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Leydig Cells Express Neural Cell Adhesion Molecules In Vivo and In Vitro[1]

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ABSTRACT

The neural cell adhesion molecule (NCAM) polypeptides are expressed by numerous tissues during embryonic development, where they are involved in cell-cell interactions. In the adult, NCAM expression is confined to a few cell types, including neurons and peptide-hormone-producing cells. Here we demonstrate that the Leydig cells of the adult rat, mouse, and hamster testes express NCAM as well. Western blotting showed that an NCAM of approximately 120 kDa was present in the adult testes of all three species investigated. This form was also found in freshly isolated mouse Leydig cells and in Leydig cells after 2 days in culture. After 4 days in culture, mouse Leydig cells expressed additional NCAM isoforms of approximately 140 and 180 kDa, indicating changes in alternative splicing of NCAM primary transcripts. Also, NCAM mRNA of all isoforms, as detected by S1-nuclease protection assays, increased with time in culture. The expression of the cell adhesion molecule NCAM by adult Leydig cells may explain the aggregation of Leydig cells in clusters in rodent testes, which could be a prerequisite for functional coordination of groups of Leydig cells. Furthermore, the presence of this neural and endocrine marker may indicate a closer relationship between Leydig cells and neural and peptide-hormone-producing cells than is considered to exist at the present time.

INTRODUCTION

Processes of cell-to-cell adhesion and cell-cell recognition are complex and involve a variety of specialized surface molecules. One of the best-studied of these is the family of neural cell adhesion molecules (NCAMs), first found in the nervous system [1–3]. These glycoproteins are members of the immunoglobulin superfamily and exist in three major isoforms of approximate apparent molecular masses of 180, 140, and 120 kDa [1–4]. They are transiently expressed during ontogeny in a variety of tissues and organs [1–3, 5] and are therefore considered to be involved in morphoregulatory processes. The principal NCAM isoforms are derived by alternative splicing of the primary transcript of a single NCAM gene and diversity forms [6]. NCAM-120 is exclusively located on the outer surface and anchored to the membrane by phosphatidyl inositol, whereas the transmembrane isoforms NCAM-140 and NCAM-180 differ in their cytoplasmic domains [4]. The major NCAM isoforms are expressed in different proportions by different cell types. In adult animals, in addition to neurons, all peptide-hormone-producing cells examined in previous studies express a specific isoform of the NCAM (140 kDa [7, 8]). The Leydig cells of the testis, like peptidergic endocrine cells (e.g., pituitary cells), produce a number of peptide hormones (e.g., inhibin; substance P; transforming growth factor [TGF]-alpha; POMC peptides) besides steroids [9–11; for review, see 12]. Since these cells conspicuously adhere to each other by forming cell clusters, we examined the possible expression of NCAMs in adult testes of rodents. We also studied NCAM expression in mouse Leydig cell cultures.

MATERIALS AND METHODS

Animals

Adult, 3–5-mo-old male Sprague-Dawley rats, mice (NMRI, breeding colony at the University of Ulm and Charles River, Extertal, Germany), and golden (Syrian) hamsters (Charles River, Wilmington, MA; tissues were generously provided by Dr. A. Bartke, Carbondale, IL) were housed under standard conditions with free access to food and water. Animals were decapitated under deep CO_2 anesthesia. Testes were removed and either frozen immediately in liquid nitrogen or dry ice (rat, mouse, and hamster testes for Western blotting), fixed in 4% paraformaldehyde/phosphate (0.01 M, pH 7.3)-buffered saline (PBS) for 8 h (rat and mouse testes for cryostat sectioning and subsequent immunocytochemistry), or immersed into Bouin's fixative overnight (rat, mouse, and hamster testes for subsequent embedding in paraffin and immunocytochemistry). The cerebellum, a tissue containing all three major NCAM isoforms, served as a positive control in S1-nuclease assays and Western blots.

Isolation and Culture of Mouse Leydig Cells

Mouse Leydig cells from adult mice were purified by use of discontinuous Percoll gradients as described by Sharpe and Fraser [13] with modifications. In brief, testes were de-

