiferous tubules cut into small pieces were transferred in DMEM, 0.5% bovine serum albumin, 20 mM Hepes, pH 7.4. Large aggregates were removed by decantation. DNase I and collagenase A (Boehringer Mannheim) were added at final concentrations of 100 μg/ml and 1 mg/ml, respectively. After incubation at 32°C for 20 minutes, cells were pelleted by low speed centrifugation, washed twice, resuspended in the same medium, and passed through a filter with 40 μm-pores (Falcon, Becton-Dickinson, Lincoln Park, NJ, USA) pushed by the piston of a 5 ml syringe.

Purification of spermatids and pachytene spermatocytes

Total germ cells prepared from twenty mice were loaded in the Beckman (Palo Alto, CA, USA) elutriation rotor JE-5.0, at a flow rate of 7 ml/minute and a constant speed of 2,000 rpm. Cells were collected in 11 fractions of 400 ml each, obtained by changing the flow rate from 7 to 50 ml/minute at constant speed. Fraction purity was checked by microscopic analysis after Hoechst 33253 staining.

Coculture of 15P-1 and germ cells

Optimal conditions for the coculture of the 15P-1 gene trap clones with germ cells were identical to those previously determined for the progression of pachytene spermatocytes through meiosis (Vincent et al., 1998). Briefly, the three most important variables were (1) to use 15P-1 cells in active exponential growth phase, (2) to add the germ cells to a suspension of 15P-1 cells after trypsinization, (3) to plate the cell mixture onto a glass substrate (Lab-Tek Chamber Glass Slides, Nunc Inc, Naperville, IL, USA).

Primary Sertoli cell cultures

Primary Sertoli cell cultures were established from 3 week-old mice according to published methods (Steinberger and Jakubowiak, 1993). They were maintained for 20 hours in DMEM with 10% fetal calf serum at 32°C. Attached germ cells were removed by hypotonic treatment (20 mM Tris-HCl, pH 7.4, for 2 to 5 minutes at 20°C) and the remaining Sertoli cells were washed with culture medium before further treatments. Before exposure to NGF, the cells were maintained for 24 hours in medium containing 0.5% fetal calf serum.

Assays of β-galactosidase activity

The enzymatic activity was measured in cell extracts by using the Galacto-Light Kit (Tropix, Bedford, MA, USA) according to the

manufacturer's instructions and was detected in situ by staining in 1.0 mg/ml X-Gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in phosphate-buffered saline (pH 7.4).

Transfection

Experiments were performed with Fugene 6 according to the supplier's instructions (Boehringer Mannheim).

Reverse transcription and PCR amplification

Quantitation of RNA amounts in cell extracts was done by comparison with that of *Hprt* reverse transcripts amplified as an internal standard in the same reaction mixture. Total RNA (1 μg) prepared using the Total RNA Isolation Kit (Boehringer Mannheim) was reverse transcribed using the MuLV reverse transcriptase according to the supplier's instructions. PCR amplification was performed with the Taq DNA polymerase (Boehringer Mannheim), using the following oligonucleotide primers (polymerization conditions indicated in parenthesis). *Fra1*: 5′-gaccagactccgagaggc-3′ and 5′-gataggccagaggtcggg-3′ (94°C, 30 seconds: 30 cycles, 59°C, 30 seconds, 72°C, 30 seconds). *Fos*: 5′-caacgccgactacgaggc-3′ and 5′-cttctgccgatgctctgcg-3′ (94°C, 30 seconds: 30 cycles 59°C, 30 seconds, 72°C, 30 seconds). *TK:* 5′-accgagacctggccacc-3′ and 5′-ccataggtgaagatctccc-3′ (94°C, 30 seconds: 30 cycles 56°C, 30 seconds, 72°C, 30 seconds). *TrkA:* 5′-gaatgtgacgtgctgggc-3′ and 5′-gcgcagagacggtgctg-3′ (94°C, 30 seconds: 30 cycles 57°C, 30 seconds, 72°C, 30 seconds). *TrkB:* 5′-cctggctgaagtggcatg-3′ and 5′-cacgatgcctggagaagg-3′ (94°C, 30 seconds: 30 cycles 57°C, 30 seconds, 72°C, 30 seconds). *TrkC*: 5′-caccctgacgtgcattgc-3′ and 5′-gttgccacgcaccacaaac-3′ (94°C, 30 seconds: 30 cycles 57°C, 30 seconds, 72°C, 30 seconds). *p75*^{NTR}: 5′-ctgcctggacagtgttac-3′ and 5′-ccaagatggagcaatagac-3′ (94°C, 30 seconds: 30 cycles 55°C, 30 seconds, 72°C, 30 seconds). Hprt: 5′-gctggattacattaaagcactg-3′ and 5′-aagggcatatccaacaacaaac-3′ (94°C, 30 seconds: 20 cycles 60°C, 30 seconds, 72°C, 30 seconds). For the *Hprt* internal controls, a sample was taken after only 5 minutes of reverse transcription. Image analysis and densitometric measurements were performed using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, USA).

