Steroidogenic Factor 1, an Orphan Nuclear Receptor, Regulates the Expression of the Rat Aromatase Gene in Gonadal Tissues

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In a concerted analysis of the genes encoding three mouse steroid hydroxylases, we identified and characterized a transcriptional regulatory protein, designated steroidogenic factor 1 (SF-1), that contributes to the coordinate expression in adrenocortical cells. SF-1, an orphan member of the nuclear receptor family, binds to PyCAAGGPyCPu motifs upstream of the steroid hydroxylases to regulate their expression. In the present study, we extend these findings by examining the role of SF-1 in regulation of the rat P450 aromatase gene in gonadal tissues.

The 5′-flanking region of the rat aromatase gene was isolated by a polymerase chain reaction-based approach, using primers corresponding to the 5′- and 3′-ends of a published aromatase sequence. DNA sequence analysis revealed three differences between our sequence and the previously published sequence, including a 44-base pair (bp) insertion. Moreover, the transcription initiation site, as determined by primer extension analysis, differed from that previously proposed. The new transcription initiation site is located 23 bp 3′ of a putative TATA box. When a revised rat sequence was compared to that of the human aromatase PII promoter by BESTFIT analysis, a region of about 300 bp was identified that was 80% conserved between the two promoters. A potential SF-1 site, CCAAGGTCA, was identified at position −82 within this region.

An oligonucleotide probe containing this putative SF-1 site was used in gel mobility shift assays. Consistent with previous studies, a specific complex was observed with nuclear extracts from gonadal steroidogenic tissues but was absent with nuclear extracts from nonsteroidogenic tissues. The role of SF-1 in this steroidogenic cell-specific complex was next addressed more directly. Bacterial extracts containing an SF-1-glutathione S-transferase fusion protein interacted specifically with the putative SF-1 site, and polyclonal antisera against SF-1-glutathione S-transferase specifically abolished the complex formed with nuclear extracts from rat ovaries or R2C rat Leydig tumor cells. Finally, the aromatase SF-1 element increased expression of an SV40 promoter/luciferase construct in transient transfection experiments in a steroidogenic cell-selective manner. Collectively, these studies implicate SF-1 in the regulation of steroid hydroxylase gene expression in nonadrenal tissues, significantly extending previous studies in adrenocortical cells. (Molecular Endocrinology 7: 776–786, 1993)

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INTRODUCTION

The enzyme cytochrome P450 aromatase (aromatase) catalyzes the conversion of androgens to estrogens, including estradiol 17β (1). Aromatase expression is tissue specific. The ovary is the primary site of aromatase activity in the sexually mature female, and estrogen synthesis is restricted to the granulosa cells of developing preovulatory follicles and to luteal cells (2). Superimposed on this tissue-specific regulation, aromatase expression in the ovary is modulated by several hormones and growth factors (3). The primary regulators of estradiol 17β production are the pituitary gonadotropins, FSH and LH, which enhance aromatase activity in granulosa cells via a cAMP-dependent signal transduction pathway (3).

Two different cell types are required for the conversion of cholesterol to estrogen in the rat ovary: theca

and granulosa cells. Both cells express the initial enzymes in this pathway, cholesterol side-chain cleavage ($P450_{ssc}$), which converts cholesterol to pregnenolone, and 3β-hydroxysteroid dehydrogenase, which metabolizes pregnenolone to progesterone. Thecal cells further convert progesterone to androgens through successive 17-hydroxylation and C17,20 lyase reactions catalyzed by steroid 17α-hydroxylase ($P450_{c17}$). Thecal cells have very low aromatase levels and consequently do not convert androgens to estrogens. In contrast, granulosa cells cannot metabolize progesterone to androgen because they lack $P450_{c17}$ activity but aromatize androgens derived from neighboring thecal cells to produce estrogens (3).

The molecular dissection of the promoter regions of genes encoding the steroidogenic enzymes can afford insight into the molecular basis for the different steroidogenic capacities of theca and granulosa cells, as well as the mechanisms by which hormones and growth factors regulate steroidogenic enzyme synthesis. Analysis of the mouse adrenocortical steroid hydroxylases identified a number of tissue-specific promoter elements with related AGGTCA motifs that interacted with a shared protein seen only with nuclear extracts from steroidogenic cells (4–7). The sequence of a cDNA encoding this protein, designated steroidogenic factor 1 (SF-1) (8), indicated that it was the mouse homolog of the *Drosophila* orphan nuclear receptor, *fushi tarazu* factor 1, (FTZ-F1), which regulates expression of the *fushi tarazu* homeobox gene in early development (9, 10). SF-1 is related to the embryonal long terminal repeat binding protein (ELP) identified in undifferentiated mouse embryonal carcinoma cells (11). The presence of potential SF-1 binding sites in the promoter regions of nonadrenal steroidogenic enzymes and the expression of SF-1 in ovary and testis suggested that SF-1 might also contribute to steroidogenic enzyme gene expression in gonadal tissue.

Analysis of the rat aromatase promoter sequence identified a potential SF-1 binding site at position −82. In this report, we demonstrate that this putative regulatory element interacts with SF-1 and increases the activity of a heterologous promoter, thereby directly implicating SF-1 in the regulation of steroidogenic enzyme genes in gonadal tissue.

RESULTS

The Rat Aromatase Promoter Contains a Potential SF-1 Site

The rat aromatase promoter has been previously cloned and used in promoter/reporter studies (12, 13). We synthesized this promoter fragment by polymerase chain reaction (PCR), using liver DNA as a template and oligonucleotide primers corresponding to the ends of the previously reported rat aromatase promoter. The amplified product was cloned, and the sequence determined for three separate clones (aro1a, aro1b, and aro1c). Additionally, two clones (aro2a and aro2b) from a second PCR reaction were cloned and sequenced. The sequences of these clones were identical and departed from the published sequence at three positions: 1) an insertion of GTGTGTGT at position −352 to −345; 2) a G to A transition at position −277; and 3) a 44-base pair (bp) insertion at positions −212 to −169 (Fig. 1a). PCR on genomic DNA with different (nested) primers and with one of the primers specific to the 44-bp insertion confirmed the presence of the 44-bp fragment (data not shown). Primer extension analysis with ovarian poly(A)$^+$ RNA indicated a major transcription start site at a position 23 bp downstream from a putative TATA box (Fig. 2). The revised sequence for the rat aromatase promoter was compared by BESTFIT analysis (14) to the human PII aromatase promoter (16), and a region of more than 300 bp was found to have 80% similarity between the two promoters (Fig. 1B). This conserved region included the sequence CCAGGTCA, at position −89 to −74 within the rat promoter. Similar sequences have been shown to be important in the coordinated expression of steroidogenic enzymes in the mouse adrenocortical cells (4–8). Accordingly, we examined whether this element interacted with SF-1 and whether it acted as a cell-selective regulatory element.

