Characterization of a Premeiotic Germ Cell-specific Cytoplasmic Protein Encoded by Stra8, a Novel Retinoic Acid–responsive Gene

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Abstract. The full-length cDNA corresponding to Stra8, a novel gene inducible by retinoic acid (RA) in P19 embryonal carcinoma cells, has been isolated and shown to encode a 45-kD protein. Both Stra8 mRNA and protein were induced in cells treated by all-*trans* and 9-*cis* retinoic acids. Two-dimensional gel analysis and dephosphorylation experiments revealed that the two stereoisomers of RA differentially regulate the phosphorylation status of the Stra8 protein, which was shown to exist in differently phosphorylated forms. Subcellular fractionation and immunocytochemistry studies showed that the Stra8 protein is cytoplasmic. During mouse embryogenesis, Stra8 expression was restricted to the male developing gonads, and in adult mice, the expression of Stra8 was restricted to the premeiotic germ cells. Thus, Stra8 protein may play a role in the premeiotic phase of spermatogenesis.

RETINOIDS have been shown to regulate various physiological functions (for reviews see Blomhoff, 1994; Sporn et al., 1994; Kastner et al., 1995*a*; and references therein). Their effects are mediated through two families of receptors that act as ligand-inducible transcriptional regulatory proteins. The three retinoic acid (RA)[1] receptors (RARα, β, and γ) bind all-*trans* (T-RA) and 9-*cis* RA (9C-RA), while the three retinoid X receptors (RXRα, β, and γ) bind only 9C-RA (for review see Chambon, 1994, 1996; Leid et al., 1992; Mangelsdorf et al., 1994, 1995). Retinoids are known to be required for vertebrate reproduction, and in the male, for the maintenance of normal testicular structure and function. Retinol deficiency leads to cessation of spermatogenesis and degeneration of the seminiferous tubules (Thompson et al., 1964; Howell et al., 1963), which have also been found in RARα-null mutant mice (Lufkin et al., 1993; for review see Kastner et al., 1995*a*). Furthermore, it has been reported that RA affects survival and proliferation of primordial germ cells (Koshimizu et al., 1995). The identification of RA-regulated genes specifically expressed in germ cells would therefore be helpful in providing molecular markers for further analysis of their development and understanding the role of RA in spermatogenesis.

Using the P19 pluripotent embryonal carcinoma (EC) cell line as a model system, we have previously isolated a number of novel RA-responsive genes, collectively designated as the Stra genes (Bouillet et al., 1995*a*,*b*; Oulad-Abdelghani et al., 1996; Roy et al., 1995; Taneja et al., 1996). We describe here the cloning of full-length Stra8 cDNA and the partial characterization of the corresponding novel protein. The Stra8 gene encodes a cytoplasmic protein that is differentially phosphorylated in P19 cells upon addition of T-RA or 9C-RA. Interestingly, Stra8 expression is restricted to the male developing gonad during mouse embryogenesis, and in the adult, the Stra8 protein appears to be expressed in testis premeiotic germ cells.

Materials and Methods

Cell Culture and RA Treatment

P19 cells were cultured and maintained as monolayers in DME enriched with 5% FCS (Rudnicki et al., 1988). F9 EC cells and D3 embryonic stem (ES) cells were cultured as described (Lufkin et al., 1991; Wang et al., 1985). RA was added as an ethanol solution at a final concentration of 1 μM to induce differentiation. Control cells were treated with an equal volume of ethanol. At appropriate incubation times, the cells were washed with PBS, scraped, and recovered by centrifugation.

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1. *Abbreviations used in this paper*: dpc, day postcoitum; Dhh, Desert hedgehog; EC, embryonal carcinoma; ES, embryonic stem; ISH, in situ hybridization; RA, retinoic acid; RAR, retinoic acid receptor; T-RA, all-*trans* RA; 9C-RA, 9-*cis* RA; RT, reverse transcription.

DNA Library Screening and Sequencing

The initial 256-bp Stra8 cDNA fragment (Bouillet et al., 1995*a*) was used as a probe to screen an oligod(T)-primed λZapII cDNA library prepared from P19 cells cultured as monolayers for 24 h in the presence of 1 μM T-RA. 10^5 phage plaques were screened using conventional techniques (Maniatis et al., 1982). Positive plaques were isolated and in vivo excision was performed according to the manufacturer (Stratagene, La Jolla, CA). The resulting pBluescript SK^- plasmids corresponding to the full-length cDNA were prepared and sequenced on both strands using the DyeDeoxy terminator cycle sequencing on an automated DNA sequencer (AB1373A; Applied Biosystems, Inc., Foster City, CA).

Antibody Production and Purification

Using PCR, Stra8 cDNA sequence was subcloned in the expression vector pET15b (Novagen, Madison, WI) to obtain a fusion protein containing six histidine residues at the NH_2 terminus. Transformation of *Escherichia coli* BL21 (DE3), preparation, and purification of bacterially expressed Stra8 protein were carried out as described (Oulad-Abdelghani et al., 1996), and the recombinant protein was used to immunize rabbits. The anti-Stra8 polyclonal antiserum was purified on an affinity column prepared by binding the Stra8 recombinant protein to a sulfolink column (Pharmacia, Uppsala, Sweden). The affinity-purified antibody preparation was dialyzed against PBS containing 20% glycerol and stored at −20°C.

Nuclear, Cytosolic, and Cytoskeletal Extracts

Nuclear and cytosolic extracts were prepared from subconfluent P19 cells treated or not with 1 μM RA for 24 h as described by Rochette-Egly et al. (1991). To prepare detergent-soluble and -insoluble fractions, P19 cells were extracted with ice-cold Triton buffer (25 mM Hepes, pH 7.4, 2 mM $MnCl_2$, 0.1% protease inhibitor cocktail, 0.5% Triton X-100) as described (Gronowski and Bertics, 1994), and the mixture was centrifuged at 100,000 *g* for 1 h.

