Phosphorylation of Rat Spermatidal Protein TP2 by Sperm-specific Protein Kinase A and Modulation of Its Transport into the Haploid Nucleus*

Kolthur S. Ullas and Manchanahalli R. S. Rao‡

From the Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Transition protein 2 (TP2), which is expressed during stages 12–15 of mammalian spermiogenesis, has been shown to undergo phosphorylation immediately after its synthesis. We reported earlier that TP2 is phosphorylated *in vitro* at threonine 101 and serine 109 by the salt extract of sonication-resistant (elongating and elongated) spermatid nuclei and the protein kinase phosphorylating TP2 was identified to be protein kinase A (PKA). We now report that the cytosol from haploid spermatids but not from premeiotic germ cells is able to phosphorylate recombinant TP2 *in vitro* at threonine 101 and serine 109. The kinase present in the haploid spermatid cytosol that phosphorylates TP2 has been identified to be the sperm-specific isoform of protein kinase A (Cs-PKA). Reverse transcription-PCR analysis indicated that Cs-PKA was present in the haploid spermatids and absent from premeiotic germ cells. The rat Cs-PKA transcript was amplified and sequenced using the isoform-specific primers. The sequence of rat Cs-PKA at the N terminus differs from mouse and human by one amino acid. Western blot analysis using specific anti-Cα1 antibodies revealed that Cα1-PKA is absent in haploid spermatid cytosol. We have also established an *in vitro* nuclear transport assay for the haploid round spermatids. Using this assay, we have found that the cytoplasmic factors and ATP are absolutely essential for translocation of TP2 into the nucleus. Phosphorylation was found to positively modulate the NLS dependent import of TP2 into the nucleus.

The process of spermiogenesis in mammals, wherein the haploid round spermatids mature into highly condensed spermatozoa, can be broadly divided into three phases. In the first phase, encompassing stages 1–10, the round spermatids are transcriptionally active and contain nucleosomal chromatin. The second phase (stages 12–15) involves the replacement of nucleosomal histones by transition proteins TP1, TP2, and TP4.[1] Finally, in the third phase, the transition proteins are replaced by protamines P1 and P2 during stages 16–19 (1, 2). It is quite interesting to note that the transformation of nucle-

ohistone type of chromatin into nucleoprotamine fiber is not complete, and ~10–15% of sperm chromatin retains nucleosomal histones, probably in the context of nucleosomal structure (3–7). These unexpected observations have raised several interesting questions as to the nature of chromatin domains, which resist this replacement process, and the possible significance of the chromatin domains that retain nucleosomal structure during early embryonic development. Over the last several years, we have been studying the DNA binding and condensing properties of the spermatid protein TP2 and have described in detail its molecular anatomy with respect to the nature and identity of novel zinc finger domains, the nuclear localization signal, and also the role of phosphorylation in DNA condensation (8, 9). Based on our earlier observations, we have proposed a model describing the possible sequence of events from the time of TP2 synthesis in the cytoplasm and subsequent initiation of chromatin condensation upon deposition on chromatin, implicating distinct roles for the phosphorylation event and zinc finger domains (9). According to this model, phosphorylation of TP2 at its C-terminal basic domain prevents the premature condensation of DNA, which allows the protein to scan the entire genome and bind to GC-rich sequences. Subsequent dephosphorylation at the basic domain leads to the initiation of chromatin condensation.

Meistrich's group has generated knockout mice for both TP1 and TP2, and they have observed abnormal chromatin condensation in sperm isolated from the knockout mice (10, 11). More recently, they have also generated TP1$^{-/-}$/TP2$^{-/-}$ double-knockout mice, which are infertile.[2] These phenotypes of the knockout mice stress the biological importance of the two proteins in mammalian spermatogenesis, particularly in the global chromatin remodeling process. In our previous study, we mapped, by site-directed mutagenesis, the major phosphorylation sites in TP2 to Thr-101 and Ser-109 in the C-terminal third of the protein, which were shown to be phosphorylated by protein kinase A (8). These experiments were done using the high salt extract of the sonication-resistant spermatid nuclei, and the involvement of protein kinase A was further demonstrated by *in vitro* experiments employing recombinant TP2 and its phosphorylation-site mutants and purified commercial protein kinase A. The PKA holoenzyme consists of two catalytic subunits bound to two regulatory subunits (R_2C_2). Three genes encode different isoforms of somatic catalytic subunits. They are 1) Cα, which is ubiquitously expressed in most of the tissues (11–13), 2) Cβ, expressed at lower levels than Cα in many tissues (11, 13), and 3) Cγ, which is a transcribed transposon and is expressed only in primate testis (14, 15). More recently, a sperm-specific isoform of Cα, termed Cs-PKA, has been reported (16). Green *et al.* (17) showed that when the testicular tubules were labeled with carrier-free [^{32}P]ortho-

‡ To whom correspondence should be addressed: Prof. M. R. S. Rao, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India. Tel.: 91-80-2932547; Fax: 91-80-3000118; E-mail: mrsrao@biochem.iisc.ernet.in.

[1] The abbreviations used are: TP, transition protein; PKA, protein kinase A; Cs-PKA, spermatid-specific PKA isoform; Cα1-PKA, somatic PKA isoform; FACS, fluorescence-activated cell sorting; GST-2K, GST-2K, glutathione *S*-transferase with PKA recognition site; PKI, cAMP-dependent protein kinase inhibitor peptide; NLS, nuclear localization signal; SV40, simian virus 40.

[2] M. L. Meistrich, personal communication.

phosphate, the radioactivity associated with TP2 was much higher in the whole cell extract than that associated with TP2 in sonication-resistant nuclei. They interpreted this observation to suggest that TP2 undergoes phosphorylation immediately after its synthesis in the cytoplasm. With this observation in mind, we were curious to examine the nature of the protein kinase present in the round spermatid cytoplasm that is involved in TP2 phosphorylation. In the present study, we investigated this question and demonstrated that the recently discovered sperm-specific isoform of the catalytic subunit of protein kinase A (Cs-PKA) is involved in the phosphorylation of TP2. Because the phosphorylation sites happen to be near the nuclear localization signal of TP2, we also examined the effect of phosphorylation on its import into the nucleus and found that the transport is modulated by phosphorylation but is not required for its import into the nucleus.

EXPERIMENTAL PROCEDURES

Preparation of Round Spermatid Cytosol Extract—Haploid round spermatids were isolated from adult testicular cells of Wistar rats (60 days old) by the technique of centrifugal elutriation as we have described previously (18). The isolated cells were washed in ice-cold phosphate-buffered saline, and an aliquot was fixed with ice-cold 70% ethanol and stained with ethidium bromide (25 μg/ml) to evaluate the purity by FACS analysis. Cytosol was prepared from round spermatids and also from the total testicular cells of 25- and 35-day-old rats by lysing the cells in hypotonic buffer containing 0.75 mM magnesium acetate, 0.15 mM EGTA, and 3 mM dithiothreitol in 5 mM HEPES buffer, pH 7.3, supplemented with protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, bestatin, leupeptin, pepstatin A, and aprotinin; 1 μg/ml; Sigma) followed by ultracentrifugation at 100,000 × *g* for 90 min at 4 °C. The cytosol was concentrated by ultrafiltration to yield a protein concentration of 25–30 μg/ml, and aliquots were stored at −70 °C.