The Potential SF-1 Site at −82 Interacts with SF-1

We first analyzed the ability of the potential SF-1 site to interact in gel mobility shift assays with nuclear extracts from steroidogenic and nonsteroidogenic cells. Gel mobility shift studies were performed with an end-labeled, double-stranded 20-mer oligonucleotide corresponding to positions −87 to −68 (see *Materials and Methods*) and nuclear extracts from several tissues. Extracts were prepared from two estrogen-producing tissues: 1) ovaries treated with PMSG for 2 days (see *Materials and Methods*); and 2) rat R2C Leydig tumor cells. Note that R2C cells express the aromatase gene, and primer extension analysis indicated that the aromatase gene is transcribed from the same position as in ovary (data not shown). Extracts were also made from the following nonsteroidogenic tissues: rat liver, human HeLa cervical carcinoma cells, and rat GH_3 pituitary tumor cells. A prominent complex (complex I) was formed by steroidogenic cell extracts but was absent in gel shifts with the nonsteroidogenic cell extracts (Fig. 3). Additional complexes of lesser mobility were formed by all of the extracts (complex II and other bands).

The specificity of the complexes was analyzed in competition experiments. Formation of complex I was competitively inhibited by 100-fold excess of unlabeled SF-1 oligonucleotide. (Fig. 3). As shown in Fig. 4, complex I or II was not inhibited by an unlabeled AP-1 site (lane 6) or by a fragment from the rat PRL promoter (lane 7), but were competitively inhibited by a 139-bp fragment from the aromatase promoter containing the SF-1 site (lane 8). In contrast, an idealized SF-1 binding

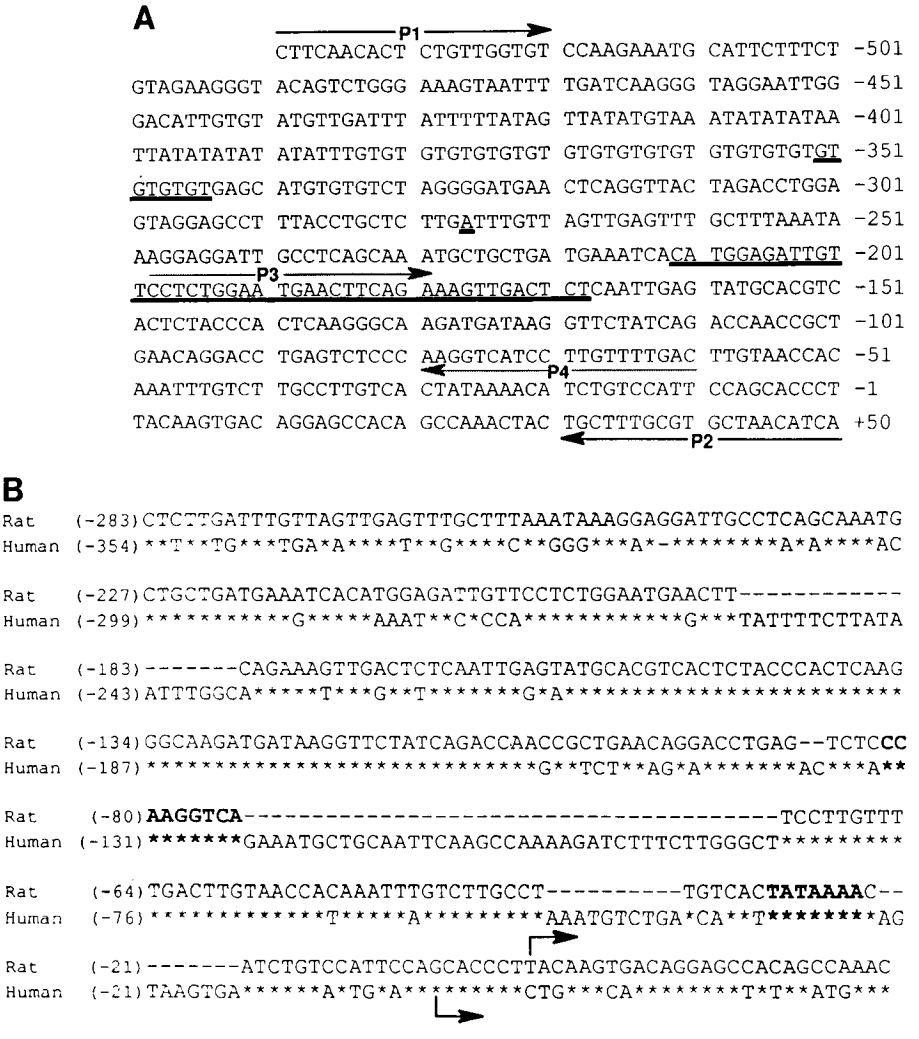

Fig. 1. Sequence of Rat Aromatase Promoter

A, Sequence of rat aromatase promoter isolated from Wistar-Furth rat genomic DNA by PCR. The sequence which differs from that previously published (13) is *underlined*. Transcription initiates at position +1. The corresponding positions for the primers for initial amplification (P1 and P2) and nested amplification (P3 and P4) are shown. The GeneBank/EMBL data bank accession number is Z11815. B, BESTFIT comparison of the rat aromatase promoter with the human aromatase PII promoter. Identical bases are denoted by *asterisks*, and gaps are denoted by *dashes*. The SF-1 site (CCAAGGTCA) and TATA box are indicated by *bold type*. *Arrows* denote site of transcription initiation.

site derived from the SF-1 site at −40 in the mouse $P450_{scc}$ promoter inhibited complex I but did not affect complex II (lane 5). This is consistent with previous studies in which the −40 element formed only complex I in gel shift assays with steroidogenic cell extracts (5, 6).

Two approaches were then taken to confirm that the potential SF-1 site actually bound SF-1. First, gel mobility shift assays were performed with the aromatase −82 probe and bacterial lysates containing an SF-1-glutathione S-transferase (GST) fusion protein (SF-1[+]-GST). As shown in Fig. 5A, recombinantly expressed SF-1[+]-GST interacted with the aromatase probe to form a prominent shift complex, and the binding displayed the same sequence specificity as rat tissue extracts shown in Fig. 4. As shown in Fig. 5B, extracts from bacteria expressing the SF-1 cDNA in the reverse orientation (SF-1[−]-GST) showed no interaction.