Immunocytochemistry and Immunohistochemistry

The full-length open reading frame of Stra8 was cloned in the sense orientation downstream of the SV-40 promoter into the expression vector pSG5 (Green et al., 1988) and transfected into COS-1 cells. Immunocytochemistry was performed in 96-well plates after fixation of the transfected cells with 2% paraformaldehyde for 4 min and permeabilization with 0.1% Triton X-100 for two times at 10 min each. Cells were then incubated with the affinity-purified anti-Stra8 antibody. The secondary antibody was biotinylated, and staining was performed using Vectastain ABC-Elite and DAB substrate kits (Vector Laboratories, Burlingame, CA).

Immunohistochemistry was performed on 10-μm cryostat sections of testis fixed with acetone, using the Vectastain ABC-Elite and DAB substrate kits. Slides were counterstained with haematoxylin and eosin.

Electron Microscopy

Adult male CD1 mice were anesthetized and perfused intraaortically with a fixative mixture (0.5% glutaraldehyde, 4% paraformaldehyde in PBS). Testes were removed and cryoprotected by immersion in 15% buffered sucrose, and then in 25% buffered sucrose overnight before being embedded in Tissue-Teck (Miles Laboratories, Inc., Elkhart, IN) and frozen. Cryostat sections (30 μm) were collected onto gelatin/chrome-alum–coated slides, treated with acetone for 5 min at 4°C, and then treated with 4% formaldehyde in PBS for another 5 min at 4°C. Labeling and detection were performed by using the Vectastain ABC-Elite and DAB kits. Stained sections were then fixed with 2.5% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide for 30 min, and dehydrated with ethanol and propylene oxide. After overnight infiltration in Epon resin, sections were flattened between microscope slides, polymerized at 60°C for 24 h, and finally glued onto plastic blocks. Thin-sections were cut and collected on 200-mesh uncoated grids and examined with an electron microscope (208; Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV without counterstaining.

SDS-PAGE, Two-dimensional PAGE, and Western Blotting

Protein extracts were analyzed by SDS-PAGE on a 12% polyacrylamide gel as described in Laemmli (1970). Two-dimensional gel electrophoresis (IEF in the first dimension and SDS-PAGE in the second dimension) was performed as described in Oulad-Abdelghani et al. (1991). Western blot analyses were carried out according to standard techniques (Towbin et al., 1979) with purified anti-Stra8 polyclonal antibody. An mAb against cellular retinoic acid–binding protein II (anti-CRABPII 1CRA4C9, manuscript in preparation) and a polyclonal anti-actin (Sigma Chemical Co., St. Louis, MO) were also used. Secondary antibodies were conjugated with HRP and revealed using an ECL kit (Amersham Intl., Little Chalfont, UK).

RNA Extraction and Reverse Transcription–PCR Analysis

Total RNA from cultured cells and organs was prepared according to Auffray and Rougeon (1980). Reverse transcription (RT)–PCR were carried out as described (Bouillet et al., 1995*a*). Oligonucleotide primers used in this study were 5′-GCCAGAATGTATTCCGAGAA-3′ (nucleotides 429–448) and 5′-CTCACTCTTGTCCAGGAAAC-3′ (nucleotides 1079–1060). Amplification products were separated on 2% agarose gels, transferred onto Hybond N membranes (Amersham Intl.), and revealed by Southern blotting (Maniatis et al., 1982).

In Situ Hybridization

The Stra8 cDNA sequence cloned in pBluescript SK^- (Stratagene) was used in T7 polymerase in vitro transcription reactions including digoxigenin-11-UTP (Boehringer Mannheim GmbH, Mannheim, Germany) or [^{35}S]CTP (Amersham Intl.) to produce antisense riboprobes (manufacturer's reagents and instructions). Probe length was reduced by a 45-min alkaline hydrolysis with $NaCO_3$ (pH 10.2). In situ hybridization on cryosections was carried out as described in Décimo et al. (1995).

Results

Stra8 cDNA and Putative Protein Sequences

Using a differential subtractive hybridization cloning strategy based on biotin-streptavidin affinity and PCR, we isolated 50 partial cDNA clones corresponding to transcripts from RA-inducible genes in P19 cells (Bouillet et al., 1995*a*). One of these clones, referred to as Stra8 (256 bp), was subsequently used as a probe to screen an oligod(T)-primed cDNA library from RA-treated P19 cells. Several positive clones were isolated, the longer being 1,455 nucleotides in length (Fig. 1). This cDNA, which was sequenced on both strands, contains an open reading frame of 393 amino acids starting with an ATG codon at nucleotide 102 and terminating by a TAA stop codon at nucleotide 1281. The sequence 5′ of the initiation site contains an in-frame TGA stop codon at nucleotide position 12. Two putative polyadenylation signals were found in the 3′ untranslated region at positions 1309 and 1435.

The Stra8 protein contains a 51–amino acid domain that is rich in glutamic acid (38 out of 51 amino acids are glutamic acid), conferring a high acidity to the Stra8 protein. In this domain glutamic acids form stretches of two to 10 residues separated by one or two different amino acids. In particular, four EEEG repeats were found in this domain. Glutamic acid-rich domains are found in several proteins such as the centromere autoantigen protein B, troponin T, or neurofilaments L, M, and H. The deduced Stra8 protein sequence does not exhibit any significant homology with sequences of the Swissprot and NBRF databases outside of this glutamic acid–rich domain. Several putative phosphorylation sites for protein kinases A and C, casein kinase 2, and proline-dependent kinases are present in Stra8 protein (Fig. 1; Kemp and Pearson, 1990).