5′ cloning and sequence determination

The RACE technique (5′ rapid amplification of cDNA ends; Frohman et al., 1988) was used for the characterization of sequences located at the 5′ end of the βgeo insert in the gene trap clones. First strand cDNA synthesis was performed from 2 μg of total RNA, using an oligonucleotide primer in the 5′ region of the β-geo insert (5′-gggcctcttcgctattacgc), with the AMV reverse transcriptase and deoxynucleotide solution according to the manufacturer's instructions (Boehringer Mannheim). The first strand cDNA was then purified from non incorporated nucleotides and primers by the High Pure PCR Product Purification Kit (Boehringer Mannheim), in which the elution buffer was replaced by 10 mM Tris-HCl buffer, pH 8. Terminal transferase was then used to add a homopolymeric A-tail to the 3′ end of the cDNA. The tailed cDNA was amplified by PCR using a second primer in the *β-geo* sequence located upstream of the first one used (5′-atgtgctgcaaggcgattaag-3′) and an oligo-dT anchor primer provided by the manufacturer. The resulting cDNA was further amplified using a third nested oligonucleotide primer in the β-geo sequence (5′-agggttttcccagtcacgacg-3′) with the same anchor primer. 5′ RACE PCR products were cloned in the pTAG vector using LigATor Kit (R&D System, Abingdon, UK). Sequencing of the cloned cDNAs on both strands was performed using the DNA cycling sequencing kit (Perkin Elmer, Foster City, CA, USA) according to the manufacturer's instructions.

Protein extraction and western blot analysis

Cells were lyzed for 15 minutes on ice in TNET solution (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing protease inhibitors (100 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A) and phosphatase inhibitors (5 mM NaF, 2 mM Na orthovanadate). The lysate was then centrifuged to remove cellular debris (15 minutes, 14,000 rpm, 4°C). Protein concentration was estimated by the BCA reaction (Pierce, Rockford, IL, USA). Four micrograms of proteins in Laemmli buffer were loaded onto a 10% acrylamide gel, electrophoresed, and transferred onto nitrocellulose. Phosphorylated ERK1 and ERK2 proteins were revealed with anti-active Map kinase antibodies (dilution 1/10,000, Promega, Madison, WI, USA) and anti-rabbit-HRP secondary antibodies (Bio-Rad, Hercules, CA, USA), using ECL reagent (Amersham Life Science, Buckinghamshire, UK).

RESULTS

Establishment of the exon trapping library

In order to tag expressed loci in the established Sertoli cell line 15P-1, cells were infected with the Rosaβgeo retrovirus (Friedrich and Soriano, 1991) and seeded in 96-well plates in the presence of a relatively low concentration of geneticin (200 μg/ml). After a maximum of one month of selection with media changes every fifth day, the resistant cells were expanded. The initial seeding density had been adjusted to a value such that the frequency of the selected event was less than one per well. We generated in this way a library of about 2,000 neo^r clones. Equivalent number of cells from each well were then seeded in quadruplicate 96-well plates and preserved by freezing. A first round of screening conducted on 700 clones by culturing 2 series of plates in parallel, respectively with and without addition of total mouse germ cells at a ratio of 50 to 1 relative to 15P-1 neo^r cells. β-Galactosidase activity was determined in lysates prepared after overnight incubation.

As shown in Fig. 1 for a representative sample, β-galactosidase activity in a large majority of the clones either was not changed, or showed only limited variations after exposure to germ cells. Six clones for which a greater than 2-fold increase and one for which a 4-fold decrease in activity were registered were kept for further studies. Induction factors initially observed were limited to a 3- to 8-fold range (Fig. 1B). It was, however, subsequently found that the culture conditions in 96-well plates, convenient for large-scale screening, are in fact less than optimal. Greater variations in enzymatic activity were recorded (Fig. 2A) when cultures were performed under the conditions that we had independently determined as optimal for the recognition by 15P-1 of germ cells (Rassoulzadegan et al., 1993; Vincent et al., 1998). Important variables are the actively growing state of 15P-1, the culture substrate, glass being more efficient than plastic, and the way the initial mixtures of germ cells and 15P-1 cells are prepared (see Materials and Methods).

In order to ascertain that the whole procedure generated clonal derivatives, three isolates were subcloned by limiting dilution in a 96 well-plate, and for each one, five subclones were tested again for the effect of germ cells on β-galactosidase activity, with concordant results in each series (data not shown). Definitive proof of clonality was, however, provided by the structure of the transgene, once established by Southern blot analysis and 5′-RACE extension (see below), evidencing in each case a unique integration of the transgene.