In order to examine further whether the apparent SF-1 binding activity in the rat R2C cell and ovarian nuclear extracts was related to the mouse SF-1 protein, extracts were preincubated with a polyclonal antimouse SF-1 antiserum before addition of rat SF-1 probe. Complex formation in both extracts was abrogated by addition of immune, but not preimmune, serum (Fig. 6). These studies indicate that rat gonadal tissues express a protein immunologically similar to murine SF-1 which specifically interacts with an SF-1 like sequence within the rat aromatase promoter.

The SF-1 Site Acts as a Cell-Selective Regulatory Element

The studies described above confirmed that SF-1 in gonadal extracts bound the aromatase −82 sequence

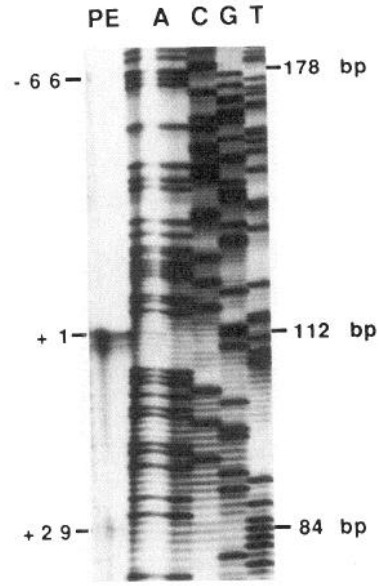

Fig. 2. Primer Extension Analysis from Ovarian Poly(A)$^+$ RNA

RNA was isolated from PMSG-primed rats (see *Materials and Methods*) and 12 μg used for primer extension. The major product is designated +1. Minor products were detected at positions −66 and +29. The ACGT sequencing ladder was run in adjacent lanes in order to size the product.

but said nothing about the functional import of this interaction. SF-1 is a major positive regulator of steroid hydroxylase gene expression in adrenocortical cells (6, 8), but its role in gonadal expression remains undefined. We therefore assessed the effect of the aromatase −82 SF-1 sequence on the expression of the SV40 promoter/luciferase reporter, pGL2-Promoter (Promega, Madison WI). Transient transfection experiments were performed in two cell types: rat R2C Leydig tumor cells, which express SF-1; and human HeLa cells, which lack SF-1. As shown in Fig. 7, addition of one or two copies of the aromatase promoter −82 SF-1 element increased luciferase expression in R2C cells about 3-fold and 7-fold, respectively. In contrast, the presence of SF-1 sites did not enhance pGL2 expression in HeLa cells. In fact, we reproducibly observed a modest inhibition with the 2xSF-1/GL2 construct in HeLa cells in four separate experiments. These results document that the −82 SF-1 site acts as a cell-selective regulatory element, strongly implicating SF-1 in steroidogenic enzyme expression in gonadal tissues.

Analysis of the Structure and Expression of Rat SF-1

The gel mobility shift and transfection studies cited above indicated the presence of a protein in rat gonadal tissue that was structurally and immunologically related to mouse SF-1. To establish that the rat SF-1 homolog was expressed in R2C cells, we performed PCR amplification of an R2C cell cDNA using degenerate oligonucleotide primers derived from two conserved regions within the DNA-binding domain and the putative ligand-binding domain of mouse SF-1 (8), mouse ELP (11), and *Drosophila* FTZ-F1 (10; see *Materials and Methods*). After gel purification, PCR products of the appropriate size were cloned and sequenced (Fig. 8). Sequence alignment (14) with mouse sequence revealed 96.5% identity at the nucleotide level and 99% identity at the level of the predicted amino acid level.

Using the homologous rat SF-1 cDNA as a probe, we examined the expression of rat SF-1. Consistent with previous results in the mouse, SF-1 transcripts were detected in R2C cells and ovary, but not in liver or GH_3 cell RNA (data not shown). To examine possible factors that affect SF-1 expression in rat gonadal tissue, we compared steady state SF-1 mRNA levels in RNA from ovaries of 22-day-old immature female rats and from animals treated with PMSG for 48 h to induce development of preovulatory follicles. As expected (15), aromatase mRNA levels were low in untreated immature female rats and were markedly induced by PMSG injections (Fig. 9). In contrast, SF-1 was already expressed at relatively high levels in the immature ovary and was induced approximately 3-fold by PMSG treatment.

DISCUSSION

Recent studies have established SF-1 as a global regulator of steroid hydroxylase gene expression in adrenocortical cells (4–8). An important unresolved issue is the role of SF-1 in the expression of steroidogenic enzymes in nonadrenal cells. In this report, we have used the rat aromatase gene to test the generality of SF-1 regulation of steroid hydroxylases. Sequence analysis revealed the existence of a putative SF-1 site within a region of the rat aromatase promoter that displayed significant similarity with the human PII aromatase promoter (16). The aromatase SF-1 site bound to nuclear proteins which existed in steroidogenic (ovary, R2C cells) but not nonsteroidogenic (HeLa, GH_3 cells) tissue extracts. This site was also shifted by bacterial extract containing a mouse SF-1 fusion protein, and competition studies indicated that the activity present in the rat extracts was similar to bacterially expressed mouse SF-1. Furthermore, the binding activity of the rat gonadal extracts was specifically blocked by mouse SF-1 antisera. Finally, a PCR-generated partial rat cDNA revealed that rat SF-1 is clearly expressed in gonadal tissue. This sequence was shown to interact with SF-1 and to enhance the activity of a heterologous promoter in a cell-selective manner. Future studies will examine the contribution of SF-1 to aromatase gene expression in the context of the native aromatase promoter.

Although the predominant interaction with the aromatase −82 element reflects SF-1 binding, additional complexes in gel shift assays suggest that other proteins interact at this site, although with less reproducibility than SF-1 (compare Figs. 4 and 6). Multiple com-