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capsulated and mechanically dispersed by repetitive pipetting in Dulbecco's modified Eagle's medium (DME; Flow Laboratories, Bonn, Germany), with the diameter of the pipette tip successively reduced. Undispersed tissue and tubule fragments were allowed to settle, and the supernatant (containing Leydig cells) was filtered through sterile gauze and centrifuged at 120 × *g* for 10 min. The pellet was resuspended in glucose-enriched PBS (6 g/L; G-PBS). Subsequently, 10 ml of the cell suspension (corresponding to the yield from 6 testes) was loaded onto a discontinuous gradient of Percoll (Pharmacia, Freiburg, Germany) in G-PBS. The gradient consisted of three layers (each 10 ml) with specific weights of 1.08, 1.06, and 1.05 g/ml. After centrifugation at 1100 × *g* for 25 min, the purified Leydig cells accumulating on top of the 1.08-g/ml layer were aspirated, washed in G-PBS containing 0.03% BSA, resuspended in culture medium (DME containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 20 μg gentamicin/ml), and counted in a hemocytometer. The yields of Leydig cells were in the range of 5–8 × 10^5 cells per animal, and the purity ranged from 75% to 95% with 3β-hydroxysteroid dehydrogenase staining used as a criterion [13]. Cells were cultured in 48-well plates (Costar, Cambridge, MA; for immunocytochemistry), in glass chamber slides (Lab-Tek, Nunc, Wiesbaden, Germany; for in situ hybridization), or in 60-mm plastic dishes (Greiner, Nuertingen, Germany; for Western blotting and S1-nuclease assay). They were cultured at 34.5°C in an atmosphere of 5% CO_2:95% air for 2 or 4 days.

cDNA and cRNA Probes Used for S1-Nuclease Assays and In Situ Hybridization Histochemistry

As described previously [14], we used the mouse NCAM cDNA DW 22-H/E [15, 16] for cDNA probe synthesis. A uniformly labeled single-stranded probe was then prepared as described [17], with use of an oligonucleotide that hybridizes to nucleotides (nt) 2054–2070 [15]. The labeled primer-extended NCAM subclone was digested with *Hin*dIII, yielding two fragments, which were separated on 5% polyacrylamide gels (containing 8.3 M urea). The 485-nt single-stranded antisense fragment was isolated and rerun on a 1% agarose gel. Subsequently, the DNA fragments were transferred electrophoretically to NA 45 DEAE-cellulose membrane (Schleicher and Schüll, Dassel, Germany). Purified fragments were then used for S1-nuclease protection assays. This probe spans 19 nt of exon 10, the entire exons 11 and 12, and 110 nt of exon 13, with the insertion of the extra exon a_{15}/AAG (15 nt and 3 nt) between exon 12 and exon 13.

cRNA Synthesis

For cRNA synthesis, we used the cDNA clone pM 1.3 (gift of Dr. C. Goridis, Marseilles, France [18]) in the pGEM-1 vector (Promega Biotec, Madison, WI) as a template [14]. Synthesis of ^{35}S-labeled cRNA (specific activity 8 × 10^8 cpm/μg) for in situ hybridization was carried out according to the T7-polymerase protocol of Promega Biotec using 107 μCi of alpha-^{35}S-CTP (specific activity 37 TBq/mM), and the NCAM cDNA clone pM 1.3 was cut with *Sau*3A. The resulting cRNA probe (243 nt) was complementary to exons 13–14.

S1-Nuclease Protection Assay

To detect specific NCAM mRNA in mouse Leydig cells, S1-nuclease protection assays [17] were performed as described previously [14, 19]. Cells (freshly isolated, or after 2 or 4 days in culture) were homogenized, and total RNA was isolated as described previously by Lahr et al. [19] by a modified guanidinium thiocyanate-CsCl method [20]. A total of 20 μg of RNA (determined photometrically) was hybridized with an excess of ^{32}P-labeled cDNA probe (5 × 10^4 cpm; specific activity 1 × 10^8 cpm/μg) in 75% formamide, 400 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 7.4) [16] for 16 h at 58°C. Hybridization was terminated by digestion with 680 U S1-nuclease (Angewandte Gentechnologie Systeme, Heidelberg, Germany) for 2 h at 37°C. Samples were extracted with phenol, precipitated with ethanol, and separated electrophoretically on 5% polyacrylamide gels (8.3 M urea). Gels were dried and exposed to x-ray film at −70°C with use of intensifying screens.

In Situ Hybridization Histochemistry

Cultured mouse Leydig cells, grown on glass chamber slides, were used for in situ hybridization histochemistry, carried out as described previously [14, 19]. In brief, cell cultures were fixed with 4% paraformaldehyde in 0.01 M PBS and were air-dried. Cells were then prehybridized at 50°C for 3 h with 1 ml hybridization solution (50% formamide; 0.75 M NaCl; 25 mM PIPES, pH 6.8; 25 mM EDTA; five-strength Denhardt's solution; 0.2% SDS; 10 mM dithiotreitol; 250 μg/ml denatured herring sperm DNA [Boehringer, Mannheim, Germany]; and 10% dextran sulfate). Cells were hybridized at 50°C overnight with 5 ng of labeled cRNA probe (specific activity 9.5 × 10^8 cpm/μg) in 150 μl hybridization solution in a humidified chamber. After hybridization, cells were treated for 30 min at 37°C in a buffer containing 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 μg/ml RNase A. They were then incubated for 30 min at 37°C in the same buffer without RNase A and washed twice with double-strength SSC (0.3 M sodium chloride; 30 mM sodium citrate, pH 7.0) at 50°C for 15 min. As a control, cells were treated with RNase A before hybridization (for details see [14]). Slides were rinsed in ascending alcohol solutions, air-dried, and dipped in Ilford K2 emulsion diluted 1:2 with distilled water. They were exposed for 3–6 wk at 4°C and developed with Kodak D19 (Eastman Kodak, Rochester, NY) at 16°C for 4 min. The cells were lightly stained with hemalaun (procedure according