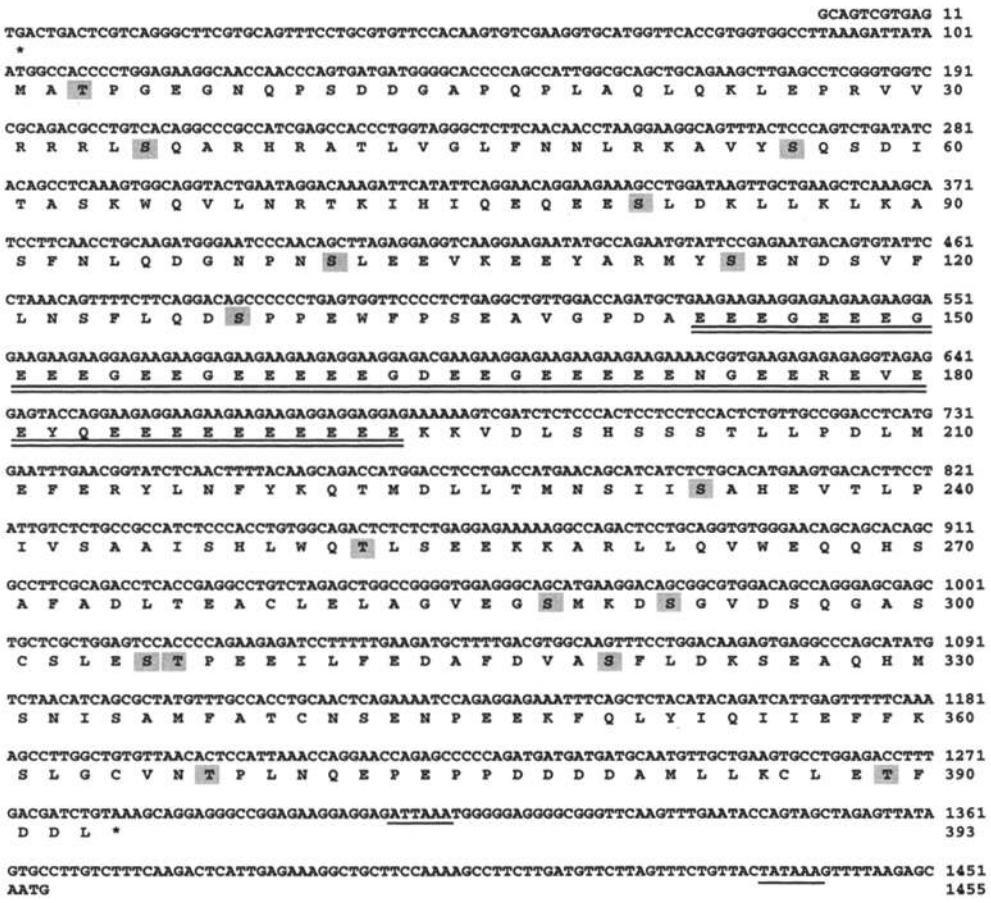

Figure 1. Nucleotide and deduced amino acid sequence of the mouse Stra8 cDNA. Numbers (*right*) refer to the position of nucleotides and amino acids. Putative phosphorylation sites for protein kinases A and C, casein kinase 2, and proline-dependent kinases (Kemp and Pearson, 1990) (*shaded boxes*). The glutamic acid–rich domain (*double underline*). Putative polyadenylation sites (*underlined*). (*Asterisks*) Stop codons. These sequence data are available from Genbank/EMBL/DDBJ under accession number Z75287.

Stra8 Gene Expression in EC Cells, ES Cells, and Adult Organs

The regulation of Stra8 gene expression by RA in P19, F9, and ES cells was investigated using the RT-PCR technique. Stra8 transcripts accumulated in P19, F9, and ES cells upon T-RA treatment and, in P19 cells, even more strongly upon 9C-RA treatment (Fig. 2 *A*). Control experiments (not shown) on the same RNA samples did not show any significant variation in the content of the invariant 36B4 RNA (Bouillet et al., 1995*a*). Kinetics experiments in P19 cells treated with either 10^{-8} or 10^{-6} M T-RA showed that Stra8 transcript accumulation starts as early as 2 h after T-RA addition and that it reaches a plateau level by 12 h (not shown; see Bouillet et al., 1995*a*).

RT-PCR was also used to investigate the possible expression of the Stra8 gene in several mouse adult tissues, including brain, heart, lung, liver, kidney, spleen, ovary, and testis. Stra8 gene expression was clearly restricted to the testis (Fig. 2 *B*).

Characterization of the Stra8 Protein and Its Phosphorylated Forms

The Stra8 protein was expressed in *E. coli*, purified, and used to produce rabbit polyclonal antibodies. Cytosolic extracts from P19 cells treated or not with 1 μM RA were subjected to SDS-PAGE, and the presence of the Stra8 protein was investigated by Western blotting. An immunoreacting species with an apparent molecular mass of 46 ± 1 kD, consistent with that expected for the Stra8 cDNA-deduced protein (45 kD), was detected (Fig. 3). As its cognate mRNA, the Stra8 protein was induced by both T-RA and 9C-RA. Note that no significant immunoreactive Stra8 protein was detected in P19 cell nuclear extract (not shown), indicating that this protein is essentially cytoplasmic (see below).

