Stra3 / lefty, a retinoic acid-inducible novel member of the transforming growth factor-β superfamily

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ABSTRACT We report the structure, chromosomal localization and expression features of Stra3, a novel mouse gene whose expression is upregulated by retinoic acid in P19 embryonal carcinoma cells. The Stra3 cDNA sequence, which encodes a novel member of the TGF-β superfamily, corresponds to, but extends more 3' than the recently published lefty sequence (Meno et al., 1996, Nature 381: 151-155). The Stra3/lefty protein, which exhibits characteristics of secreted proteins, is synthesized as a precursor of 42 kDa and secreted after cleavage, suggesting that it may function as an intercellular signaling molecule. There are four exons in the Stra3/lefty gene and its 5' flanking region contains, among other putative regulatory elements, an unusual retinoid response element consisting of two half sites arranged as a palindrome, with an 8 base pairs spacer. We also show that Stra3/lefty is ectopically induced in the endodermal and ectodermal layers following in vivo administration of retinoic acid to gastrulating mouse embryos.

KEY WORDS: gastrulation, mouse development, retinooids, left-right asymmetry, secreted factors

Introduction

The transforming growth factor-β (TGF-β) superfamily is a large group of structurally related secreted proteins which have been shown to be involved in growth and differentiation in both vertebrates and invertebrates (for review, see Kimelman 1993; Kingsley 1994; Wall and Hogan 1994). These proteins are first synthesized as large precursors that undergo proteolytic cleavage to generate the secreted mature carboxy-terminal peptides, which harbor 7 to 9 conserved cysteine residues. TGF-β-related proteins are active as dimers and function as intercellular signals through receptors which are complexes of transmembrane serine/threonine kinases (Massagué et al., 1994). A number of TGF-β-related gene products have been reported to have mesoderm-inducing activity in amphibian embryos, the most effective being activins (e.g. Harland 1994; Klein and Melton 1994). The expression of several TGF-β family members appears to be regulated by retinoic acid (RA) in different embryonal carcinoma cell lines (Gudas et al., 1994). RA regulates gene expression through two families of receptors which act as ligand-dependent transcriptional regulatory proteins. The three retinoic acid (RA) receptor isotypes (RARα, β and γ) bind all-trans and 9-cis RA, while the three retinoid X receptor isotypes (RXRα, β, and γ) bind 9-cis RA only. These receptors bind as heterodimers to sequences known as RA-response elements (RARE) which are located in the regulatory regions of target genes (reviewed in Chambon 1994,1996; Mangelsdorf et al., 1994,1995; Kastner et al., 1995).

In a differential screening experiment aimed at isolating new RA-inducible genes in P19 embryonal carcinoma cells (Bouillet et al., 1995), we have isolated a cDNA sequence, Stra3, which encodes a novel murine member of the TGF-β superfamily. This cDNA partly overlaps the recently described lefty sequence, whose product has been suggested to play a role in the establishment of left-right asymmetry in the mouse embryo (Meno et al., 1996). We report here the primary structure of the Stra3/lefty protein and of the corresponding gene, as well as its expression pattern in adult and embryonic mouse tissues. Our data suggest that a RARE located in the promoter region may be responsible for the RA induction of this gene. We also show that the Stra3/lefty protein is synthesized in cultured cells as a precursor and secreted after cleavage, and that the Stra3/lefty gene is inducible by RA in vivo in gastrulating mouse embryos.

Results

Cloning and characterization of Stra3

We previously reported a subtractive hybridization strategy that led to the isolation of cDNA clones corresponding to RA-inducible transcripts in P19 embryonal carcinoma cells (Bouillet et al., 1995). One of these clones, Stra3, was used as a probe to screen an

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The sequence homology of Stra3 with other members of the TGF-β family was investigated. The gene contained four exonic sequences (E1, EII, EIII and EIV) of 358, 247, 246 and 789 bp, respectively, encoding the entire cDNA sequence and separated by three introns of 1180, 215, and 358 bp (Fig. 1A). All the intron-exon junctions correspond to consensus splice sites (Fig. 2A). The 1.8 kb genomic sequence located upstream of exon I was determined and found to display features of eukaryotic gene promoters (Fig. 2B). The presence of a consensus TATA box (TATAAA) at position -14 from the start of Stra3 cdNA and of a consensus CT signal (CTNCCNAGNC) (Larsen et al., 1995) suggest that this region may contain the promoter of the Stra3 gene.

