Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization

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Abstract We have identified the sites of transcription of the apolipoprotein D (apoD) gene in the rabbit by in situ hybridization. We show here for the first time that *1*) apoD mRNA production does not correlate with the sites of steroid hormone production in adrenal glands nor male genital tissues; and *2*) the expression of the apoD gene is clearly higher in white than in gray matter throughout the central nervous system (CNS). Specifically, apoD mRNA was abundant near blood vessels and was expressed mostly in fibroblast-like cells, in particular in the testis, the efferent ducts, the ductus epididymis, the lung, and the subarachnoid space of the CNS. Other positive cell types were endothelial cells of adrenal sinusoidal capillaries and glial cells of the CNS. We detected apoD mRNA in both the adrenal cortex and medulla. White but not gray matter showed high levels of apoD mRNA throughout both the rabbit CNS and in human brain. The red pulp of spleen showed a strong hybridization. In prepubertal rabbits apoD mRNA levels were moderate in both testis and epididymis. Epididymal but not testicular expression increased with the onset of puberty and epididymal levels always exceeded those of the testes in animals showing spermatogenesis. Thus, the variation in levels of apoD mRNA among organs in vivo, that we and others have previously reported, can be explained by transcription being not only characteristic of cell type, with a few common cell types producing in each organ, but transcription also varied among cells of the same lineage.—**Provost, P. R., L. Villeneuve, P. K. Weech, R. W. Milne, Y. L. Marcel, and E. Rassart.** Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization. *J. Lipid Res.* 1991. **32:** 1959–1970.

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Human apolipoprotein D (apoD) was isolated and partially characterized by McConathy and Alaupovic (1, 2), and subsequently by Drayna et al. (3), Weech et al. (4, 5), and Albers et al. (6). It is a glycoprotein of apparent M_r 29,000 and in the blood plasma it is mainly distributed in the high density lipoproteins (HDL) both in the human (5 and references therein) and the rabbit (7).

ApoD was found associated with lecithin:cholesterol acyltransferase (LCAT) in plasma (6, 8–11) and it may stabilize its enzymatic activity (12). The human apoD cDNA was cloned and sequenced (3, 13), and the analysis of the corresponding amino acid sequence revealed that apoD belongs to the $\alpha 2\mu$-globulin superfamily (13). Other members of this superfamily are known to transport small hydrophobic ligands in a pocket formed by an antiparallel β-strand structure (14–19). Therefore, apoD was postulated to transport a substrate or a product of the LCAT reaction (13). However, on the basis of data from comparative sequence analysis, detailed molecular modeling, and preliminary binding studies, Peitsch and Boguski (20) hypothesized that a heme-related compound may be a preferred ligand for apoD. They showed that purified human apoD binds cholesterol poorly relative to bilirubin (20). Moreover, Balbin et al. (21) found that the amino acid sequence of a progesterone-binding protein, GCDFP-24, present in high concentration in gross cystic disease fluid (22), matched perfectly with the sequence of the human apoD protein. Consequently, a possible role of apoD in the transport of steroid hormones has been proposed. The apoD gene expression was also shown to be stimulated in peripheral nerve regeneration (23–25), but its physiological ligand in this process is still unknown. However, it is obvious that

Abbreviations: apoD, apolipoprotein D; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; bp, base pair; kbp, kilobase pair; PBS, phosphate-buffered saline; CBG, corticosteroid-binding globulin; GCDFP, gross-cystic-disease-fluid protein; CNS, central nervous system; DHT, 5α-dihydrotestosterone (5α-androstane-17β-ol-3-one); DHEA, dehydroepiandrosterone (androst-5-ene-3β-ol-17-one).

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there is still considerable ambiguity in our understanding of the role and function of apoD. Moreover apoD may have a different function at each or several of its sites of expression.

On the basis of apoD cDNA cloning, sequence, and biochemical characterization of the protein, we showed that the rabbit is an excellent model in which to study apoD (7). We have shown by quantitative analysis (7) that the apoD gene is expressed in all the 16 rabbit organs that we studied, with the major sites of expression being spleen, adrenal glands, lung, brain, and testes (including epididymis). Smith, Lawn, and Wilcox (26) have performed in situ hybridization on rhesus monkey tissues with the human apoD probe. They found that the gene was expressed mainly by interstitial and connective tissue fibroblasts in many peripheral tissues, and also by neuroglial cells and scattered neurons in the brain. Cells expressing apoD were often found associated with blood vessels or capillaries. However, they did not analyze some of the major apoD expressing organs, such as adrenal glands, lung, and the ductuli efferentes and the ductus epididymis of the male genital system.

The apoD gene is unique among the apolipoprotein genes being expressed throughout the body and producing high levels of mRNA in organs other than the liver and intestine, the central organs of lipoprotein metabolism (7). These observations led us to hypothesize that apoD may have a local function in peripheral organs in addition to, or in conjunction with a potential role in the circulation.

In this report we have addressed two main problems: *1*) to find the sites of transcription of the apoD gene in all of the organs of the normal rabbit that we identified as containing major amounts of apoD mRNA, and to determine whether or not there was a characteristic common to these different organs. Of special interest to us were the organs that have not been studied before in any species by this technique, e.g., the lung, the entire CNS, and adrenal glands. *2*) The second problem was to test the hypothesis that a main role of apoD is to transport steroid hormones, by determining whether or not there is a correlation between the cells or tissues that transcribe apoD mRNA and the cells that are believed to synthesize steroid hormones in the adrenal gland and male genital system.

MATERIALS AND METHODS

Animals and biological samples

The male rabbits were purchased from "Ferme de sélection Cuniqur" (St-Valérien, Qué., Canada). The animals were separated into four groups (prepubertal, early and late pubertal, and adult rabbits) on the basis of the testicular morphology. The seminiferous tubules of the prepubertal animal had undifferentiated Sertoli cells and contained degenerating gonocytes and no spermatocytes. No differentiated Leydig cells were seen in the testis. The early pubertal testis contained differentiated Sertoli cells and Leydig cells. Spermatocytes were seen in some seminiferous tubules indicating that a low level of spermatogenesis occurred in this tissue. In contrast, spermatogenesis was seen in all the seminiferous tubules of the late pubertal tissue, but in most tubules, the process was incomplete and high levels of degenerating germinal cells were observed. Only some tubules contained spermatozoids. However, the adult tissue showed high levels of complete spermatogenesis and spermatozoids.

