0021-972X/97/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 82, No. 6
Printed in U.S.A.

Steroidogenic Factor 1 Messenger Ribonucleic Acid Expression in Steroidogenic and Nonsteroidogenic Human Tissues: Northern Blot and *in Situ* Hybridization Studies

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ABSTRACT

Steroidogenic factor-1 (SF-1), a tissue-specific orphan nuclear receptor, regulates the genes of several steroidogenic enzymes, Mullerian inhibiting substance, and the gonadotrophins. Also, this transcription factor is crucial for hypothalamic, adrenal, and gonadal organogenesis in the mouse. We recently cloned the human SF-1 (hSF-1) complementary DNA (cDNA) and now report the distribution of this factor's messenger RNA (mRNA) in human tissues. Northern blot analyses of peripheral tissues revealed high hSF-1 mRNA expression in the adrenal cortex and the gonads, but no hSF-1 mRNA was detected in the placenta. High hSF-1 mRNA expression also was seen in the spleen. In this tissue, in addition to the main transcript of 3.5–4 kb seen in the adrenal and gonads, two additional transcripts of 4.4 kb and 8 kb were noted. The additional 4.4-kb transcript also was seen in several peripheral tissues and various components of the brain. However, adult liver and heart showed only the 4.4-kb transcript. In the human brain, hSF-1 mRNA expression was widespread, including several components of the limbic system. *In situ* hybridization studies confirmed the strong expression of hSF-1 mRNA in adrenal cortex, ovary, testis, and the spleen, primarily within reticuloendothelial cells. Thus, in the human, the hSF1 mRNA is present in both steroidogenic and nonsteroidogenic tissues, albeit not in the placenta. In the central nervous system, the expression of hSF-1 mRNA is widespread. It is composed of several different mRNA species distributed in a tissue-specific fashion. These findings suggest that hSF-1 may play a role in reticuloendothelial/immune cell maturation and/or function, as well as nervous system development and/or neurosteroid biosynthesis. (*J Clin Endocrinol Metab* **82:** 1799–1806, 1997)

STEROIDOGENIC FACTOR 1 (SF-1), a tissue and cell-specific orphan nuclear receptor, plays a pivotal role in the transcriptional regulation of several genes coding for steroidogenic enzymes (1–6). In addition, SF-1 regulates the genes coding for Mullerian inhibiting substance (7), oxytocin (8), the α-subunit of the glycoprotein hormones (9, 10), the β-gene promoter of luteinizing hormone (11, 12), DAX-1 (13), and steroidogenic acute regulatory protein (StAR) (14). *In situ* hybridization studies in mouse embryos demonstrated that SF-1 was expressed in the urogenital sinus and the developing adrenal cortex, gonads, and diencephalon (15). Targeted disruption of the SF-1 gene in mice established its essential role in the organogenesis of these tissues (10, 16–21). Thus, these mice had agenesis of their adrenal glands, gonads, and ventromedial nucleus of the hypothalamus, resulting in complete congenital adrenal insufficiency and male-to-female sex reversal.

To determine the role of SF-1 in the human, we previously cloned and sequenced the human SF-1 (hSF-1) complementary DNA (cDNA) by heterologous probing of a λgt11 fetal adrenal cDNA library using a PCR-amplified ^{32}P-labeled mouse SF-1 cDNA probe that did not include the region coding for the zinc finger domain (22). Sequence analysis revealed an entire open reading frame, most of the 5′-untranslated region, and the complete 3′-untranslated region, including the poly-A signal and tail. The derived amino acid sequence showed a high degree of homology (>95%) was found to the bovine and murine sequences. The zinc fingers, the FTZ-1 box, and the AF2 domains showed 100% conservation of the derived amino acid residues with a lesser degree of homology in the ligand binding/dimerization domains (regions I and II) (22).

In this study, we defined the sites of hSF-1 messenger RNA (mRNA) expression in human tissues by both Northern blot and *in situ* hybridization analyses.

Received September 17, 1996. Revised December 6, 1996. Accepted February 20, 1997.

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Materials and Methods

Materials

Restriction enzymes, the rapid DNA ligation kit, Pwo DNA polymerase, and PCR nucleotide mix were purchased from Boehringer Mannheim Co. (Indianapolis, IN). Multiple tissue Northern blots and ExpressHyb hybridization solution were purchased from Clontech (Palo Alto, CA). Cloning vector pBluescript II KS ±, expression vector pBK-CMV, and competent cells XLI-Blue MRF′ were purchased from Stratagene (La Jolla, CA) and Sephadex G-50 spin columns from Lofstrand Laboratories (Gaithersburg, MD).