**Chromosomal mapping of Stra3/leaky**

The chromosomal location of the Stra3 gene was determined by fluorescence in situ hybridization (FISH) on metaphase chromosome spreads, using a 20 kb biotinylated genomic probe (see Materials and Methods). FISH on WMP mouse, which harbors a Rb(1;11) Robertsonian translocation, revealed a signal in the distal region of chromosome 1, corresponding to band 1F (Fig. 3).

**Stra3 gene expression in P19 and ES cell lines and in adult organs**

The regulation of Stra3 gene expression by retinoic acid in P19 and ES cells was investigated using a semi-quantitative RT-PCR technique (Bouillet et al., 1995). Stra3 transcripts were detected in untreated P19 and D3 ES cells, but a strong increase in transcripts...
levels was found upon RA treatment (Fig. 4A). We have also used RT-PCR to investigate the expression of the Stra3 gene in several adult mouse tissues including brain, heart, lung, liver, kidney, spleen, female genital tract and testis. Stra3 was found to be expressed in all of these organs except in brain and liver (Fig. 4B). Control experiments (not shown) on the same RNA samples did not show significant variation in the content of the invariant 36B4 RNA (Bouillet et al., 1995).

Since another RA-inducible gene (Stra8) was previously shown to be expressed in premeiotic germ cells (Oulad-Abdelghani et al., 1996b), we performed immunohistochemistry with an anti-Stra3 antibody to localize the distribution of the corresponding protein on adult mouse testis sections (see Materials and Methods). Stra3 labeling was restricted to elongated cells which can be easily identified as spermatids (Fig. 5). The signal detected in the interstitial spaces was unspecific, since it also appeared in control experiments with adsorbed antibody solution, under conditions where there was no spermatid staining (not shown). Furthermore, the Stra3 protein was found in some seminiferous tubules only (Fig. 5), suggesting that its expression is restricted to certain stages of the spermatogenic cycle.

Processing and secretion of the Stra3 protein

Most of the members of the TGF-β family are synthesized as precursor molecules with an amino-terminal signal sequence and a prodomain. This precursor is usually cleaved at a dibasic (RXR) site to release a mature protein. As the Stra3 protein is a member of this family and contains a putative signal sequence, we analyzed the synthesis and processing of the Stra3 protein. COS-1 cells were transfected with a pSG5-based expression vector (Green et al., 1988) containing the full length Stra3 cDNA. Total cellular proteins as well as proteins released in the medium were characterized by SDS-PAGE and western blotting (see Materials and Methods). An immunoreacting polypeptide of 42 kDa, consistent with the mass of the full protein predicted from the cDNA, was detected in the transfected cells only (Fig. 6A). In contrast, a polypeptide of 33 kDa was detected in conditioned medium obtained from COS cells transfected with the Stra3 expression vector, and was not found in the medium of control cells. We did not detect the presence of the Stra3 precursor protein in total P19 cell protein extracts, probably due to a rapid turnover of the protein (Fig. 6B). However, as with transfected COS-1 cells, an immunoreactive species of 33 kDa was detected in the medium of RA-treated P19 cells. This result suggests that the Stra3 protein is expressed as a precursor of 42 kDa and cleaved to release a mature protein of 33 kDa in the medium.

Identification of an unusual RA response element in the Stra3/lef ty gene

The 5’ flanking region of exon I contains, among other potential regulatory elements, a sequence consisting of two hexameric motifs AGGTCC and TGACCT arranged as an imperfect palindrome (inverted repeat, IR) with an 8bp spacer (IR6; positions -51 to -70 in Fig. 2B). To analyze whether this element is able to bind RARs, we performed electromobility shift assays using an IR6-containing oligonucleotide (positions -42 to -81) as a probe and the bacterially-purified RARα1 protein. A retarded complex was obtained with the RARα1 protein, and this complex could be competed out by an excess of unlabeled oligonucleotide (Fig. 7A).