The human brain samples were provided by the Brain Bank (Douglas Hospital Research Centre, Verdun, Qué., Canada). Subjects 1 and 2 were, respectively, 69 and 53 year old women. Neuropathological examination of brain samples revealed normal histological structures for the subject 1 brain and some lesions of multiple sclerosis in the brain of subject 2. None of the white matter samples studied here contained apparent plaques of demyelination.

Tissue preparation

The rabbits were perfused with 1 × PBS (1 × PBS: 140 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) followed by 4% paraformaldehyde in 1 × PBS, pH 7. The collected tissues were fixed overnight in 4% paraformaldehyde in 1 × PBS at 4°C before their dehydration in graded alcohols. Subsequent manipulations were performed with RNAse-free materials. The organs were embedded in paraffin using a LY-120 tissue processor (Innovative Medical System Corp.) and a paraffin dispensor (Lipshaw, model 224, Detroit, MI). The resulting blocks were cut in sections of 5 μm, mounted onto APES-coated slides (APES: 3-aminopropyltriethoxysilane; Sigma).

Probes

The two probes for in situ hybridization consisted of ^{35}S-labeled RNA prepared from our rabbit apoD-cDNA (APOD-RAB-6 cDNA cloned in Bluescript KS$^+$ vector) (7). The probe for specific hybridization with apoD mRNA was antisense with respect to the mRNA, but a sense transcript was used as a probe of nonspecific RNA binding. The antisense RNA probe was prepared with T7-RNA-polymerase (Pharmacia) and *Hin*dIII-linearized plasmid. The T3-RNA-polymerase (Pharmacia) and the same cloned DNA, cut with *Pst*I, were used to synthesize the sense RNA probe. Transcriptions were run as described (27) using [α-^{35}S]UTP (Amersham). After digestion with DNaseI (FPLC-pure, Pharmacia) and purification by ethanol precipitation, the probes were resuspended in 50 mM dithiothreitol.

In situ hybridization

At least two paraffin blocks were prepared from two late pubertal animals for all the organs studied. Depending on the organ, three or four series of in situ hybridizations were performed. Each of them led to the identification of the same structures by the antisense apoD-RNA probe. The specificity of each round of in situ hybridization was verified by parallel hybridizations with the sense apoD-RNA probe giving background. Additional blocks were prepared with genital tissues from an adult animal. These tissues were hybridized in parallel with the late pubertal genital tissues.

In situ hybridization was performed basically as described (28). Briefly, after deparaffinization and rehydration, the sections were incubated successively with: 0.2 M HCl, distilled water, 2 × SSC at 70°C (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), distilled water, Proteinase K, 0.2% glycine in 1 × PBS, 1 × PBS, 4% paraformaldehyde in 1 × PBS, 1 × PBS, acetic anhydride (diluted 1:400 in 0.1 M triethanolamine pH 8), and 1 × PBS. The sections were then dehydrated in graded alcohols and air-dried. The 1 × hybridization buffer contained 300 mM NaCl, 10 mM Tris-HCl, 10 mM $NaPO_4$ (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin, 40% formamide, 10% dextran sulfate, 1 mg/ml total RNA, and 50 mM dithiothreitol. The heat-denatured probes in 1 × hybridization buffer were pipetted over each group of dried sections. After an overnight incubation at 50°C, the samples were washed at 37°C in 0.5 M NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA. After a treatment with RNaseA, the sections were washed again with the same buffer at 37°C, followed by 2 × SSC and then, 0.1 × SSC, both at room temperature. After dehydration in graded alcohols and air drying, the sections were exposed to Kodak Xar-5-X-OMAT film for a few days, then coated with Ilford K5 emulsion, and finally, exposed in the dark at 4°C for 2–3 weeks. After development, the slides were stained with Ehrlich's hematoxylin/$LiCO_3$ only or followed by eosin.

Northern blot analysis

Total RNA was extracted by the acid-guanidium thiocyanate-phenol-chloroform method (29) from various organs. After denaturation by glyoxal, the RNA samples were electrophoresed in 1.1% agarose gel and transferred to Nytran membranes. The filters were hybridized at 60°C as described (30). The DNA probes were prepared by the random-primed oligonucleotide method (31), using the 1 kbp *Eco*RI insert isolated from the clone pAPOD-RAB-6 (7) as the matrix (rabbit apoD probe), and the 800 bp *Eco*RI fragment purified from the pAPOD6 clone (provided by Dennis Drayna; Genentech, Inc. (3)) (human apoD probe). After hybridization, the membranes were washed in 2 × SET for 1 min at room temperature (1 × Tris-EDTA-salt buffer (SET): 30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA), followed by 2 × SET containing 0.2% SDS at 60°C for 30 min, followed by 1 × SET containing 0.2% SDS at 60°C for 30 min and finally, in 1 × SET for 1 min at room temperature. Filters were exposed to Kodak Xar-5-X-OMAT film at −70°C with a Cronex Lighting Plus intensifying screen (E. I. du Pont de Nemours & Co., Wilmington, DE).

RESULTS

Major steroid hormone production sites and male sexual tissues

On adrenal gland sections, the apoD probe hybridized strongly to cells that are associated with the sinusoidal capillaries (**Fig. 1, A and B**) which pass through the stroma of the cortex. These cells have an elongated nucleus and are most likely endothelial cells. Throughout the cortex the steroid-secreting cells were clearly negative with the apoD probe. ApoD mRNA was detected in the capillary-associated cells of the zona glomerulosa, as well as in the corresponding cells of the zona fasciculata and the zona reticularis (Fig. 1, A–D). In the medulla, capillary endothelial cells were also positive (Fig. 1, C and D). Among the cells that composed the endothelium of large vessels, only some of these were positive. Interestingly, all the medullar cells with a round nucleus appeared negative on our sections. Some of these negative cells must be chromaffin cells.