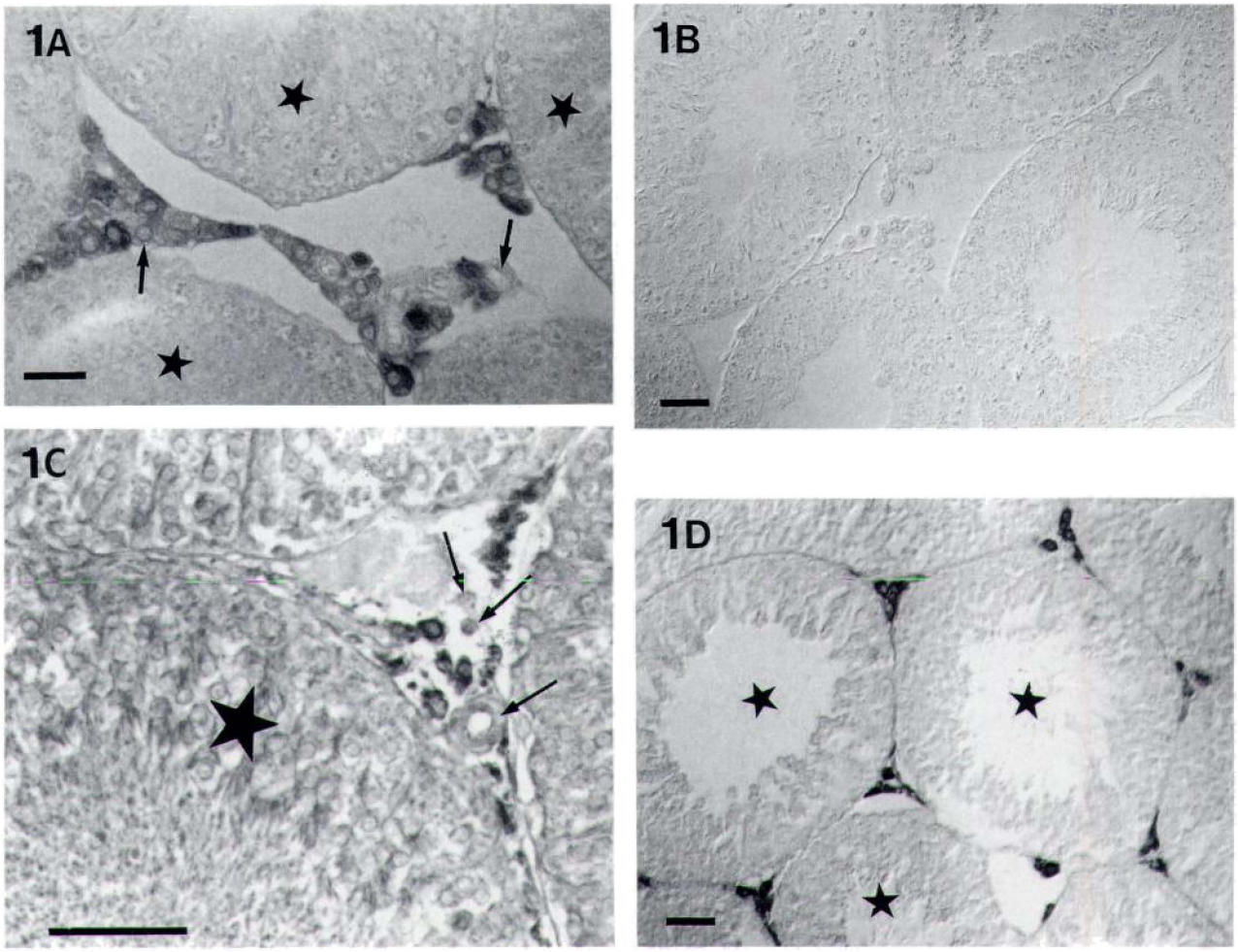


FIG. 1. Immunocytochemical analysis of NCAM in adult rodent testes. Immunolabeling with a polyclonal antiserum raised against a GST-NCAM-Ig domain fusion protein and a mAb against NCAM-bound PSA showing immunoreactive Leydig cells of adult rodent species. A) Paraffin section of a mouse testis; PAP method; NCAM antiserum, 1:500; Bar = 20 μm. B) A consecutive section of the one shown in A, used as a control (omission of the NCAM antiserum). Bar = 40 μm. C) Paraffin section of a rat testis; ABC method; mAb 735; dilution 1:1000; Bar = 100 μm. D) Paraffin section of a hamster testis; ABC method; NCAM antiserum; dilution 1:500; Bar = 40 μm. Asterisks mark the unstained tubular compartment; arrows point to unstained interstitial cells and unstained blood vessels.