Methods

Northern blot analyses. The hSF-1 cDNA was cloned and sequenced as previously described (22). Prehybridization and hybridization were car-

ried out in ExpressHyb hybridization solution, according to the manufacturer's protocol. The stringency of the washing conditions used in the Northern blot experiments is as follows: Low-stringency wash was done at room temperature × 30 min with 3 changes of wash solution containing sodium chloride 300 mmol/L, sodium citrate 300 mmol/L (2 × SSC), and 0.1% sodium dodecyl sulfate. High-stringency wash was done at 50 C × 40 min with 2 changes of wash solution containing 0.2× SSC, 0.1% SDS.

The probe was prepared by PCR amplification of hSF-1 cDNA using Pwo DNA polymerase and PCR nucleotide mix in Perkin-Elmer Gene Amp PCR system 9600 (Perkin-Elmer Corp., Foster City, CA). The primers used for the PCR amplification of the probe were: forward primer, 5′-GCAGAAGAAGGCACAGATTC-3′; and reverse primer, 5′-TCACCAGGATGTGGTTATTC-3′.

The probe was radiolabeled with Klenow DNA polymerase-directed incorporation of ^{32}P deoxycytidine 5′-triphosphate, primed by random deoxynucleotide monophosphate hexamers. The probe was purified by removal of unincorporated ^{32}P-labeled deoxycytidine 5′-triphosphate by Sephadex-G-50 spin column.

In Situ hybridization. The template for the sense probe was generated by Xho-1 digestion and that for the antisense probe by Xba-1 digestion of pBluescript SKII ± containing the 3.109-kb hSF-1 fragment. These cDNA template fragments were gel purified for *in vitro* transcription to generate the sense and antisense RNA probes. The sense probe template spanned the entire hSF-1 cDNA. The antisense probe template was located in the 3′ untranslated region and spanned nucleotides 2355–3109 of the hSF-1 cDNA.

Human tissues (liver, pituitary, ovary, and testes) included fresh-frozen necropsy specimens from young adults (having died suddenly as a result of trauma; obtained through the National Disease Research Interchange, Philadelphia, PA) and fresh-frozen surgical specimens from patients undergoing nephrectomy at the NIH Clinical Center (spleen, kidney, and adrenal). Patients gave informed consent to the disposition of their surgically removed tissues under a protocol approved by the NIH Clinical Center Institutional Review Board. Aside from the diagnosis of presumed renal tumor, the patients were free of endocrine or renal disease and were 43–48 yr old. Rhesus monkey ovaries were obtained from healthy animals undergoing ovariectomy for a protocol approved by the NICHD Animal Committee. Two to five independent specimens were evaluated for each tissue type. Tissue was snap-frozen and stored at −70 C until sectioning. Sections of 10-μm thickness were cut at −15 C and thaw-mounted onto poly-L-lysine-coated slides and stored at −70 C until use.

^{35}S-labeled RNA probes were synthesized to SA ~ 2 × 108 dpm/μg, as previously described (23). Equal concentrations of labeled probe (106 cpm/100 μL) were applied to all sections, which were then hybridized and washed as previously described (23). Sense and antisense probes were used in parallel experiments to evaluate nonspecific signal level. Sections were exposed to NTB2 nuclear emulsion for 2 weeks, developed, and counterstained with hematoxylin and eosin for microscopic evaluation and photography.

Results

hSF-1 mRNA is expressed in both steroidogenic and nonsteroidogenic human tissues

A hSF-1 expression plasmid, containing our hSF-1 cDNA, produced functional hSF-1 that, as expected, stimulated a StAR promoter-luciferase reporter construct, 5-fold, in HeLa cells (data not shown). The human StAR promoter-luciferase reporter construct was kindly donated by Dr. Jerome F. Strauss III, of the University of Pennsylvania.

The expression of hSF-1 mRNA in human tissues was examined both by Northern blot and *in situ* hybridization analyses using a hSF-1 cDNA and a cRNA probe, respectively.

Northern blot analysis of multiple peripheral tissues revealed a single message size of approximately 3.5 kb in the steroidogenic tissues, *i.e.* adrenal cortex, ovary, and testis (Fig. 1, A and B). However, a nonsteroidogenic tissue, the spleen, also showed strong expression of the hSF-1 message with a signal intensity less than that of the adrenal cortex but higher than or similar to that of the gonads. Also, several lymphoid tissues, including the lymph nodes, thymus, appendix, and peripheral blood leukocytes, showed an hSF-1 transcript of a signal intensity less than that of the spleen (Fig. 1D). In keeping with these data, human lymphoid and myeloid cancer cell lines, a lymphoid B cell line (Burkitt's lymphoma), the myeloid cell lines HL-60 (promyelocytic leukemia) and K-562 (chronic myelogenous leukemia), also expressed hSF-1 mRNA (Fig. 1E). In the spleen, the main transcript, as well as two additional transcripts of 4.4 kb and 8 kb, were noted. The myeloid and lymphoid cancer cell lines, several tissues of the immune system, skeletal muscle, and pancreas showed the 4.4-kb transcript in addition to the main transcript. However, the heart and adult liver showed only a single 4.4-kb transcript (Fig. 1C).