To assay for RAR transcriptional activation, COS-1 cells were transfected with a reporter construct consisting of the IR6-containing region (nucleotides +15 to -262, see Fig. 2B) linked to the SV40 early promoter and the CAT gene (PBLCAT5; Boshart et al., 1992), and
a RARα1 expression vector. No significant increase in CAT activity was observed when cells were transfected with the reporter and expression vectors in the absence of RA, or in the presence of RA but without cotransfection of the RARα1 expression vector (Fig. 7B). However, when cotransfected cells were treated with RA (1μM), a 4-fold increase in CAT activity was observed (Fig. 7B). These results suggest that the IR8 element can act as a retinoic acid response element (RARE) to mediate transactivation by RARα1.

**Ectopic RA-induced expression of Stra3/lefty in mouse embryos**

In a previous report, Stra3/lefty expression was analyzed by whole-mount *in situ* hybridization and shown to be restricted to early post-implantation development (Meno et al., 1996). Our expression analysis is in good agreement with these data. Expression of Stra3/lefty was first detected at the onset of gastrulation (6.0-6.5 dpc). The transcripts were restricted to the primitive streak and adjacent mesoderm (Meno et al., 1996; Figs. 8A and 9A,C). Note that both the posterior and anterior extremities of the primitive streak and adjacent mesoderm do not express this gene. In addition, Stra3/lefty transcripts were detected in a small region of the visceral endodermal layer at the anterior pole of the egg-cylinder (Figs. 8A and 9A,C). Transverse sections showed that the transcripts are not found in the ectodermal layer (Fig. 9C). As described previously (Meno et al., 1996), Stra3/lefty expression appeared to be completely shut off before the headfolds appear. At the beginning of somitogenesis (from 3-4 somite pairs), Stra3/lefty transcripts were expressed asymmetrically on the left side of the embryo in (i) the lateral plate mesoderm, from the base of the allantois to the caudal boundary of the precardiac area, and (ii) the floor plate of the neural tube (Fig. 8B), except in its most caudal part where the expression was symmetrical (Meno et al., 1996; our unpublished data). This transient expression became undetectable at about 6-7 somite pairs.

We performed *in situ* hybridization on cryosections in order to analyze Stra3/lefty expression at later stages of development. The only region showing some signal was the genital ridge and developing gonad, whose cells were labeled at 9.5 dpc (not shown) and later stages (Fig. 8C,D: 12.5 dpc). After sexual differentiation, Stra3/lefty transcripts were only detected in the male gonad (data not shown).

In order to investigate whether exogenous RA may interfere with Stra3/lefty expression during gastrulation, T-RA was administered orally to pregnant females at 6.25 dpc (see Materials and Methods). Embryos were collected 12 h after T-RA administration and analyzed by whole-mount *in situ* hybridization. In such samples, Stra3/lefty expression was only detected in the male gonad (Fig. 9B). As described previously (Meno et al., 1996), Stra3/lefty expression was completely shut off before the headfolds appear. At the beginning of somitogenesis (from 3-4 somite pairs), Stra3/lefty transcripts were expressed asymmetrically on the left side of the embryo in (i) the lateral plate mesoderm, from the base of the allantois to the caudal boundary of the precardiac area, and (ii) the floor plate of the neural tube (Fig. 8B), except in its most caudal part where the expression was symmetrical (Meno et al., 1996; our unpublished data). This transient expression became undetectable at about 6-7 somite pairs.

![Fig. 3. Mapping of the Stra3/lefty gene to WMP mouse chromosomes.](image)

Two partial metaphases show the specific hybridization signal on distal chromosome 1 (arrowheads), in the Robertsonian Rb(1;11) translocation.

![Fig. 4. Expression of the Stra3 gene in P19 and ES cells, and in adult organs.](image)

Total RNA was extracted and analyzed by RT-PCR. Amplification products were analyzed by Southern blotting. **(A)** P19 cells were incubated for 24 h with ethanol (c) or with 1μM T-RA. D3 ES cells were grown for 24 h in the presence of ethanol (c) or for 12 h, 24 h and 48 h in the presence of 10 nM T-RA. **(B)** Stra3 RNA expression in adult mouse organs. F.G.T., female genital tract.
embryos, ectopic activation of Stra3/lef ty was observed in some visceral endodermal cells over the entire distal surface of the egg cylinder (Fig. 9A,B and D, unfilled arrowheads). Ectopic expression was also seen in some of the underlying ectodermal cells (Fig. 9D, filled arrowheads). Furthermore, labeling extended into more anterior regions in RA-treated embryos (Fig. 9A,B: note the absence of a sharp anterior expression boundary in the primitive streak of RA-treated embryos); but this extension was less marked in the lateral mesodermal regions than in the primitive streak.