to Mayer) and eosin (H.-E.) or Giemsa stain and were examined with a Zeiss Axioplan microscope (Oberkochen, Germany).

Western Blotting

Western blotting was performed as previously described [13]. In brief, frozen samples were thawed, homogenized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% sucrose and 2% SDS, and sonicated before heating (100°C for 5 min). Samples (15 μg of protein per lane) were separated electrophoretically on 7.5% SDS-PAGE. Proteins were blotted onto nitrocellulose membranes and probed with a well-characterized rabbit NCAM antiserum [21] (generously donated by Dr. G. Rougon, Marseilles, France), directed against the N-terminal end of the NCAM (used at a 1:1000 dilution; incubation overnight at 4°C). Immunoreaction was detected with the avidin-biotin complex (ABC) method (nonradioactive detection) [14] according to the manufacturer's instructions (Vectastain ABC kit; Camon, Wiesbaden, Germany). For the detection, a biotinylated goat anti-rabbit IgG antiserum (1:500; 1 h 20°C; Camon) was used as second antibody, followed by incubation with the ABC reagent and incubation with 0.01% H_2O_2, 0.075% 3,3′-diaminobenzidine-tetrahydrochloride dihydrate solution (DAB; Aldrich, Milwaukee, WI; diluted in 0.05 M Tris-HCl; pH 7.6), and 0.03% $NiCl_2$.

Immunocytochemistry

The localization of the NCAM in the testes and in cultured Leydig cells was examined with two different antisera and a monoclonal antibody (mAb): (1) the polyclonal antiserum used for Western blotting [21] was used for immu-

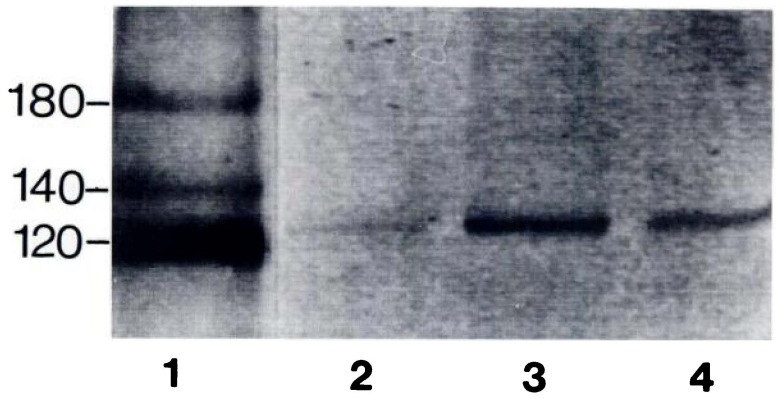


FIG. 2. Western blot analysis of testicular NCAM. Testicular homogenates from adult rat (lane 2), mouse (lane 3), and hamster (lane 4) contain NCAM with approx. molecular mass of 120 kDa. Rat cerebellum contains NCAM-180, -140, -120 (lane 1). Molecular masses of markers are indicated at left. Dilution 1:1000; ABC method.