Northern blot analysis did not reveal a signal in human placenta after a 16-h exposure (Fig. 1C). A weak signal was detected after an 8-week exposure (data not shown).

Northern blot analysis of several human central nervous system (CNS) components, including the spinal cord, revealed widespread presence of an hSF-1 message of 3.5–4 kb. An additional mRNA signal of 4.4 kb also was widely expressed. The hSF-1 mRNA concentration was highest in the caudate and subthalamic nuclei and also in the thalamus. hSF-1 mRNA also was present in the hippocampus, corpus callosum, and cerebral cortex (Fig. 2, A and B).

In situ hybridization studies, using an hSF-1 antisense probe, confirmed and expanded the data obtained by Northern blot analysis. A high level of hSF-1 signal intensity was detected in all the three zones of the adrenal cortex, whereas no signal was detected in the adrenal medulla (Fig. 3, A–D). No hybridization was detected in the kidney or liver (data not shown). SF-1 mRNA localization was investigated in both human and rhesus monkey ovary sections, and findings were identical in both species. In the ovary, hSF-1 mRNA was abundant in granulosa and theca cells at all stages of follicular development (Fig. 4, C and D), except for primordial follicles. hSF-1 message also was present in corpora lutea (data not shown). Testicular SF-1 mRNA was most abundant in clusters of large round cells with abundant eosinophilic cytoplasm containing fine granular basophilic stippling around large, pale blue nuclei (Fig. 4, A and B). These cells have the typical appearance of steroidogenically active Leydig cells and are localized in clusters in the interstitial compartment, as are Leydig cells (Fig. 4A); thus it is concluded that SF-1 mRNA is concentrated in Leydig cells. SF-1 hybrid signal also is specifically detected in a diffuse distribution within the seminiferous tubules (Fig. 4, A and B). Given this diffuse pattern, it cannot be determined whether the grains are localized over Sertoli cells or spermatocytes or both cell types, and thus, it is concluded simply that SF-1 mRNA is present in the germinal epithelium of the human testis. In the spleen, hSF-1 mRNA expression had a reticular pattern, and the signal was concentrated in the red pulp, primarily within reticuloendothelial cells (Fig. 5, A–D).