The Stra8 protein contains several putative serine and threonine phosphorylation sites (Fig. 1). To investigate whether Stra8 phosphorylated forms could exist, cytosolic extracts from P19 cells (treated or not with 1 μM T-RA or 9C-RA) were analyzed by two-dimensional gel electrophoresis and Western blotting (Fig. 4). The Stra8 protein exhibited a migration typical of phosphoproteins (Creighton, 1990). Nine phosphorylated forms of Stra8 (1–9) could be detected in the different extracts. All of these phosphorylated forms disappeared when the P19 cell extracts were treated with alkaline phosphatase and more basic polypeptides of the same molecular mass appeared (not shown). The nine phosphorylated forms were detected in P19 cells treated with T-RA, whereas only forms 2 and 3 were clearly found in cells treated with 9C-RA (Fig. 4). Note also that the labeling corresponding to forms 2 and 3 was much less intense in T-RA–treated cells than in 9C-RA–treated cells.

Cytoplasmic Localization of Stra8 Protein

To confirm the cytoplasmic localization of Stra8 protein,

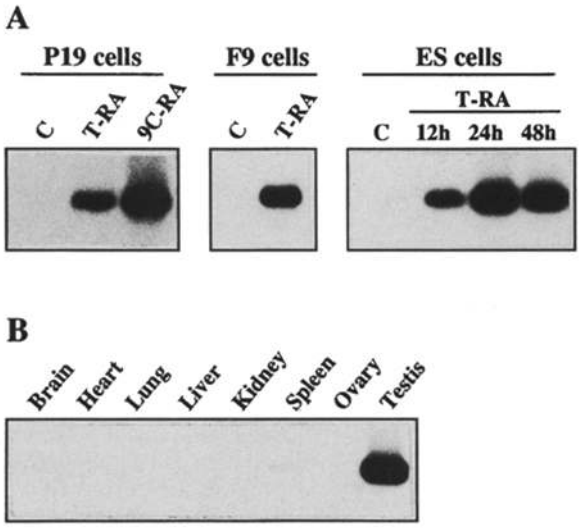

Figure 2. Expression of Stra8 in P19, F9, and ES cells and adult organs. Total RNA was isolated from cells (*A*) and adult organs (*B*) and analyzed by RT-PCR and Southern blotting. (*A*) P19 and F9 cells were treated for 24 h with either ethanol (*C*), 1 μM T-RA, or 1 μM 9C-RA. ES cells were grown for 24 h in the presence of ethanol (*C*) or for 12, 24, and 48 h in the presence of T-RA (10 nM). (*B*) Stra8 RNA expression in adult mouse organs.

the cDNA sequence of Stra8 was cloned in the pSG5 expression vector and transfected into COS-1 cells, and the expressed protein was immunocytochemically detected using anti-Stra8 antibody. The cytoplasm of transfected cells was highly labeled (Fig. 5 *A*), while no significant signal was detected in the nucleus.

As glutamic acid stretches are found in some proteins of the cytoskeleton, such as Neurofilaments (Levy et al., 1987)

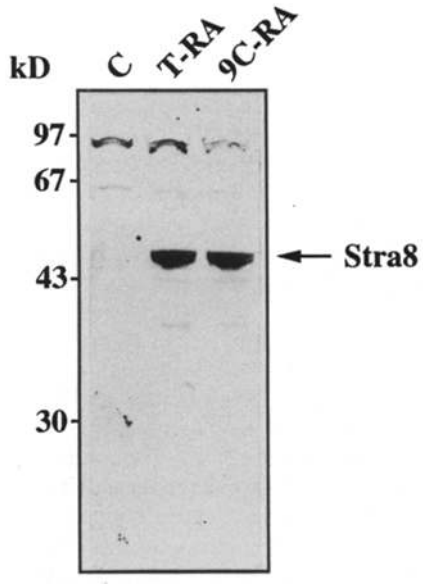

Figure 3. Immunodetection of endogenous Stra8 protein in P19 cells. Cytosolic extracts from P19 cells incubated for 24 h with either ethanol (*C*), 1 μM T-RA, or 1 μM 9C-RA were subjected to SDS-PAGE and Western blotting using a polyclonal antibody raised against bacterially expressed Stra8 protein.

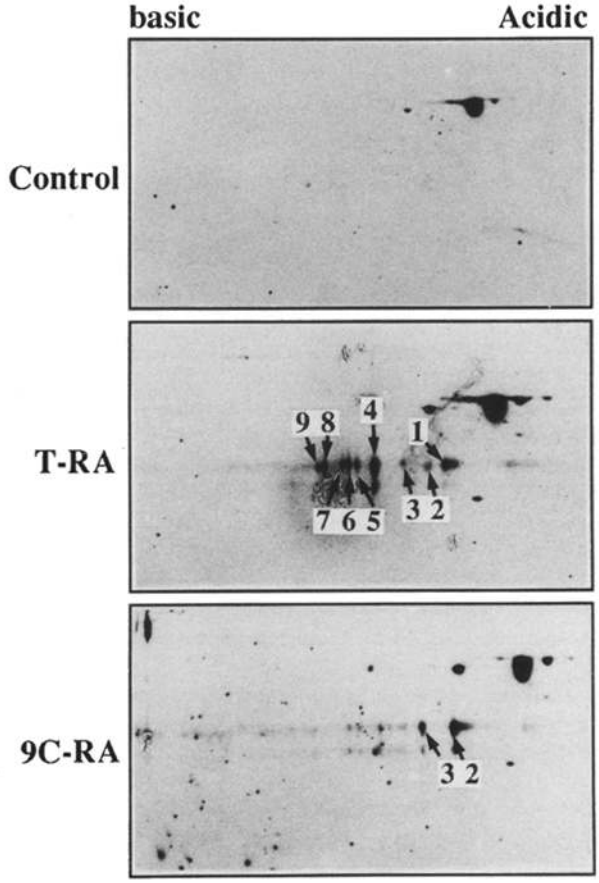

Figure 4. Immunodetection of phosphorylated forms of the Stra8 protein. Cytosolic extracts from P19 cells treated for 24 h with ethanol (*Control*), 1 μM T-RA, or 1 μM 9C-RA were subjected to two-dimensional gel electrophoresis (IEF and SDS-PAGE) and Western blotting using an anti-Stra8 antibody. The phosphorylated forms of Stra8 protein (1 to 9) are indicated. The thick spots observed in the upper right side correspond to an artifactual cross-reaction between the secondary antibody and an abundant P19 protein.

