The mouse transcription factor Stat4 is expressed in haploid male germ cells and is present in the perinuclear theca of spermatozoa

Gilles Herrada\textsuperscript{1,3} and Debra J. Wolgemuth\textsuperscript{1,2,3,4,*}

Departments of 1 Genetics and Development and 2 Obstetrics and Gynecology, 3 The Center for Reproductive Sciences and 4 The Columbia Cancer Center, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA

*Author for correspondence (e-mail: djw3@columbia.edu)

SUMMARY

STAT (signal transducer and activator of transcription) proteins have been shown to be essential transcription factors which mediate biological effects of cytokines. Although most of the STATs have been shown to be widely expressed, Stat4 mRNA has been detected in only a few tissues, including the testis. In the present study, immunoblot analysis confirmed that the presence of Stat4 protein was similarly restricted, with the highest level observed in testis. In situ hybridization, immunoblot, and immunohistochemistry analyses revealed that in the testis, Stat4 was abundantly and exclusively expressed in male germ cells which have completed meiosis, at the round and elongating spermatid stages. Cytolocalization at various times of spermatid differentiation showed that the level of Stat4 protein increased in parallel in both cytoplasm and nuclei. No specific nuclear translocation that would have been an indicator of Stat4 activation was observed at any stage of spermatogenic differentiation. Interestingly, the Stat4 transcription factor was localized to the condensing perinuclear theca of spermatids, a localization that was confirmed by selective biochemical extraction of thecal proteins. Since the theca is known to depolymerize in the cytoplasm of the oocyte during the hours following fertilization, we hypothesized that sperm Stat4 would represent an original paternal contribution to the fertilized egg which may be involved in the onset of zygotic transcription.

Key words: Stat4, Perinuclear theca, Sperm, Transcription factor

INTRODUCTION

The recent characterization of the pathway of signal transduction of the cytokines has permitted the identification of two new families of intracellular effector proteins, the JAKs (Janus tyrosine kinase) and the STATs (signal transducer and activator of transcription) (reviewed by Ihle, 1995; Schindler and Darnell, 1995). The JAK-STAT signal transduction pathway is now considered as the major mediator of the functional response of cells to most, and probably all cytokines. Cytokines are defined as a family of approximately 30 soluble proteins which promote diverse and fundamental aspects of cell communication in a variety of processes, including immunity and hematopoiesis as well as embryonic, gonadal, mammary, and neuronal development (reviewed by Ihle, 1995; Schindler and Darnell, 1995). Recent data have also suggested that the JAK-STAT pathway can be directly involved in cell proliferation (Mui et al., 1996; Chin et al., 1996).

To date, four JAK and seven STAT genes have been cloned in various mammals, including mouse, human and sheep. A signaling pathway involving the hopscotch-marelle genes, whose activating signal is still unknown, has been recently identified in \textit{Drosophila} as a homologue of the mammalian JAK-STAT pathway (Hou et al., 1996; Yan et al., 1996) and a STAT-like protein has been reported in the frog (Schindler and Darnell, 1995). The JAK-STAT pathway is thus widely present among animal species.

Unphosphorylated STAT proteins are latent transcription factors confined to the cytoplasm. A cascade of kinase activities initiated by cytokines results in the phosphorylation of STATs, at which point they dimerize, translocate into the nucleus, and are capable of activating transcription. Interactions have been demonstrated between the JAK-STAT pathway and other cellular signaling proteins, such as the tyrosine kinase receptors of EGF and PDGF (Fu and Zhang, 1993; Zhong et al., 1994b; Ruff-Jamison et al., 1995; Leaman et al., 1996; David et al., 1996; Yamamoto et al., 1996), and the RAS pathway (Zhang et al., 1995; David et al., 1995). Moreover, constitutive activation of the JAK-STAT cascade was shown to be correlated with cell line transformation by the tyrosine kinase oncogenes \textit{Scr} (Yu et al., 1995), \textit{v-abl} (Danial et al., 1995), its related chimeric oncogene \textit{Bcr/Abl} (Carlsson et al., 1996), as well as the HTLV 1 virus (Migone et al., 1995). Most of the \textit{Stat} genes have been reported to be ubiquitously expressed (reviewed by Ihle, 1995; Schindler and Darnell, 1995). Mouse \textit{Stat4} is the remarkable exception, since the highest level of expression is observed in testis, where two transcripts of 3.3 and 3.6 kb are detected (Yamamoto et al., 1994; Zhong et al., 1994a). Lower levels of the 3.3 kb \textit{Stat4} transcript have also been reported for a restricted number of tissues, such as spleen, thymus, lung, skeletal muscle, and bone marrow, and...
in a few cell lines of myeloid, erythroid, and lymphoid types (Yamamoto et al., 1994; Zhong et al., 1994a). Recent studies have focused on the possible role of STAT4 protein in T helper type 1 lymphocytes and in natural killer cells, as well as its activation by interleukin 12 (IL-12) (Jacobson et al., 1995; Bacon et al., 1995). Consistently, STAT4 expression in activated lymphocytes correlates with the appearance of the IL-12 receptor (Bacon et al., 1995). Tyrosine phosphorylation of STAT4 (presumably on Tyr693) in lymphocytes is likely to be catalyzed by the JAK2 and TYK2 kinases, which are also activated by IL-12 (Bacon et al., 1994). Interestingly, Xu et al. (1996) have shown that the transcriptional activity of STAT4 is more likely to result from the cooperative binding of multiple STAT4 homodimers onto adjacent sites of lower affinities than from the binding of one STAT4 homodimer to its optimal binding site (TTCCGGGA). However, the molecular mechanisms underlying the biological activity of STAT4 remain to be determined in vivo.