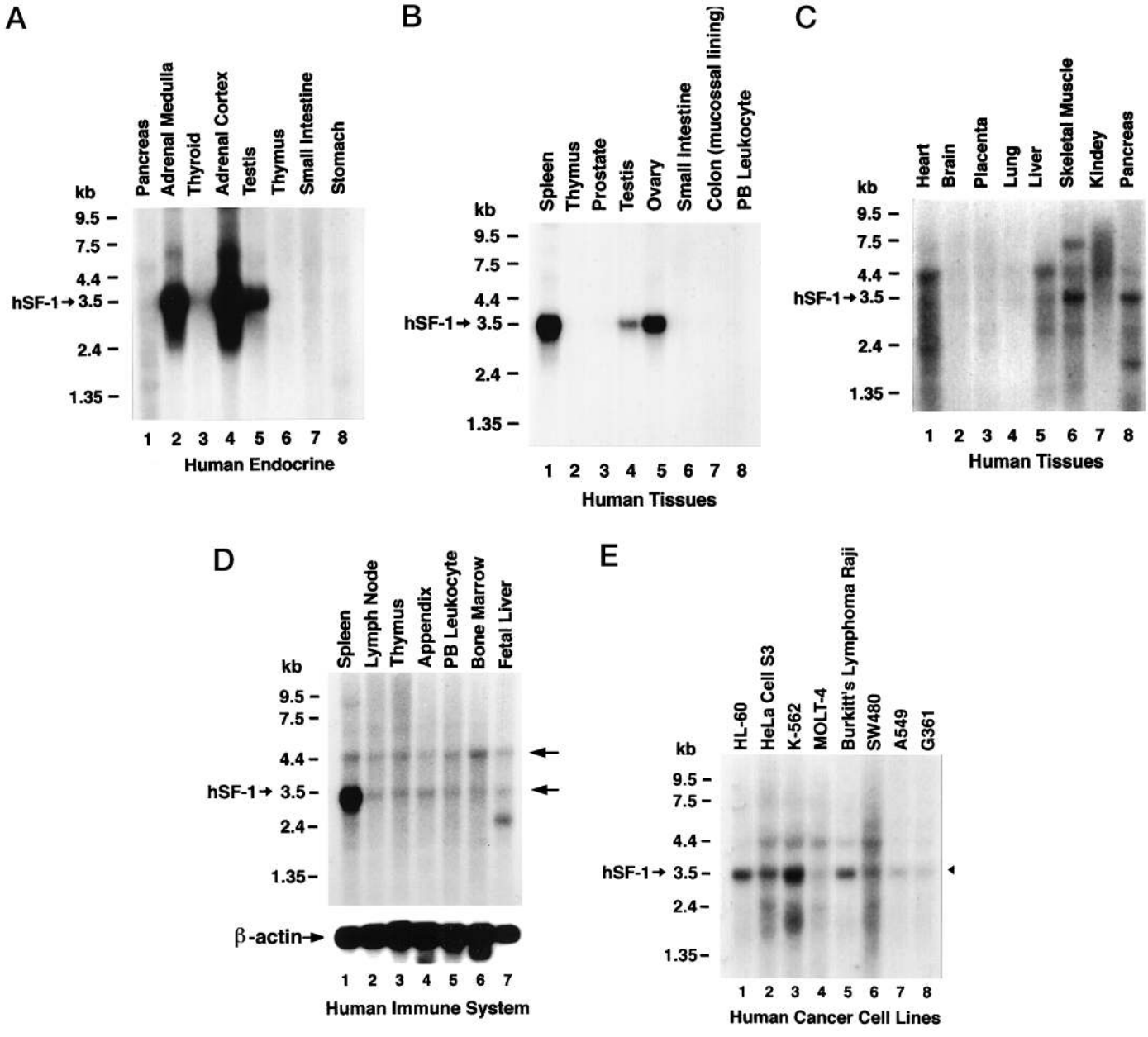

FIG. 1. hSF-1 mRNA is expressed in multiple human tissues. Human multiple tissue Northern blots were probed using ^{32}P-labeled hSF-1 cDNA, as described in *Materials and Methods*. Each lane contains 2 μg of pure poly A$^+$ RNA. The exposure time for the endocrine system Northern blots (blot A) and the multiple-tissue Northern blots (blots B and C) was 16 h; for the immune system and cancer cell line Northern blots (blots D and E), it was 24 h.

Discussion

Previous studies have demonstrated that SF-1 is important for the transcriptional regulation of genes involved in steroid biosynthesis (1–6, 24) and crucial for the organogenesis of the adrenal glands, gonads, and the ventromedial nucleus of the hypothalamus in the mouse (15–17, 19–21). In this study, we demonstrate, by both Northern blot and *in situ* hybridization analyses using a hSF-1 probe, that in humans, this transcription factor is expressed not only in steroidogenic but also in nonsteroidogenic tissues. Furthermore, we have found that its expression in the CNS in humans is widespread, rather than limited to the hypothalamus as seen in the mouse (15, 19, 20).

SF-1 in the human was expressed clearly in the adrenal cortex, the ovary, and the testis. The adrenal cortex had higher hSF-1 mRNA intensity than the gonads. Expression of hSF-1 also was seen in the adrenal medulla by Northern blot analysis. However, this expression probably reflects the presence of hSF-1 in adrenal cortical rests within the medullary tissue. Histological studies have shown the presence of islands of adrenal cortical tissue within this portion of the gland (25), and this was confirmed by our *in situ* hybridiza-

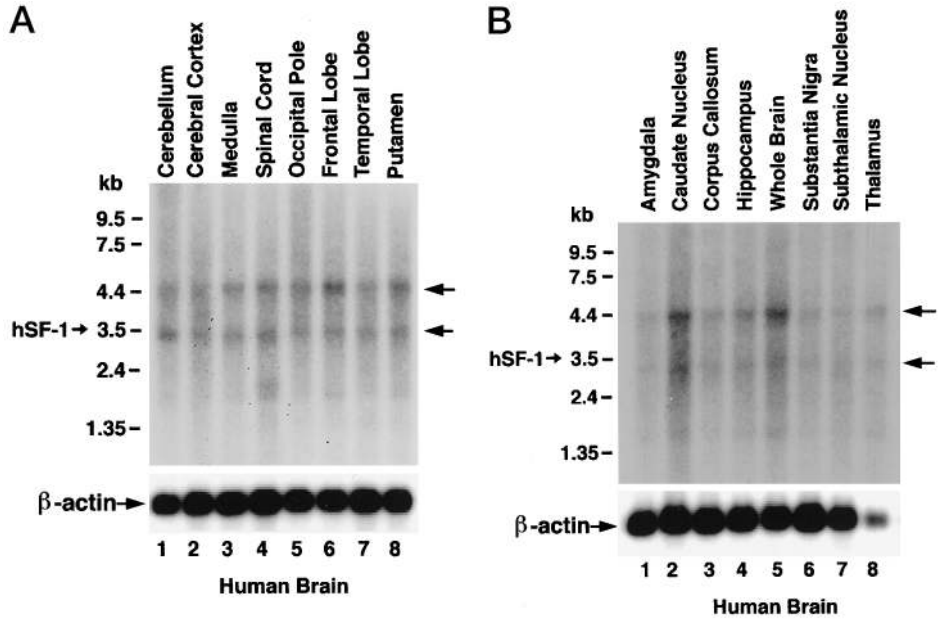

FIG. 2. hSF-1 mRNA is expressed in components of the human brain. Human brain multiple-tissue Northern blots were probed using ^{32}P-labeled hSF-1 cDNA, as described in *Materials and Methods*. Each lane contains 2 μg of pure poly A$^+$ RNA. The exposure time was 24 h.

tion studies showing hSF-1 mRNA expression in steroidogenic but not chromaffin cells.