In testicular tissues, relatively low levels of apoD mRNA were detected in interstitial connective tissue that supports the seminiferous tubules, both in late pubertal (Fig. 1, E and F) and adult rabbits (not shown). While the positive cells were difficult to characterize, they could be of fibroblastic origin. Some Leydig cells were negative on our sections, but the expression of the gene in other cells of this lineage cannot be ruled out because positive interstitial cells were often located near Leydig cells. There were no positive cells in the seminiferous tubules, neither spermatocytes nor Sertoli cells (Fig. 1, E and F). Low to moderate levels of apoD gene expression occurred in the stratum vasculare of the tunica albuginea (**Fig. 2**), but high levels were observed close to arteries (not shown). Again, the positive cells were most likely fibroblasts. No difference in the site of apoD gene expression was observed between late pubertal and adult testes. However, levels of apoD mRNA were higher in late pubertal tissues for all of the above-mentioned sites when compared with their adult counterparts (Fig. 2) but this difference could have been due to individual variation.

The antisense apoD probe hybridized strongly with cells of the connective tissue surrounding the ductuli efferentes (not shown) and the ductus epididymis, both in late pubertal (Fig. 1, G and H and Fig. 2) and adult

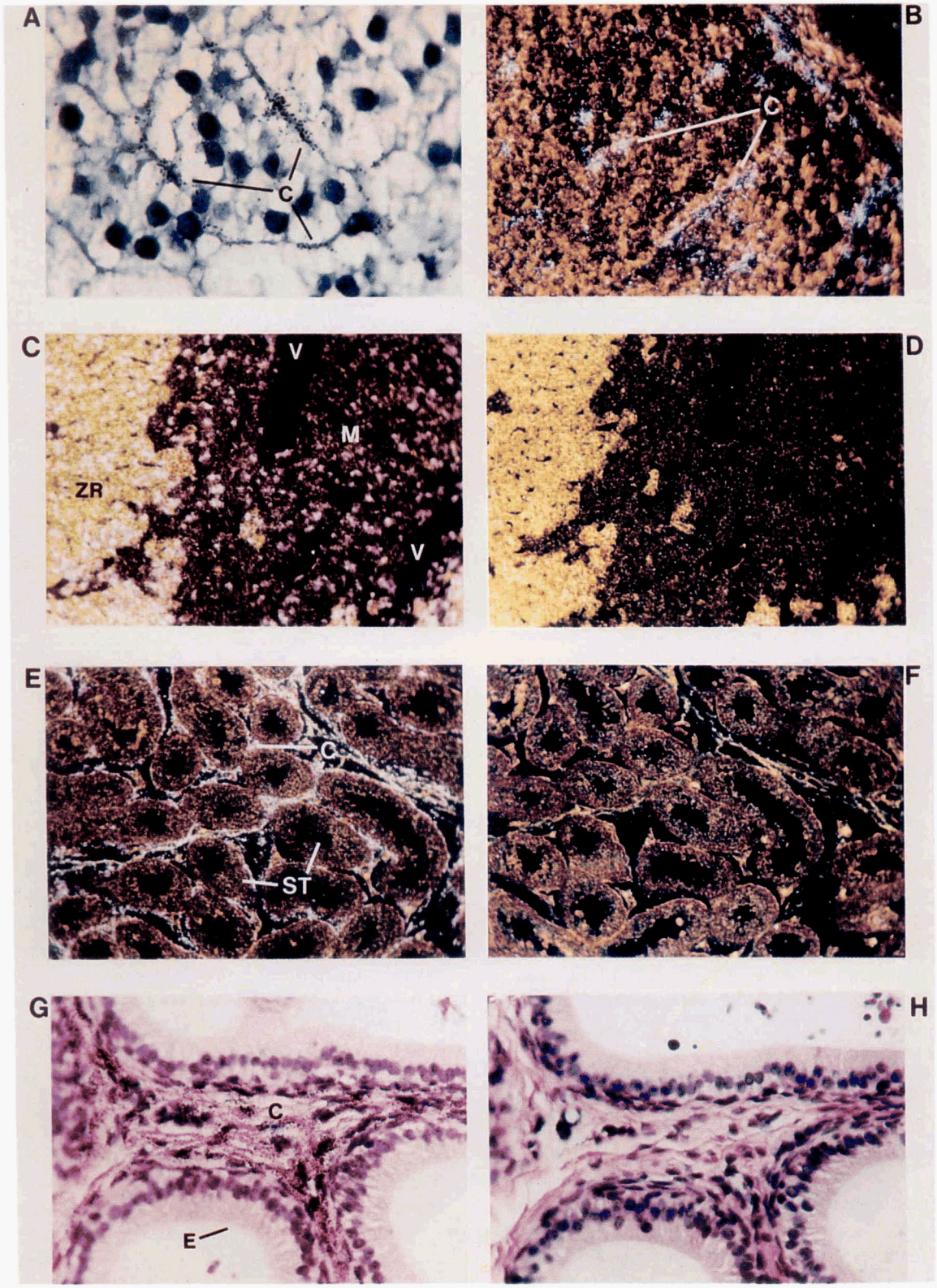

Fig. 1. Rabbit apoD mRNA synthesis in the major steroid hormone production sites and male sexual tissues. All tissues were isolated from late pubertal rabbits. A, B, C, E, and G were hybridized with the rabbit antisense apoD probe, whereas D, F, and H were probed with the sense apoD labeled RNA as control. Slides A and B were exposed to emulsion 14 days and the others for 20 days. A: Zona fasciculata of the adrenal cortex. Note the positive cells along the sinusoidal capillaries (C). Magnification × 1000. B: Dark-field photograph of zona glomerulosa and zona fasciculata of the adrenal cortex. The positive cells are localized along the capillaries (C). Magnification × 400. C and D: Dark-field photograph of the adrenal medulla (M) and zona reticularis (ZR). (V) medullar veins. Magnification × 100. E and F: Dark-field photograph of seminiferous tubules (ST) and interstitial connective tissue (C) in the stroma of the testis. Magnification × 100. G and H: Ductus epididymidis (E) and connective tissue (C) of the epididymis. Magnification × 400.