In Vitro and In Gel Kinase Assay—Approximately 6–8 μg of cytosolic proteins and 3 μg of recombinant TP2 or GST-2K (containing the PKA site) were incubated in a buffer containing 20 mM Tris-Cl, pH 7.5, 20 mM NaCl, 1 mM benzamidine, 10 mM MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, and 10 μM zinc sulfate for 30 min at 37 °C. 3–5 μCi of [γ-^{32}P]ATP was included in all the reactions. For inhibition assays, increasing amounts of PKI peptide (Sigma) was preincubated with cytosol before the addition of protein substrate. The reaction was stopped by the addition of 2× Laemmli SDS-PAGE buffer and heating at 95 °C for 5 min. The samples were then run on a 15% SDS-PAGE, stained with Coomassie brilliant blue, destained, dried, and subjected to autoradiography.

In gel kinase assay was performed as described previously (19, 20) with minor modifications. Briefly, about 35–40 μg of cytosolic protein from round spermatids was run on a 12% SDS-PAGE with the resolving gel containing 0.02% recombinant TP2. After the electrophoretic run, the gel was washed with 50 mM Tris-Cl, pH.8.0, containing 20% propanol and the proteins were subsequently denatured in 50 mM Tris.Cl, pH 8.0, containing 1 mM dithiothreitol and 6 M guanidinium hydrochloride for 60 min. Proteins were then slowly renatured over a period of 12–16 h with 8–10 changes in a buffer containing 50 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, and 0.05% Tween 20. The gel was equilibrated in 1× kinase buffer for 20 min at room temperature and then incubated in the same buffer containing 10–20 μCi of [γ-^{32}P]ATP. After washing with buffer to remove unincorporated ATP, the gel was dried and subjected to autoradiography. Cytosolic proteins run on a gel without TP2 served as a negative control.

Western Blot Analysis with Anti-Cα PKA Antibodies and Analysis of Cα 1 and Cs-PKA Isoforms by RT-PCR—Cytosolic preparations (6–8 μg protein) from round spermatids and testicular cells from 25- and 35-day-old rats were run on a 12% SDS-PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane. The blotted proteins were probed using anti-Cα-PKA antibodies (Calbiochem) raised against an N-terminal peptide (residues 7 to 21). Anti-rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody with diaminobenzamidine as the substrate. For RT-PCR analysis, total RNA was isolated from round spermatids and testicular cells of 25- and 35-day-old rats using the TRIzol (Invitrogen) method. Reverse transcription was carried out using the common reverse primer for both the isoforms Cα-1 and Cs-PKA, 5′-ACT TTG GTA TGA AGG CTT CCA CCT-3′, and the PCR amplification was done using the isoform-specific forward primers (5′-

AACGCCGCCGCCGCCAAGAA-3′ for Cα1-PKA and 5′-GTTCTATCTG-CCCCTACCCTGC-3′ for Cs-PKA) and the common reverse primer as described by San Agustin *et al.* (16). To obtain 5′ end sequences of rat Cs-PKA, 5′-GACTGAGTGATGGCTTCCA-3′ was used as the forward primer and 5′-CCTTGAAGGAGAATTCAAGTTTG-3′ as the nested reverse primer. The DNA sequences of the amplicons were determined in an ABI 377 automated DNA sequencer (Applied Biosystems).

In Vitro Nuclear Transport—Intracellular transport of exogenously added recombinant TP2 *in vitro* with digitonin-permeabilized cells was studied according to the method of Adam *et al.* (21) with minor modifications. Briefly, the round spermatids obtained after centrifugal elutriation were washed with ice-cold transport buffer, 20 mM HEPES, pH 7.3, 100 mM potassium acetate, 5 mM potassium acetate, 2 mM dithiothreitol, 1 mM EGTA and protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatinA, E-64, bestatin, leupeptin, and aprotinin; 1 μg/ml; Sigma). The cells were then permeabilized in transport buffer containing 40 μg/ml digitonin (Sigma) at 0 °C for 5 min and subsequently washed with cold transport buffer. 1.5 μg of wild-type and phosphorylation-site mutant TP2 were used for *in vitro* transport assay. *In vitro* transport was initiated in 50 μl transport buffer containing cytosol, TP2, and 1 mg/ml BSA and supplemented with 0.5 mM ATP, 0.5 mM GTP, 5 mM creatine phosphate, and 20 units/ml creatine phosphokinase. The import reaction was allowed to proceed for 30 min at 30 °C. In the indicated experiments, import reaction was carried out in transport buffer in the presence or absence of ATP regenerating system. Nuclear import assay in cytosol without supplementing ATP regenerating system was also carried out as one of the controls. After the import reaction, the cells were washed with transport buffer, smears were prepared on glass slides, and the localization of TP2 was visualized by immunofluorescence using anti-TP2 antibody as described previously (7). Where indicated, the cytosol was preincubated with myristoylated PKI$_{(14–22)}$ inhibitor peptide (Calbiochem) for PKA inhibition in nuclear import assay.

Expression of Wild-Type TP2 and TP2-GFP Fusion Constructs in HeLa Cells—Rat TP2 cDNA was cloned into pEGFP-C2 vector (BD Biosciences Clontech) at EcoRI/ApaI sites such that TP2 was fused in frame at the C terminus of enhanced green fluorescent protein. HeLa cells were transfected with wild type TP2-cDNA or the fusion GFP-TP2 construct using LipofectAMINE PLUS reagent (Invitrogen) according to the protocol supplied by the manufacturer. HeLa cells were grown on cover slips in a 24-well plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO$_2$ 24 h after transfection, the cells were washed with 1× Dulbecco's phosphate-buffered saline (Sigma) and fixed with 4% paraformaldehyde. Expressed proteins were visualized in an Axio vision (Carl Zeiss) fluorescence microscope.

Immunolocalization of Nucleolin—Localization pattern of nucleolin in HeLa cells grown on cover slips and elutriated round spermatids was studied by immunofluorescence, using anti-nucleolin (C23) antibodies (Santa Cruz Biotechnology). Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1%Triton X-100, blocked with phosphate-buffered saline containing fetal bovine serum (1%) and probed with anti-nucleolin antibodies. Donkey anti-goat IgG conjugated with rhodamine was used as the secondary antibody.