Although the highest level of STAT4 expression was observed in the testis of adult mice, to date no data relevant to its possible role in this tissue have been reported. The adult testis is a complex tissue with highly organized associations of different cell types, such as the somatic Leydig, myoid, and Sertoli cells, and the proliferating germ cells. Spermatogenesis begins when a pool of progenitor cells, the spermatagonia, differentiate into meiotic spermatocytes, haploid spermatids, and, finally, spermatzoa. Successful completion of spermatogenesis is hypothesized to be the result of the convergence of both intrinsic differentiation programs and a highly orchestrated signaling network between somatic and germ cells.

The aim of this study was to begin to determine the role of STAT4 in the process of spermatogenesis. The cellular site of expression of STAT4 in mouse testis was localized, at both the RNA and protein levels, to spermatids, male germ cells which have completed meiosis. We further demonstrated the presence of the STAT4 protein in sperm cells, more specifically in the perinuclear theca of spermatzoa. These findings provide new insight into the possible function of STAT4, in particular suggesting an intriguing possible role in spermatogenesis at fertilization for a transcription factor expressed in male postmeiotic germ cells.

MATERIALS AND METHODS

Sources of tissues and antibodies

Swiss Webster mice (Charles River, Wilmington, DE) were used as the source of wild-type tissues, including sperm cells. The mouse mutant strain atrichosis (at) (A1TEBE/le w/a distalbe) was obtained from the Jackson Laboratories (Bar Harbor, ME). Adult mice were older than 2 months. For in situ hybridization and immunohistochemistry analyses, freshly dissected testes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C, then washed for 30 minutes in PBS, saline solution (0.85% NaCl), 50% saline/ethanol, and 70% ethanol/water, prior to paraffin embedding. Alternatively, testes were fixed in Histochoice MB (Amresco, Solon, OH) overnight at 4°C, and processed as above. A polyclonal anti-C-terminal STAT4 rabbit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Stat1 antibody was kindly provided by Dr Chris Schindler (Shuai et al., 1992).

RNA probes

A cDNA containing a 1 kb sequence of the mouse Stat4, cloned in pBluescriptII-SK+, was a generous gift of Dr James Ille. RNA probes generated from this plasmid detected two RNA bands of 3.3 and 3.6 kb in northern blot analysis, by using a total testicular RNA fraction, in agreement with published data (Yamamoto et al., 1994; Zhong et al., 1994a). For in situ hybridization analysis, anti-sense and sense [35S]UTP (DuPont, NEN, Wilmington, DE) riboprobes were generated from the linearized plasmid, by using either T7 or T3 RNA polymerase (Promega Biotech., Madison, WI).

In situ hybridization analysis

Paraffin embedded testes were cut into 5 μm sections and analyzed by in situ hybridization, using the procedures described by Jaffe et al. (1990), and Chapman and Wolgemuth (1992). Briefly, rehydrated sections were prehybridized at 50°C for 2-6 hours in 50% formamide, 0.6 M NaCl, 120 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.02% Ficoll (M₄, 400,000), 0.02% polyvinylpyrrolidone, 0.1% BSA (bovine serum albumin) fraction V, 0.5 mg/ml denatured salmon sperm DNA, and 0.6 mg/ml yeast total RNA. The hybridization mixture was identical to the prehybridization solution with the addition of 10 mM DTT (dithiothreitol), 0.1% SDS, and 10⁵ dpm/ml of 35S-labeled riboprobe. After hybridization at 50°C for 16 hours, the slides were washed in, successively, formamide wash buffer (50% formamide, 1x SSC, and 10 mM DTT) for 30 minutes at 50°C, 0.5x SSC at room temperature (RT) for 30 minutes, 3.5x SSC containing 20 μg/ml RNase A for 30 minutes at RT, 3.5x SSC for 10 minutes twice at RT, and 0.1x SSC at 65°C for 2 hours. The sections were then dehydrated and coated with a nuclear track emulsion, type NBT-2 (Kodak). After 2-3 weeks of exposure at 4°C, slides were developed, stained with hematoxylin and eosin, mounted and viewed on a Leitz photomicroscope under epiilluminescence optics. Specificity of the labeling with the antisense probe was confirmed by comparison with sections hybridized with the sense probe, which gave an estimation of the background signal.