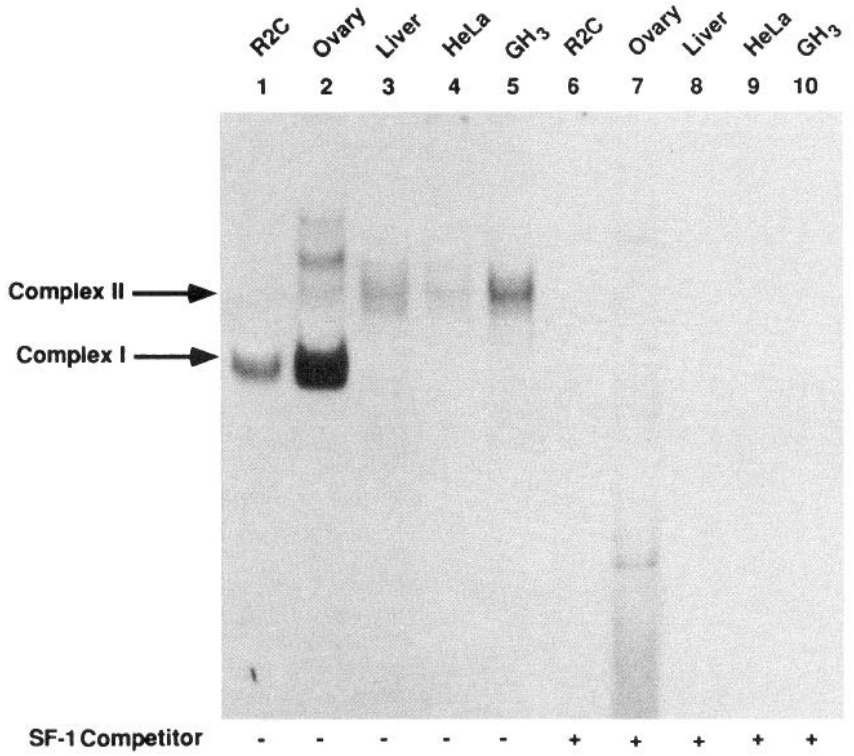

Fig. 3. The Rat Aromatase SF-1 Site Forms a Major Complex (Complex 1) with Nuclear Extracts from Steroidogenic (R2C Cell, Ovary) But Not from Nonsteroidogenic (Liver, HeLa, GH_3) Tissues

Binding reactions were performed with 15 μg each extract as described in *Materials and Methods* in the absence (−) or presence (+) of an approximately 100-fold molar excess (120 ng) of unlabeled specific SF-1 competitor.

plexes have been seen with other SF-1 sites containing AGGTCA sequences, the half-sites for nuclear receptor proteins of the estrogen receptor subtype (17). Studies with adrenocortical cell extracts defined discrete interactions reflecting the binding of chicken ovalbumin upstream promoter transcription factor (6) and NGFI-B (18), orphan members of the nuclear receptor family. These results thus suggest a complicated interplay among various receptors interacting with compound response elements to regulate steroid hydroxylases.

Similarly, it is evident that SF-1, although an important contributor, is not the sole determinant of cell-selective expression of the steroidogenic enzymes. Two SF-1-regulated steroid hydroxylases, steroid 21-hydroxylase and 11β-hydroxylase, are expressed in the adrenal but not the gonads, despite high levels of SF-1 in gonadal cells. Conversely, aromatase is not expressed in the adrenal cortex despite high levels of SF-1. It will be important to identify and characterize other promoter elements that restrict steroidogenic enzyme expression to the appropriate subsets of steroidogenic tissues.

Another unresolved question is the role of SF-1 in hormonal induction of steroidogenic enzymes. The results in Fig. 9 suggest that gonadotropin treatment increases SF-1 expression in the preovulatory follicle in a manner that coincides with the marked increase in aromatase expression. These findings are consistent with a role of SF-1 in gonadotropin induction of aromatase. However, SF-1 is clearly expressed in the ovary at a time when aromatase is not expressed. Further analysis of this role of SF-1 will require examination of the intraovarian location of SF-1 expression before and after gonadotropin treatment. Also, the level and activity of SF-1 protein needs to be examined in addition to the measurement of SF-1 mRNA levels. In light of the finding that gonadotropins act via a cAMP-dependent signal transduction pathway (2, 3), SF-1 activity could be regulated by phosphorylation, rather than absolute levels of SF-1 mRNA and protein. It is important to note that previous studies in adrenocortical cells showed no major effects of corticotropin or 8-Br-cAMP on SF-1 expression (Parker, K., unpublished observations). Additionally, we have recently shown that SF-1 expression is essentially normal in Y1 mutants that are severely impaired in their cAMP-dependent protein kinase activity (19). Further studies will be required to reconcile these findings and to elucidate the role (if any) that SF-1 plays in the hormonal regulation of the steroidogenic enzymes.

MATERIALS AND METHODS

Cell Culture

R2C rat leydig tumor cells and GH_3 rat pituitary tumor cells were purchased from American Type Culture Collection (Rock-

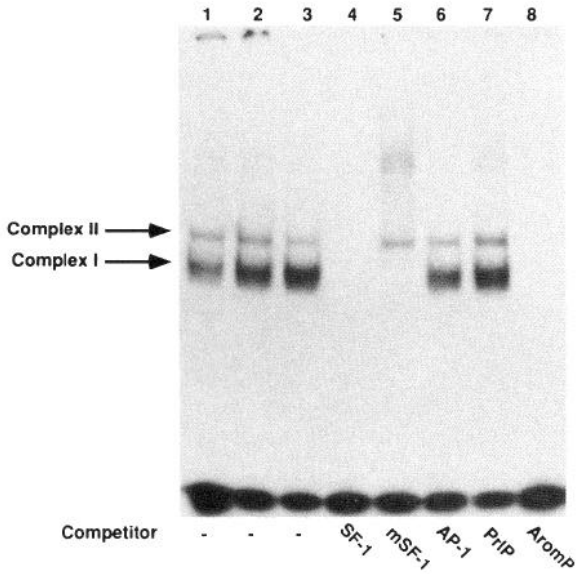

Fig. 4. Specificity of Binding to the Rat Aromatase SF-1 Site

Gel mobility shift assays were performed in the presence of 1.2 ng labeled SF-1 oligonucleotide and 100 ng unlabeled competitors. See *Materials and Methods* for description of various oligonucleotides. mSF-1, Modified SF-1; AP-1; AP-1 consensus binding site; PrlP, fragment from rat PRL promoter; aromP, fragment from rat aromatase promoter. The reactions shown in lanes 1, 2, and 3 contained 200, 500, and 1000 ng poly(dI-dC)/poly(dI-dC), respectively. Reactions shown in lanes 4–8 contained 250 ng poly(dI-dC)/poly(dI-dC).

ville, MD). HeLa human cervical carcinoma cells were generously provided by Dr. S. Preston (Department of Physiology, University of Connecticut Health Center). Cells were cultured in Joklik's modified minimum essential medium (GIBCO, Grand Island, NY) supplemented with 7.5 mM HEPES, pH 7.0, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO). R2C cells were grown in 100-mm culture dishes in media supplemented with 0.5 mM $CaCl_2$, 15% horse serum, and 2.5% fetal calf serum (GIBCO). HeLa cells were cultured similarly, except that the serum supplement was 10% bovine calf serum (HyClone, Logan, UT). GH_3 cells were cultured in suspension in media supplemented with 2.5% fetal calf serum and 12.5% horse serum (GIBCO).