or Troponin T (Fyrberg et al., 1990), we examined whether the Stra8 protein could be a component of the cytoskeleton. P19 cells were extracted using Triton X-100, and the detergent-soluble and -insoluble (cytoskeletal) fractions were subjected to SDS-PAGE and anti-Stra8 immunoblotting. An immunoreactive species corresponding to the molecular mass of Stra8 was clearly detected in the Triton X-100–soluble fraction of RA-treated P19 cells only (Fig. 5 *B*). In control experiments carried out on the same extracts, the soluble CRABPII was also found only in the Triton-soluble fractions, whereas actin, which is known to be both soluble and associated with the cytoskeleton, was detected in both Triton-soluble and -insoluble fractions (Fig. 5 *B*). Thus, Stra8 protein does not appear to be a component of the cytoskeleton.

Restricted Expression of Stra8 mRNA and Protein in the Mouse Testis

Using an antisense RNA probe, ISH was performed to determine the expression pattern of the Stra8 gene in mouse

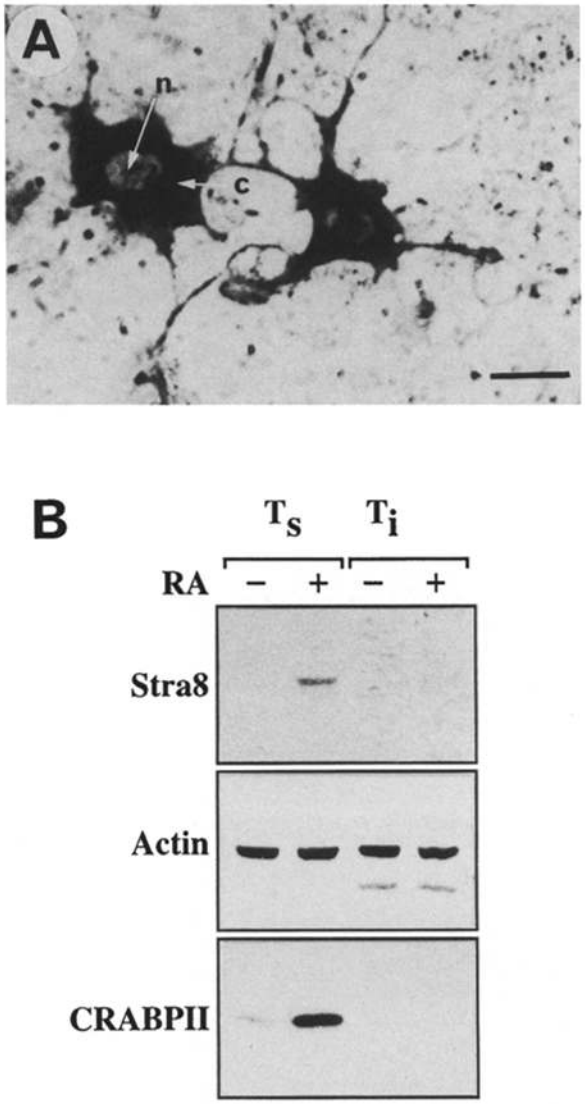

Figure 5. (*A*) Localization of Stra8 protein by immunocytochemistry in transfected COS-1 cells. COS-1 cells transfected with a Stra8 expression vector were stained by the HRP method with anti-Stra8 antibody. (*B*) Detection of Stra8 protein in Triton-soluble and -insoluble (cytoskeleton) fractions. P19 cells incubated for 24 h with ethanol (control) or with 1 μM T-RA were extracted with 0.5% Triton buffer and centrifuged at 100,000 *g* for 1 h. The pellet and supernatant were analyzed by SDS-PAGE and Western blotting using antibodies against Stra8, CRABPII, or actin as indicated. *c*, cytoplasm; *n*, nucleus; T_s, Triton-soluble; T_i, Triton-insoluble. Bar, 20 μm.

embryos and adult tissues. ISH was performed on mouse embryos at various developmental stages (6.5 d postcoitum [dpc] to neonates), and no signal was detected elsewhere than in the developing gonads (Fig. 6 *A*; data not shown). Stra8 transcripts were found in the genital ridges at 12.5 dpc, i.e., before the differentiation of the gonadal anlage (data not shown). Later in development, Stra8 transcripts were strongly expressed in some cells of developing testes (Fig. 6, *A* and *B*; 14.5 dpc). On the other hand, no signal was detected in the developing ovaries at 16.5 dpc (data not shown). ISH of newborn testis sections showed heterogeneous Stra8 labeling among seminiferous tubules. Indeed, individual tubules were either strongly, weakly, or not labeled (Fig. 6 *C*). Similar heterogeneous labeling was observed in prepubertal (1–4-wk-old) and adult (5-mo-old; Fig. 6 *D*) testes. Stra8 transcripts were clearly restricted to the basal cell layer of the seminiferous tubules (Fig. 6 *E*). The considerable variation of signal intensities between individual tubules (Fig. 6 *D*) suggests that Stra8 gene expression is restricted to certain stages of the spermatogenic cycle (Russell et al., 1990). Consistent with the RT-PCR results, no ISH signal was detected on adult ovary sections (Fig. 6 *F*), thereby confirming that Stra8 expression is specific to male germ cells.