Immunoblot analysis

Wild-type adult tissue and precipitated sperm protein fractions (see Sperm protein fractionation) were solubilized in tissue lysis buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 150 mM NaCl, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF) at 4°C. Extracts were then centrifuged at 10,000 g for 20 minutes to pellet unsolubilized debris and nuclei. Protein concentration of the supernatants was determined with Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, CA), using BSA as the standard. Extracts were diluted in Laemmli sample buffer (Laemmli, 1970), boiled for 2 minutes, and resolved by 10% or 7.5% SDS-PAGE. When specified in the legend, tests from 7- to 17-day-old and adult mice, and epididymal sperm (see Sperm protein fractionation) were directly homogenized in Laemmli sample buffer. Gels were electrophoresed onto nitrocellulose membrane. Membranes were blocked by soaking in Blotto (3% dried fat free milk) in TBS (Tris buffered saline) for 30-60 minutes, incubated with the antibody diluted in Blotto at RT for 2 hours, washed three times in TBS for 5 minutes, incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) diluted in Blotto for 1 hour, and washed three times in TBS-0.05% Tween-20 for 5 minutes. The chemiluminescence blotting substrate POD kit (Boehringer Mannheim, Indianapolis, IN) was used for the detection step, according to the manufacturer’s recommendations.

Immunohistochemistry analysis

Sections of adult testis, fixed in 4% paraformaldehyde or Histochoice (Amresco, Solon, OH) were deparaffinized in xylene, and progressively rehydrated in a graded series of ethanol/water mixtures. Sections were boiled in 10 mM sodium citrate, pH 6.0, for 5 minutes twice in a microwave oven and cooled for 15-20 minutes. Sections were then exposed to 0.3% H2O2 in methanol to destroy any endogenous peroxidase activity from the tissue, washed in PBS, and blocked in
blocking solution (1% goat serum containing PBS) for 1 hour at RT. Anti-STAT4 antibody diluted from 1:50 to 1:1,000 in blocking buffer was applied, and incubations were carried out overnight at 4°C in a humidified chamber. Sections were rinsed in PBS and further processed, by using a horseradish peroxidase-based Vectastain Elite ABC detection kit (Vector, Burlingame, CA), according to the recommendations of the manufacturer. Sections were reacted with the DAB (3,3 diaminobenzidine tetrahydrochloride) substrate. Color development was monitored under a dissecting microscope. Reactions were stopped by rinsing extensively in water. When indicated, sections were counterstained with hematoxylin and mounted. Purified rabbit IgG (Sigma, St Louis, MO) was used as primary antibody in the negative control.

Sperm protein fractionation

Sperm cells were recovered from the lumen of the epididymis and solutions of increasing denaturing strength (adapted from Bellve et al., 1993). Freshly dissected epididymis (including caput, corpus and cauda epididymis) from 3- to 4-month-old males were minced in 10 ml of ice-cold PBS, and kept at 4°C for 15 minutes with constant agitation. The supernatant was filtered through a 74 µm mesh and centrifuged at 5,000 g for 20 minutes. Pellets were washed in 1 ml of ice-cold PBS and twice in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, which led to the lysis of contaminating blood and epithelial cells. The purity was monitored and the number of the resulting spermatooza evaluated by microscopy. Typically, 10⁶ cells were obtained from 6 epididymides. Sperm cells were resuspended in 1 ml of tissue lysis buffer without DTT (see Immunoblot analysis) and incubated at 4°C for 15 minutes, which solubilized the plasma membrane and cytoplasm while the nucleus and tail remained intact. After pelleting at 15,000 g for 30 seconds, the supernatants (containing the solubilized proteins of the ‘mild lysis’ fraction) were precipitated overnight at −20°C with 3 volumes of ethanol. The resulting pellets were washed twice in 1 ml PBS and resuspended in 50 mM Tris-HCl, pH 7.4, 1% SDS, and 1 mM PMSF. Extraction of mouse sperm with 1% SDS solubilized acrosomes, separated tails from nuclei and lysed any remaining nuclei of contaminating cells. Proteins from the ‘SDS’ fraction were ethanol precipitated as above. Decapitated sperm cells were pelleted and washed three times in PBS at RT to remove all trace of SDS. Sperm nuclei were recovered separately from the tails by centrifugation through a 37% sucrose (w/v), 50 mM Tris-HCl, pH 8.0, cushion. The pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 25 mM DTT, and incubated at RT for 20 minutes with constant vortexing. Cetyltrimethylammonium bromide (CTAB) (1%, w/v) was then added and incubated for 20 minutes. The nuclei were pelleted at 10,000 g for 15 minutes and the supernatant (DTT-CTAB fraction) was ethanol-precipitated. DTT-CTAB extraction specifically induced the depolymerisation of the perinuclear theca by reduction of the disulfide bonds. Nuclei were washed twice in 50 mM Tris-HCl, pH 8.0. The remaining nuclear proteins and proteins of the perinuclear matrix were finally extracted in 50 mM Tris-HCl, pH 8.0, 132 mM CaCl₂, 88 mM MgCl₂, and 50 µg/ml DNase I, at 37°C for 1 hour, and ethanol precipitated. All ethanol precipitated proteins fractions were recovered by centrifugation at 10,000 g for 20 minutes and processed as described for immunoblot analysis.