Cloning and Sequencing of Rat Aromatase Promoter

Genomic DNA was prepared from 2 g Wistar-Furth rat liver as described (20). Two oligonucleotide primers were synthesized which corresponded to the ends of the published sequence of the rat aromatase promoter (13). These oligonucleotides are designated:

P1, 5′-CTTCAACACTCTGTTGGTGT-3′; and
P2, 5′-atagaattcTGATGTTAGCACGCAAGCA-3′.

Each primer contained an *Eco*R1 site at the 5′-end (lower case letters). PCR was performed using the GeneAmp kit (Perkin-Elmer/Cetus, Norwalk, CT) and 100 pmol of each primer, 2.5 U Amplitaq, and 8 μg genomic DNA in a 100-μl reaction. PCR was performed by incubating samples at 94 C for 5 min, followed by 20 cycles of 1 min at 94 C, 1 min at 60 C, and 1 min at 72 C. After removal of mineral oil by $CHCl_3$ extraction, two samples were pooled and precipitated with 0.5 vol 7.5 M ammonium acetate and 2.5 vol 95% ethanol. The product was resolved on a 4% acrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) and viewed by ethidium bromide staining under UV transillumination. A single band of approximately 600 bp was cut from the gel and eluted by incubation at 37 C for 12 h in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. The product was precipitated, washed in 80% ethanol, dried, and reamplified by three cycles of PCR as described above and cloned directly into pCR1000 using the TA Cloning System (Invitrogen, Thousand Oaks, CA). Clones with appropriately sized inserts were sequenced on both strands using the Sequenase kit (United States Biochemical, Cleveland, OH).

Primer Extension Analysis

RNA was isolated from PMSG-primed rat ovaries (see below) and R2C cells by the RNAzol method (Tel-Test, Inc.). Whole ovaries were trimmed of connective tissue and homogenized in RNAzol (1 ml/ovary) with a tissue grinder and then sonicated for 30 sec. Near confluent R2C cells were scraped from 100-mm dishes, pelleted by centrifugation, and lysed in RNAzol (200 μl/10^6 cells) by vortexing for 30 sec. Poly $(A)^+$ RNA was isolated from 1.5 mg total RNA exactly as described (21) using oligo dT cellulose (Pharmacia, Piscataway, NJ) chromatography. A 30-mer, 5′-ACATCTTGTGGTATTTTGCCTCAGAAGGAA-3′ (corresponding to nucleotides +83 to +112), was labeled by T4 kinase and purified on a Sephadex G-25 spin column. Primer extension was performed as described (21) using 5×10^5 cpm primer, 15 μg poly$(A)^+$ RNA, and 3000 U Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD). Products were analyzed on an 8% acrylamide-7 M urea sequencing gel. Sequencing ladders were run in adjacent lanes to determine the size of primer extension products.

Nuclear Extract Preparation

Ovarian and liver nuclear extracts were obtained using a modification of the method described by Gorski *et al.* (22). All buffers except PBS contained the protease inhibitors PMSF (0.5 mM), leupeptin (0.5 μg/ml), pepstatin A (1.0 μg/ml), and benzamidine (50 μM) added immediately before use. Ten 23-day-old rats were injected ip with 20 IU PMSG and killed 48 h later. The ovaries were trimmed of connective tissue, rinsed in ice-cold PBS, and minced with a sterile razor. The tissue was homogenized on ice in 7 ml homogenization buffer (10 mM HEPES, pH 7.9, 2 M sucrose, 25 mM KCl, 10% glycerol, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and 0.5 mM dithiothreitol) with 10 strokes of the B pestle. The homogenate was layered over a 5-ml cushion of homogenization buffer and the nuclei pelleted by centrifugation in a swinging bucket rotor at 90,000 $\times$ *g* for 30 min at 2 C. The supernatant was carefully removed and the clean nuclei resuspended in 1.2 ml lysis buffer (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.2 mM EDTA, 0.5 mM spermidine, and 0.5 mM dithiothreitol) and transferred to a Beckman 1.5-ml microfuge tube (Beckman Instruments, Palo Alto, CA). Nuclei were gently mixed in lysis buffer for 45 min at 4 C using a Roto-Torque rotator (Cole-Parmer Instrument Co., Chicago, IL). Nuclear debris was pelleted by centrifugation at 100,000 $\times$ *g* for 1 h at 2 C in a Beckman TLS55 swinging bucket rotor. The supernatant was concentrated to 100 μl using a Centricon-10 microconcentrator (Amicon, Danvers, MA); 1.9 ml 20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM spermidine, and 0.5 mM dithiothreitol were added, the extract reconcentrated to 70–100 μl in a Centricon-10, and protein concentration determined by the Bradford method (29). Extract was frozen on dry ice and stored at −70 C. R2C, HeLa, and GH cell nuclear extracts were prepared as described (23).

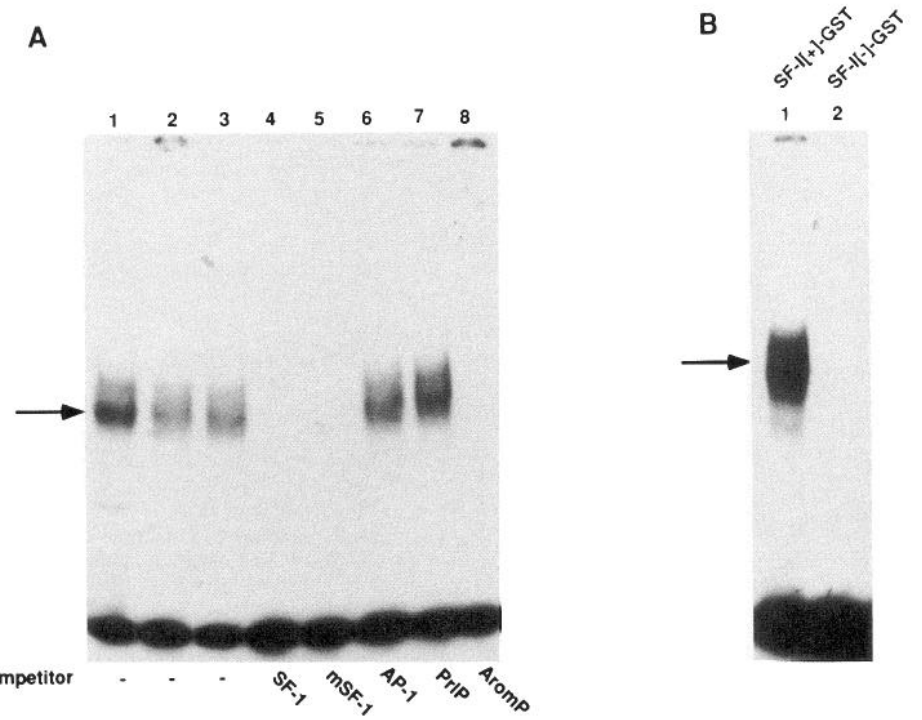

Fig. 5. SF-1 Binding Activity of Bacterial Extracts Containing a Mouse SF-1/GST Fusion Protein

A, Specificity of mouse SF-1/GST fusion protein binding. Refer to Fig. 4 for explanation of conditions. B, Binding to the rat aromatase SF-1 site was observed with extract from bacteria harboring a plasmid with the SF-1 cDNA in the sense orientation (SF-1[+]-GST), but not in the antisense orientation (SF-1[−]-GST).