We have also used the anti-Stra8 antibody to analyze the distribution of the corresponding protein in the adult mouse testis (Fig. 7 *A*). The staining observed in the tubules from adult testis was consistent with the results of in situ hybridization experiments (Fig. 6 *D*). Indeed, expression of the Stra8 protein was restricted to the peripheral layer of the tubule, although not all of the cells from this layer were labeled. This suggested that Stra8 protein was not present in Sertoli cells, since the cytoplasmic processes of these cells extend in the luminal portions of the seminiferous tubule. As previously reported for CRBPI (Kato et al., 1985), immunoreaction with a cytoplasmic protein expressed in Sertoli cells results in a signal extending radially toward the lumen, which was never observed with anti-Stra8 antibody. Note that no specific Stra8 protein staining could be detected in the interstitial cells (Leydig cells). That Stra8 is not expressed in Sertoli cells was confirmed by immunohistochemistry at the EM level on cryosections of adult testis. An intense immunoperoxidase reaction could be detected in the cytoplasm of germ cells located in direct contact with the basal lamina and displaying features of spermatogonia (these cells harbor a large round or oval nucleus). The immunostained cells were detected as isolated cells (Fig. 7 *C*) or groups of cells (Fig. 7 *B*), presumably corresponding to the progeny of a single stem cell. Note that in Fig. 7 *C*, the section was across the cytoplasm of the spermatogonia and did not show any part of the nucleus, thus explaining why the whole cell appeared immunostained. Sertoli cells (which can be easily recognized by their typical nucleus and a prominent reticular nucleolus) were not immunostained (Fig. 7 *C*). Thus, although the present optical resolution is not good enough to precisely identify which type of germ cells express Stra8, Stra8 expression is clearly restricted to premeiotic germ cells (spermatogonia and possibly preleptotene spermatocytes).

Discussion

Using a differential subtractive hybridization cloning procedure, we have previously isolated several murine cDNA clones corresponding to RA-induced genes (Bouillet et al., 1995*a*). One of these partial cDNAs, Stra8, was used as a probe to screen an oligod(T)-primed cDNA library from RA-treated P19 cells. A full-length Stra8 cDNA that encodes a putative protein with a molecular mass of 45 kD was cloned. The deduced Stra8 protein does not appear to be related to any of the protein sequences already present in the databases, with the exception of a glutamic acid–rich