Identification of the trapped cellular promoters was performed by 5′-RACE amplification, cloning and sequencing of the region 5′ of the β-geo splice acceptor in the fusion transcripts. Results for the seven clones are summarized in

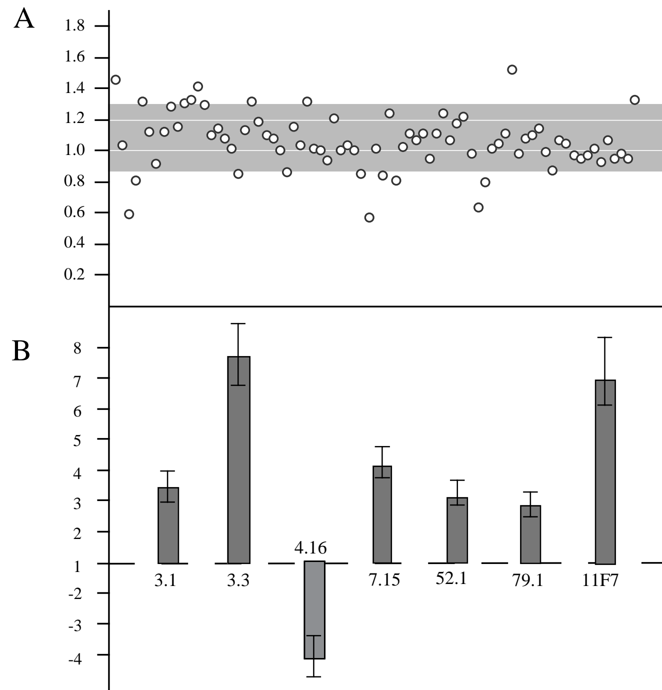

Fig. 1. Screening of the gene-trap library. (A) Values recorded in a representative series corresponding to one duplicated 96-well plate. Indicated values are ratios of β-galactosidase activity in extracts of cells after overnight incubation with added germ cells to control cultures. The broken line indicates the mean value of the ratios and the shaded area the standard error extent. (B) The same ratios are indicated by bars (average and s.e.m. on 2-4 independent repeats) for the seven clones whose ratios of activity with and without exposure to germ cells fall clearly outside the range defined in A. The negative value indicates the extent (–fold) decrease in activity for clone 4.16.

Table 1. Mouse loci in which β-geo expression was modulated in the presence of germ cells

Gene trap clone	GenBank locus name	Gene product
3.1	-	Unknown
3.3	Not determined*	
4.16	D49732‡	Mouse lamin A/C
7.15	RATBG§	Rat transforming growth factor beta receptor III
52.1	RANBP7§	Human Ran binding protein 7 (importin 7)
79.1	RNU31463§	Rat nonmuscle myosin heavy chain-A
11F7	AF017128‡	Mouse Fra1 transcription factor

*Retroviral insertion in the most 5′ part of the transcribed region (see text).
‡Identical to sequence in library.
§At least 90 per cent identical to sequence in the library.

Table 1. The analysis was inconclusive for only one of them (3.3), for which only transgene sequences were repeatedly amplified, suggesting that insertion had occurred immediately 3′ of a cellular promoter. Among the other six genes, data banks searches showed one unknown gene and five whose sequences were either completely identical to a known mouse gene, or more than 90% identical to a human or a rat gene whose murine homologue was not present in the data base. Whenever the genomic structure of the gene was available, it was clear that integration of the provirus had occurred in the first intron of the gene. In all cases it resulted in a fused RNA in frame with the upstream exon of the gene. Subsequent studies were then focused on clone 11F7, in which the transgene was inserted in the *Fra1* gene, of specific interest since modulation of expression of the AP1 complex would be expected to mediate secondary events in the Sertoli-germ cell interaction.

Response to purified spermatids and NGF

We determined the response of 11F7 cells to the addition of purified fractions of germ cells prepared by elutriation centrifugation (Meistrich, 1977). This technique allows essentially for the fractionation to a purity of 80 to 90% of three types of germ cells, the pachytene spermatocytes, round spermatids and elongated spermatids. Neither pachytene spermatocytes nor elongated spermatids had any effect on β-galactosidase synthesis, whereas exposure to round spermatids resulted in a 30- to 40-fold increase in activity (Fig. 2B). On the basis of this result, we searched for a possible effect of NGF, based on previous studies which identified NGF as a likely mediator in the function of the seminiferous epithelium. Produced by post-meiotic germ cells, NGF is assumed to interact with receptors on the surface of the Sertoli cells. RT-PCR determinations (not shown) confirmed that NGF mRNA was present in the round spermatid fractions. β-galactosidase activity in 11F7 extracts, was found to increase in a dose-dependent manner after overnight incubation in the presence of NGF (Fig. 2C). In situ staining for β-galactosidase activity evidenced a strongly positive reaction in 11F7 cells that had been incubated overnight in the presence of either total germ cells (50 germ cells per 11F7 cell) or NGF (100 ng/ml) (Fig. 2D). On the other hand, cell culture medium conditioned by overnight incubation of total germ cells had no effect on the level of β-galactosidase in 11F7 extracts (not shown). A likely, although trivial explanation is that this negative result is due to an insufficient concentration of NGF in the conditioned medium. It may also reflect a more complex situation, with factors secreted by other types of germ cells modulating the response to NGF of the Sertoli cell, a point that will clearly require further studies.

Induction of *Fra1* expression by NGF is a general feature of Sertoli cells

Further analysis demonstrated that the effect of NGF on the expression of the *Fra1* promoter is indeed a property of Sertoli cells, and not a unique property of the gene trap clone 11F7, thereby validating the gene trap approach as a way to detect modulation of gene expression in the Sertoli-germ cell cross talk. Semi-quantitative RT-PCR determination in extracts prepared from 15P-1 cells and from freshly established primary Sertoli cell cultures with and without addition of the neurotrophin demonstrated in all cases an increased amount of *Fra1* mRNA after overnight exposure to NGF (Fig. 3).