RESULTS

Phosphorylation of TP2 by Round Spermatid Cytosol—We have shown previously that rat TP2 is phosphorylated *in vivo* by metabolic labeling with [^{32}P]orthophosphate and *in vitro* by the protein kinase A present in the high salt extract of sonication-resistant spermatid nuclei (8). The PKA phosphorylation sites were localized to the C-terminal third of the molecule and were identified to be Thr-101 and Ser-109, among which Ser-109 happens to be the predominant site (Fig. 1). Sonication-resistant spermatid nuclei represent a mixture of both elongating (stages 12–15) and elongated (stages 16–19) spermatids. As mentioned earlier above, the two major basic nuclear proteins present in the elongating spermatids (stages 12–15) are TP1 and TP2 and in the elongated spermatids (stages 16–19) are protamines P1 and P2. Because the nuclei from which the salt extract was prepared contain TP2 bound to chromatin, we believed it necessary to study the phosphorylation of TP2 in the cytosol of round spermatids, particularly because TP2 is believed to undergo phosphorylation immediately after its synthesis (17). For this purpose, we have isolated the haploid round spermatids from total testicular cells by the technique of

V8 PROTEASE SITE

FIG. 1. **Schematic representation of phosphorylation sites in rat TP2.** *, phosphorylation sites at the C-terminal basic domain; glutamate at the 86th position is indicated.
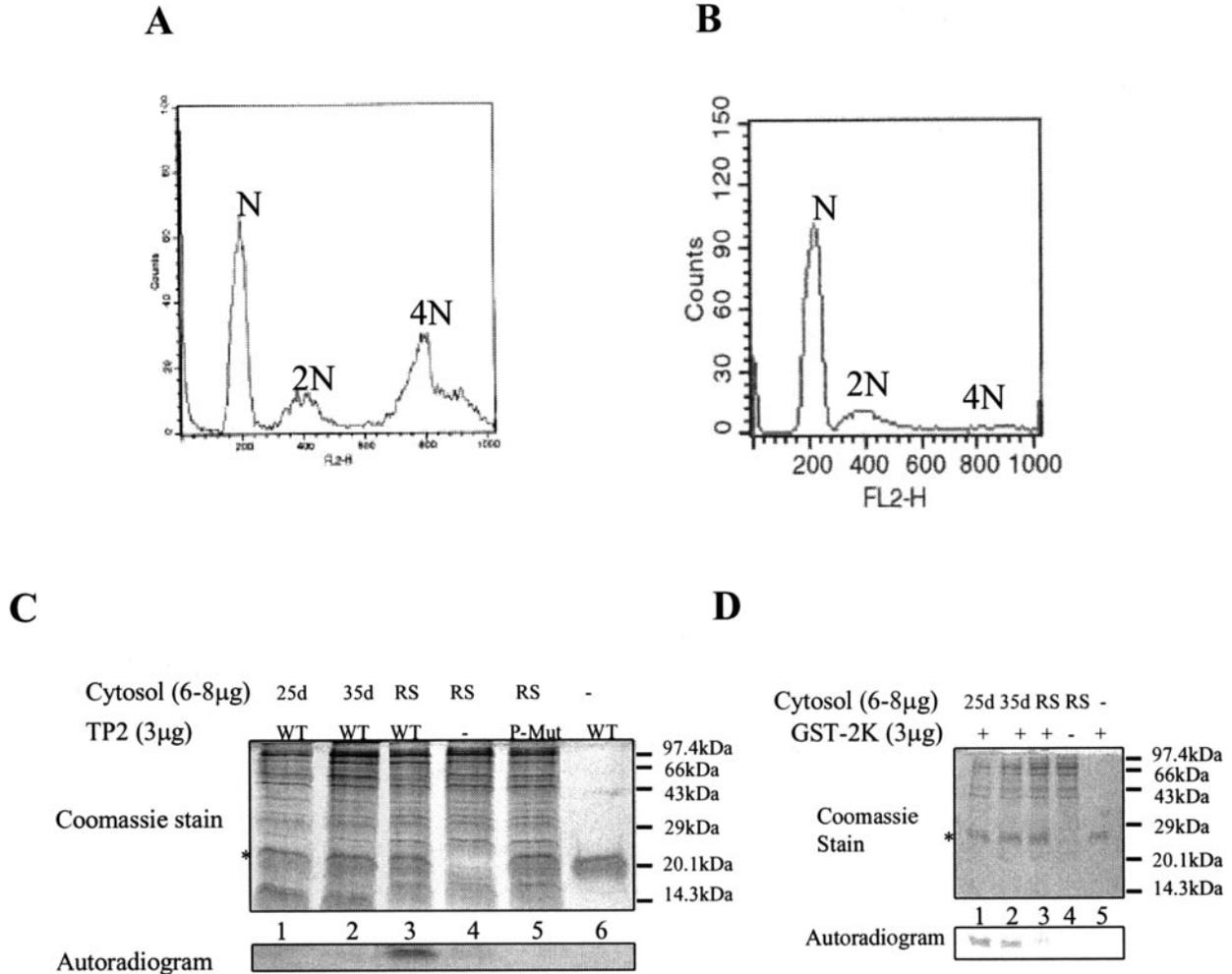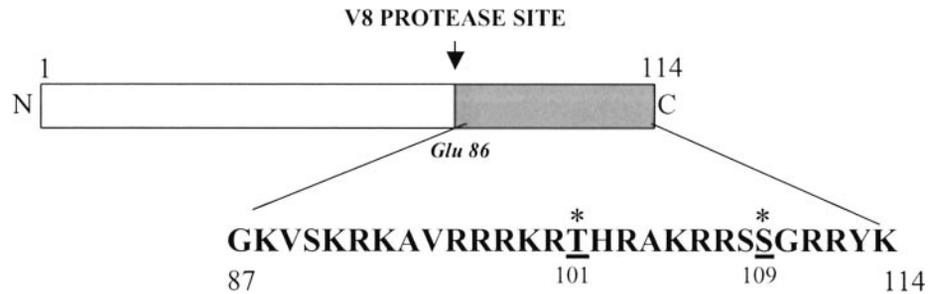
GKVSKRKAVRRRKRTHRAKRRSSGRRYK

87   101   109   114

A

B

C

D

FIG. 2. **Isolation of round spermatids and *in vitro* kinase assay.** *A*, FACS profile of total testicular cells from adult rat. *B*, FACS profile of elutriated round spermatids from adult rat. *N*, haploid cells; *2N*, diploid cells; *4N*, tetraploid cells. *C*, *in vitro* kinase assay of recombinant TP2 (3 μg) was carried out as described under "Experimental Procedures," and the samples were run on SDS-PAGE and subjected to autoradiography. The lanes indicate kinase assays performed with cytosols (6–8 μg) prepared from 25-day-old testicular cells (*lane 1*), 35-day-old total testicular cells (*lane 2*) and elutriated round spermatids (*RS*) (*lanes 3*, *4*, and *5*). *Lane 4* is the minus TP2 control, whereas *lane 5* shows phosphorylation-site mutant TP2 (T101A, S109A) used as substrate instead of the wild type TP2. *Lane 6* shows recombinant TP2 alone. *D*, *in vitro* kinase assay of GST-2K (3 μg) was performed using the cytosols (6–8 μg) prepared from 25-day-old rat total testicular cells (*lane 1*), 35-day-old rat total testicular cells (*lane 2*) and elutriated round spermatids (*RS*) (*lanes 3* and *4*). *Lane 4* is the minus GST-2K control, whereas *lane 5* shows GST-2K alone. *, TP2 and GST-2K bands in *C* and *D*, respectively. TP2, 13 kDa (with an anomalous mobility at 20–25 kDa); GST-2K, 27 kDa.