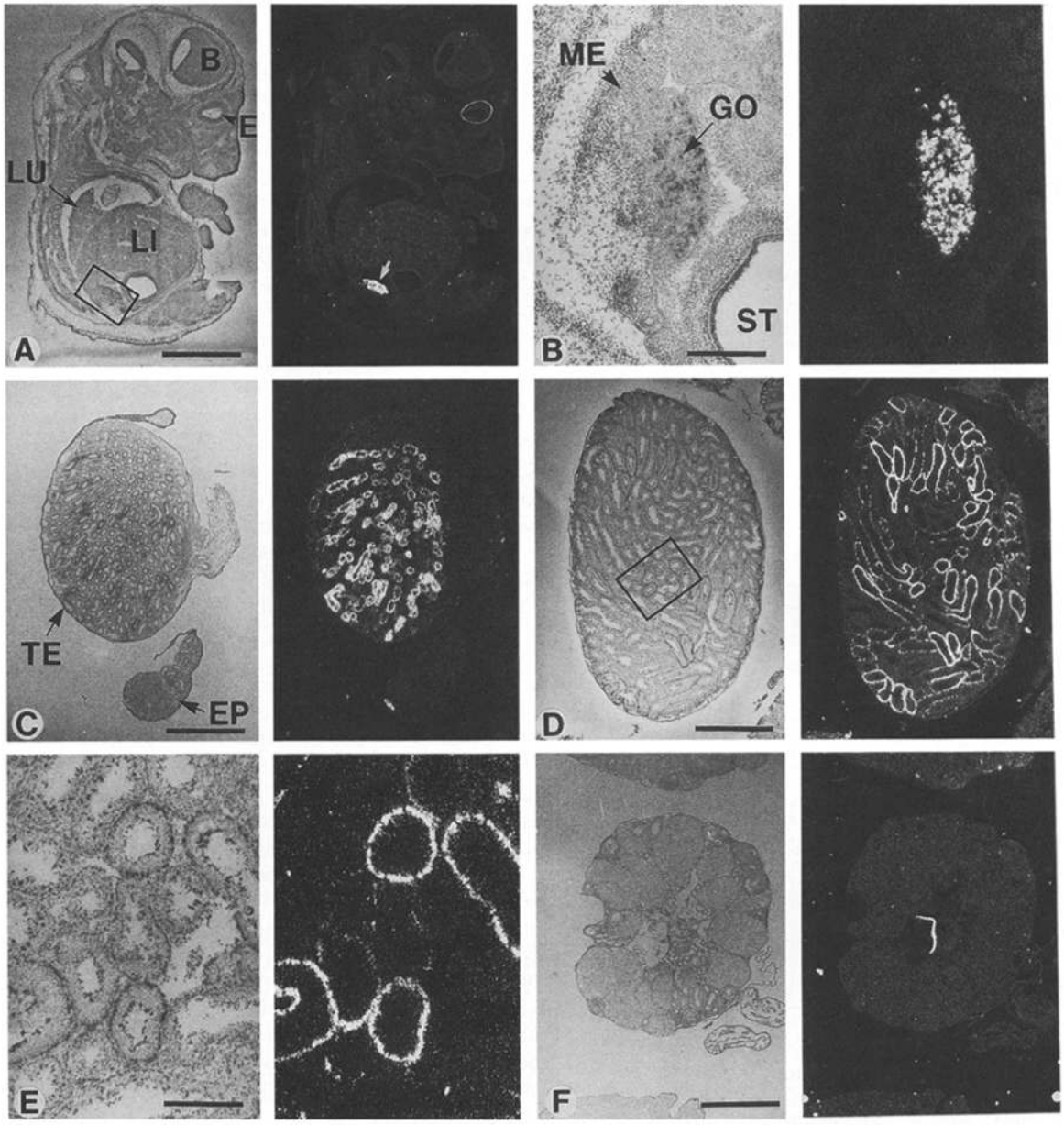

Figure 6. In situ hybridization analysis of the expression of Stra8 transcripts. (*A*) Sagittal section of a 14.5-dpc mouse fetus showing a signal restricted to the developing gonad (*white arrow*). (*B*) High power magnification of the gonad (*boxed in A*) that has histological features of a developing testis and shows heterogeneous labeling, presumably in germ cells. *GO*, gonad; *ME*, differentiating mesonephros; *ST*, stomach. (*C*) Section of a newborn testis (*TE*). Note the variable signal intensities among individual tubules, and the absence of signal in the epididymis (*EP*). (*D*) Section of an adult (5 mo) testis. (*E*) High power magnification of the field boxed in *D* showing both labeled and unlabeled seminiferous tubes. Note that the labeling was always restricted to the basal cell layer. (*F*) Section of an adult ovary. The weak signal in the upper right side corresponds to an artifact and was not reproducibly observed. In all cases, the lefthand panels are bright-field views showing the histology, and the righthand panels are dark-field views showing the hybridization signal grain in white. Bars: (*A*) 2.5 mm; (*B*) 350 μm; (*C*) 600 μm; (*D*) 1.4 mm; (*E*) 330 μm; (*F*) 600 μm.

domain. Subcellular fractionation studies of P19 cell proteins and immunocytochemistry have revealed that the Stra8 protein is localized in the cytoplasm, and they have also shown that this protein may not be a component of the cytoskeleton. In addition, Stra8 protein was enriched in membrane-deprived cytosolic fraction (Fig. 3) and was not detected in membrane preparations (not shown). This suggests that Stra8 protein may be a cytoplasmic soluble protein rather than a structural protein.

Two-dimensional-gel analysis and dephosphorylation experiments have revealed that Stra8 can exist in several phosphorylation states. The phosphorylated forms that are present after RA treatment differ depending on the RA stereoisomer that is used. Seven of these forms are found essentially only in T-RA–treated cells, and the two others (forms 2 and 3; Fig. 4) are found in cells treated with both isomers, although at a much higher relative level in 9C-RA–treated cells. Since it is known that T-RA used at a