Time course of *Fos* and *Fra1* induction in Sertoli cells

In fibroblasts, *Fra1* expression induced by growth factors follows that of *Fos* after a delay of about 60 minutes (Cohen and Curran, 1988). Furthermore, NGF induces *Fos* expression in PC12 cells with the same kinetics as serum and growth factors (Cohen and Curran, 1988; Bartel et al., 1989). We were

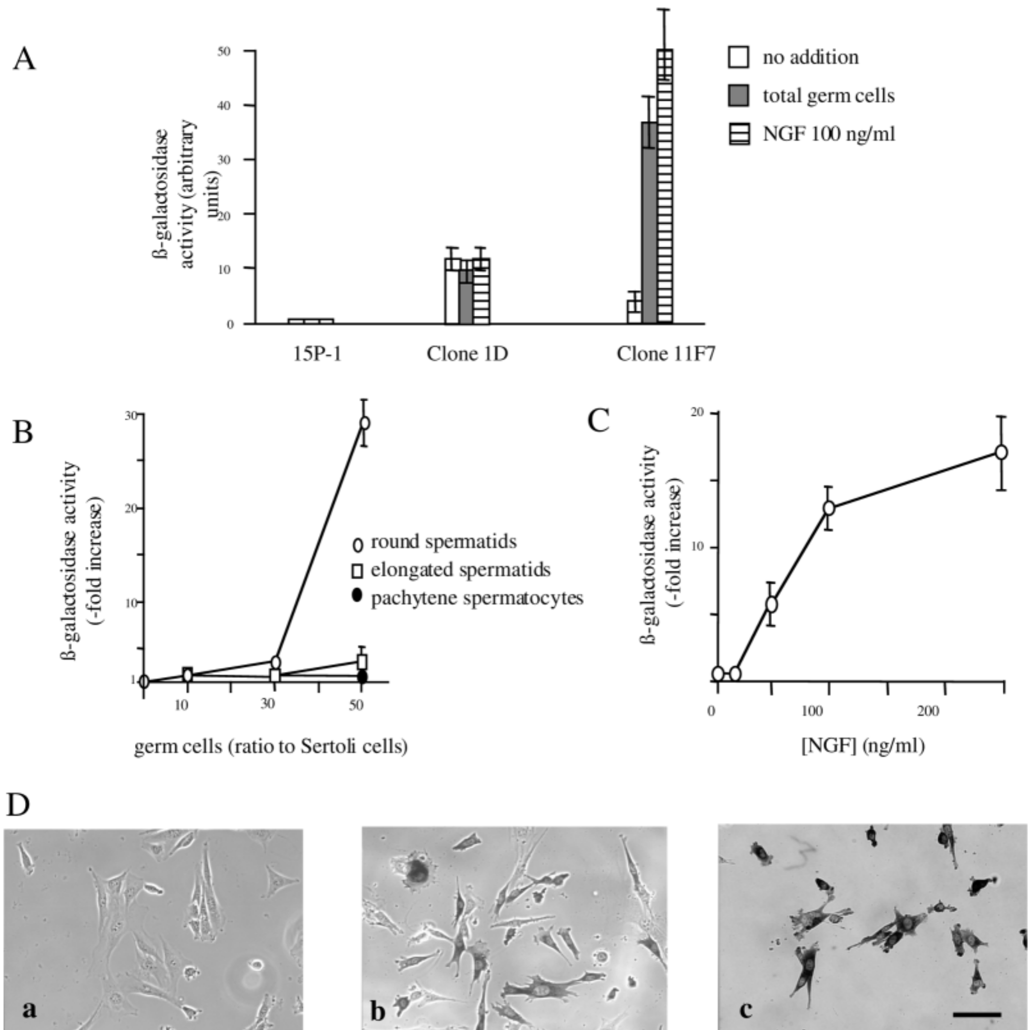

Fig. 2. Induction of β-galactosidase synthesis in clone 11F7 by round spermatids and by NGF. In these and the following experiments, culture conditions were optimized for Sertoli-germ cell interaction (see main text and Materials and Methods). (A) Enzymatic activity in extracts prepared from 15P-1, from a representative non responding clone (1D), and from clone 11F7. To parallel exponentially growing cultures were added either a 50-fold excess of unfractionated germ cells, or NGF at a concentration of 100 ng/ml, or fresh culture medium. After overnight incubation, enzymatic assays were performed on crude lysates. (B) Induction of *β-geo* expression was measured on 11F7 cells as in A, but using 80-90 per cent pure preparations of round spermatids, elongated spermatids and pachytene spermatocytes prepared by elutriation centrifugation. (C) NGF dose-response curve. Same experiment as in A with increasing concentrations of NGF. Standard error of the mean of 2-3 independent measurements. (D) X-Gal staining of β-galactosidase positive cells: left to right, 11F7 cells in standard cell culture medium, in medium supplemented with NGF and after overnight coculture with total germ cells under the same conditions as in A.