centrifugal elutriation. FACS analysis of the isolated round spermatids is shown in Fig. 2*B*. They were 92% pure; the remaining 8% was contributed by diploid (*2N*) cells. Fig. 2*A* also shows the representation of haploid, diploid, and tetraploid cells in the total testicular cells from which round spermatids were isolated. The results of *in vitro* kinase experiments with recombinant TP2 as the substrate and cytosol preparations from round spermatids and total testicular cells from 25- and 35-day-old rats are shown in Fig. 2*C*. The germ cells in the testis of 25-day-old rats represent premeiotic spermatogonia and a few early pachytene spermatocytes; those

from 35-day-old rats contain a predominant population of pachytene spermatocytes (about 30%) and premeiotic germ cells. Postmeiotic haploid round spermatids appear in the seminiferous tubules of rats at around 40 days. As can be seen from Fig. 2*C*, TP2 was phosphorylated only by the round spermatid cytosol (*lane 3*) but not by the cytosol preparation of 25- or 35-day-old rat testicular cells (*lanes 1* and 2). The figure also shows that the phosphorylation-site mutant (T101A and S109A) of TP2 is not phosphorylated by the round spermatid cytosol. Thus, it is obvious that even the round spermatid cytosol phosphorylated the same serine and threonine residues
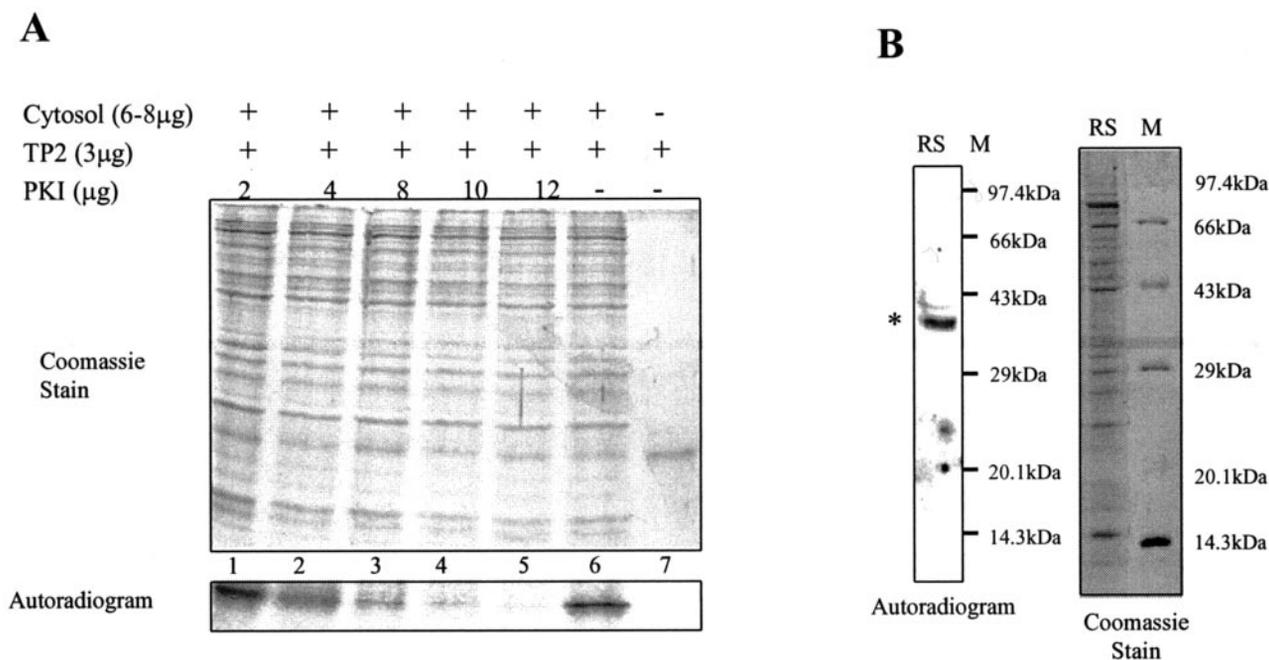
A

B

FIG. 3. **Inhibition of *in vitro* kinase assay by PKA inhibitor peptide and In-gel kinase assay.** *A*, *in vitro* kinase assay of recombinant wild type TP2 (3 μg) was carried out as described under "Experimental Procedures," and the samples were run on SDS-PAGE and subjected to autoradiography. *Lanes 1–5* represent kinase assays performed with cytosol (6–8 μg) prepared from elutriated round spermatids (*RS*) in the presence of increasing amounts of PKI peptide (specific inhibitor of the catalytic subunit of PKA). *Lane 6* indicates the assay performed in the absence of the inhibitor peptide, whereas *lane 7* shows the mobility of recombinant wild type TP2. *B*, in-gel kinase assay was carried as mentioned under "Experimental Procedures" using round spermatid cytosol (30–40 μg) as the source of kinase and recombinant, wild-type, unphosphorylated TP2 (0.02% in the resolving gel) as the substrate. The autoradiogram shows a 40-kDa band, which lights up in the *RS* lane, indicating the molecular mass of the kinase that phosphorylates TP2. *M*, protein molecular mass markers. *, covalently modified PKA.

that we had observed earlier with the salt extract of sonication-resistant spermatid nuclei. The lack of phosphorylation of TP2 by the cytosol preparation of 25- or 35-day-old rat testicular cells was rather surprising. To rule out any artifacts in our assay and also to examine whether these cytosol preparations contain PKA, we carried out similar assays using GST-2K (containing PKA site) as the substrate instead of TP2. The results presented in Fig. 2D clearly demonstrate that GST-2K is phosphorylated by all the cytosol preparations, indicating that PKA is indeed present in the cytosols of 25- and 35-day-old testicular cells but does not phosphorylate exogenously added TP2. To demonstrate that PKA is phosphorylating TP2 in the round spermatid cytosol, we carried out phosphorylation assay in the presence of increasing concentrations of PKI peptide, a specific inhibitor of PKA. As can be seen in Fig. 3A, there was a progressive inhibition of phosphorylation of TP2 with increasing concentrations of PKI peptide, thus confirming that PKA present in round spermatid cytosol is indeed phosphorylating the TP2. We also carried out in-gel kinase assay to find out the molecular size of the kinase present in the round spermatid cytosol that is phosphorylating TP2. The result of such an experiment showed in Fig. 3B reveals a doublet of around 40 kDa, which agrees with the molecular mass of known PKA isoforms. Appearance of a doublet probably suggests covalent modification of the PKA (see below).