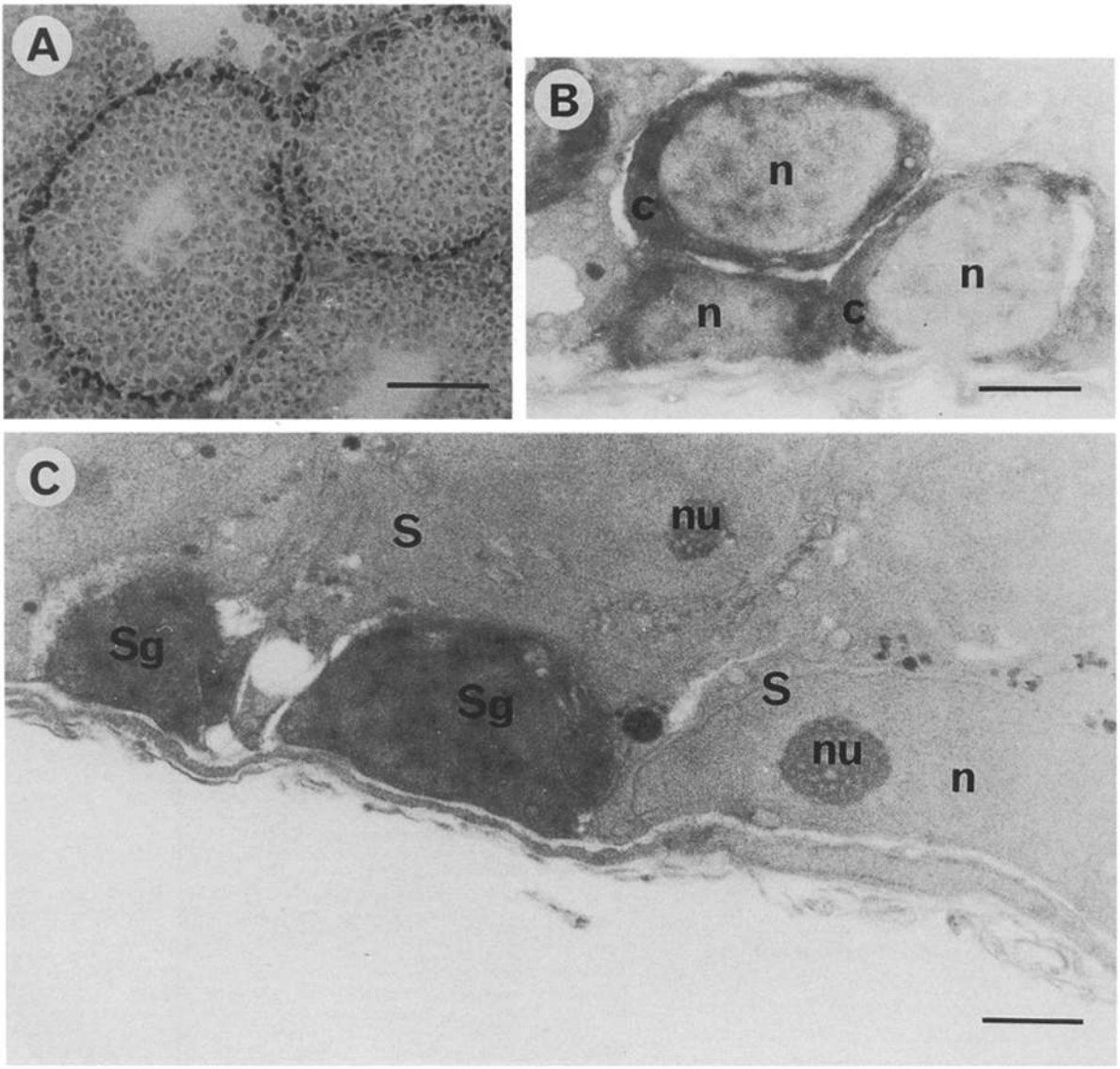

Figure 7. Stra8 protein localization in the testis. (*A*) Stra8 protein localized by immunohistochemistry with an anti-Stra8 antibody on 10-μm cryosections of testis from adult mouse. (*B* and *C*) EM localization of Stra8 protein on cryosections of adult testis, without counterstaining. The dark immunoperoxidase staining was restricted to the cytoplasm of putative spermatogonia (see text). Note that there was no positive signal detected in Sertoli cells (*C*, *right*). *c*, cytoplasm; *n*, nucleus; *nu*, nucleolus; *S*, Sertoli cell; *Sg*, spermatogonia. Bars: (*A*) 160 μm; (*B* and *C*) 2.5 μm.

high concentration can be converted in vivo into its 9-*cis* isomer (Levin et al., 1992; Heymann et al., 1992), it is possible that these two latter phosphorylated forms could in fact be specifically induced by 9C-RA. Thus, T-RA and 9C-RA may not only differentially control gene expression at the RNA level (Durand et al., 1992), but also differentially regulate posttranslational modifications. This action of T-RA or 9C-RA on protein phosphorylation may reflect effects on kinases and/or phosphatases. Interestingly, the expression of the gene encoding alkaline phosphatase has been shown to be regulated by RA in RCT-1 and F9 cells (Heath et al., 1992; Gianni et al., 1993), whereas RA induces a decrease in the expression of $p34^{cdc2}$, a serine/threonine kinase that has an important role in controlling cell cycle progression (Gaetano et al., 1991). RA has also been shown to increase the phosphorylation of RARβ1 and RARβ3 isoforms (Rochette-Egly et al., 1992). Thus, Stra8 may provide a good model to study protein phosphorylation during RA-induced differentiation.

In the adult mouse the expression of Stra8 RNA appears to be restricted to the testis. In situ hybridization and immunocytochemistry demonstrate that the expression of Stra8 is limited to the basal layer of seminiferous tubules where the Sertoli and premeiotic germ cells are localized. Immunoelectron microscopy reveals that Stra8 protein is found only in the cytoplasm of cells that lie in close contact with the basal lamina, and these cells were identified as spermatogonia and possibly preleptotene spermatocytes. Stra8 gene is not expressed in all of the tubules present in a given testis section, suggesting that its expression depends on the stage of the spermatogenic cycle. A similar

restricted expression has already been observed for several genes such as RXRα, CRBPI, or TAK1 (Kastner et al., 1995*b*; Rajan et al., 1990; Hirose et al., 1995). This can be explained by the fact that the process of spermatogenesis is synchronized, with waves of activity occurring sequentially along the length of each tubule (Russell et al., 1990).

Stra8 expression is limited to the premeiotic germ cells of the adult testis. To date, c-kit gene is the only gene that was shown to have this specificity of expression in the seminiferous epithelium (Manova et al., 1990). In contrast, it should be noted that c-kit gene is also expressed in Leydig cells at all ages examined (Manova et al., 1990), whereas Stra8 expression was never detected in these cells. This makes Stra8 a very interesting marker of premeiotic germ cells and should prove to be very useful in identifying this population in studies of germ cell development.

In the mouse embryo, Stra8 transcripts were detected only in the male developing gonad from 12.5 dpc. Several other genes have previously been shown to exhibit a male-specific expression in the gonad. Sry, which is located on the Y chromosome, is thought to be the major factor of sex determination and is expressed in the Sertoli cell precursors of the developing gonad from 10.5 to 12 dpc, at the time when testis begins to form (Koopman et al., 1990; Hacker et al., 1995). Desert Hedgehog (Dhh) begins to be expressed in the pre-Sertoli cells of the developing male gonad at 11.5 dpc, and this expression persists in the adult testis, whereas no expression is detected in the female gonad (Bitgood et al., 1996). Since Dhh begins to be expressed shortly after the activation of Sry, it has been proposed that it may be a direct target gene for Sry. Interestingly, male Dhh homozygous null mutants are infertile and harbor a gross germ cell deficiency that can already be detected at 18.5 dpc (Bitgood et al., 1996). The expression of another gene displaying male-specific transcription in the Leydig cells, *Patched*, has been shown to be lost in Dhh null mutants (Bitgood et al., 1996). Since Stra8 expression starts shortly after the onset of Dhh expression, it would be interesting to study its expression in Dhh null mutant embryos between 12.5 and 18.5 dpc to determine whether Stra8 could be a component of Dhh signaling pathway.

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