therefore led to ask (i) whether *Fos* is induced by NGF in Sertoli cells, and (ii) what are the time courses of *Fra1* and *Fos* expression. As shown in Fig. 4, the answer to the first question was that *Fos* showed a discrete peak of expression within the first 1 hour after NGF induction. *Fra1* showed a more complex profile, with a clearly biphasic kinetics (Fig. 4). A first burst of expression concomitant with *Fos* expression, about 1 hour after NGF addition, was followed by a return to the initial basal level of expression and a late increase to the higher values recorded in previous experiments after overnight exposure, starting at about 16 hours and peaking around 20 hours after NGF addition. By contrast, after serum induction in parallel 15P-1 cultures, the expected early induction of *Fra1* was detected during the first hour, but RNA then remained at a low and roughly constant level for the following 24 hours (Fig. 4B). This delayed increase may result from a requirement for the intermediary activation of other gene(s). A clear prediction would be that protein synthesis inhibitors should prevent the late increase in *Fra1* RNA, and indeed preliminary results indicate that addition of cycloheximide 15 minutes before that of NGF completely suppressed the increase in *Fra1* RNA 24 hours later.

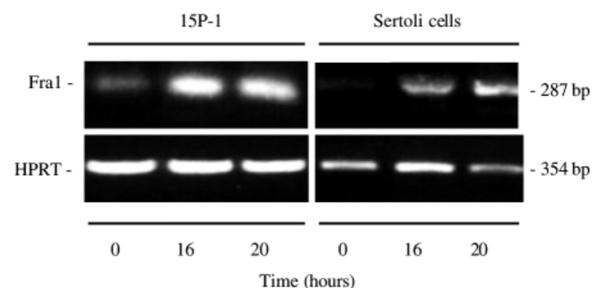

Fig. 3. *Fra1* expression is induced by NGF in 15P-1 and in Sertoli cells. 15P-1 and freshly prepared Sertoli cells in primary cultures were treated with NGF, and RNA was extracted at the indicated time. It was reverse transcribed using oligo-dT primers, and a fraction of the cDNA preparation (2 out of 20 µl) was used for PCR determination of *Fra1* RNA (upper panels), with *Hprt* amplification as an internal control (lower panels). Size of the products given in brackets closely correspond to the values expected from the sequence.

The TrkA receptor and the ERK1-ERK2 MAP kinase kinase pathway are implicated in the control of *Fra-1* expression

The two receptors p75^{NTR} and TrkA respond to NGF with different affinities and the concentrations used in our experiments were sufficient to activate both the low affinity p75^{NTR} and the high affinity TrkA. To determine which receptor was involved in *Fra1* regulation, and in view of the somewhat controversial results reported in the literature, we first proceeded to an analysis of their expression in 15P-1 cells, primary Sertoli cell cultures and whole testis, by RT-PCR assays using oligonucleotide primers specific either for the tyrosine kinase domain common to the Trk family, or for the p75^{NTR} receptor (Fig. 5). Expression at messenger level of at least one of the Trk receptors was evidenced in whole testis RNA, in primary culture Sertoli cells and in 15P-1 cells. p75^{NTR} mRNA, on the other hand, was detectable neither in Sertoli nor in 15P-1 cells, although it was clearly present in total testis extracts. To further precise the Trk receptor(s) present in Sertoli cells, we designed couples of oligonucleotide primers specific for *TrkA*, *TrkB* and *TrkC* mRNAs, located in the 5′ parts of their respective sequences. Results indicated that the three genes are all expressed in the testis but that *TrkA* is the only one transcribed in Sertoli cells.

The conclusion that TrkA signaling was responsible for *Fra1* induction was independently confirmed by the observation of high levels of *Fra1* RNA in transfected cells expressing a constitutively activated form of the receptor. The *Trk5* oncogenic mutant results from a deletion of 50 amino acid residues in the extracellular domain of *TrkA* (Coulier et al., 1990). Experiments were performed both on 15P-1 cells transiently expressing *Trk5* and *neo*^{r} after transfection and on pools of clones selected in geneticin medium. RT-PCR assays demonstrated in all cases elevated levels of *Fra1* expression in the absence of NGF stimulation (Fig. 6).

One of the main pathways of signal transduction by tyrosine kinase receptors is the ERK1-ERK2 MAP kinase kinase pathway, required for the induction by NGF of the differentiation of PC12 cells (Pang et al., 1995). We thus determined whether the ERK1-ERK2 kinases were activated by assaying the degree of phosphorylation of the two proteins after exposure to NGF (Fig. 7A). Western blot experiments were performed with antibodies specific to the phosphorylated forms of the proteins. A somewhat irregular background of phosphorylation was observed at time 0, clearly related with the fact that, unlike the standard fibroblast cell lines often used in this type of experiment, Sertoli cell cultures are not completely arrested in the absence of serum. In spite of that, an increase of the phosphorylated form was consistently observed upon NGF addition, with a peak about 30 minutes after addition of NGF. The same results were observed when freshly isolated Sertoli cells were used in the same conditions (Fig. 7B), except that the maximum of phosphorylation was reached after only ten minutes of treatment with NGF. This difference in kinetics between two widely different types of cells in culture could not be explained at this stage, but it was highly reproducible. These results were confirmed by using the MAP kinase kinase pathway inhibitor PD 098,059 (Alessi et al., 1995). Treatment of 15P-1 cells with the inhibitor 30 minutes before NGF addition to the culture medium completely suppressed *Fra1* mRNA accumulation (Fig. 7C).