Discussion

We have cloned and characterized the RA-responsive Stra3 gene, whose cDNA was previously reported under the name of lefty (Meno et al., 1996), and which encodes a new member of the TGF-β superfamily. The primary structure of the Stra3/lef ty protein shows most of the conserved residues characteristic of this family, particularly the seven invariant cysteine residues. In TGF-β2, nine cysteine residues are involved in intrachain and interchain disulfide bonds and are important for the structure and dimerization of the protein (Daopin et al., 1992; Schlunegger and Grütter 1992). By analogy with the structure of TGF-β2, intrachain disulfide bonds could occur in Stra3 between Cys-253 and Cys-266, Cys-265 and Cys-318, Cys-295 and Cys-353, and Cys-299 and Cys-355. However, Stra3 encodes a glutamine residue at amino acid position 317, where most of the other TGF-β like molecules contain a cysteine. This change could be significant, since structural studies of TGF-β2 have demonstrated that this cysteine is involved in a disulfide linkage between two molecules (Schlunegger and Grütter 1992) and participates in the formation of a functional dimeric protein. The dimer, however, is stabilized by a variety of other interactions, including hydrophobic interactions and hydrogen bonding. Consistent with this amino acid change in Stra3 protein, we have not detected any stable Stra3 dimer by SDS-PAGE under non-reducing conditions (data not shown). However, one cannot

Fig. 6. Stra3 is a secreted protein. Proteins from cells or conditioned media were prepared as described in Materials and Methods and subjected to SDS-PAGE and western blotting. (A) Stra3 immunodetection in transfected COS cells and their conditioned media. (B) Immunodetection of endogenous Stra3 protein in P19 cells incubated for 24 h with or without 1μM RA. Abbreviations: C, control (non-transfected) cells; T, transfected cells; TP, total cell proteins; MP, proteins from the medium.

Fig. 5. Stra3 protein detection in the testis. Stra3 protein was localized by immunohistochemistry with an anti-Stra3 antibody on cryosections of adult mouse testis. Dark immunoperoxidase staining can be observed in elongated spermatids. The weak staining detected in interstitial spaces is non-specific (see Results).
Members of the TGF-β superfamily with highly related sequences are grouped into distinct subfamilies (TGF-β, activin, dpp or 60A subfamilies; for review, see Kingsley 1994; see also Fig. 1B). The sequence identity between Stra3/Lefty and other TGF-β like proteins (19% to 37%) is not high enough to connect it to anyone of these subfamilies in particular. Thus, Stra3/Lefty may define a novel type of TGF-β related protein.

Proteins of the TGF-β family are typically synthesized as inactive dimers that undergo proteolytic cleavage to generate mature carboxy-terminal segment that forms the ligand molecule (Celeste et al., 1990; Barr 1991). The cleavage site is usually located near the first conserved cysteine. The Stra3 protein contains an amino-terminal signal sequence and a putative N-glycosylation site, consistent with the idea that the Stra3 protein, as most of the TGF-β family members, is secreted. Our analysis of Stra3 expression and processing in COS and P19 cells indicates that this protein is synthesized as a precursor of 42 kDa and cleaved to release a mature protein of 33 kDa. From these results, we propose that the multibasic site RGKR at position 74 could be the proteolytic cleavage site (Barr, 1991). Our data are consistent with those reported by Meno et al., (1996) in other cell types. Thus, there appears to be an important difference between Stra3/Lefty and other TGF-β family members where the cleavage site is located further downstream (releasing only the conserved carboxy-terminal region). It would be interesting to investigate whether the long N-terminal domain characteristic of the Stra3/Lefty protein could be involved in any specific function.