In situ hybridization studies of normal architecture adrenal gland showed similar distribution of SF-1 mRNA signal in all the three zones of the adrenal cortex. However, within each cortical zone, the signal distribution was heterogeneous (Fig. 3, C and D). Sesano *et al.* (26), using immunohistochemistry, also demonstrated the heterogenous distribution of the signal of Ad4BP, the bovine homolog of SF-1, within each of the three cortical zones of normal, neoplastic, and atrophied human adrenal glands. Interestingly, in our *in situ* hybridization studies of a normal nodular variant of the adrenal gland, we detected a very high SF-1 gene expression in the proliferative nodules (Fig. 3, A and B). These data suggest that, in addition to regulation of steroidogenesis, SF-1 also may have a role in regulating the growth and proliferation of adult adrenal cortical cells.

Parallel examinations of rhesus and human ovary for SF-1 mRNA localization, by *in situ* hybridization studies, revealed an identical distribution in the two species. These studies demonstrated that hSF-1 mRNA was abundant in granulosa cells at all stages of follicular development, except for primordial follicles, and also was present in corpora lutea. Both the theca interna and externa cells surrounding the graafian follicles also expressed hSF-1 mRNA. In addition, a thecoma seen in a section of Rhesus monkey ovary also showed strong expression of hSF-1 mRNA, suggesting active steroidogenesis in this tissue. hSF-1 mRNA, however, also was seen in atretic follicles, which normally are not steroidogenic. Takayama *et al.* (27) recently correlated the expression of steroidogenic enzymes to the expression of immunoreactive Ad4BP in the human ovary. Although there was such a correlation, Ad4BP expression also was seen in preantral and atretic follicles and degenerating corpora lutea, where no steroidogenesis was detected. These data collectively suggest a broader role for hSF-1, beyond steroidogenesis, perhaps in differentiation and/or apoptosis processes of the ovary. Data from studies in the rat with *in vitro* cultured granulosa cells are compatible with this process (28).

In the adult testis, hSF-1 expression was seen in both the interstitial cells and the inner border of the seminiferous tubules, suggesting expression in the steroidogenic Leydig cells and the germinal epithelium. Previous studies in the embryonic mouse testis and postnatal day-15 rat Sertoli cells also show expression of SF-1 mRNA in the seminiferous tubules of the mouse and the Sertoli cells of the rat (15). In immunohistochemical studies in the rat testis, using Ad4BP antiserum, immunoreactivity was detected in the cells surrounding the seminiferous tubules but not within the tubules (3). This discrepancy is probably a species-specific difference or could be explained as incongruous expression of hSF-1 mRNA and protein. Our results suggest that, in the human testis, in addition to steroidogenesis, hSF-1 also may have a role in the function of spermatogenesis.

Northern blot analysis of the human placenta did not reveal hSF-1 message after a 16-h exposure; however, a weak signal was noted after 8 weeks of exposure (data not shown). SF-1 message expression in the bovine (6) and human placenta (29) was reported previously using the highly sensitive RT-PCR technique. The apparently low expression of hSF-1 in human placenta suggests that it may not have a major role in placental steroidogenesis. This view is supported by previous studies that demonstrated expression of SF-1 mRNA in BeWo human choriocarcinoma cells only by the highly sensitive RT-PCR technique (14), nonexpression of the StAR gene in the placenta (30), expression of P450 side-chain-cleavage enzyme in the placenta of SF-1- deficient mice (21), use of SFRE-deficient aromatase promoter 1.1 for placental aromatase gene transcription (31), and regulation of placental p450scc gene transcription by a 55-kd protein that is expressed in the placenta but not in the adrenal cortex (32). The data from these studies, along with our own data, suggest that alternative pathways of steroid metabolism or func-