DISCUSSION

A primary aim of this work was to establish a convenient tool

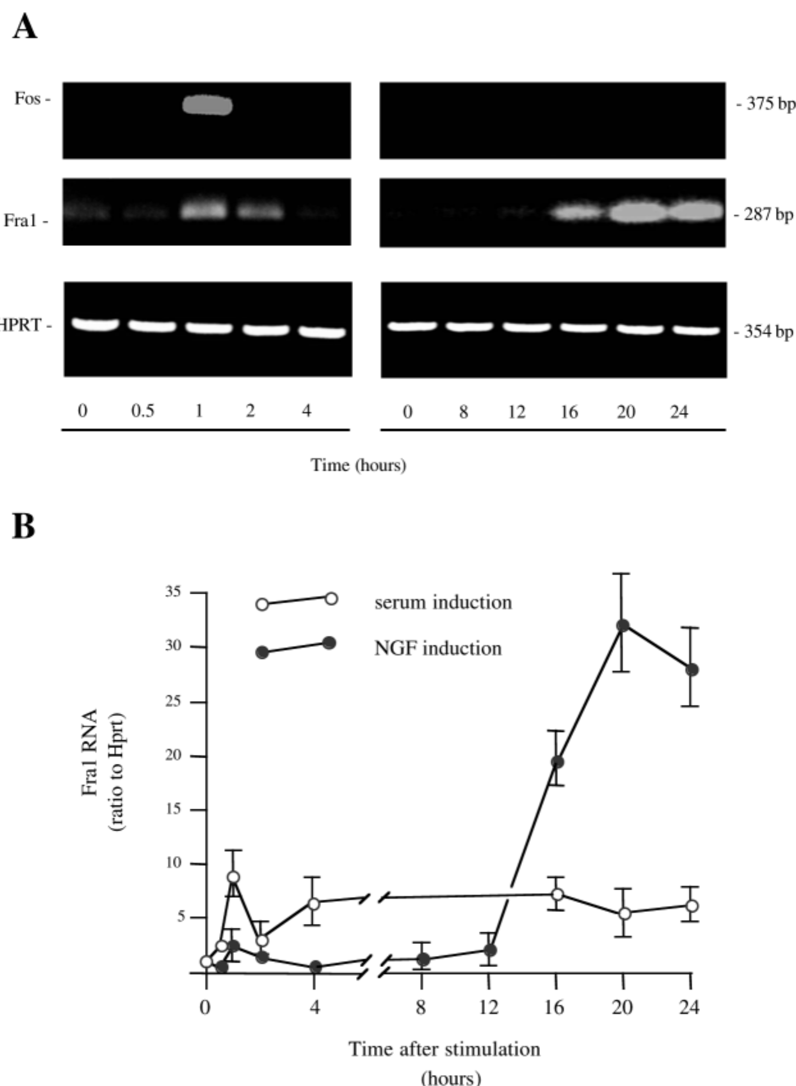

Fig. 4. Time-course of *Fos* and *Fra1* induction after NGF treatment of 15P-1 cells. (A) RNA was prepared at the indicated times after addition of NGF (100 ng/ml) and processed as described in the legend of Fig. 3. (B) Comparative time course of *Fra1* induction after NGF stimulation (100 ng /ml) and after serum stimulation. One set of cultures was analyzed as in A. A parallel set was incubated for 24 hours in DMEM medium without serum supplemented with BSA. Medium was changed at time 0 for DMEM supplemented with 20% serum. RNA was extracted at the indicated times and processed as in A. Values shown (mean ± s.e.m.) are the densitometric measurements of the bands corresponding to the amplified product of *Fra1* RNA. Open circles: serum induction; closed circles: NGF induction.