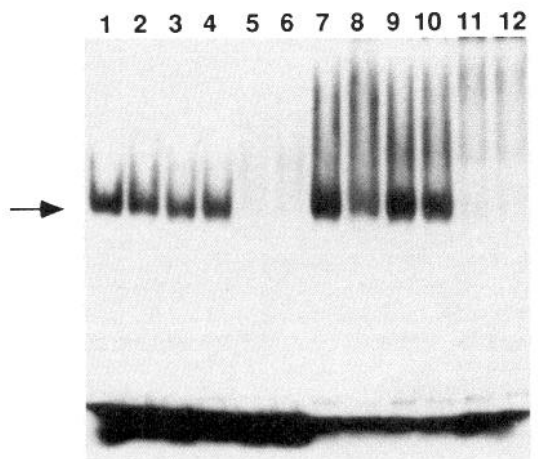

Fig. 6. Inhibition of SF-1 Binding by Mouse SF-1 Antisera

Gel mobility shift assays were performed as described in *Materials and Methods* with R2C cell (lanes 1–6) or ovarian (lanes 7–12) nuclear extracts. Nuclear extracts were preincubated with the following reagents for 30 min at room temperature before addition of probe: buffer only, lanes 1, 2, 7, and 8; preimmune serum, lanes 3, 4, 9, and 10; anti-SF-1 antisera, lanes 5, 6, 11, and 12.

Synthesis of SF-1[+]-GST Fusion Protein and Anti-SF-1/GST Antiserum

A 403-bp fragment of the mouse SF-1 cDNA, matching positions 432–801 of the ELP sequence (11), which includes the entire zinc finger DNA-binding domain and a region designated the FTZ-F1 box (24), was cloned downstream of the GST gene in the plasmid pGEX-1λT (Pharmacia) and expressed in *Escherichia coli* as previously described (25). The fusion protein was partially purified by affinity chromatography and used for immunization as described (25). After immunization of rabbits and boosting, the 5-week bleed, which showed activity against purified SF-1 in Western blot assays, was collected. This polyclonal antiserum was then used in gel mobility shift assays as described below.

Gel Mobility Shift Assay

Two complementary oligonucleotides containing the putative SF-1 element were synthesized and represented the following portion of the rat aromatase promoter: 5′-GTCTC**CCAAGGTCA**TCCTTG-3′, corresponding to positions −87 to −68. Ten picomoles of the upper strand were labeled with ^{32}P by T4 kinase and purified on two successive 1-ml Sephadex G-25 spin columns. The oligonucleotide was then coprecipitated with 2 μg poly dI-dC/poly dI-dC (Pharmacia) in 100 mM NaCl and 2.5 vol 95% ethanol, washed with 80% ethanol, and air dried. The pellet was resuspended in 20 μl annealing buffer (10 mM Tris-Hcl, pH 7.5, 1 mM EDTA, 25 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) containing 40 pmol of the complementary strand as described (26). Annealing was performed by heating the reaction to 90 C for 3 min, then gradually reducing the temperature to 30 C over 3 h in a programmable thermal controller (M.J. Research USA/Scientific Plastics, Ocala, FL). Unlabeled oligonucleotide competitor was synthesized by annealing 2 nmol of each oligo in 30 μl annealing buffer with no carrier DNA. The modified SF-1 contained the sequence 5′-GTCTC**CCAAGGCTA**TCCTTG-3′. The aromatase promoter fragment corresponded to positions −249 to −61 and was obtained by PCR using 20-mer primers and rat genomic DNA, followed by gel purification using Prep-a-Gene (BioRad, Richmond, CA).The AP-1 site was obtained with the AP-1 protein binding detection system (BRL). The PRL pro-

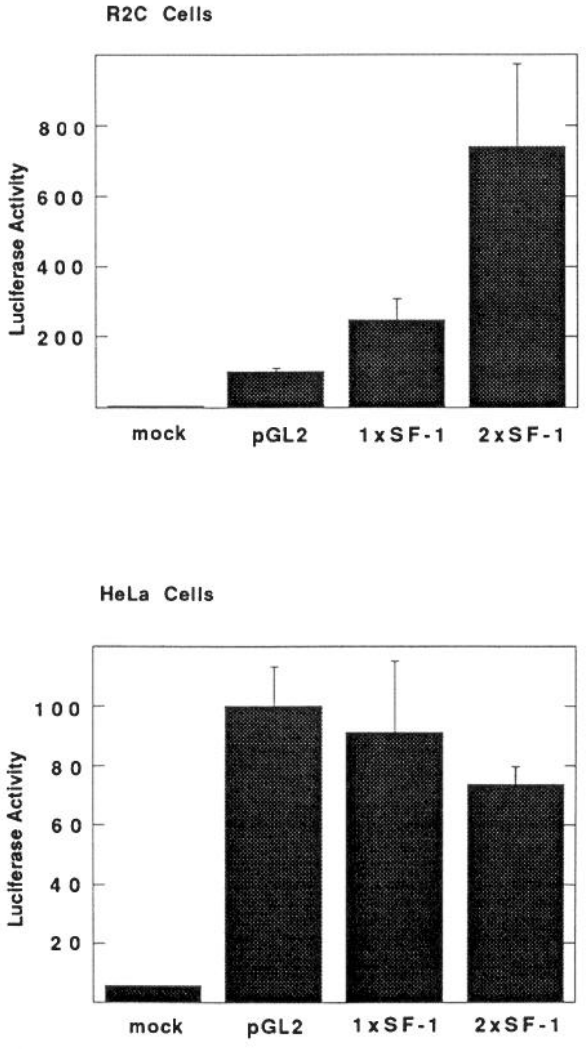

Fig. 7. Activity of SF-1 Sites in R2C Cells and HeLa Cells

Cells were transfected as described in *Materials and Methods* with no DNA (mock), Promega luciferase construct containing an SV40 promoter (pGL2), pGL-2 containing 1 SF-1 site (1xSF-1), or pGL2 containing 2 SF-1 sites (2xSF-1). Each determination represents the mean + SE of triplicate determinations and is presented as a percentage of the pGL2 value. This experiment is representative of four experiments.

moter fragment was obtained by annealing complementary oligonucleotides representing the following sequence: 5′-TATAGGAATTGGGGATGAAA-3′.