RESULTS

Distribution of Stat4 protein in adult tissues

Although the tissue distribution of expression of Stat4 mRNA had been previously reported at the RNA level (Yamamoto et al., 1994; Zhong et al., 1994a, the expression of Stat4 protein had not been evaluated. Immunoblot analysis of cellular extracts from various tissues was carried out to determine the relative abundance of Stat4 at the protein level. Stat4 protein levels essentially paralleled those of the mRNAs: highest levels of Stat4 protein were observed in the testis, and lower levels observed in spleen and thymus (Fig. 1). Although longer exposures detected Stat4 at very low levels in lung (data not shown), no signal was seen in skeletal muscle and bone marrow in which Stat4 mRNA had been reported (Yamamoto et al., 1994). Parallel immunoblot analysis with Stat1, which was detected at high levels in spleen and thymus and at lower levels in almost all the tissues tested (Fig. 1), underscored the specificity of the Stat4 expression pattern. Stat1 protein was weakly detected in adult mouse testis (Fig. 1), and immunohisto-

![Fig. 1. Immunoblot analysis of Stat4 and Stat1 expression in various mouse tissues.](image)
Chemical analysis of its distribution revealed uniform staining among all cell types, barely distinguishable from the background (data not shown).

The Stat4 protein sometimes appeared as a doublet of 90 and 93 kDa (data not shown), similar to that detected in lymphocytes and suggested to be due to serine/threonine phosphorylations (Bacon et al., 1995). However, no unique testis-specific polypeptide was observed (Fig. 1), indicating that the testis-specific 3.6 kb mRNA transcript did not produce any obvious corresponding testis-specific Stat4 protein. Interestingly, a fairly abundant band of ~65 kDa was reproducibly detected, exclusively in immunoblots and immunoprecipitates of spleen lysates (Fig. 1 and data not shown). It is not known whether this band represents a degradation product of Stat4 or a truncated form of Stat4, but relevant to this work, it was never detected in the testis.

Fig. 2. Cellular localization of Stat4 transcripts in the testis by in situ hybridization. Hybridization was made with α-35S-labeled antisense Stat4 riboprobe, and the exposure time was 2 weeks. Sections were photographed under epiluminescence optics. (A,C-H and B) Sections from normal, and a/at mutant testes, respectively. (C-H) The spermatogenic stages of the seminiferous epithelium are indicated by Roman numerals. Cell types are designated as follows: Leydig cells (lc), Sertoli cells (sc), peritubular myoid cells (mc), round spermatids (rs), elongated spermatids (es), pachytene spermatocytes (ps), spermatagonia (sg), diplotene spermatocytes (ds), dividing secondary spermatocytes (ss). Bars, 100 μm (A,B); 50 μm (C-H).
Presence of Stat4 in the perinuclear theca

Cellular localization of Stat4 expression in mouse testis

Although Stat4 mRNA and proteins were shown to be abundantly expressed in the testis, the cellular localization of this expression was not known. To more precisely determine the cellular localization of the Stat4 transcripts in adult mouse testis, in situ hybridization analysis was performed on histological sections of testes from normal and germ cell-deficient mutant strains of mice (discussed by Wolgemuth and Watrin, 1991). As evidenced in Fig. 2A and B, Stat4 transcripts were detected in all tubules of normal adult testes, while no hybridization above the background level was observed in the germ cell-deficient testes, indicating a germ cell expression of Stat4 mRNA.

The developmental program of spermatogenesis in the adult mouse testis has been well characterized at the histological level. Spermatogonia, the stem cells, are located at the periphery of the tubules, while more differentiated cells are located towards the lumen. Since male germ cells develop in a temporally defined progression along the tubule, there exists a characteristic association of germ cells in particular stages of spermatogenesis. In the mouse, this progression of the cycle of the seminiferous epithelium had been divided into twelve

Fig. 3. Immunoblot analysis of Stat4 expression in prepuberal and adult mouse testes. Samples contained 50 μg of total protein obtained from post natal day 7, day 17, and adult testis lysates (see Materials and Methods). The sizes of the proteins markers (kDa) are indicated on the right side of the figure, and the arrow on the left side specifies the position of the Stat4 protein

Fig. 4. Immunohistochemical localization of Stat4 protein in paraformaldehyde-fixed testis sections. Brown staining was generated using a DAB-peroxidase immuno-detection, and testis sections were counter-stained with hematoxylin, which stains the nucleus blue. The spermatogenic stages of the seminiferous epithelium are indicated by roman numerals. Cell types are designated as follows: round spermatids (rs), elongated spermatids (es), pachytene spermatocytes (ps). Bar, 50 μm.
stages, each with a characteristic set of spermatogenic cells in association with one another (Oakberg, 1956; Russell et al., 1990). Determination of the stage of a specific tubule allows for more precise identification of the spermatogenic cells present in the tubules.