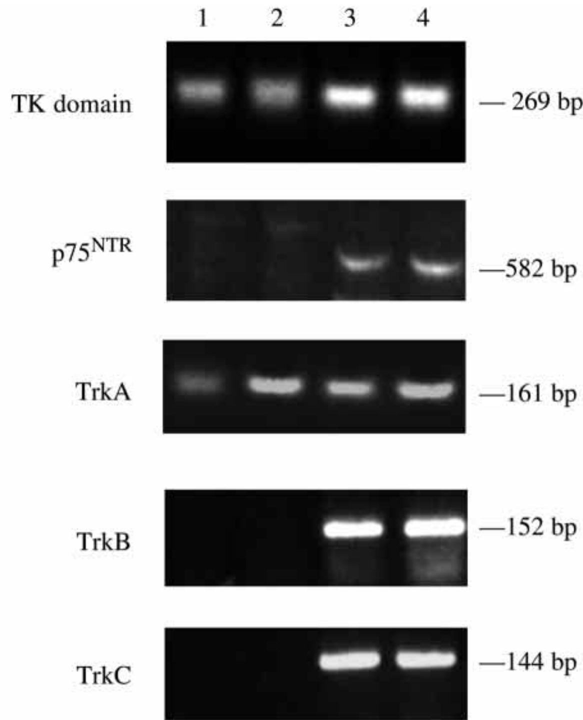

Fig. 5. Neurotrophin receptors mRNAs in total testis and Sertoli cell RNA. RNA was prepared from primary culture Sertoli cells (lane 1), 15P-1 cells (lane 2), whole testis (lane 3), and brain (lane 4) and reverse transcribed using the oligo-dT primer. One tenth of the reaction was used in PCR assays using oligonucleotide primers designed to amplify the tyrosine kinase domain common to the Trk family members ('TK domain'), the p75^{NTR} receptor ('p75'), and to discriminate between the TrkA, TrkB and TrkC tyrosine kinase receptors (see Materials and Methods). Size of the products given as in Fig. 3.

Fig. 6. Expression of Trk5 increases *Fra1* mRNA accumulation. 15P-1 cells were cultivated in serum free medium supplemented with 0.2% BSA (lane 1). Parallel cultures were maintained in the same medium after transfection with DNA of plasmid pDm-78 including the *Trk5* oncogenic mutant of *TrkA* (Coulier, 1990) and the *neo*^{r} selection marker. RNA was prepared either 48 hours after transfection (lane 2), or after two weeks of growth in geneticin containing medium (lane 3). Reverse transcription from oligo-dT primers and semi-quantitative PCR measurements were performed with the Fra1 oligonucleotide primers (upper panel) and the *Hprt* primers as control (lower panel).

for the identification of some of the genes involved in a complex intercellular signalisation network such as the cross-talk between a Sertoli cell and its associated germ cells at their successive differentiation stages. This method relies on the fact that one of the partner cells is established as a differentiated cell line. A library of gene trap clones can then be generated, each one corresponding to the random integration of the *βgeo* reporter in a chromosomal 'trapped' locus, different in each clone. A simple enzymatic assay will then quantitatively reveal the activity of the promoter, and thus, its possible modulations under a set of predetermined experimental conditions. The method has obvious inborn limits. First, inducible genes will be identified only if they are already expressed at a basal level in the absence of stimulation. This basal level may, however, be quite low, and still, be sufficient to confer resistance to concentrations of geneticin not exceeding 200 μg/ml. A second limit is the work load involved, which limits the number of genes analyzed. Screening several hundred, if not thousands of genes is possible, but these numbers remain far below the range explored by the microarray technology (Lipshutz et al., 1999). Beside a much lower cost, however, the interest of the gene

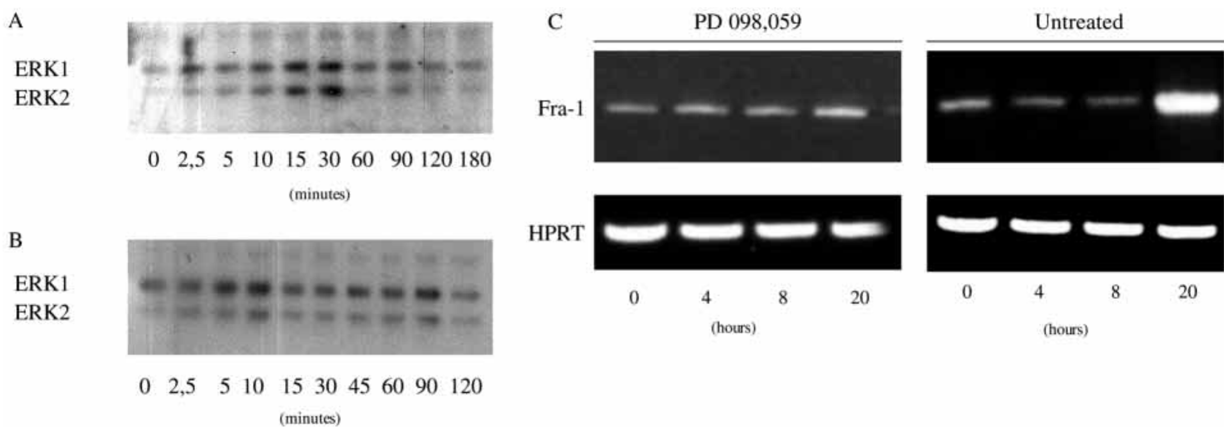

Fig. 7. The effect of NGF on *Fra1* messenger accumulation is mediated by the ERK1-ERK2 MAP kinase kinase pathway. (A and B) Western blot analysis using antibodies directed against the phosphorylated forms of ERK1 and ERK2 proteins. (A) NGF induction in 15P-1 cells that have been first maintained for 48 hours in serum-free medium supplemented with 0.2% bovine serum albumin; (B) the same in Sertoli cell primary cultures after 24 hours in medium supplemented with 0.5% serum. At the indicated times after addition of NGF (100 ng/ml), protein extracts were analyzed by western blotting using antibodies specific of the phosphorylated forms of the proteins. (C) In the same experiment, RNA was extracted at the indicated times after NGF addition at the same concentration to 15P-1 cultures either pretreated of not for 30 minutes with Park Davis 098,059 phosphorylation inhibitor (30 μM). After reverse transcription using oligo-dT primers, 2 out of 20 μl of reaction mixture were used in semi-quantitative PCR measurements of the *Fra1* and *Hprt* control RNAs.