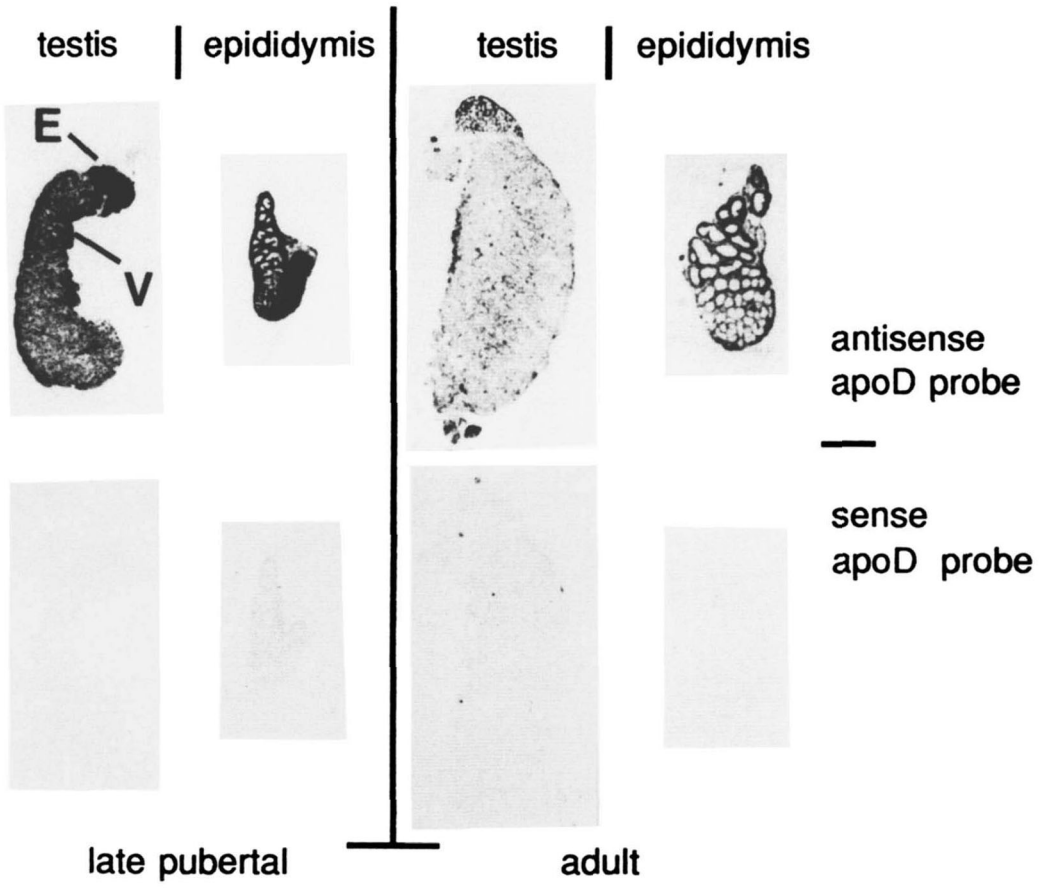

Fig. 2. ApoD mRNA levels in male sexual tissues of late pubertal and adult rabbits. The autoradiographs of tissue sections from late pubertal or adult rabbits were made on Kodak Xar-5 X-OMAT film after in situ hybridization but before coating with emulsion. The sections were hybridized with the antisense or the sense (control) rabbit apoD probe as indicated. Five-day exposures are shown; (E), Ductuli efferentes and the region of transition to ductus epididymidis; (V), stratum vasculare of the tunica albuginea.

animals (Fig. 2). Again, these cells are probably fibroblasts. The epithelial cells of ductuli efferentes and ductus epididymis showed no hybridization with the apoD probe. The arteriolar endothelial cells appeared clearly negative and the capillary endothelial cells were also probably negative. It is of interest to note that the levels of apoD mRNA varied throughout the epididymis of both late pubertal and adult animals, but the precise segments of the duct around which the gene is strongly expressed have not been identified. (See adult epididymis panel in Fig. 2. The late pubertal epididymis panel is overexposed, but the same variation appeared by microscopic visualization; not shown.)

Interestingly, the levels of apoD mRNA were significantly higher in ductuli efferentes and the epididymis than in the testes, both during late puberty and in the adult animal (Fig. 2). In other words, apoD mRNA levels were relatively low near the site of steroid hormone synthesis from cholesterol. Northern blot analyses have been performed with RNA extracts prepared from one early pubertal and one prepubertal rabbit, as judged by the morphology of the testicular tissues (see Materials and Methods section). The results from the early pubertal animal demonstrated again that the apoD mRNA concentration was higher in the epididymis than in the testis (**Fig. 3**). However, in the prepubertal animal, the epididymis contained similar or lower apoD mRNA levels than the testicular tissues (Fig. 3). Thus, elevated expression of the apoD gene in the epididymis seemed to be restricted to animals showing spermatogenesis, but it did not correlate with the presence of mature spermatozoids in the epididymis because only the adult rabbit had this characteristic.