Approximately 100 tubules from each of 5 experiments were staged and examined for the expression of Stat4 mRNA. Our results clearly showed that Stat4 transcripts were localized to germ cells in the middle layer of tubules at stages ~IV (Fig. 2D) to VIII (Fig. 2E) and in the layers of cells lining the lumen at stages IX (Fig. 2F), XI (Fig. 2G), XII (Fig. 2H), I (Fig. 2C), and terminating at stage ~IV (Fig. 2D). These cells were identified as spermatids. The strongest signal was restricted to the transition stages, VIII (Fig. 2E) and IX-XI (Fig. 2F and G), when the late round spermatids differentiate into early elongating spermatids. No signal above background was observed in either more peripheral germ cells, such as spermatogonia and spermatocytes, or in intra- or extratubular somatic cell types. These results demonstrated that expression of Stat4 mRNA is restricted to the post-meiotic haploid germ cells, from the mid-round spermatid to the elongating spermatid stages. The specificity of signal was further reinforced by the very low background observed in controls with the sense probe (data not shown).

A typical feature of postnatal testis development in animals such as mouse, rat or human, is the progressive appearance of increasingly differentiated germ cells, in addition to the somatic and spermatogonial stem cells already present at the time of birth (Nebel et al., 1961; Bellve et al., 1977; Wolgemuth and Watrin, 1991). This progression of spermatogenic differentiation in prepuberal testes allows for the enrichment or elimination of particular types of germ cells. Immunoblot analysis of protein extracts from day 7, day 17 and adult testes indicated that Stat4 was only detected in the adult samples (Fig. 3). The earliest age at which low amounts of Stat4 were detected was 20 days, when germ cells have just entered the haploid stage (data not shown). These results indicated that Stat4 is expressed most abundantly in postmeiotic germ cells, in agreement with the RNA analysis.

The anti-Stat4 antibody was also used to localize Stat4 protein in histological sections of adult mouse testis. DAB peroxidase reaction product was detected in the same cell types in which the mRNA had been localized by in situ hybridization, from the mid-round (at stage IV) to elongating spermatid stages (Fig. 4A-F). Darkest staining was consistently localized in germ cells at the transition from round to elongating spermatid stages (Fig. 4D-F).

Interestingly, in all sections observed, staining was detected in both the cytoplasm and nucleus. Since nuclear localization of STAT proteins has been linked to their initial phosphorylation/activation, the presence of Stat4 in the nuclear compartment in round spermatids was examined in detail (Fig. 4A-D). Stat4 protein was first detected in round spermatids in stage IV tubules, apparently equally distributed in the nucleus and the cytoplasm (Fig. 4B). Staining intensity progressively and uniformly increased in the round spermatids as they differentiated into elongating spermatids (Fig. 4C-D). Careful examination of cells at different stages did not reveal any obvious nuclear translocation of a cytoplasmic pool of Stat4 protein at any specific step of round spermatid development. As it is a tyrosine-phosphorylated form of Stat4 that is thought to localize to the nucleus, we attempted to test for the presence of phosphorylated tyrosines. Although our data are inconclusive, neither the 4G10 (UBI, NY) nor PY20 (Santa Cruz, CA) anti-phosphotyrosine antibodies were able to detect tyrosine-phosphorylated Stat4 in immunoblot analysis of nuclear extracts, in anti-Stat4 immunoprecipitates, or in immunoprecipitates of whole testis lysates (data not shown). However, in control experiments using vanadate-activated splenocytes (in which the inhibition of phosphatase activity induced a burst of tyrosine phosphorylation in these cells), we were able to detect tyrosine-phosphorylated proteins, in this case, Stat1 (data not shown).

In experiments evaluating conditions for immunohistochemical analyses, we noted that the use of a non-cross linker fixative (Histochoice) provided excellent resolution of Stat4 cytolocalization, although the efficacy of antibody recognition of Stat4 was less robust under these conditions and the cellular morphology was less well preserved. Seminiferous tubules from Histochoice-fixed testis exhibited the same pattern of expression of Stat4 in round and elongating spermatids, as did paraformaldehyde-fixed specimens, and the staining was similarly observed in both cytoplasmic and nuclear compartments.