nocytochemistry of cryostat sections and Leydig cells in culture; (2) another polyclonal rabbit antiserum raised against a purified glutathione-*S*-transferase (GST) fusion protein containing all five NCAM-Ig-domains (coded for by mouse cDNA sequence position 212–1635 [15], including extra exon π [16]) was used for staining of Bouin's-fixed and paraffin-embedded testicular sections; (3) a monoclonal antibody (mAb 735 [22]) directed against homopolymeric alpha (2–8) *N*-acetylneuraminic acid (polysialic acid; PSA) linked to NCAMs was also used for staining of Bouin's-fixed and paraffin-embedded testicular sections. Both the ABC method [23] and the peroxidase-anti-peroxidase (PAP) method [24] were performed on paraformaldehyde-fixed Leydig cells (4% in 0.01 M PBS, pH 7.3), cryostat sections, and deparaffinized Bouin's-fixed paraffin sections. The mAb [25, 26] recognized the PSA epitope of NCAMs in paraffin sections. Only the NCAM antiserum raised against the fusion protein works well in paraffin sections. Therefore, for the other NCAM antiserum we used 10–15-μm-thick cryostat sections of paraformaldehyde-fixed (4% in PBS; pH 7.3) mouse and rat testes. For the PAP method, cryostat and deparaffinized sections were preincubated with 2% normal swine serum (NSS) in Tris-buffered saline (TBS: 0.05 M Tris, 150 mM NaCl; pH 7.6) before incubation with the NCAM antisera (diluted in TBS with 2% NSS; 1:1000 or 1:500) overnight at 4°C. Subsequently, the sections were incubated with a swine anti-rabbit IgG antiserum (diluted in TBS with 2% NSS; 1:50; Dakopatts, Hamburg, Germany) and then with rabbit PAP complex (Dakopatts; 1:100; for details see [19]). For the ABC method, preincubation with 0.01 M PBS (pH 7.3) containing 0.5% BSA, a biotinylated secondary antiserum (goat anti-rabbit IgG; 1:500 diluted in PBS with 1% BSA; Camon), and a commercial ABC kit (Vectastain; Camon) were used. Immunoreaction was visualized in both cases with 0.01% H_2O_2 and 0.05% DAB solution (in 0.05 M Tris-HCl; pH 7.6). Controls were performed by (1) omission of the first antisera and (2) incubation with normal rabbit serum instead of the specific antisera. For detection of the PSA epitope by the mAb, we used deparaffinized 5-μm-thick sections of Bouin's-fixed testes (rat, mouse, and hamster), which were incubated with the mAb (1:1000) overnight. For detection with the PAP method, a secondary rabbit anti-mouse IgG antiserum (1:50) followed by mouse PAP (1:100) was used (both from Sternberger-Meyer Immunocytochemicals Inc., Jarretsville, MD). For detection with the ABC method, a biotinylated horse-anti-mouse IgG antiserum (1:500; Camon) and a commercial ABC kit (Vectastain; Camon) were used. Immunoreaction was visualized with DAB. Besides omitting the primary antibody, we performed controls by incubating with another commercial IgG mouse mAb of different spec-

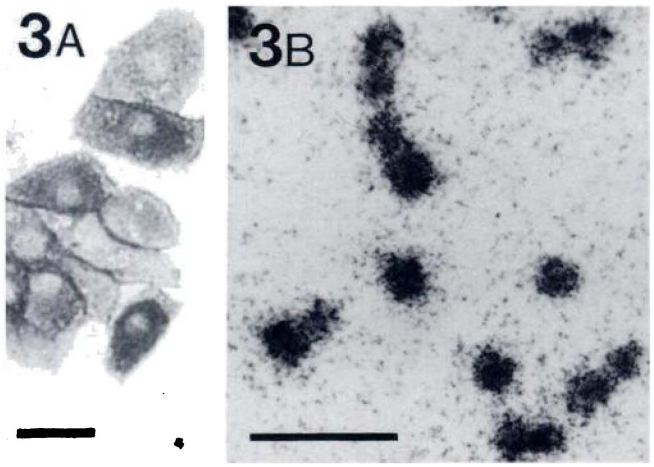


FIG. 3. NCAM immunoreactivity of cultured Leydig cells isolated from mouse testis and NCAM in situ hybridization. A) Cultured Leydig cells, especially at contact sites of neighboring cells are NCAM-immunoreactive (4 days in culture; ABC method; dilution 1:1000; bar = 25 μm). B) Leydig cells in culture possess NCAM mRNA (2 days in culture; bar = 50 μm).

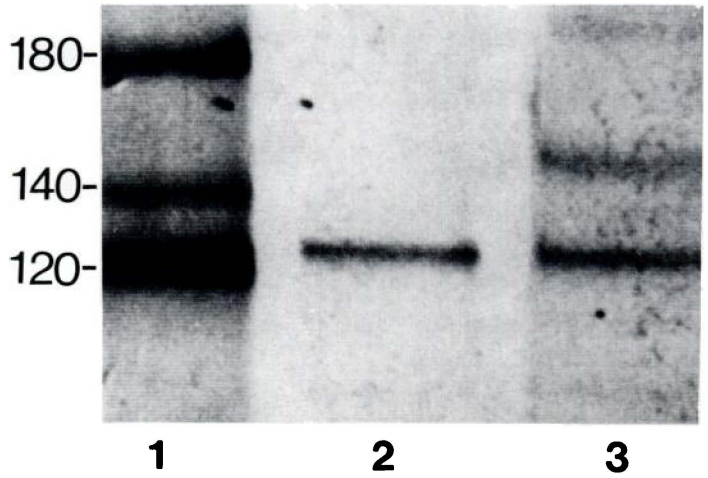


FIG. 4. Western blot analysis of mouse Leydig cell NCAM. NCAM with a molecular mass of approximately 120 kDa is present in freshly isolated mouse Leydig cells (lane 2); after 4 days in culture additional NCAM forms of approximately 140 and 180 kDa are found (lane 3). Rat cerebellum contains NCAM of 180, 140, and 120 kDa (lane 1). Molecular masses of markers are indicated at left. Dilution 1:1000; ABC method.

ificity (anti-tyrosine hydroxylase; 1:1000, Boehringer) and with mouse IgG (1:1000; Sigma, Munich, Germany). Sections were examined with a Zeiss Axioplan microscope.