Other major tissues of apoD gene expression

In the lung, apoD mRNA was detected in peribronchiolar and periarteriolar connective tissues or fibroblasts (**Fig. 4, A and B**). The bronchiolar epithelium, the arteriolar endothelial cells, and the smooth muscle cells of the arterial wall were negative, but the tunica intima of some arteries, composed of connective tissue, hybridized with the antisense apoD RNA probe. Tissues surrounding the veins throughout the lungs did not hybridize to any significant extent. Thus, the apoD gene expression around

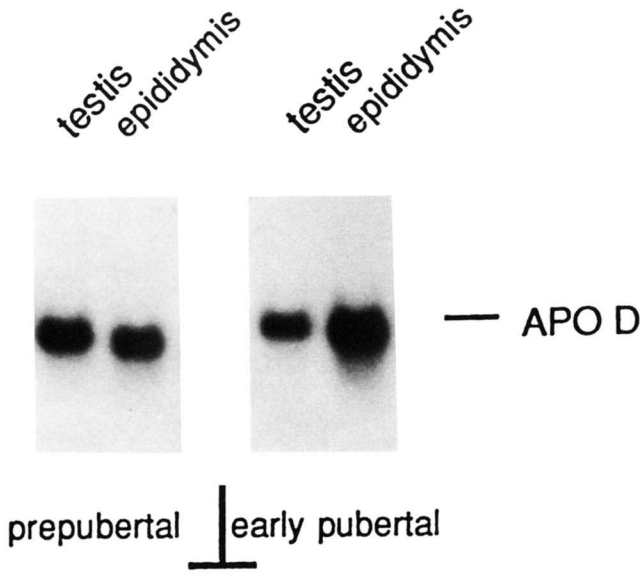

Fig. 3. Northern blot analysis of apoD mRNA levels in male sexual tissues of prepubertal and early pubertal rabbits. Total RNA extracted from various tissues (5 μg) was loaded on an agarose gel, electrophoresed, and transferred to Nytran membranes. The filter was hybridized with ^{32}P-labeled rabbit apoD cDNA, washed, and autoradiographed (16 h exposure). The tissues were from prepubertal or early pubertal rabbits, as indicated (see Materials and Methods). The testicular RNA extracts were prepared from a transverse section of the organs. Variations of total RNA amounts from sample to sample did not exceed 1.5-fold as determined by slot blot, hybridization with poly-dT probe (7), and densitometry (not shown).

vessels seemed to be restricted to arteries. The alveolar walls showed few grains, close to background. Nevertheless, the expression of apoD gene in this area cannot be ruled out from out experiments.

ApoD mRNA in rabbit CNS was mainly found in the subarachnoid space and white matter in brain, cerebellum, rachidian bulb, and spinal cord. The expression in the subarachnoid space was again associated most probably with fibroblasts, with a particularly high level of apoD mRNA around arteries (Fig. 4, C and D). We saw no apoD hybridization in the endothelial cells. In addition to fibroblasts, the contribution of pial cells to the production of the messenger RNA cannot be excluded in the subarachnoid tissues. In white matter, scattered glial cells showed a moderate to high level of hybridization to the apoD probe throughout the CNS (Fig. 4, E and F, **Fig. 5, A–D,** and data not shown). These cells could be astrocytes or oligodendrocytes. The presence of some very scattered positive neuroglial cells in the brain gray matter can be suspected from our sections, but their apoD mRNA levels were barely above background. Expression in neurons cannot be ruled out for the same reason. We saw some very scattered positive cells in the molecular and the granular layers of the cerebellum, and in the gray matter of the rachidian bulb and the spinal cord (not shown). We tentatively identified these cells as neuroglia, but some of these could be neurons. However, the number of positive cells in the gray matter was very low when compared to that in the white matter. Thus, the rabbit white matter appeared to be a major site of apoD mRNA production (Fig. 5, A–D). This interesting observation was confirmed by Northern blot analysis of human white and gray matter-RNA samples. RNA extracts were prepared from the brain samples of two subjects and the Northern blot was probed with the ^{32}P-labeled human apoD cDNA (Fig. 5, E). Results showed that human white matter is also a major site of apoD gene expression, in contrast to gray matter. Thus, the pattern of apoD gene expression in rabbit brain tissues is like that of the human. No focus of strong apoD gene expression was detected in the granular layer of the rabbit cerebellum (Fig. 5, A and B) in contrast to the rhesus monkey cerebellum, as reported in the literature (26). Glial cells from the white matter of the rabbit cerebellum, as well as those of the rachidian bulb and spinal cord, showed a hybridization signal even higher than the corresponding cells in the brain (not shown).

When we used the in situ hybridization technique to examine apoD gene expression in another major producer organ, the spleen, the red pulp showed very strong hybridization with the antisense apoD probe, particularly near the venous sinuses, the capsule, and trabeculae (Fig. 4, G and H). The reticular cells of the splenic cords are probably the positive cells, although macrophages cannot be excluded. Tissues of the capsule, trabeculae, and white pulp showed a signal slightly higher than the background.

DISCUSSION

Expression of the apoD gene was found mainly in connective and interstitial tissue fibroblasts in nearly all the tissues examined (Figs. 1 and 4), with higher levels of apoD mRNA often seen in cells near arteries or capillaries. ApoD mRNA production was not, however, restricted to fibroblasts. Capillary endothelial cells seem to be the site of apoD gene expression in adrenal glands. In addition, glial cells of the brain white matter also expressed the gene, and it cannot be excluded from our results that other cell types could also produce apoD mRNA in the CNS and other organs. These observations, except that concerning the adrenal glands, confirm the major data of Smith et al. (26).

Connective tissue cells did not express the gene equally in all sites. Thus, our studies demonstrate that apoD gene expression is not only a function of cell type but also of the local tissue environment in which the cells are found. In spleen, fibroblasts of the capsule and trabeculae produced little or no apoD mRNA (Fig. 4, G and H) in contrast to the high levels found in the connective tissue surrounding the ductus epididymis (Fig. 1, G and H),