However, the intracellular localization of Stat4 did not appear as homogeneous as previously observed in paraformaldehyde-fixed sections. In round spermatids, DAB staining was concentrated in an intranuclear structure that resembled the nucleolus, as well as in the developing acrosome, visualized as a characteristic ~120 degree arc bordering the nuclei of round spermatids from stages VII-VIII (Fig. 5A). When elongating spermatids from stage I tubules line the edge of the lumen, the intranuclear staining disappeared and the darkest staining followed the discrete comma-like shape of the condensing nuclei (Fig. 5B). When sections of tubules from successive stages are compared (Fig. 5C, stages I-XI), the progressive change of shape of the perinuclear staining and its early acrosome-like formation appeared to be characteristic of the perinuclear theca, a thin and dense structure enclosed between the nucleus and the acrosome of the developing spermatids and spermatooza. Staining of the perinuclear theca from elongating spermatids decreased in intensity in stages II-III to IV, and was no longer detected around the dense nuclei of late spermatids about to be released in the lumen (Fig. 5C, compare elongating spermatids from stages II-III and VIII). Previous studies in mouse (Bellve et al., 1990) and rat (Oko, 1995) have described similar patterns for the localization of other thecal proteins. Stat4 was also detected in the residual bodies, which result from the elimination of the remaining cytoplasm of the maturing male gamete (Fig. 5A and C, stage VIII).

**Immunoblot analysis of total and fractionated proteins of mouse sperm**

Since elongating spermatids at the latest stages of differentiation did not exhibit immunohistochemical localization of Stat4 in the presumed perinuclear theca, it was important to determine if Stat4 protein was still present in these cells. Since the absence of detection of Stat4 in the theca of late elongating spermatids and terminally differentiated spermatooza could be explained by the inaccessibility of the antibody to an antigenic motif deeply enclosed in this extremely compact
structure, known to be highly resistant to detergents (Bellve et al., 1993), the presence of Stat4 was directly examined in total extracts of sperm cells isolated from the epididymis. Immunoblot analysis of total lysate from epididymal sperm revealed a band at 90 kDa, co-migrating with the testicular Stat4 band (Fig. 6A). Stat4 was clearly much less abundant in sperm compared to total testis extracts (Fig. 6A).

To confirm that Stat4 was indeed present in the sperm theca, and not due to the potential presence of lymphocytes and/or spermatids, which are usual contaminants of ejaculated sperm, sperm proteins were sequentially extracted by submitting epididymal sperm to increasingly robust solubilizing and denaturing conditions. This procedure allows for the progressive isolation of proteins from more and more internal structures of the sperm cell (Bellve et al., 1993; see Materials and Methods; outlined in Fig. 6B). When the four fractions so obtained were tested for the presence of Stat4 protein by immunoblot analysis, a 90 kDa band was only detected in fraction 3 (Fig. 6C, fraction 3), corresponding to the proteins composing the perinuclear theca. Furthermore, the Stat4 protein was still detected as a tight doublet, as previously observed in testis, spleen and thymus extracts (Fig. 1 and data not shown). Since no signal was observed in either fraction 1 and 2 (Fig. 6C), the possibility that the presence of Stat4 in sperm extracts was due to contaminating cells is highly unlikely, as these cells should have been totally lysed by the 1% SDS. The absence of Stat4 in the nuclear fraction (Fig. 6C, fraction 4) also suggested that Stat4 is eliminated from the intranuclear compartment during the DNA compaction process and no longer present in the dense sperm nucleus.

DISCUSSION

It has been hypothesized that the Stat gene family arose via the tandem duplication of an ancestral gene, followed by the duplication of the original pair of genes on two other chromosomes (Copeland et al., 1995; Ihle, 1996). The Stat1 and Stat4 genes co-segregate and have been mapped to the proximal region of chromosome 1 (Yamamoto et al., 1994; Copeland et al., 1995). They are ~50% similar at the amino acid level, making them more closely related to one another than the other Stat pairs (compared to 19% for Stat2/Stat6, and 24% for Stat3/Stat5) (Yamamoto et al., 1994; Copeland et al., 1994). The very restricted pattern of expression of Stat4 (e.g. testis, spleen, thymus) contrasts with the nearly ubiquitous expression of Stat1 in many tissues (Fig. 1) and cell lines (Schindler and Darnell, 1995) examined. Furthermore, in the testis, Stat4 exhibited a high level of expression in a restricted population of cells. Results from immunoblot, in situ hybridization, and immunohistochemistry analysis clearly revealed the presence of Stat4 in male germ cells, from the mid-round to the early elongating spermatid stages. At these stages, the male germ cells are haploid and undergo dramatic morphological modifications, including assembly of the tail, formation of the acrosome, and the remodeling and condensation of the nucleus.

Previous studies (Zhong et al., 1994a,b; Yamamoto et al., 1994) had demonstrated the existence of an additional Stat4 transcript of 3.6 kb in testis, while the 3.3 kb transcript was also expressed in spleen, thymus, lung, bone marrow and skeletal muscle. A tight doublet of apparent molecular mass of 90 and 93 kDa was detected with the anti-Stat4 antibody, with only quantitative differences observed among positive tissues, similar to what was also found in lymphocyte culture (Bacon et al., 1995). Thus, we have no evidence for the existence of a testis-specific form of Stat4 protein, as might have been predicted by the presence of a longer transcript.