Primer extension experiments did not allow us to determine unequivocally the transcription initiation site (our unpublished results). However, analysis of the 5' flanking sequence of the Stra3 gene revealed the presence of a consensus TATA-box (TATAAA) and a consensus CT signal (CTCNNAAGC), which are widely found in eukaryotic promoters (Larsen et al., 1995), suggesting that this region may contain the promoter of the Stra3 gene. The region upstream of the putative TATA box contains an element consisting of two PuGGTCA-related hexameric motifs (AGGTCC and TGGACT) arranged as an imperfect inverted repeat with a 8bp spacer (IR8). Electromobility shift and cotransfection assays have revealed that RARα1 can bind to an oligonucleotide probe containing this IR8 element and can activate transcription from an IR8-containing reporter gene. Thus, this IR8 element appears to be an atypical RARE, since most of these RAREs which have been described consist of direct repeats with 1, 2 or 5bp spacers (DR1, DR2, and DR5). However, a number of elements showing different arrangements of repeated motifs have also been characterized as putative RAREs (for review and refs, see Chambon 1996; Gronemeyer and Laudet 1996). In this respect, we note that the Stra3 IR8 element is related to the RARE identified in HIV-1, which consists of an inverted repeat of two hexameric motifs separated by a 9bp spacer (Orchard et al., 1994). Our results suggest that IR8 represents a RA response element which may be responsible for the RA-inducible expression of the Stra3 gene in P19 cells.

We have used in situ hybridization to analyze Stra3/Lefty expression pattern during development. The early expression of Stra3/Lefty in the anterior visceral endoderm, at the onset of gastrulation, was not described in the previous study of Meno et al., (1996), who only reported expression in the primitive streak mesoderm. In keeping with their study, we detected asymmetrical (left-sided) expression of Stra3/Lefty during early somitogenesis, which ap-
Fig. 8. In situ hybridization analysis of Stra3/lefy expression during development. (A) Whole-mount in situ hybridization of a late primitive streak-stage embryo (−6.75 dpc). The arrow is pointing to the visceral endoderm-expressing cells. (B) Whole-mount in situ hybridization of a 5 somite-stage embryo (−8.5 dpc; dorsal view) showing the asymmetrical expression on the left side. The unlabeled arrow indicates the signal in the floor plate. (C and D) Dark-field and bright-field views, respectively, of a histological section through the developing gonad of a 12.5 dpc embryo, hybridized to a 5S-labeled Stra3/lefy riboprobe. The signal grain appears in white in panel C. Abbreviations: b, brain; g, gonad; k, kidney; l, liver; lpm, lateral plate mesoderm; m, mesonephros; ps, primitive streak; s, stomach.

Seemed to be quickly shut off at the 6-7 somite-stage. The only structure where Stra3/lefy expression resumed at later stages was the genital ridge, and then the developing male gonads. It will be of interest to investigate whether this phase of expression and the expression seen in spermatids cells of the adult testis reflect a single or distinct functions.

We have also analyzed whether the Stra3/lefy gene may respond to RA when it is first expressed during early gastrulation at 6.0-6.5 dpc. In normal embryos, the transcripts are restricted to the middle region of the primitive streak and the adjacent newly formed mesoderm, as well as to a patch of anterior visceral endodermal cells. We analyzed Stra3/lefy transcript distribution 12 h after maternal administration of T-RA at 6.25 dpc. Interestingly, Stra3/lefy was ectopically activated, both in endodermal and ectodermal cells, in the entire distal region of the egg-cylinder. Furthermore, expression extended more anteriorly in the primitive streak of RA-treated embryos, but appeared less widespread in the adjacent lateral mesodermal wings.