Identification of PKA Isoform Present in Round Spermatid Cytosol—George Witman's group (16, 22) has identified and studied the expression pattern of the sperm-specific isoform of the Cs-PKA in mouse testis. Cα-1 (somatic) and Cs-PKA (sperm-specific) isoforms have a stage-specific and mutually exclusive expression pattern during spermatogenesis in the mouse. Cα-1 PKA is expressed only in somatic cells, spermatogonia, and preleptotene spermatocytes. The report by San Agustin *et al*. (22) shows that Cα1 disappears from the spermatogenic cells coincident with the onset of meiosis. Immuno-

histochemical analysis using anti-mouse Cs-PKA specific antibodies revealed that the maximal expression of Cs isoforms starts from late pachytene stage spermatocytes. Furthermore, Cs-PKA has also been shown to be present in the cytoplasm of round and elongating spermatids (22). They have also shown that Cs expression is absent in somatic cells and undifferentiated germ cells, including spermatogonia and preleptotene, leptotene, zygotene, or early pachytene spermatocytes. Cs-PKA has also been detected in human and pig. These two isoforms differ from each other in their N-terminal amino acid sequence (see Fig. 5). Because Cα1-PKA-specific antibodies are available (raised against N-terminal peptide residues 7 to 21, which does not cross react with Cs-PKA), we set out to examine the nature of PKA present in the cytosol of rat round spermatids. Fig. 4, *A* and *B*, describe the results of Western blot analysis in which it is clear that the Cα PKA specific antibodies did not recognize the PKA present in the round spermatid cytosol, but it did react with the Cα1-PKA isoform present in the cytosol of 25- and 35-day-old testicular cells. We then examined the expression pattern of Cα1-PKA and Cs-PKA isoform messenger RNA by RT-PCR analysis using isoform-specific primer sets designed at the 5′-end of their mRNAs (mouse). These results are shown in Fig. 4, *C* and *D*. The transcript corresponding to Cα1-PKA isoform (940 bp) was detected only in the testicular cells of 25- and 35-day-old rats but not in round spermatids of rat. On the other hand, the Cs-PKA isoform (1.2 kb) transcript was detected only in round spermatids. Thus, these results confirm the mutually exclusive expression pattern of Cα1 and Cs-PKA in rat germ cells that has also been reported in the case of mouse, ovine, and human (16, 22). Furthermore, these results also suggest that Cs-PKA is responsible for TP2 phosphorylation in round spermatid cytosol. The Cα1 and Cs-PKA isoforms in mouse also differ in the type of covalent modifications at their N termini (acetylation in the case of Cs-PKA and myristylation of Cα1-PKA). This N-terminal covalent modification
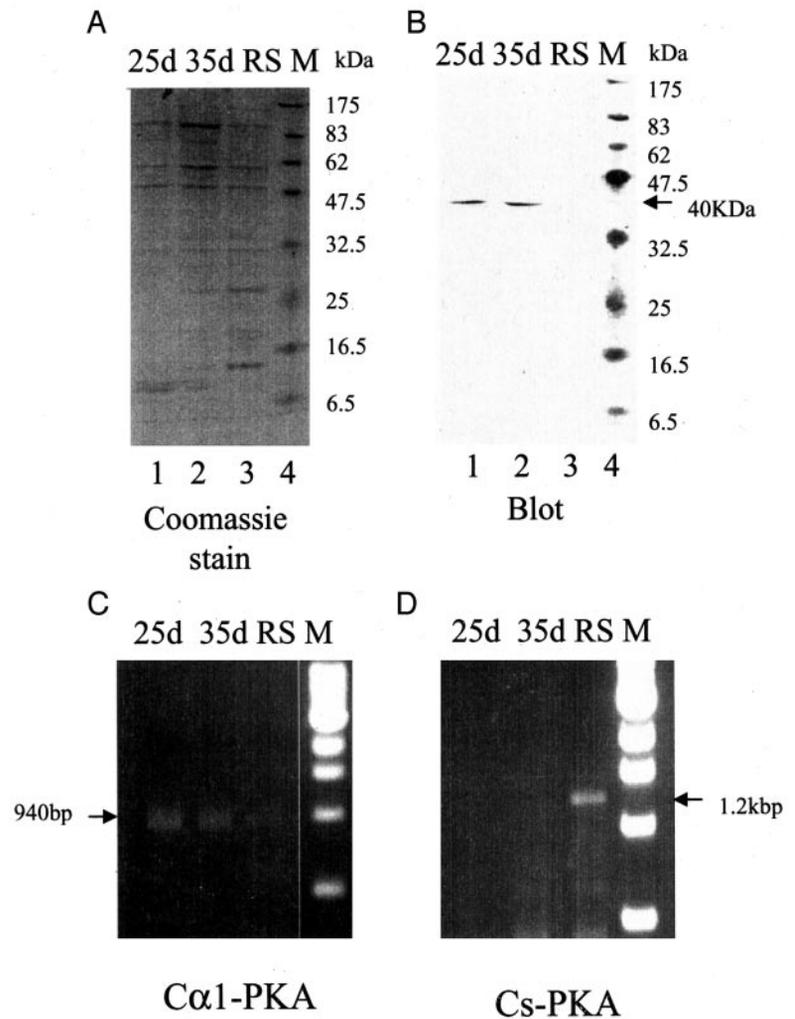
FIG. 4. **Western-blot and RT-PCR analysis of Cα 1-PKA and Cs-PKA.** Western blot analysis and RT-PCR analysis were carried out to identify the kinase involved in the phosphorylation of TP2 and to study the expression patterns of Cα1 and Cs-PKA isoforms. *A*, Coomassie blue-stained SDS-PAGE image of the cytosolic extracts (6–8 μg). *B*, Immunoblot using anti-Cα PKA antibodies. *Lane 1*, 25-day-old rat testis cytosol; *lane 2*, 35-day-old rat testis cytosol; *lane 3*, round spermatid cytosol; *lane 4*, pre-stained molecular mass markers. *C* and *D*, RT-PCR analysis. *C*, RT-PCR with Cα 1-PKA specific upstream and common downstream primers (see "Experimental Procedures") amplifying a 940-bp product. *D*, RT-PCR with Cs-PKA-specific upstream and common downstream primers amplifying a 1.2-kb product. *25d*, *35d*, and *RS* indicate the RNA samples from 25-day-old rat testis, 35-day-old rat testis, and round spermatids respectively. *M*, molecular size markers.

might explain the doublet we observed in in-gel kinase assay (Fig. 3*B*). A striking observation that can be noticed in the present study is that the recombinant TP2 was not phosphorylated by the cytosols of 25- and 35-day-old rat testicular cells even though they do contain Cα1-PKA.