In a typical assay (20), 10 μg nuclear extract were adjusted to 20 μl final vol with buffer containing the final concentrations: 10 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 250 mM spermidine, 1 mM dithiothreitol, 100–200 ng poly (dI-dC)/poly (dI-dC), and competitor if indicated. After incubation for 15 min on ice, 0.1 pmol (1.24 ng) labeled probe was added, and the reaction was gently mixed and incubated for 20 min at room temperature. Products were resolved by electrophoresis at 150 V in 0.25× TBE at 4 C in a 4.5% polyacrylamide gel. Gels were dried and exposed to x-ray film.

Transfection Studies

The luciferase construct pGL2-Promoter was obtained from Promega and prepared for cloning by digestion with *Sma*I and dephosphorylation by bacterial alkaline phosphatase. The double-stranded SF-1 fragment used for the gel mobility shift assays was synthesized. Fifty picomoles of each strand were phosphorylated by T4 kinase and purified by two successive 1-ml Sephadex G-25 spin columns. After precipitation with $MgSO_4$ and ethanol, the oligos were annealed as described above. The double-stranded oligonucleotide was ligated into the prepared pGL2-Promoter vector and the recombinant clones isolated and sequenced. Clones 1XSF-1/GL2 and 2XSF-1/GL2 were confirmed by sequencing. All DNA was banded on cesium chloride before use (20).

Cells were freshly plated 3 days before use at a low density (about 20% confluent) and given fresh media the night before use. Cells were washed gently with media and then harvested with a Teflon cell scraper. After washing once in ice-cold PBS, R2C cells were resuspended to 7–8 × 10^6 cells/ml in PBS and incubated on ice for 5 min. HeLa cells were likewise treated but resuspended at a density of 10^7 cells/ml. Five hundred microliters of cells, 20 μg of the luciferase construct, and 40 μg pSVβ (Clontech, Palo Alto, CA), a β-galactosidase expression vector used to correct for transfection efficiencies, were combined in an electroporation cuvette and electroporated by two 1-msec pulses (120 V, 950 μF, R2) using an Electro Cell Manipulator 600 (BTX, San Diego, CA). Cells were immediately decanted into fresh media and cultured for 48 h. All constructs were assayed in triplicate transfections.

Cells were processed similarly to the method described by Brasier *et al.* (27). Cells were scraped, washed in ice-cold PBS, and resuspended in 25 μl PBS. Cells were lysed by addition of 250 μl luciferase lysis buffer (1% Triton-X100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol) and incubation for 2 min on ice. Lysates were cleared by centrifugation at 7000 × *g* for 2 min at 4 C and the supernatant transferred to a clean microfuge tube. D-Luciferin (potassium salt; Sigma, St. Louis, MO) was dissolved in sterile H_20 at a stock concentration of 5.5 mM. At the time of assay, it was diluted to 1 mM in luciferase assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate monobasic, 15 mM potassium phosphate dibasic, pH 7.8, 1 mM dithiothreitol, and 5 mM ATP). The luciferase assay was performed by addition of 50 μl lysate and 100 μl 1 mM D-luciferin to 380 μl luciferase assay buffer and measuring light units in a Monolight 411 luminometer (Analytical Luminescence, Laboratories San Diego, CA). β-Galactosidase activity was assayed exactly as described in 100 μl cell lysate (28). Protein concentrations of the lysates were determined by the Bradford assay (29). Luciferase activity was corrected for both protein concentration and β-galactosidase activity.

Cloning of Partial cDNA for Rat SF-1

Degenerate oligonucleotide primers were designed using stategies described previously (30, 31) based on sequences for *Drosophila* FTZ-F1 (9), murine embryonal long terminal repeat binding protein (11), and murine SF-1 (8).

The upstream primer,

5′-gcggaattcTG(C/T)CC(A/C/T)GT(C/G/T)TG(C/T)GG(A/C/G/T)GA(C/T)AA-3′,

was made to a conserved amino acid sequence in the DNA-binding domain. The downstream primer,

5′-gcggaattcTG(A/C/G/T)A(A/G)(C/G/T)A(A/G)(A/G/T)GTCAT(C/T)TG(G/T)TC-3′,

was based on a conserved amino acid sequence in the putative ligand-binding domain. Both primers possess *Eco*R1 restriction enzyme sites at their 5′-ends. A cDNA was synthesized from 1 μg R2C cell poly(A)$^+$ RNA using Moloney murine leukemia virus reverse transcriptase (BRL) and oligo $d(T)_{20}$, and purified by Sephadex G-50 spin column. Ten microliters of cDNA were amplified by 25 cycles of PCR using 200 pmol of each degenerate primer in a 50-μl reaction using the conditions described for the GeneAmp kit. The products were

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  1 G GTG TCG GGC TAC CAC TAC GGG CTG CTC ACG TGC GAG AGC TGC AAG GGC TTC   52
  1   Val Ser Gly Tyr His Tyr Gly Leu Leu Thr Cys Glu Ser Cys Lys Gly Phe   17

 53   TTC AAG CGC ACA GTC CAG AAC AAC AAG CAT TAC ACG TGC ACC GAG AGT CAG  103
 18   Phe Lys Arg Thr Val Gln Asn Asn Lys His Tyr Thr Cys Thr GluSer  Gln   34

104   AGC TGC AAA ATC GAC AAG ACG CAG CGT AAG CGC TGT CCC TTC TGC CGC TTC  154
 35   Ser Cys Lys Ile Asp Lys Thr Gln Arg Lys Arg Cys Pro Phe Cys Arg Phe   51

155   CAG AAG TGC CTG ACG GTG GGC ATG CGC CTG GAA GCT GTG CGT GCT GAT CGA  205
 52   Gln Lys Cys Leu Thr Val Gly Met Arg Leu Glu Ala Val Arg Ala Asp Arg   68

206   ATG CGG GGC GGC CGG AAC AAG TTT GGG CCC ATG TAC AAG AGA GAC CGG GCC  256
 69   Met Arg Gly Gly Arg Asn Lys Phe Gly Pro Met Tyr Lys Arg Asp Arg Ala   85