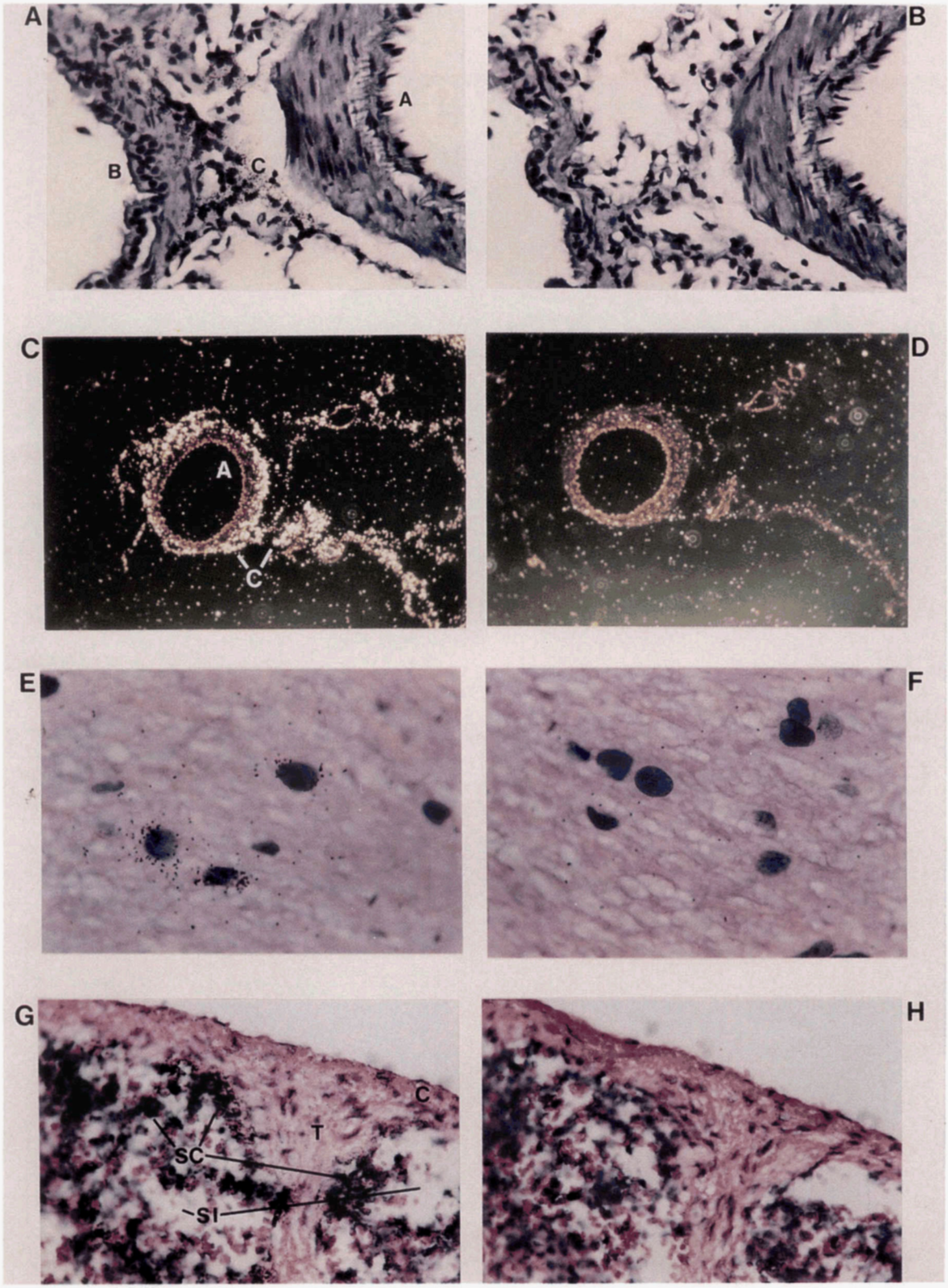

Fig. 4. Cellular characterization of apoD gene expression at the major sites of apoD mRNA production. All tissues were isolated from late pubertal rabbits. A, C, E, and G were hybridized with the rabbit antisense apoD probe, whereas B, D, F, and H were probed with the sense apoD labeled RNA as control. Slides G and H were exposed to emulsion 20 days and the others for 14 days. A and B: Bronchioles (B) and pulmonary arteriole (A) of the lung. Note the positive signal in peribronchiolar and periarteriolar connective tissue (C). Magnification × 400. C and D: Dark-field photograph of artery (A) of the subarachnoid space of the brain and connective tissue (C) surrounding the artery. Magnification × 100. E and F: Labeled glial cells in white matter of the cerebellum. Magnification × 1000. G and H: Spleen; venous sinuses (SI) and splenic cords (SC) of the red pulp, trabecula (T), and capsule (C). The positive signal in the splenic cord reticular connective tissue area is very strong. Magnification × 400.