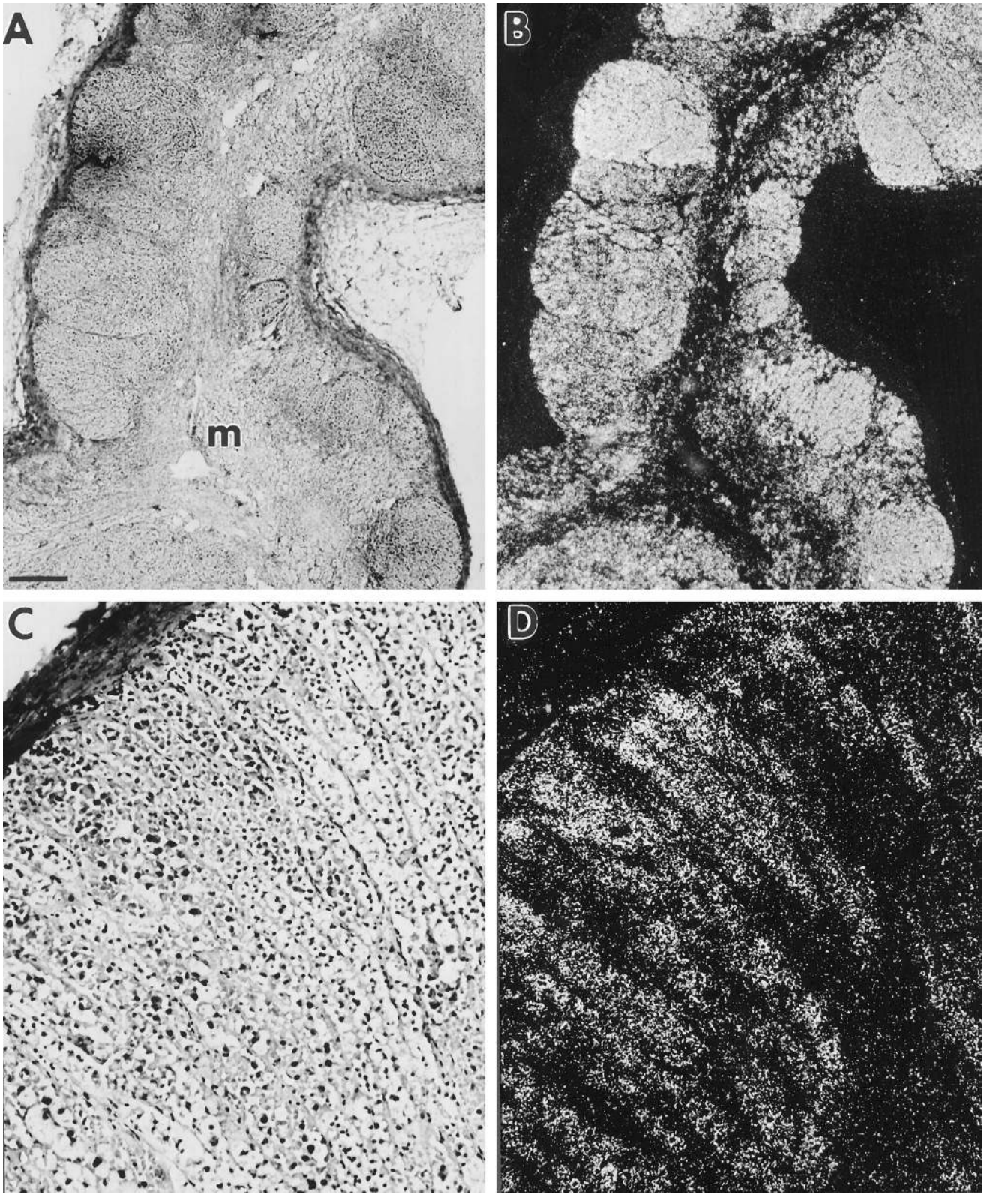

FIG. 3. hSF-1 transcripts in human adrenal glands by *in situ* hybridization. Low magnification, paired, bright- and dark-field micrographs of a nodular adrenal are shown in A and B. Little hybrid signal is detected in the adrenal medulla (m). Panels C and D show higher magnification views of the normal human adrenal cortex, with hSF-1 signal arrayed in orderly fascicles. Bar = 200 μ in A and B and 50 μ in C and D.

