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A Steroid Receptor Coactivator, SRA, Functions as an RNA and Is Present in an SRC-1 Complex

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Summary

Nuclear receptors play critical roles in the regulation of eukaryotic gene expression. We report the isolation and functional characterization of a novel transcriptional coactivator, termed steroid receptor RNA activator (SRA). SRA is selective for steroid hormone receptors and mediates transactivation via their amino-terminal activation function. We provide functional and mechanistic evidence that SRA acts as an RNA transcript; transfected SRA, unlike other steroid receptor coregulators, functions in the presence of cycloheximide, and SRA mutants containing multiple translational stop signals retain their ability to activate steroid receptor–dependent gene expression. Biochemical fractionation shows that SRA exists in distinct ribonucleoprotein complexes, one of which contains the nuclear receptor coactivator steroid receptor coactivator 1. We suggest that SRA may act to confer functional specificity upon multiprotein complexes recruited by liganded receptors during transcriptional activation.

Introduction

Nuclear receptors are members of a structurally and functionally related family of ligand-activated and sequence-specific eukaryotic transcription factors. By modulating the transcription of target genes in response to their own ligands and other afferent signals, they play key physiological roles in the regulation of development, metabolism, and reproduction. Receptor activation is a multifaceted cascade of events that results in the binding of the receptor to specific regulatory DNA sequences and culminates in the modulation of target gene expression (Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995). Common to nearly all nuclear receptors is the activation function AF2 in the distal carboxyl terminus of the ligand-binding domain (LBD). A highly conserved amphipathic helix in AF2 has been shown to be important for ligand binding and hormone-dependent transactivation (Danielian et al., 1992; Vegeto et al., 1992; Lanz and Rusconi, 1994). The variable amino-terminal domain of nuclear receptors is extended in the type I or "classical" receptor subclass comprising the receptors for androgens (AR), estrogens (ER), glucocorticoids (GR), mineralocorticoids (MR), and progestins (PR). This modulatory domain contains a strong and autonomous transactivation function (AF1) that has been shown to be critical for target gene specificity (Tora et al., 1988).

The role of activated nuclear receptors is to direct the assembly and stabilization of a preinitiation complex in a transcriptionally permissive environment at the promoter of a target gene. This involves the functional interaction of the receptor with factors contained in the transcription preinitiation complex (Tsai et al., 1987; Beato and Sanchez-Pacheco, 1996) and with other DNA-bound transcription activators (Jonat et al., 1990). Such interactions are necessary but not sufficient for accurate regulation of transcription. Initial findings that distinct receptors interfere with or "squelch" each other's transactivation (Meyer et al., 1989) indicated that common limiting factors were involved. Several biochemical and genetic screens have since identified a number of proteins that interact with activated receptors. By fulfilling a number of functional criteria, these coregulators have been defined as coactivators for nuclear receptors (Horwitz et al., 1996; McKenna et al., 1999): they significantly enhance transactivation without altering basal transcriptional activity; when overexpressed, they specifically reverse squelching between different receptors; and they contain autonomous, transferable activation domains.

Coactivators that have recently received considerable attention are members of the SRC gene family and the "cointegrators" p300 and CBP. Steroid receptor coactivator 1 (SRC-1) was cloned in our laboratory as a general coactivator for nuclear receptors (Onate et al., 1995) and has been termed variously as p160/NCoA-1 (Kamei et al., 1996) or ERAP-160 (Halachmi et al., 1994). Highlighting the critical physiological role of coactivators, the targeted deletion of SRC-1 causes partial hormone insensitivity (Xu et al., 1998). Other nuclear receptor coactivators have been subsequently identified and characterized that are structurally and functionally related to SRC-1 (McKenna et al., 1999 and references therein). The cointegrators CREB-binding protein (CBP; Chrivia et al., 1993) and the closely related adenovirus E1A-associated p300 (Eckner et al., 1994) are well characterized as general coactivators that interact not only with multiple nuclear receptors but with a wide variety of other transcriptional activators.

To date, the majority of receptor-interacting factors have been identified by genetic screens, such as the yeast two-hybrid system, typically using the LBD of a given nuclear receptor as bait. This approach has led to the identification of multiple AF2 coactivators with common structural and functional features. The "classical" type I steroid receptors, however, exert transactivation via their amino-terminal transcription activation function, AF1. For some steroid receptors, AF1 and AF2 have a distinct pattern of cell and promoter specificity (Bocquel et al., 1989; Tasset et al., 1990). Reasoning that specificity in steroid receptor–mediated transactivation might be provided by factors that associate with the poorly conserved AF1, we searched for coregulators

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that interact with the amino-terminal AF1 domain of human PR (hPR). We report here the cloning and characterization of a novel transcription coregulator termed steroid receptor RNA activator (SRA). We provide functional and mechanistic evidence that SRA acts as an RNA transcript and exists in a ribonucleoprotein complex that contains the AF2 coactivator SRC-1.

Results

Characterization of *SRA*

In an attempt to find cofactors that interact with steroid hormone receptors, our laboratory used different functional domains of the hPR as baits in a yeast two-hybrid screening system. We previously reported the isolation and characterization of a protein, SRC-1, that interacts with the hPR-LBD (Onate et al., 1995). We performed a similar screen of a human B-lymphocyte library with the AF1-containing amino terminus of hPR_A (corresponding to amino acids 165–567 of hPR_B). Primary sequence analysis of two positive clones from this screen indicated a short open reading frame (ORF). The 3′ extension of reverse-transcribed skeletal muscle poly(A)$^+$ RNA identified an extended ORF with sequence identical to the 5′ ORF from the lymphocyte library.