Several genes such as nodal (Varlet et al., 1997), Lim-1 (Barnes et al., 1994; Shawlot and Behringer, 1995), Otx-2 (Simeone et al., 1993; Ang et al., 1994) HNF-3β (Ang et al., 1993; Monaghan et al., 1993) or Hesx-1 (Thomas and Beddington, 1996) are expressed in the visceral endoderm of gastrulating embryos. Loss-of-function of some of these genes (Ang and Rossant, 1994; Weinstein et al., 1994; Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996), as well as chimera studies (Varlet et al., 1997) and other experimental strategies (Ang and Rossant, 1993; Thomas and Beddington, 1996) suggested that the anterior visceral endoderm may be responsible for the patterning of the most rostral structures in the embryo. It has been shown that RA administration during late gastrulation-early organogenesis induces a lack or a truncation of anterior structures (Cunningham et al., 1994; see Conlon 1995 for review and refs; Simeone et al., 1995; Avantaggiato et al., 1996). Hence, endogenous retinoids may act as inducers of posterior structures during embryogenesis. Our data show that administration of excess RA during early
Fig. 9 Retinoic acid induces ectopic activation of Stra3/lefty in gastrulating embryos. (A) Whole-mount in situ hybridization analysis of Stra3/lefty transcript distribution in 6.75 dpc mouse embryos. Top row: control embryos. The arrowheads point to the sharp anterior expression boundary in the primitive streak and adjacent mesoderm. Bottom row: embryos that received a dose of excess RA (by maternal gavage) 12 h previously (see Materials and Methods). All embryos are oriented with the distal part of the egg-cylinder towards the bottom, and the anterior side towards the left. (B) Higher magnification of one of the RA-treated embryos. Note the abnormal presence of labeled cells in the whole distal region of the egg-cylinder and the absence of a clear boundary of expression towards the anterior part of the primitive streak. (C) Transverse section through a control embryo, showing expression in a discrete area of the anterior visceral endoderm, as well as in the primitive streak and lateral mesodermal wings. (D) Transverse section through an RA-treated embryo. Note the presence of ectopically labeled endodermal and ectodermal cells (unfilled and filled arrowheads, respectively). Abbreviations: egg-cylinder axes: an, anterior; po, posterior; pr, proximal; di, distal; ec, ectoderm; em, embryonic region; en, visceral endoderm; ex, extra-embryonic region; me, mesoderm; paf, posterior amniotic fold; ps, primitive streak.

gastrulation can alter the spatially-restricted expression of Stra3/lefty, and support the idea that endogenous sources of retinoids may regulate this expression during normal development. Gene disruption (knockout) experiments will be required to identify which aspects of embryonic patterning are under the control of Stra3/lefty signaling.

Materials and Methods

Cell culture and RA treatment
P19 cells were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) enriched with 5% fetal calf serum (Rudnicki et al., 1988). D3 ES cells were cultured as described (Lufkin et al., 1991). To induce differentiation, all-trans RA (T-RA) was added to a final concentration of 1μM (P19 cells) or 10nM (ES cells) from a 1mM stock solution in ethanol. In control cultures, ethanol was added to a final concentration of 0.1%. At appropriate incubation times, the cells were washed with PBS, scraped and recovered by centrifugation.

RNA extraction and RT-PCR analysis
Total RNA from cultured cells and organs was prepared (Auffray and Rougeon 1980), and reverse transcription-polymerase chain reactions (RT-PCR) were carried out as described (Bouillet et al., 1995). Oligonucleotide primers used in this study were 5'-CACAGAGGCGGTGGGAAAG-3' (nucleotides 562 to 579) and 5'-GTTCCTGGCCCATTTCA-3' (nucleotides 897 to 881 on the reverse strand). Amplification products were separated on 2% agarose gels, transferred onto Hybond N membranes (Amersham) and revealed by Southern blotting (Maniatis et al., 1982).

DNA cloning and sequencing
The 220 base pairs (bp) Stra3 cDNA fragment (Bouillet et al., 1995) was used as a probe to screen an oligo(dT)-primed λZap II cDNA library prepared from P19 cells cultured as monolayers for 24 h in the presence of 1μM T-RA. Positive plaques were isolated and in vivo excision was performed according to the manufacturer (Stratagene). The longest pBluescript SK plasmid cDNA was sequenced on both strands using the DyeDeoxy terminator cycle sequencing on an ABI373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Genomic fragments were obtained by screening a mouse genomic library prepared from D3-ES cell DNA in λEMBL3 (Frischauf et al., 1983). The nucleotide sequence of Stra3 cDNA and genomic DNA have been submitted to Genbank/EMBL and assigned accession numbers AJ000082 and AJ000083, respectively.

Fluorescence in situ hybridization chromosomal mapping
Metaphase spreads were prepared from a WMP male mouse, in which all the autosomes except chromosome 19 were in the form of metacentric Robertsonian translocations (Bonhomme and Guénét, 1989). Concana- lin A-stimulated lymphocytes were cultured at 37°C for 72 h with 5-bromodeoxyuridine added for the final 6 h of culture (60 mg/ml of medium) to ensure a chromosomal R-banding of good quality.