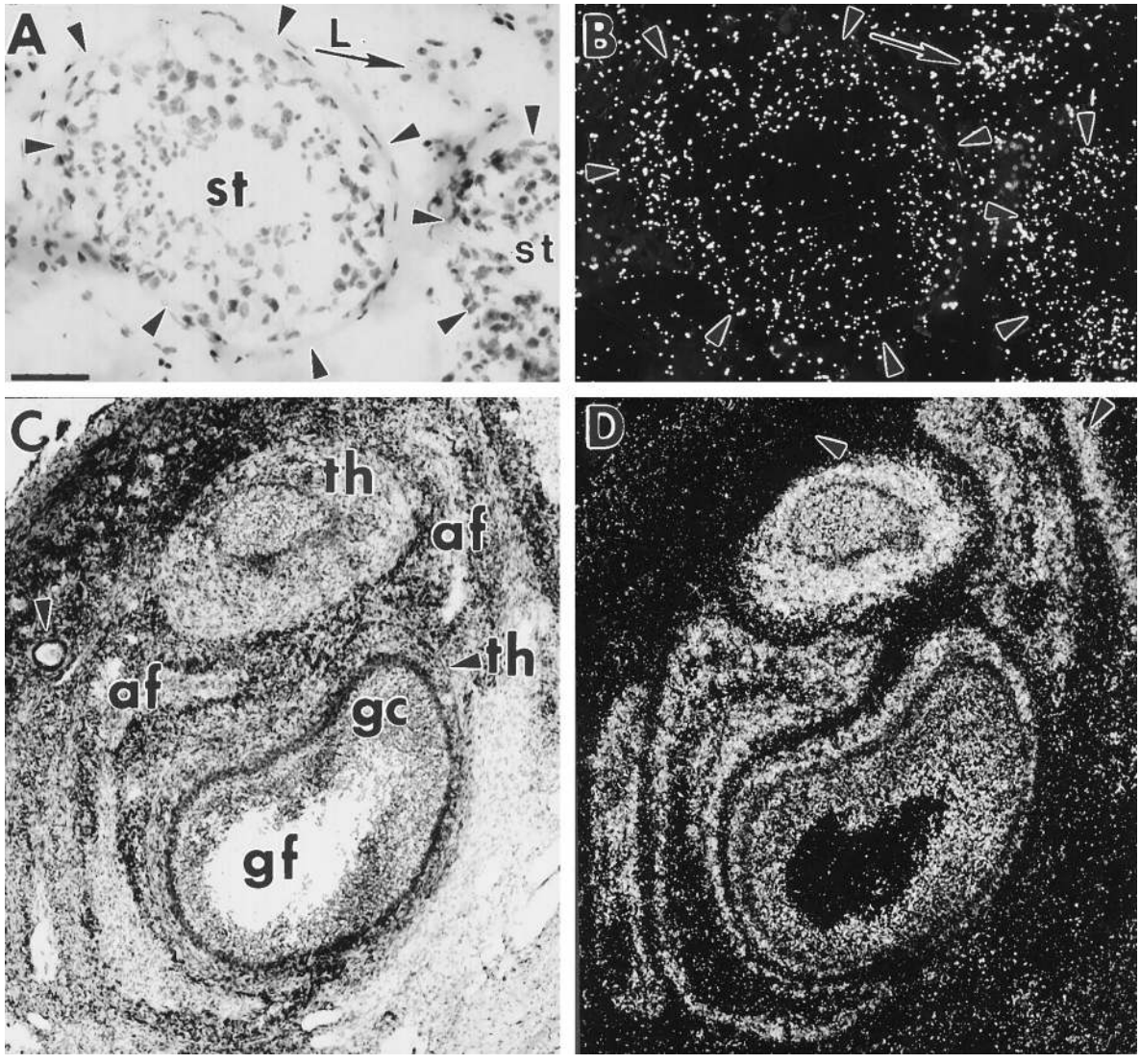

FIG. 4. hSF-1 mRNA localization in testis (A and B) and ovary (C and D) by *in situ* hybridization. In the human testis, hSF-1 hybrid signal is detected along the *inner border* of the seminiferous tubules (st) and in Leydig cells (L). The *arrows* in A and B point to a cluster of Leydig cells (L). *Arrowheads* delimit the seminiferous tubules (st) in A and B. SF-1 mRNA localization was identical in the human and rhesus monkey ovary; a rhesus ovary section is shown because more different types of follicles are found close together in the rhesus ovary, which is much smaller than the human. In the ovary, hSF-1 transcripts are abundant in granulosa (gc) and theca (th) cells. This section contains a single healthy-appearing graafian follicle (gf) with a well-formed theca and some deformed, presumably atretic follicles (af), as well as an interesting follicle consisting of hyperplastic theca and small granulosa compartment, presumably a thecoma, which displays extremely intense hSF-1 expression. The small primordial follicle (*arrowhead*) demonstrates no hSF-1 mRNA. Bar = 50 μ for A and B and 200 μ for C and D.

tional homologues of hSF-1 may be operational in human placental steroidogenesis.

We detected a widespread expression of hSF-1 mRNA in components of the human brain, by Northern blot analysis. In addition to the main message of 3.5–4 kb, also seen in peripheral tissues, a message of 4.4 kb also was detected. The hSF-1 mRNA concentration was highest in the caudate and subthalamic nuclei; furthermore, hSF-1 mRNA was detected in the amygdala, hippocampus, corpus callosum, and brain cortex. In contrast to our findings in the human, SF-1 expression was found to be quite limited in animal brains. In the cow brain, the Ad4BP message was detected only by RT-PCR (6), whereas in the mouse brain, SF-1 expression was restricted to the hypothalamus and pituitary (10, 15, 16, 20). The widespread expression of SF-1 mRNA in the human brain, as opposed to the restricted expression in the hypothalamus and pituitary in mice, suggests a broader role for this transcription factor in the human. It is tempting to speculate that in the human, SF-1 may have a role in the regulation of neurosteroid biosynthesis and/or in the development of the CNS. At the present time, we can only speculate about the additional message found in the brain. This could be an alternatively spliced or polyadenylated product of the hSF-1 gene, or an altogether different, but highly homologous, molecule.