We have also amplified the entire open reading frame of the rat Cs-PKA isoform and determined its complete nucleotide sequence, which has been submitted to GenBank. The sequence at the 5′ end and the derived amino acid sequence of rat Cs-PKA isoform are shown in Fig. 5 along with the corresponding sequences of mouse, ovine, and human Cs-PKA and the somatic isoform Cα1-PKA. In all of these species, the first 15 amino acids of the N terminus of Cα1−PKA are replaced by a seven-amino acid stretch in the Cs-PKA isoform. It is interesting to note that there is a single nucleotide substitution in the Cs-PKA isoform of all four species at different positions, resulting in a change of one amino acid residue. San Agustin *et al.* (16, 22) observed that antibodies against the Cs-PKA isoform of mouse (raised against residues 1 to 7) did not cross-react with the Cs-PKA isoform of ovine, probably because of this single amino acid change. They have also shown that the Cα1-PKA and Cs-PKA isoform mRNAs are generated by the mechanism of alternate transcription start sites (16), and the two diverse first exons are joined into the rest of the open reading frame. The nucleotide sequence of rat Cs-PKA and Cs-PKA of mouse, human, and pig beyond the first seven amino acids were found to be identical to that of Cα1-PKA.

In Vitro Import of TP2 into Round Spermatid Nucleus and Effect of Phosphorylation—Phosphorylation of many nuclear proteins plays an important role in their import from the cytoplasm, particularly modulating the function of NLS (23–26). We have identified previously that the NLS sequence of TP2 resides between the residues 87 and 95 belonging to the class of monopartite module that is present in SV40 T antigen. Because phosphorylation by protein kinase Cs-PKA isoform takes place on Thr-101 and Ser-109, which are 10 residues immediately downstream of the NLS sequence, we were curious to know the effect of phosphorylation, if any, on the import of TP2 into the round spermatid nucleus. Normally, these experiments are done after transient transfection of the desired construct in the respective cell lines. However, we were unsuccessful in establishing a working transfection protocol for round spermatids using the now well-established procedures for various cell lines. Furthermore, there is also no report in the literature describing such a procedure. Therefore, we resorted to the alternate approach of using nuclear import assay with digitonin-permeabilized round spermatids, which have been used extensively in the literature to understand the mechanism of nuclear import (21). In these assays, we have monitored the import of exogenously added recombinant wild-type TP2 and its phosphorylation-site mutant by indirect immunofluorescence. The results of these experiments are presented in Fig. 6. Fig. 6, *B–G*, shows the immunofluorescence pattern of the localization of exogenously added recombinant TP2. When permeabilized round spermatids were incubated with transport buffer alone in the absence and presence of the ATP regenerating system, respectively, the protein was localized in the form of a ring decorating the outer membrane of the nuclear enve-

MOUSE Cs-PKA

ATG GCT TCC AGC TCC AAC GAT GTG AAA GAG TTC CTA GCC AAA GCC AAG GAA GAT TTC CTG

$_1$M   A   S   **S**   S   N   D $_8$V   K   E   F   L   A   K   A   K   E   D   F   L $_{20}$

OVINE Cs-PKA

ATG GCT TCC AAC CCC AAC GAT GTG AAA GAG TTC CTA GCC AAA GCC AAG GAA GAT TTC CTG

$_1$M   A   S   N   **P**   N   D $_8$V   K   E   F   L   A   K   A   K   E   D   F   L $_{20}$

HUMAN Cs-PKA

ATG GCT TCC AAC TCC AGC GAT GTG AAA GAG TTC CTA GCC AAA GCC AAG GAA GAT TTC CTG

$_1$M   A   S   N   S   **S**   D $_8$V   K   E   F   L   A   K   A   K   E   D   F   L $_{20}$

RAT Cs-PKA

ATG GCT TCC AAC TCC AAC GAT GTG AAA GAG TTC CTA GCC AAA GCC AAG GAA GAT TTC CTG

$_1$M   A   S   <u>N   S   N</u>   D $_8$V   K   E   F   L   A   K   A   K   E   D   F   L $_{20}$

RAT Cα1-PKA

ATG GGC AAC GCC GCC GCC GCC AAG AAG GGC AGC GAG CAG GAG AGC GTG AAA GAG TTC CTA

$_1$M   G   N   A   A   A   A   K   K   G   S   E   Q   E   S $_{16}$V   K   E   F   L $_{20}$

F<small>IG</small>. 5. **Comparison of the nucleotide and N-terminal amino acid sequences of mouse, rat, ovine, and human Cs PKAs and rat Cα 1 PKA.** The mouse, ovine, and human Cs-PKA sequences were obtained from San Agustin *et al.* (2000). The non-consensus nucleotide and amino acid residues in mouse, ovine, and human Cs-PKA are *highlighted*. The rat Cs-PKA transcript was amplified; the sequence was determined and submitted to GenBank (GenBank accession number AY375243). The residues in rat Cs-PKA indicate the consensus sequence, which are *underlined*. The identical amino acids in rat Cs-PKA and Cα 1-PKA are indicated in a *smaller font*.

lope. However, when the same transport assay was done in the presence of cytosolic proteins, TP2 was translocated into the nucleus (Fig. 6*E*). This nuclear import of TP2 in the presence of cytosol is dependent on the presence of ATP regenerating system (Fig. 6*D*). These experiments clearly demonstrate that transport of TP2 into the round spermatid nucleus is mediated by cytoplasmic protein components and is energy-dependent.

We would also like to draw attention here to the nature of fluorescence pattern (foci) observed within the round spermatid nucleus in our import assay (Fig. 6*E*). We had shown earlier, in transient transfection assays, that TP2 preferentially localizes to nucleolus in COS-7 cells. Just to make sure that preferential localization to nucleolus is not an isolated observation, we have repeated transfection assays with HeLa cells and find that even in HeLa cells, TP2 preferentially localizes to nucleoli (Fig. 6*H*). Furthermore, even the TP2-GFP fusion protein localizes preferentially to nucleoli in HeLa cells (Fig. 6*I*), ruling out artifacts caused by immunofluorescence procedures. Nucleoli have been speculated to be inactive in round spermatids and to disassemble during the elongation phase when transition proteins are expressed during spermiogenesis. Immunolocalization of nucleolin was studied, using anti-nucleolin antibody, in HeLa cells (Fig. 6*J*) and elutriated round spermatids (Fig. 6*K*). As can be seen from the figure, although nucleoli were seen in HeLa cells (Fig. 6*J*), such nucleolar structures were not observed in the round spermatid nucleus (Fig. 6*K*). This indicates that the foci seen in the case of wild-type TP2, within the round spermatid nucleus, are not nucleoli and might probably represent preferential localization of TP2 to GC-rich DNA.