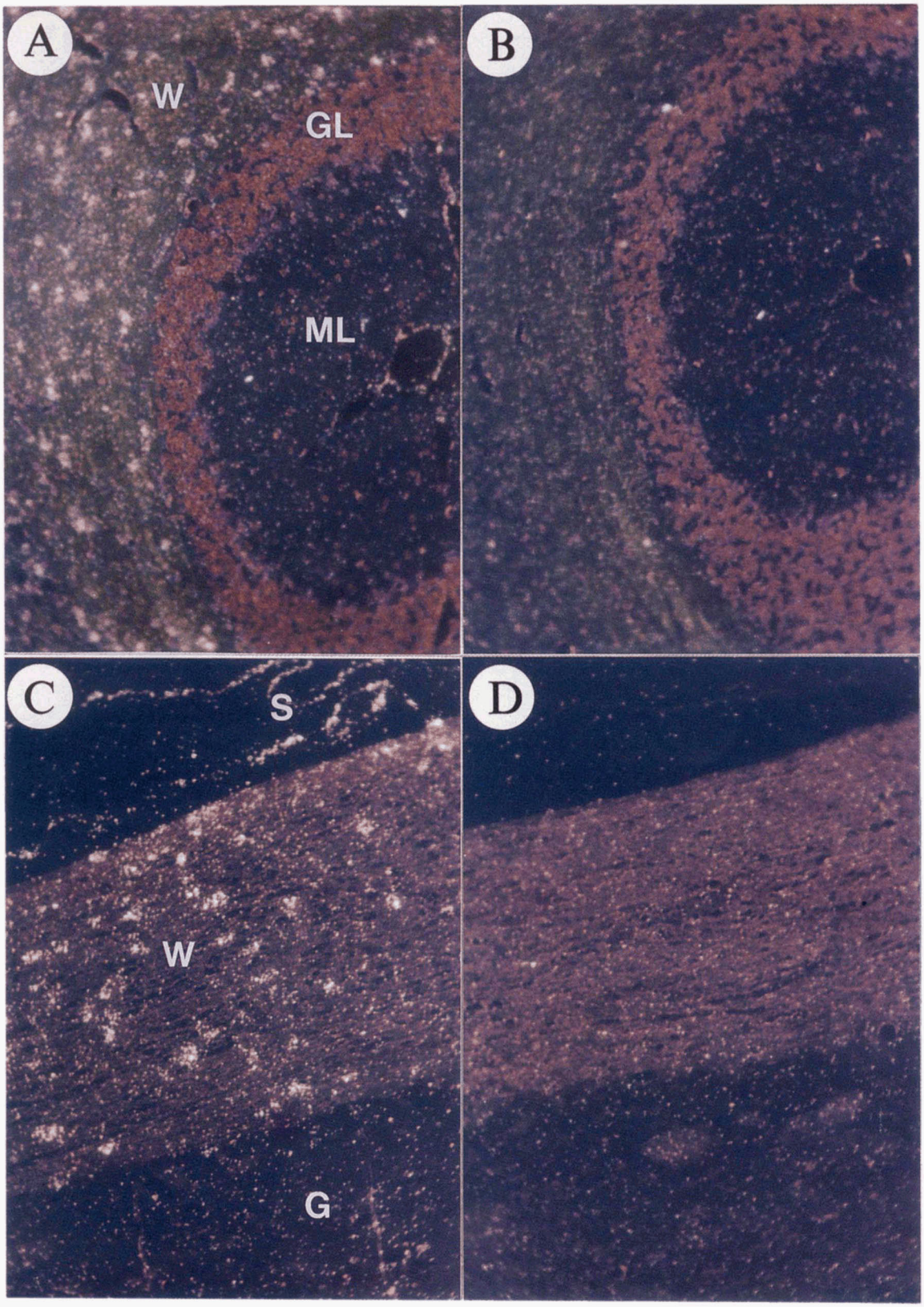

Fig. 5.

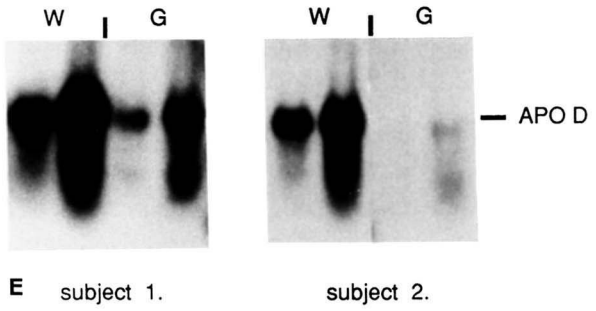

Fig. 5. Comparison of apoD mRNA levels between white and gray matters of rabbit and human central nervous system. A to D: Dark-field photographs of the rabbit cerebellum (A and B) and spinal cords (C and D), after in situ hybridization with the antisense apoD probe (A and C) and the sense apoD probe (B and D); 14-day exposure. Magnification × 100. (ML), molecular layer and (GL), granular layer of the cerebellar gray matter; (W), white matter; (G), gray matter; (S), subarachnoid space. E: Northern blot analysis of apoD mRNA levels in human brain. Total RNA extracted from gray matter (G) and white matter (W) of human brain (5 and 15 μg each) were loaded on an agarose gel, electrophoresed, and transferred to Nytran membranes. The filters were hybridized with ^{32}P-labeled human apoD cDNA, washed, and autoradiographed (16 h exposure).

although both tissues were stained by van Gieson's method, indicating that both produced collagen fibers (not shown). We have also presented evidence that apoD gene expression could be modulated during sexual maturation in epididymal connective tissue. ApoD expression in vivo would therefore appear to be linked to specific metabolic pathways that exist in the cell and tissue environment rather than to a function common to all cells of a given lineage.

ApoD was postulated to transport a substrate or a product of the LCAT reaction (13). However, the nature of the apoD ligand is unknown. First, in addition to the well-defined substrates and products of the LCAT (unesterified cholesterol, lecithin, esterified cholesterol, lysolecithin), the existence of other substrates or products is possible. For example, it was postulated that the esterification of some steroid hormones, that was observed in HDL_3 in vitro, was catalyzed by LCAT (32, 33). Second, a role of apoD in conjunction with the LCAT reaction remains speculative.

The postulated role of apoD in the transport of cholesterol from peripheral organs (26) seems to be unlikely for several reasons. First, it has been claimed that the transport of cholesterol from cellular membrane to HDL can be achieved by simple diffusion (34). Second, because a ratio of one molecule of ligand for one molecule of apoD is expected, the amount of apoD protein required for this transport would be very high. Finally, we have shown in a recent paper (35) that the pattern of apoD gene expression in fibroblast cultures in vitro was not altered by the addition of exogenous cholesterol or by a homozygous mutation affecting the LDL-receptor gene. Thus, the nature of the apoD ligand and, consequently, the role of the protein are still unknown.

This study is mainly descriptive but our findings can allow us to speculate about the role of the protein. However, the presence of apoD mRNA may not necessarily lead to the translation of the message and the secretion of the protein.

Since it was shown that apoD and GCDFP-24 are the same protein (21), a role for apoD in steroid hormone binding and transport in the testis and the adrenal gland could be envisaged. While former studies on GCDFP-24 revealed that this protein can bind pregnenolone and progesterone in vitro, testosterone, DHT, and DHEA can also bind to apoD with low affinity (36, 37). We have shown here that, in male genital tissues, there is a poor correlation both anatomically and developmentally between apoD gene expression and steroid hormone synthesis. Epididymal connective tissue, which is not a site of steroid hormone synthesis from cholesterol, produced higher amounts of apoD mRNA than the testicular connective tissue which contains Leydig cells (Fig. 2). Moreover, important apoD gene expression in male genital tissues precedes spermatogenesis and, the onset of puberty is not associated with an increase in the testicular level of apoD mRNA (Fig. 3). While these observations would be consistent with a non-hormonal ligand for apoD, they neither favor nor exclude a role for apoD in steroid hormone transport in male genital tissue. We cannot rule out the possibility that the increase in epididymal apoD mRNA levels in early pubertal animals could be associated with the start of spermatogenesis in the testis.