The unexpected detection of hSF-1 transcripts in the spleen demonstrates that the role of this transcription factor may not be limited to steroidogenic tissues and the brain in the hu-

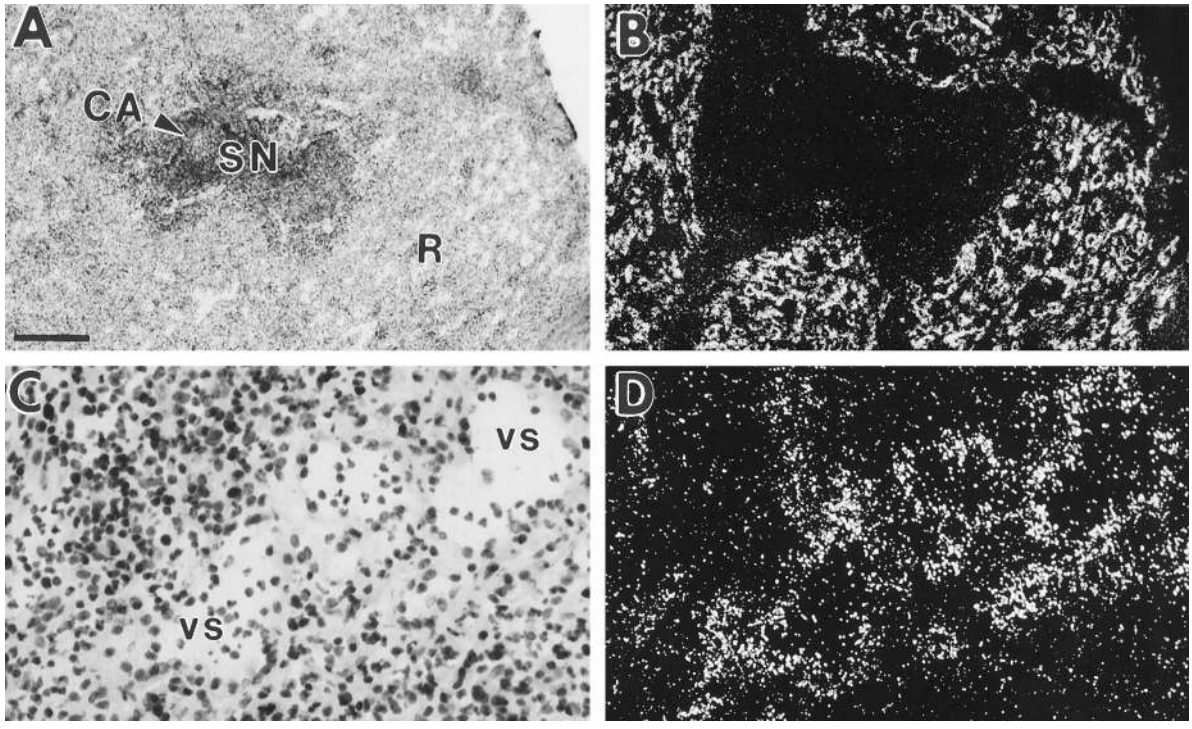

FIG. 5. hSF-1 transcripts in human spleen by *in situ* hybridization. Paired, bright- and dark-field illuminations of human spleen at low (A and B) and high (C and D) magnification reveal that hSF-1 transcripts are selectively concentrated in the reticuloendothelial network of the spleen. SN, splenic nodule (Malphigian corpuscle); vs, venous sinus; CA, central artery; R, red pulp. Bar = 200 μ for A and B and 50 μ for C and D.

man. The Northern blot analysis of the spleen revealed that the signal strength in this organ was second only to that of the adrenal cortex and greater than or equal to that of the gonads. The hSF-1 mRNA also was detected in the myeloid K-562, and HL-60 and Burkitt's lymphoma cells, which are related to the hematopoetic/immune system. The *in situ* hybridization study showed that hSF-1 transcripts were concentrated selectively in the reticuloendothelial network of the spleen. In cows, Ad4BP was not detected in the spleen by either Northern blot analysis or by RT-PCR (6). Additionally, SF-1 gene-knockout studies in the mouse did not report agenesis or dysgenesis of the spleen or any obvious immune dysfunction (19). The strong hSF-1 mRNA expression and the two additional transcripts of 4.4 kb and 8 kb seen in the spleen, together with the additional 4.4-kb transcript seen in the rest of the immune tissues and cancer cell lines, indicate that this transcription factor may have a significant role in human immune cell maturation or function.

We conclude that in the human, SF-1 may have a more comprehensive role than in previously studied animals. Its unexpected widespread expression in the brain and its strong expression in the spleen are indicative of such a broader role. It is, therefore, tempting to speculate that in the human, SF-1 may have a fundamental role in neurosteroid biosynthesis and/or brain development, as well as in hematopoetic/immune cell proliferation and/or differentiation.

Acknowledgments

We are grateful to Dr. Elizabeth Webster for helpful suggestions and to Mr. Keith Zachman for expert technical assistance.

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