A 20 kb Stra3 genomic DNA probe was biotinylated by nick-translation with biotin-16-dUTP, as outlined by the Bohringer Mannheim protocol. Hybridization to chromosome spread was performed with standard protocols (Pinkel et al., 1986, Matsuda et al., 1992). The DNA probe was mixed with hybridization solution at a final concentration of 10 ng/ml and used at 100 ng per slide. Before hybridization, the labeled probe was annealed with a 150-fold excess amount of Cot-1 DNA (GIBCO-BRL) (for 45 min at 37°C) in order to compete the specific repetitive sequences. The hybridized probe was detected by means of fluorescence isothiocyanate-conjugated avidin.
Transfection experiments and DNA mobility shift assay

The full length Stra3 cDNA was cloned downstream of the SV40 promoter into the expression vector pSGS (Green et al., 1988). Transfections were carried out in COS-1 cells by the calcium phosphate coprecipitation method (Kumar et al., 1986). For CAT assays, 5 μg of a pBLCAT5 reporter plasmid (Boshart et al., 1992) containing part of the putative Stra3 promoter (nucleotides -15 to -262 from the start of lefty cDNA) was included in each transfection sample with or without 1 μg of a mRARα1 expression vector (Zelent et al., 1989). To determine the transfection efficiency of each cell, cells were cotransfected with 1 μg of the pCh110 β-galactosidase expression vector. Cells were then washed twice with DMEM and incubated with fresh medium in the presence or absence of RA (1 μM) for 24 h. CAT assays were performed as described (Petkovich et al., 1987) and normalized by measuring the β-galactosidase activity.

Electrophoretic mobility shift assays using the purified bacterially-expressed mRARα1 protein were carried out as described (Oulad-Abdelgani et al., 1996a). The probe was a [32P]-end-labeled double-stranded oligonucleotide (nucleotides -42 to -81). Competitor DNA consisted of unlabeled probe oligonucleotide.

Antibody production

The Stra3 cDNA sequence corresponding to the 124 carboxy-terminal amino acids was amplified by PCR and subcloned in-frame in the expression vector PET11b (Novagen), in order to obtain a fusion protein containing six histidine residues at the N-terminus. The recombinant protein was expressed in E. coli BL21, purified on a Ni-NTA column (Pharmacia). The affinity purified antibody preparation was dialyzed against PBS containing 20% glycerol and stored at -20°C.

Immunohistochemistry

Immunohistochemistry was performed on 10 μm cryostat sections of testis fixed with acetic, using the Vectastain ABC-Elite and DAB substrate kits (Vector Laboratories, Burlingame, CA). Slides were slightly counterstained with eosin and haematoxylin.

SDS-PAGE and western blotting

Total protein extracts were prepared as described (Rochette-Egly et al., 1991). Proteins from the medium were precipitated by adding four volumes of acetone to one volume of medium at -20°C and recovered by centrifugation. The pellet was washed with acetic acid, dried and resuspended in loading buffer (Laemmli 1970). Protein extracts were analyzed by SDS-PAGE on a 12% polyacrylamide gel, transferred to nitrocellulose membranes (Towbin 1979), and the presence of Stra3 protein was revealed by western blotting using an ECL kit (Amersham).

Treatment of mouse embryos with retinoic acid and in situ hybridization

Mouse embryos were recovered from natural overnight mating of CD1 mice. Fertilization was assessed by detection of vaginal sperm plugs in the morning (midday being considered as 0.5 days post coitum [dpc]). T-RA (Sigma) was prepared just prior to treatment by resuspending 50 mg in 1 ml ethanol. This mixture was diluted in 9 ml of sunflower oil. The suspension was administered by oral gavage to pregnant females at 6.25 dpc, at a dose of 50 mg/kg body weight. Embryos were collected 12 h after maternal gavage and processed for in situ hybridization. The embryos were staged according to the landmarks described in Down and Davies (1993). Whole-mount in situ hybridization with a digoxigenin-labeled Stra3 riboprobe, as well as in situ hybridization on cryosections using a [32P]-labeled riboprobe, were performed as previously described (Décimo et al., 1995).

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