RESULTS

Expression of NCAMs in the Testes of the Adult Rat, Mouse, and Hamster

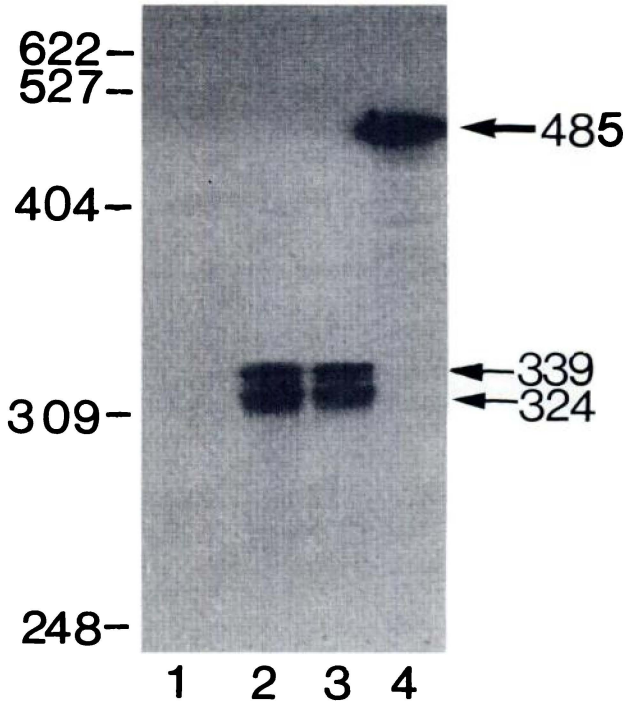
In the testes of adult mice (Fig. 1A), rats (Fig. 1C), and hamsters (Fig. 1D), only Leydig cells were immunoreactive with NCAM antisera and a mAb detecting NCAM-bound PSA. Faint immunoreactivity localized over the tubular compartment, mainly over spermatids, was observed under certain conditions, but was completely abolished by further diluting the antiserum/antibody (conditions in which Leydig cell staining persisted). Such staining was also abolished by preincubation with sera from species in which the secondary antibodies were raised. This leads us to conclude that this staining was unspecific. Immunocytochemical localization of NCAMs in sections of frozen testes was identical to NCAM localization in paraffin sections (not shown). In all controls performed, Leydig cells were unstained. In Western blots, an immunoreactive NCAM of a molecular mass of approximately 120 kDa was identified in testicular homogenates of adult rats, mice, and hamsters (Fig. 2).

II. Expression of NCAMs in Mouse Leydig Cell Cultures

NCAM immunoreaction was also present in cultured mouse Leydig cells after all culture periods examined (Fig. 3A). Results of in situ hybridization histochemistry indicate that the mouse Leydig cells cultured for 2 or 4 days (Fig. 3B) contained NCAM mRNA. Western blotting showed an immunoreactive NCAM of approximately 120 kDa in freshly isolated mouse Leydig cells and in mouse Leydig cell culture after 2 days (Fig. 4). However, after 4 days in culture, mouse Leydig cells also expressed NCAM forms of approximately 140 and 180 kDa (Fig. 4). The results of the S1-nuclease assays revealed that NCAM mRNA levels in freshly isolated mouse Leydig cells were below the detection limits of the assay, but NCAM-specific mRNA was detectable in cultured mouse Leydig cells 2 and 4 days after isolation. As shown in Figure 5, hybridization with the probe spanning parts of exon 10 and exons 11–13, followed by digestion with S1-nuclease, yielded 339-nt, 324-nt, and 93-nt fragments (93-nt fragment not shown). The major band (324 nt) indicates that the main mRNA form contains either no insertion in splice site a or only an AAG. Since no fragment of 435 nt was observed, the diversity composition exon 12-a_{15} (± AAG)-exon 13 is absent in Leydig cell NCAM mRNA. The thinner protected band of 339 nt indicates the insertion of the extra exon a_{15} followed by one or more additional alternative exons (see *Discussion*). We did not detect NCAM mRNA (using S1-nuclease assay and in situ hybridization; our unpublished results) in the tubular compartment or in whole testicular homogenates, which largely consist of seminiferous tubules.