In spleen and thymus, Stat4 is likely to be linked to the IL-12 signaling pathway in T lymphocytes (Bacon et al., 1995; Jacobson et al., 1995). Immunohistochemical analysis of Stat4 (as well as Stat1) protein distribution in adult spleen sections has shown staining in a few cells, mostly scattered in areas surrounding the oval-shaped B cell follicles (data not shown), consistent with an expression more frequent in T compared to B cells (Bette et al., 1993). We initially hypothesized that Stat4 might be similarly activated during spermiogenesis, and potentially by IL-12, the only cytokine shown to induce the phosphorylation of Stat4 in lymphocytes. However, we have been unable to detect expression of genes involved in the IL-12 signaling pathway, including the β-subunit of the IL-12 receptor (Chua et al., 1995) or the p35 and p40 subunits of IL-12 (Schoenhaut et al., 1992), by RNA blot analysis using poly(A) RNA fractions from total testis. In contrast, thymus and spleen samples exhibited expression of these genes (data not shown). Although conclusive evidence regarding the lack of the IL-12 pathway in the testis may require more sensitive detection procedures, it is not likely to be a major activator.

It is generally held that STAT protein activation involves phosphorylation/dimerization of STATs and subsequent translocation into the nucleus. However, our experiments using anti-phosphotyrosine antibodies suggested that testicular Stat4 protein was not phosphorylated (data not shown), although immunohistochemical (Fig. 4) and immunoblot (data not shown) analyses clearly indicated an abundant nuclear localization at the earliest stage of expression of Stat4. These results are in marked contrast to observations on STAT protein activation in cell cultures (Ihle, 1995; Schindler and Darnell, 1995). The kinetics of activation of STAT proteins have been reported to be extremely rapid, usually within 5 minutes. Once phosphorylated, dimerized and translocated into the nucleus, STAT proteins are dephosphorylated within a few hours, still remaining nuclear (Shuai et al., 1992). In comparison, spermiogenesis, which corresponds to all haploid stages, lasts ~14 days in the mouse (Hecht, 1986). If Stat4 is activated during spermiogenesis with kinetics similar to those observed in cultured cells, spermatids containing tyrosine-phosphorylated Stat4 may represent only a very small proportion of the total population (e.g. <3% if one considers a ratio between a maximum of 10 hours of activation and 14 days of spermiogenesis). This comparison underscores the difficulty in extrapolating the response to cytokines (or other activators) in homogeneous and synchronized cell cultures with tissues in vivo.

One of the more intriguing results of this study was the detection of Stat4 transcription factor in spermatids and, especially, in spermatozoa. A noted property of these cells is a highly compacted nucleus which is transcriptionally inactive. The high level of DNA compaction, organized through the replacement of the histones by the protamines, impedes RNA synthesis at the earliest stage of elongating spermatids (Hecht, 1986). Our results clearly indicated that Stat4 specifically localized at highest levels in the perinuclear theca of elongating spermatids and mature spermatozoa (Fig. 5). Parallel
immunohistochemical analysis of the expression of a putative transcription factor similarly present in late round and early elongating spermatids, the zinc finger protein Zfp-37 (Burke and Wolgemuth, 1992), did not reveal any signal at the level of the perinuclear theca (G. Herrada and D. J. Wolgemuth, unpublished results). Localization in the perinuclear theca does not then appear to be a common fate for transcription factors expressed in spermatids.

The perinuclear theca, described as a rigid capsule lying between the nucleus and the acrosome inner membrane, has been divided into two continuous regions, the subacrosomal layer (perforatorium in mouse and rat), and the postacrosomal sheath (or dense lamina), which are immunologically distinguishable (Oko, 1995). Our immunohistochemical results (Fig. 5) suggested that, at the latest stages at which it is detected, Stat4 is present along the full length of the nuclei of elongating spermatids. To date, the role of the perinuclear theca is largely unknown, although its particular localization, sandwiched between the acrosome and the nucleus, together with its remarkable resistance to solubilization in protein-denaturing agents, has suggested a role in the maintenance of the structure of the acrosome-nucleus (Bellve et al., 1990; Oko, 1995).

Only a limited number of thecal proteins have been identified. Among the better characterized are the rat PERF 15 protein, which shares similarities to a family of lipid binding proteins (Oko and Morales, 1994), the mouse thecins (Bellve et al., 1990), and a group of abundant insoluble basic proteins, including cyclin I and II, described in bovine and human sperm (Longo et al., 1987; Hess et al., 1993, 1995). The
presence of calmodulin has also been reported in the sperm theca of several mammals (Kann et al., 1991). In round spermatids from rat testis, thecal proteins have been immunologically detected in both cytoplasmic and nuclear compartments, and at the highest level along the developing acrosome (Oko, 1995), as characteristically observed for Stat4 in mouse testis (Fig. 6). At the later stages, theca protein-staining concentrated in the forming periforatorium and correlated with the loss of staining over the nuclei (Oko and Clermont, 1991). The presence of Stat4 in nuclei may thus result from the normal processing of a thecal protein, and not from its activation by phosphorylation.