After establishing the import assay with wild type TP2, we carried out the assay with the double phosphorylation-site (T101A, S109A) mutant of TP2. The immunofluorescence pattern of localization of mutant TP2 is shown in Fig. 6*F*. Two important observations can be noted from this immunofluorescence pattern. First, the cytoplasmic distribution of TP2 is quite different from that of wild type TP2 (Fig. 6*E*). No definitive foci were observed in phosphorylation-site mutant TP2, as in the case of wild-type TP2, and most of the protein was localized around the nuclear envelope, as was observed with

minus cytosol control (Fig. 6*C*). A second important observation was that there were also no definite foci of phosphorylation-site mutant TP2 localization within the nucleus, as observed with wild-type TP2. Furthermore, the fluorescence pattern of phosphorylation-site mutant TP2 in the nucleus is much more diffuse than the wild-type TP2. We have also done the import experiment with wild-type TP2 in the presence of 50 μM PKI, an inhibitor of protein kinase A. As can be seen from *G* of Fig. 6, the fluorescence pattern showed an intense decoration around the nuclear envelope, which was very similar to that of the phosphorylation site mutant TP2. The cytoplasmic distribution as well as nuclear distribution was much less defined compared with the wild-type TP2. Taken together, the results of these two experiments indicate that phosphorylation positively modulates the NLS-dependent transport of TP2 into the round spermatid nucleus, as in the case of SV40 T antigen (25, 26). We have also done this import assay for shorter incubations (5, 10, and 15 min) and have observed similar differences in the fluorescence pattern of wild-type TP2 and phosphorylation site mutant TP2 (data not shown).

DISCUSSION

In the present study, we demonstrated that rat spermatide protein TP2 is phosphorylated at Thr-101 and Ser-109 residues by the sperm-specific PKA isoform, Cs-PKA, present in the cytoplasm of haploid round spermatids. The expression patterns of the somatic isoform Cα1 and the sperm-specific isoform Cs-PKA during rat spermatogenesis agrees with the pattern observed in mouse (16, 22). The activated form of Cs-PKA has also been detected in the sperm tail and is believed to be necessary for motility of sperm. However, it is not detected in ovaries and is hence male-specific. A striking observation made in the present study is that the Cα1-PKA present in the cytosol of 25- and 35-day-old rat testicular cells did not phosphorylate recombinant TP2. This result must be viewed in the context of our previous demonstration that TP2 can be phosphorylated *in vitro* by purified commercial PKA (8). It is quite likely that the different isoforms of PKA might exist as multiprotein complexes *in vivo*, as with A-kinase anchoring proteins, which may

FIG. 6. **Immunolocalization pattern of TP2 after *in vitro* import assay.** The effect of phosphorylation of TP2 near its NLS was studied by nuclear import assay using recombinant wild-type and mutant TP2 and the cytosol prepared from elutriated round spermatids. *A*, without recombinant TP2; *B*, wild type TP2 in import buffer without cytosol and ATP regenerating system; *C*, wild-type TP2 in import buffer with ATP regenerating system but without cytosol; *D*, wild-type TP2 in import buffer with cytosol and without ATP generating system; *E*, wild-type TP2 in import buffer with cytosol and ATP regenerating system; *F*, phosphorylation site mutant in import buffer with cytosol and ATP regenerating system; and *G*, wild-type TP2 in import buffer with cytosol (preincubated with 50 μM myr-PKI (14–22) inhibitor peptide) and ATP regenerating system. *H* and *I*, ectopic expression of wild-type in HeLa cells after transient transfection. *H*, wild-type TP2; *I*, TP2 fused to enhanced green fluorescent protein (pEGFP-C2-TP2). *J* and *K*, immunolocalization of nucleolin, using anti-nucleolin antibodies, in HeLa cells *(J)* and elutriated round spermatids *(K)*. A merge of 4,6-diamidino-2-phenylindole and tetramethylrhodamine B isothiocyanate fluorescence patterns, and differential interference contrast (phase contrast) images are included in *J* and *K*. Scale bar, 8 μm for *A–C* and *E–K* and 10 μm for *D*. Images in *A–G*, *J*, and *K* were obtained in Leica Confocal microscope LCS NT, whereas images in *H* and *I* were obtained in Axio vision fluorescence microscope (Carl Zeiss) using standard filters.
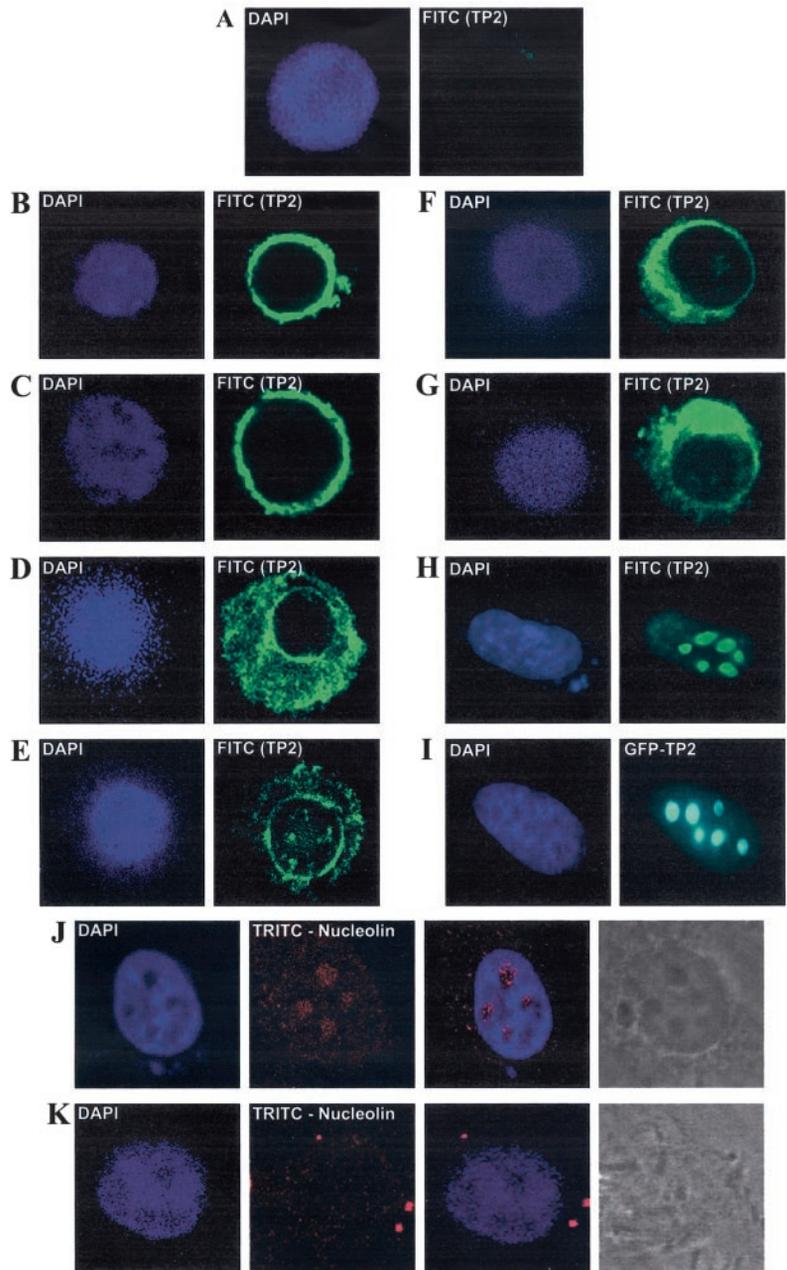
actually determine substrate specificity of phosphorylation. In this context, sperm-specific A-kinase anchoring proteins have also been recently reported in the literature (27, 28).