DISCUSSION

Although it is a typical feature of Leydig cells to form aggregates in the interstitial spaces of the testis, adhesive mechanisms involved in the formation of these Leydig cell clusters have not been well examined. It is known that gap junctions are found between adjacent Leydig cells, and recently it has been proposed that Leydig cells are able to adhere to extracellular matrix with affinities for collagen, fibronectin, laminin, and the basement membrane matrix [27]. In the present study, we describe for the first time the presence of the adhesion molecule NCAM on Leydig cells. The NCAM could mediate adhesion of Leydig cells to each



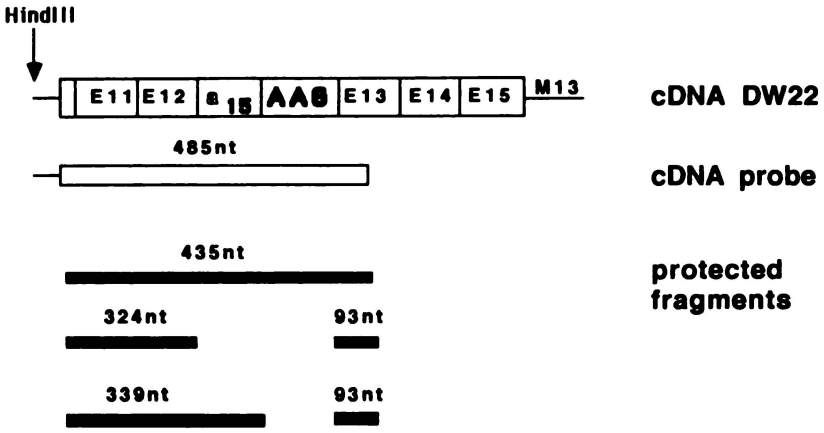


FIG. 5. Result of S1-nuclease protection assay and scheme of probe and protected fragments. Top: NCAM-specific mRNA is not detectable in RNA from freshly isolated mouse Leydig cells (lane 1), but is found after 2 and 4 days of culture (lanes 2, 3). Note the double banding (see Results and Discussion): A 324-nt fragment and a 339-nt fragment are detected (arrows), indicating the absence of extra exons a_{15} (± AAG). Twenty micrograms of RNA per lane was examined. Lane 4: probe (485-nt; thick arrow). Sizes (nt) of markers (PBR 322 × *Hpa*II) are indicated at right, sizes of the probe and the protected fragments are shown at left. Bottom: Schematic representation of the probe used for S1-nuclease protection assay and expected protected fragments (see Materials and Methods, Results, and Discussion for details).

other, as well as adhesion of Leydig cells to extracellular matrix, because NCAM binding is homophilic (autoadhesion, which could explain why neighboring NCAM-bearing Leydig cells can adhere to each other) and because via a heparin-binding region (and via laminin present in the testis [28]) the NCAM can adhere to collagen [3, 29]. The fact that Leydig cells form typical clusters in the testis appears to be of functional importance, as concluded from recent

in vitro experiments performed by Hedger and Eddy [30]. In these experiments, the ability of cultured rat Leydig cells to respond to hCG with testosterone output was greatly augmented by increasing the density of Leydig cells on the culture plate. This was apparently due to the production of a yet unknown factor by Leydig cells [30] that stimulated testosterone production. The authors suggested that a similar mechanism also exists in vivo and that a Leydig cell factor could coordinate the function of local clusters of Leydig cells. In this context, it should be mentioned that in other NCAM-expressing tissues NCAM-mediated adhesion promotes the formation of cell-to-cell channels (in chick neuroectoderm) [31] and that NCAM, while promoting contacts between cell membranes, also allows the transmission of NCAM-independent cell signals (in embryonic sympathetic neurons) [32]. In view of these data, the NCAM on Leydig cells may be a prerequisite for the formation of Leydig cell clusters and thus for a coordinated regulation of Leydig cell function in vivo.

Our data showing that staining for the NCAM is virtually absent in the tubular compartment of adult rat, mouse, and hamster testes contrast with previous reports [33–35]. These immunocytochemical studies describe that an antiserum cross-reactive with D2 (equivalent to the NCAM [35]) and another antiserum against the NCAM recognized only the head region of spermatids and residual bodies in the adult rat testis [33, 35], or weakly stained residual bodies and late spermatids in the mouse testis [34]. Although we have no clear explanation for these discrepancies, differences in techniques and specificity of the antisera may account for the conflicting results of previous studies [33–35] in which no staining of Leydig cells was reported. We have also observed faint nonspecific immunoreactivity over the tubular compartment, mainly over spermatids under certain conditions. However, this staining was abolished by further diluting the antiserum/antibody (after which Leydig cell staining persisted). Such staining was also abolished by preincubation with sera from species in which the secondary antibodies were raised. Thus we concluded that this staining is unspecific. Moreover, besides the lack of specific NCAM immunoreactivity, we did not detect NCAM mRNA nor NCAM protein in the tubular compartment.