trap approach is to provide us from the start with useful 'knocked in reporters' for the genes of interest. It is also important that, unlike other methods (microarray analysis, substractive hybridization, differential display), it does not bias towards genes with high expression levels. A third series of questions may be raised regarding the possibility of artefacts. No cell line will ever be a totally faithful reproduction of the physiology of a given cell, due to the genetic alterations associated with immortalization as well as to the rather alien environment of the cell culture, but some of them like the 15P-1 line used in the present work, maintain a significant part of the in vivo phenotype. Subsequent studies, either in the mouse or on primary cultures of freshly explanted cells, should then validate the conclusions drawn from the analysis of the gene trap clones.

The exon trapping method had initially been developed to establish patterns of gene expression during embryonic development (Friedrich and Soriano, 1991). Here we show that the same method can be applied to differentiated cells. By using only a fraction of the library, a series of seven genes were identified whose expression is either up- or down-regulated during an overnight coculture of Sertoli cells with total germ cells. Several of them appear of interest for further studies, as their variations can be related to a biological feature of germinal maturation. For instance, the induction of a non muscle myosin gene may be related with the movement of the maturing germ cells, in all likelihood driven by the Sertoli cell, from the periphery to the central part of the tubule, and at some stages in the opposite direction. Similarly, modulation of Type III TGFβ receptor expression may have a functional significance. A recent study (Lewis et al., 2000) established a possible role of this receptor in the sensitivity of the cell to inhibin, itself a regulator of Sertoli cell proliferation (Matzuk et al., 1992; Lopez et al., 1999).

As a first step, and with the primary aim to validate the gene trap approach, we have focused our studies on the induction of *Fra1* and *Fos*, two obvious candidates for a central role in transcriptional regulations. We determined that induction of *Fra1* in Sertoli cells was triggered by the post-meiotic round spermatids, with NGF as a potential mediator. Function of NGF in the testis has been previously indicated (Lonnerberg et al., 1992; Parvinen et al., 1992; Chen et al., 1997). Induction by NGF of the production by 15P-1 cells of antimicrobial proteins of the defensin family (Grandjean et al., 1997) had established the presence of a receptor, now identified as TrkA, whose role in the induction of *Fra1* was further confirmed by its constitutive high level of expression in cells expressing the activated oncogenic derivative Trk5.

In Sertoli cells as in the neuronal cell line PC12, TrkA activation leads to the downstream activation of the ERK1-ERK2 kinase kinases (Pang et al., 1995). The resulting time course of *Fra1* activation appears, however, different in Sertoli cells from that in neuronal and fibroblast cells. In serum-stimulated fibroblasts, as well as in NGF-treated PC12 cells, *Fra1* induction is an early event, although it is delayed by 30 to 60 minutes relative to *Fos* induction. In Sertoli cells, we also observed an early induction to a relatively modest level, peaking 1 hour after the addition of NGF, and concomitant with *Fos* induction. More strikingly, a second wave of transcription, of a larger amplitude, begins between 12 and 16 hours, reaching a maximum at about 20 hours. This late increase was not seen after serum induction in 15P-1 cells (Fig. 4), and it has not been reported in other cell types, after induction either by serum (fibroblasts, PC12) or by NGF (PC12). These features clearly differentiate the effects of NGF on 15P-1 Sertoli cells from those observed after serum stimulation in various cell lines, including 15P-1 (Fig. 4B) and mouse fibroblasts (data not shown). Establishing whether this delayed effect is due to a cascade of induction with the intermediary activation of other gene(s) will be the subject of further studies.

The 'knock in' *βgeo* reporter provides a convenient assay for the expression of *Fra1* and a tool for subsequent studies on the transduction of the NGF signal in Sertoli cells. Further studies will then be required to evaluate the physiological function of NGF during germinal differentiation in vivo. Establishing the patterns of expression of the neurotrophin in Sertoli cells during germinal differentiation will require a precise in situ analysis. One element suggestive of a physiological function of *Fra1* in the control of spermatogenesis is the specific expression pattern observed in the testis of a seasonal breeder, the European red fox (Cohen et al., 1993). In the mouse, in situ analysis of Fra-1 expression in the adult testis is made difficult by the complex and compact structure of the seminiferous epithelium. Preliminary data (not shown) on the first wave of spermatogenesis in the testis of the young mouse indicated that Fra-1 transcription is not turned on before its completion. This result is consistent with induction in Sertoli cells in response to post-meiotic stimuli. It is clear that definite answers on the in vivo function of NGF will depend on the availability of a targeted mutation of the gene. Since, however, the *Ngf*$^-$ homozygous genotype is lethal at an early developmental stage (Crowley et al., 1994), a conditional mutation is required, which, ideally, would affect the gene exclusively during spermatogenesis. One possible way is offered by the transgenic 'TAMERE' mice which express the Cre recombinase during meiosis (Hérault et al., 1998; Vidal et al., 1998). This strategy, however, requires the *Ngf* locus to be first modified to include properly positioned copies of the target sequence of the recombinase (*LoxP* sites).

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