We have also established, in the present study, an *in vitro* import assay for studying the uptake of nuclear proteins into the haploid round spermatids. These experiments clearly demonstrate that transport of TP2 into the round spermatid nucleus is mediated by cytoplasmic protein components and is energy-dependent. It is now fairly well established that nuclear import of proteins is a two-step process involving a dimeric complex of importin α/β, of which the α-subunit binds directly the NLS motif and serves as the adaptor to importin (29, 30). However, there are some exceptions, where importin β binds directly to an NLS sequence. The NLS-protein complexes docks onto nuclear pore complexes via importin β and are translocated through the nuclear pore in an energy-dependent manner. After translocation, high levels of Ran GTP in the nucleoplasm promote cargo release from the importin complex (31, 32). The fluorescence pattern of TP2 in the cytoplasm of round spermatids (Fig. 6*D*), showing a defined distribution pattern,

might represent the TP2-importin adapter complexes. It remains to be seen whether TP2 forms a complex with importin α/β heterodimer or interacts directly with importin β. An isoform belonging to importin β superfamily, Importin 7, has been shown to be involved in the nuclear import and chaperoning of basic, nucleic acid binding proteins, such as ribosomal proteins and histone H1 (33–35). It is generally believed that proteins larger than 45 kDa require a NLS to be targeted to the nucleus and proteins of lower molecular mass can diffuse into the nucleus. However, studies on import of histone H1 (34, 36, 37) and ribosomal proteins (33, 38, 39) have indicated that proteins and RNAs that need to be translocated into the nucleus depend on specific carrier systems, even if they are small enough for passive diffusion. It has also been hypothesized that such nuclear import mechanisms evolved to prevent undesired interactions before the cargo reaches its final destination. Such mechanisms have been specifically implicated for very basic proteins, such as histones or ribosomal proteins, which have a high tendency to precipitate and aggregate at physiological salt concentration (34). It is interesting to note that TP2 is a highly

basic protein and has a molecular mass of only 13 kDa, but it still has a defined NLS and a requirement of cytoplasmic components for its import into the nucleus, indicating that it is a regulated process. Hence, it can be speculated that even though TP2 is relatively small, it needs an efficient nuclear transport mechanism to ensure that it reaches its final destination and is not misdirected (see below). These questions are being addressed in a separate study and will be reported elsewhere.

The subnuclear foci of TP2 localization in round spermatids did not reveal the nucleolus-specific localization pattern that we had observed earlier in COS-7 cells (7). We have now shown that the nucleolar preference of localization was not an isolated observation with COS-7 cells but was also observed in HeLa cells using both wild-type TP2 and TP2-GFP fusion constructs (Fig. 6, *H* and *I*). The absence of nucleolin staining in round spermatids clearly indicated the absence of active nucleolar structures in these cells (Fig. 6*K*). Lack of such a preferential localization in nucleolus in round spermatid nucleus was initially surprising and puzzling. Earlier studies had assumed that haploid spermatids are inactive in pol II transcription and that all the mRNAs are presynthesized during the meiotic prophase (pachytene) interval. However, it is now clear that haploid spermatids are active in RNA polymerase II transcription (40). However, a systematic study of rDNA transcription has not been made; hence, the lack of pol I-mediated transcription in haploid spermatids needs to be documented. Even though nucleoli are defined organelles in a eukaryotic nucleus, there is no physical barrier between the nucleoplasm and nucleolar structure in the form of a lipid bilayer membrane. Nucleolar assembly is believed to be initiated by coalescence of the different rDNA repeats and a set of nucleolus-specific proteins (41). In the absence of such a defined organellar structure, the rRNA cistrons will be distributed all over the nucleus in round spermatid nuclei. It will be interesting to see whether TP2 bound chromatin domains that are visualized within the round spermatid nucleus in these import assays represent rDNA repeats because of their GC-richness. In a separate study to be reported elsewhere, we have observed, using chromatin immunoprecipitation assay, that the endogenous TP2 bound DNA sequences in elongating spermatids (stages 12 to 15) are indeed GC-rich.

The import assay experiments reported here have also demonstrated that the import of wild type TP2 in the presence of PKI-inhibitor peptide or the phosphorylation-site mutant (T101A, S109A) of TP2 is not as efficient as that of wild type TP2, suggesting that the NLS of TP2 might belong to the class of phosphorylation-regulated NLS, proteins that are modulated by a set of sequence motifs. For example, the CcN-motif of SV40 T antigen is regulated by dual phosphorylation by protein kinase CK2 and the cyclin-dependent kinase cdc2 (25, 26). Substitution of CK2 site by the PKA site also caused it to be regulated by PKA-mediated phosphorylation, indicating that this regulation is not kinase specific (42). As against the positive modulation of NLS function, an example of negative regulation of an NLS by phosphorylation is the cyclin-dependent kinase site-mediated inhibition of yeast TFSW15 (23). It is quite likely that such regulation of transport by phosphorylation might be influenced through the interaction of the cargo with the importin complexes in the cytoplasm. The presence of monopartite NLS in TP2 and the positive modulation of the NLS-dependent nuclear import by a phosphorylation event is very similar to that observed in the case of SV40 T antigen. Thus, the phosphorylation of TP2 seems to have an additional role in addition to the chromatin function, as has been proposed in our model (8). In this model, we proposed that phosphorylation of the basic domain of TP2 temporarily masks its condensation property to facilitate the zinc fingers to recognize and dock onto the CpG dinucleotides in chromatin. A similar phosphorylation-dephosphorylation event has been proposed for protamine deposition on sperm chromatin (43). Our model also predicts that a phosphatase should be present in elongating spermatid nucleus to dephosphorylate TP2 and to initiate chromatin condensation. In this context, it is worth mentioning that Varmuza *et al.* (44) have reported that the protein phosphatase 1cγ gene is specifically expressed in round spermatids, and mice homozygous for targeted insertion within this gene are infertile.

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