In the testes of the adult rat, mouse, and hamster, Leydig cells express an isoform of the NCAM with a molecular mass of approximately 120 kDa. Other studies suggest that this form appears to be a rather mature form of the NCAM, normally transcribed late during development; it is also found on mature oligodendrocytes [36]. Moreover, a low turnover rate of testicular NCAM-120, with the consequence of only minimal transcription, can be deduced, since we were unable to detect measurable amounts of NCAM mRNA by S1-nuclease protection assays in testicular homogenates or in freshly isolated mouse Leydig cells. Likewise, using in situ hybridization, we saw only very weak and inconsistent labeling of rodent Leydig cells in situ (unpublished data).

In general, the results of NCAM expression by Leydig cells are in accordance with our previous results concerning NCAM expression by ovarian endocrine cells [14]. Endocrine cells of the ovary and testicular Leydig cells, although classically regarded as prototypes of steroid-producing cells, are known to produce several peptides as well (substance P, TGF-alpha, inhibin) [9–12]. An NCAM of 140 kDa has been found in endocrine cells synthesizing peptide hormones (e.g., pituitary cells, pancreatic islet cells) [7, 8] and also in the ovary [14]. Moreover, NCAM-140 is the prevalent isoform in human endocrine cells and tumors [37, 38]. The reason for the difference between testicular expression of NCAM-120 (representing a mature NCAM form) and ovarian NCAM-140 could be that in the ovary NCAM-expressing endocrine cells are subjected to continuous remodelling processes, whereas NCAM-expressing Leydig cells are typically nondividing, phenotypically stable cells.

Like Sertoli cells, Leydig cells are thought to develop in the mesenchyme [39]. However, recent findings on neuropeptide expression, e.g., substance P, by Leydig cells in the adult have cast doubt on the mesenchymal origin of Leydig cells and have raised the possibility that Leydig cells could originate in the neural crest [10]. The fact that NCAMs are present on adult Leydig cells is not unequivocal evidence of the latter hypotheses, since the NCAM is known to be transiently expressed during ontogeny by a variety of tissues (for example, kidney [40]). Interestingly, after isolation and culture of mouse Leydig cells, the pattern of NCAM expression changed, implying that NCAM gene transcription was induced and additional NCAM isoforms appeared. Although NCAM mRNA levels were below the detection limit of the S1 assay in freshly isolated Leydig cells, a dramatic increase in NCAM mRNA levels occurred within 2 days in culture. After 4 days in vitro, not only NCAM mRNA was detectable in abundance, but also all three major isoforms of the NCAM (NCAM-120, -140, and -180) appeared. Another post-transcriptional modification might occur at the exon 12/13 splice junction. At this site, near the sequence coding for the proposed hinge region of the NCAM, the alternative splicing of extra exon sequences has been reported [6, 16]. Our results from S1-nuclease protection experiments indicate that, besides the main diversity form "exon 12-(± AAG)-exon 13" (corresponding to the 324-nt band) in cultured Leydig cells, additional alternative exons are present. This is the conclusion from the presence of a 339-nt band. These exons are most likely a_{48} and/or a_{42} [6] or a still unknown sequence, located downstream from extra exon a_{15}. The exact sequence of this splice site in Leydig cells is currently under investigation. The mechanism(s) responsible for the observed up-regulation of the NCAM and the changes in the alternative splicing of the primary transcript are unknown at present. One possibility is that NCAM up-regulation could be associated with de-differentiation processes of Leydig cells, which with time in culture show changes in morphology and function. However, serum supple-

mented to the culture medium seems to contain certain as yet undefined factors, which can induce NCAM up-regulation in fibroblasts [41]. Positively identified factors, which are able to up-regulate the NCAM in the absence of serum, are nerve growth factor (NGF) [42, 43] and TGF-beta [41]. Mouse Leydig cells in the present study were cultured in the presence of serum; moreover, these cells in culture themselves produce NGF (Seidl, personal communication). However, since receptors for NGF are not found on Leydig cells (Seidl, personal communication), the question of whether stimulation of NCAM expression by cultured mouse Leydig cells is due to autocrine stimulation by Leydig cells via NGF or other Leydig cell-derived factors, or to serum factors cannot be answered at the present time.

In conclusion, our results show that the NCAM is expressed by Leydig cells. The fact that the surface molecule NCAM is shared by neurons, pure peptide-hormone producing cells, and endocrine cells like ovarian granulosa or testicular Leydig cells, which produce steroids and peptides, may indicate a closer relationship of these cell types than has previously been considered to exist and may provide an explanation for the formation of Leydig cell clusters in the testis.

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