The presence of a potential transcription factor such as Stat4 in the perinuclear theca may suggest that this structure has other than a purely structural role. In most mammals, and particularly in mouse, ultrastructural studies and tracking of specific sperm antigens of newly fertilized eggs have shown that in the hours following the acrosome reaction, the whole remaining sperm body is progressively incorporated into the cytoplasm (Oura and Toshimori, 1990), including the sperm tail (Simerly et al., 1993). The perinuclear theca separates from the decondensing nucleus, releasing a ‘V’-shaped structure which appears associated with numerous vesicles forming in the adjacent ooplasm (Stefanini et al., 1969). A similar release of globular and amorphous materials had been observed over the entire postacrosomal nuclear surface of hamster sperm heads at an early stage of incorporation into the egg (Usui, 1996). Our experiments have shown that in vitro reduction of the perinuclear theca can extract the Stat4 protein without any obvious proteolytic cleavage (Fig. 6). In vivo, such a reducing power is present in the ooplasm of the fertilized oocyte and is absolutely required for the decondensation of the sperm nucleus into a male pronucleus (Perreault et al., 1984). It is therefore very likely that the Stat4 transcription factor, enclosed in the sperm head, is internalized within the oocyte at the time of fertilization.

At both the RNA (Yamamoto et al., 1994) and protein levels (Fig. 1), Stat4 expression was not detected in ovary. We therefore hypothesized that Stat4 could represent an original contribution of the sperm to the pool of transcription factors in the newly formed egg. Such a hypothesis is supported by recent findings concerning the SPE-11 protein in C. elegans (Browning and Strome, 1996). SPE-11, which is a protein specifically present in male germ cells and sperm, has been demonstrated to be an essential factor allowing for the first mitosis of the newly formed egg. The reported localization of SPE-11 is particularly interesting since this protein is concentrated in the perinuclear region of the sperm. These data suggest, first, that information provided by the spermatozoon to the oocyte may also, in some species, contain some paternal factor(s) of proteinaceous nature and, second, that proteins from sperm perinuclear structures can be released in a functional state into the cytoplasm of the egg. Another similarity between Stat4 and SPE-11 is that they both localize in the nuclear compartment of developing germ cells before nuclei condensation (Browning and Strome, 1996). It is interesting to note that SPE-11 was no longer detectable after the sperm had penetrated the oocyte, apparently because of the very small amount of protein contributed by the theca of a single sperm. Similarly, our attempts to date to immunologically detect Stat4 in fertilized eggs have not been successful.

The next important step will be to elucidate whether Stat4 can act as a transcription factor during early embryonic development. Various tyrosine kinases which have been detected in the oocyte and/or the egg, such as c-kit (Horie et al., 1991), the EGF/TGF-α receptor (reviewed by Wiley et al., 1995), and the oncogene c-abl (Iwao et al., 1993), may represent potential candidates for the phosphorylation of Stat4. The influence of several growth factors and cytokines on embryo development has been extensively described (reviewed by Adamson, 1993; Giudice and Saleh, 1995). In vitro deprivation of most of these factors or the disruption of their signaling pathway has not, however, revealed any dramatic effects on the early steps of embryo development, suggesting a high degree of redundancy among these stimulatory signals, as generally thought for the cytokine family (Tanigushi, 1995).

The recent reports of disruption of the Stat4 gene have shown an impaired IL-12 response in T cells, but not sterility (Thierfelder et al., 1996; Kaplan et al., 1996). While these results indicated that Stat4 is not absolutely essential for either spermatogenesis or preimplantation embryo development, one cannot rule out more subtle effects on these processes. It is
interesting to compare these observations with the results of recent disruptions of the Stat1 and Stat6 genes. That is, despite their expression and activation in a broad variety of tissues and cell lines, the Stat1 and Stat6 proteins appear to play a crucial role in only a limited number of cell types (Durbin et al., 1996; Meraz et al., 1996; Takeda et al., 1996; Shimoda et al., 1996). Similarly, a role for Stat4 in the activation of transcription and/or cell division in the early embryo should not be excluded.

We thank Dr James Ihle for providing the murine cDNA clone for Stat4 and Dr Chris Schindler for the anti-Stat1 antibody. We also thank Dr Ihle and Dr Anthony Bellve for stimulating discussions and very helpful comments on this work, as well as Dr Schindler and Bellve for critical reading of the manuscript. We are grateful to Xiangyuan Wang for technical assistance. G.H. was supported by postdoctoral fellowships from the French Association pour la Recherche sur le Cancer and from The Lilaror Foundation. This work was also supported in part by a grant from the NIH, R01 HD18122 to D.J.W.

REFERENCES


Nebel, B. R., Amarose, A. P. and Hackett, E. M. (1961). Calendar of


(Received 18 February 1997 – Accepted 9 May 1997)