Similar arguments could be made in the case of the adrenal cortex where the apoD gene is clearly not expressed in steroid hormone-producing cells (Fig. 1). The positive endothelial cells of the capillaries are not restricted to a particular zone or area of the gland but are found throughout the cortex and the medulla (Fig. 1). If apoD is produced and secreted, it could therefore transport molecules from the tissue to the blood, from the blood to the tissue, or from the outer cortex to the inner cortex. Potential ligands could include pregnenolone and progesterone, both of which are steroid hormone precursors; alternatively or additionally, as the gene is also expressed in the adrenal medulla, a non-hormonal molecule could be bound by apoD.

Steroid hormones (active androgens and corticosteroids especially) are unlikely to be the physiological ligand of apoD in other major sites of gene expression in normal rabbit. The apoD gene is expressed strongly in the red pulp of the spleen where reticular cells are probably the site of apoD mRNA production (Fig. 4, G and H). After the lysis of old erythrocytes that occurs in this area, the hemoglobin is phagocytozed by reticular cells and is transformed to bilirubin which returns to the circulation

via the venous sinuses. It was shown that heme-related compounds may be favorable ligands for apoD and that the protein binds bilirubin in vitro with an approximately one-to-one molar ratio (20). If the apoD is secreted in the red pulp of the spleen, it may exert its role locally by binding bilirubin molecules and transferring them into the blood for further transport by serum albumin. In the lung, peribronchiolar and periarteriolar connective tissues showed high levels of the messenger RNA. No physiological role of steroid hormones has been described in this area. However, the gene encoding the corticosteroid-binding globulin (CBG) is expressed in cells lining the basement membrane of bronchiolar epithelial cells of the human lung, as shown by in situ hybridization (38, 39), but this finding remains unexplained. Since some proteins of the $\alpha 2\mu$-globulin family can transport multiple ligands (40, 41), it is possible that apoD binds and transports more than one kind of molecule, depending on the specific ligands that are available in a given physiological compartment, as proposed by Peitsch and Boguski (20).

We have shown for the first time that apoD gene expression is stronger in the white matter than in the gray matter throughout the rabbit CNS and also in the human brain (Fig. 5). The apoD ligand is still unknown in the CNS, as in peripheral nerve where the amount of apoD protein increases strongly during regeneration (23–25). While there is no regeneration in the CNS, it is possible that the apoD plays a role in the process of synaptogenesis where it could bind the same ligand as in the peripheral nerve regeneration.

The wide variety of tissues that produce apoD mRNA would make it unlikely that steroid hormones are the exclusive physiological ligand(s) of apoD. Moreover, as apoD binds progesterone and pregnenolone with an affinity constant of around 1×10^6 l/mol, the association of progesterone with apoD in human breast gross cystic disease fluid may simply reflect the high local concentration of the hormone, whereas the function of the protein in nonpathological sites is not related to steroid hormones. Nevertheless, it has been shown that enzymes that play a role in steroid hormone maturation such as 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), aromatase, 5α-reductase, and sulfatase are expressed in a large series of peripheral tissues in humans and primates and, at least for some of these enzymes also in mouse and rat (42–44 and references therein). Thus, it cannot be excluded that the wide distribution of apoD gene expression could reflect apoD participation in steroid hormone maturation in a variety of peripheral tissues. Contrary to this idea, however, it should be noted that, with the exceptions of the adrenal gland and the testes, the major sites of expression of the apoD gene are different from those of the 3β-HSD gene (7, 43, 44).

Nothing is known about the translation of apoD mRNA and the secretion of the protein in rabbit organs. This work remains to be done and, consequently, the conclusions that arise from the present study are limited. However, Boyles et al. (45) have shown the presence of apoD protein in a wide selection of peripheral organs in the rat by immunohistochemistry. The apoD must be produced locally in rat organs since there is none or only a very low level of apoD in circulation in that species (24, 45). Although there could be some differences from one species to another, we can hypothesize that among the organs that produce apoD mRNA, the translation and the secretion of apoD messenger and protein, respectively, should not be restricted to a limited number of sites in the rabbit.

The most important finding of our study is that there is no correlation, anatomically nor developmentally, between apoD mRNA production and steroid hormone formation. This observation leads us to consider other candidate ligands for apoD. It is possible that apoD has multiple physiological ligands and functions and that both ligand and function vary according to the site of synthesis. The most likely alternative hypothesis is that no one has yet found or postulated a true high affinity physiological ligand of apoD, which would allow us to conceptually unite the different sites of expression with a common physiological role.

More extensive studies on in vitro binding of apoD will help to clarify the function of the protein. Observations and conclusions presented here lead us to consider that a large selection of ligand candidates including hormonal and nonhormonal molecules should be studied. The present work gives us an essential basis to study apoD gene modulation in vivo by factors that we expect to identify by in vitro binding studies and by experiments using fibroblast cultures.

In this study, we have shown for the first time a fine characterization of apoD gene expression in the adrenal glands, the ductuli efferentes, and the epididymis of the male genital tissues, the lung, and the entire CNS including the rachidian bulb and the spinal cord. Also, we have confirmed the major data of Smith et al. (26), specifically: *1*) the apoD gene was mainly expressed in connective and interstitial tissue fibroblasts; *2*) its expression was often higher near blood vessels; and *3*) other cell types can also produce apoD mRNA. In addition to the descriptive results, two major new conclusions evolved from our study: *1*) the apoD mRNA production did not correlate well anatomically with accepted sites of steroid hormone biosynthesis in either the male genital tissues or the adrenal glands nor with the ontogeny of male genital function; and *2*) the white matter, but not the gray matter, is a major site of apoD mRNA production throughout the CNS.

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