257   TTG AAG CAG CAG AAG AAA GCA CAG ATT CGG GCC AAT GGC TTC AAA CTG GAG  307
 86   Leu Lys Gln Gln Lys Lys Ala Gln Ile Arg Ala Asn Gly Phe Lys Leu Glu  102

308   ACC GGA CCA CCG ATG GGG GTT CCC CCG CCA CCC CCT CCC CCA CCG GAC TAC  358
103   Thr Gly Pro Pro Met Gly Val Pro Pro Pro Pro Pro Pro Pro Asp Tyr  119

359   ATG TTA CCC CCT AGC CTG CAT GCA CCG GAG CCC AGG GCC CTG GTC TCT GGC  409
120   Met Leu Pro Pro Ser Leu His Ala Pro Glu Pro Arg Ala Leu Val Ser Gly  136

410   CCA CCC AGT GGG CCG CTG GGT GAC TTT GGA GCC CCA TCT CTG CCC ATG GCC  460
137   Pro Pro Ser Gly Pro Leu Gly Asp Phe Gly Ala Pro Ser Leu Pro Met Ala  153

461   GTG CCT GGT CCC CAC GGG CCT CTG GCT GGC TAC CTC TAT CCT GCT TTC TCT  511
154   Val Pro Gly Pro His Gly Pro Leu Ala Gly Tyr Leu Tyr Pro Ala Phe Ser  170

512   AAC CGC ACC ATC AAG TCT GAG TAT CCA GAG CCC TAC GCC AGC CCC CCT CAA  562
171   Asn Arg Thr Ile Lys Ser Glu Tyr Pro Glu Pro Tyr Ala Ser Pro Pro Gln  187

563   CAG CCA GGG CCA CCC TAC AGC TAT CCG GAG CCC TTC TCA GGA GGG CCC AAT  613
188   Gln Pro Gly Pro Pro Tyr Ser Tyr Pro Glu Pro Phe Ser Gly Gly Pro Asn  204

614   GTA CCA GAG CTC ATA TTG CAG CTG CTG CAA CTA GAG CCA GAG GAG GAC CAG  664
205   Val Pro Glu Leu Ile Leu Gln Leu Leu Gln Leu Glu Pro Glu Glu Asp Gln  221

665   GTG CGT GCT GCC ATC GTG GGC TGC CTG CAG GAG CCA GCC AAA AGC CGC CCT  715
222   Val Arg Ala Arg Ile Val Gly Cys Leu Gln Glu Pro Ala Lys Ser Arg Pro  238

716   GAC CAG CCA GCG CCC TTC AGC CTC CTC TGC AGG ATG GCG GAC CAG ACC TTT  766
239   Asp Gln Pro Ala Pro Phe Ser Leu Leu Cys Arg Met Ala Asp Gln Thr Phe  255

767   ATC TCC ATT GTC GAC TGG GCA CGA AGG TGC ATG GTA TTT AAG GAG CTG GAG  817
256   Ile Ser Ile Val Asp Trp Ala Arg Arg Cys Met Val Phe Lys Glu Leu Glu  272

818   GTG GCT                                                             823
273   Val Ala                                                             274
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Fig. 8. Nucleotide and Predicted Amino Acid Sequence of Partial Rat SF-1 cDNA Clone

The zinc finger region and the putative ligand-binding domains as discussed by Tsukiyama *et al.* (11) are *underlined* at the beginning and end of the sequence, respectively.

ethanol precipitated, resuspended, and digested with 50 U *Eco*R1 in a 50-μl reaction, and resolved in a 1% agarose gel. The fragment of approximately 900 bp was excised and purified by Prep-A-Gene (BioRad). The PCR products were ligated into *Eco*R1-digested and phosphatased Bluescript KS+ (Stratagene, La Jolla CA). Clones with inserts were sequenced with the Sequenase system (United States Biochemical). One clone (rSF-1) was sequenced fully on both strands.

Analysis of SF-1 and Aromatase Gene Expression in the Rat Ovary

A rat aromatase cDNA probe was obtained by PCR, using the primers:

5′-CTTCATTAACGAGAGCCTGC-3′ (position +1132 to +1152); and
5′-ATGGGGCTGTCCTCATCTAG-3′ (position +1519 to +1499).

A single band was resolved in a 4% polyacrylamide gel, eluted as described above, and cloned into pCR1000 using the TA cloning system (InVitrogen). One clone was confirmed by sequencing and used as a probe in Northern blot hybridizations.

Control ovaries were obtained from 22- to 25-day-old rats. Preovulatory ovarian tissue was obtained 48 h after ip injection of 22- to 25-day-old rats with 20 IU PMSG (Sigma). RNA was isolated by the RNAzol procedure as described above and processed for Northern blot hybridization as described (20).

Note Added in Proof

In the March issue Fitzpatrick and Richards published the revised sequence of the rat aromatase gene promoter (*Molecular Endocrinology* **7:** 341–354, 1993). Also, they provided evidence for a functional SF-1 site in this promoter.

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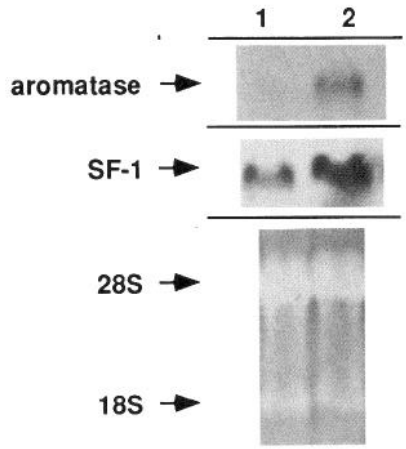

Fig. 9. Expression of Aromatase mRNA and SF-1 mRNA in Rat Ovary

RNA was isolated from immature 21-day-old rats which were untreated (lane 1) or from rats which had received one injection of 20 IU PMSG 48 h before tissue collection in order to induce large preovulatory follicles (lane 2). Aromatase and SF-1 mRNA were detected on separate Northern blots. The ethidium bromide staining of 28S and 18s rRNA used for the SF-1 blot is also shown.

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Erratum

In the article, "Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation," by Tai-Jay Chang, Barbara M. Scher, Samuel Waxman, and William Scher (*Molecular Endocrinology* **7:** 528–542, 1993), on page 528, the third sentence of the *Introduction* should read, "Glucocorticoid treatment of MEL cells undergoing erythrodifferentiation inhibits all of the features of this process that have been tested, including inhibition of the synthesis of α_1, β-major, and β-minor globins, their mRNAs, heme, and hemoglobin (11–13), and the levels of δ-aminolevulinate dehydratase and porphobilinogen deaminase."