In order to retrieve cDNAs encoding full-length SRA, we used conventional screening of three different human cDNA libraries from skeletal muscle, heart, and the HeLa S3 cell line. We obtained 13 positive clones with DNA sequences that were identical in a central region. Three variants of SRA were predicted, all containing unique 5′ and 3′ extensions beyond an identical 687 bp core sequence (Figures 1A and 2). We also screened a human genomic DNA library and found two clones with partial sequence identity with the original SRA clones. Additionally, screening of a mouse genomic DNA library identified 5 positive clones, and screening of a mouse cDNA library found 14 positive clones, of which 2 revealed 75% identity to the human SRA cDNA (Figure 2). Primary sequence analysis of clones from different human and mouse cDNA and genomic DNA libraries suggested that SRA represented a family of clones highly homologous in a core sequence but divergent in their 5′ and 3′ regions. Sequence comparison using the BLAST algorithm indicated no homologs but identified partial SRA sequences isolated as HepG2-3′UTR (accession number D16861), expressed sequence tag clones, and chromosome 5 BAC clone 319C17 (AC005214), although no functions for these sequences were described.

To determine the expression patterns of the corresponding RNA, we performed Northern analysis using a cDNA probe corresponding to the core sequence of human SRA. Major transcripts of 0.7–0.85 kilobases (kb) in length and less abundant transcripts of 1.3–1.5 kb were detected in a human multiple tissue Northern blot (Clontech) (Figure 1B), indicating that the isolated cDNAs were likely to be full length. In addition, SRA was expressed at different levels in the tissues examined; transcripts were enriched in liver and skeletal muscle but expressed at a low level in brain. Interestingly, the expression of the two messages in the 0.7–0.85 kb doublet appeared to be tissue specific in the multiple tissue blot. A cell line–specific expression of these isoforms (represented by the doublet) was also observed in a

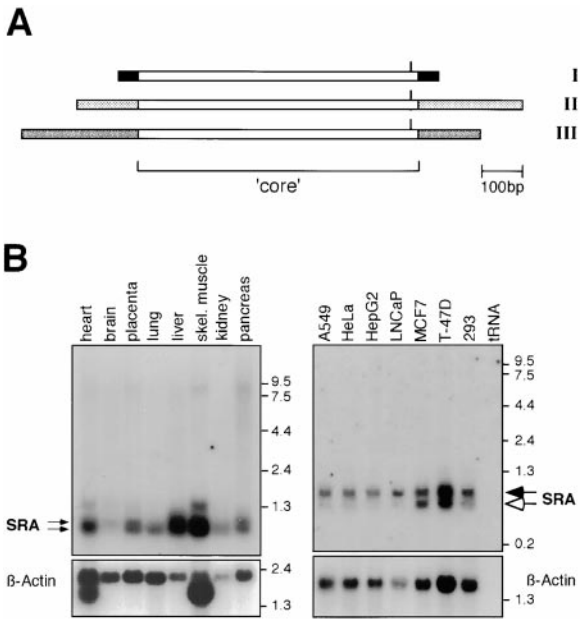

Figure 1. Characterization of *SRA* Genes

(A) Structure of three SRA isoforms (I–III) deduced from screening of different cDNA and genomic DNA libraries from human and mouse. The sequences are identical in a "core" region of 687 bp (no shadow) but are divergent in their 5′ and 3′ sequences (distinct shadings). The vertical lines indicate the location of the proposed termination codon of the putative open reading frame ORF1.

(B) Northern analysis of human *SRA* gene expression. (Left panel) Multiple tissue Northern blot, containing 2 μg of human poly(A)$^+$ RNA from each of the tissues indicated at the top, was hybridized with a cDNA probe corresponding to the core sequence of human *SRA* (A). Predominant transcripts of about 0.7–0.85 kb (double arrows) and less abundant transcripts of 1.3–1.5 kb are apparent. The blot was stripped and reprobed with β-actin to correct for RNA loading (bottom). (Right panel) Northern analysis of human tissue culture RNA probed with the longest cDNA sequence of SRA ([A], isoform III) indicates a cell line–specific expression of SRA isoforms. MCF-7 and T-47D cells have significantly higher levels of the smaller SRA transcript (open arrow) compared to other tissues, but they express similar levels of the larger transcript (filled arrowhead). Total RNA was isolated from human cell lines as indicated and 15 μg analyzed. The membrane was subsequently hybridized with β-actin probe as an internal control for loading (bottom). Size markers are indicated on the right (kb).

A549, lung carcinoma; HeLa, epitheloid cervix; HepG2, hepatoblastoma; LNCaP, metastatic prostate adenocarcinoma; MCF-7, breast adenocarcinoma; T-47D, breast ductal carcinoma; 293, transformed primary embryonal kidney.

Northern analysis of poly(A)$^+$-selected mRNA from different human tissue culture cell lines. All of the cell lines tested expressed the ~0.85 kb doublet species, whereas the smaller ~0.7 kb species was expressed at significantly higher levels in the breast cancer cell lines MCF7 and T-47D, compared to the other cell lines investigated (Figure 1B). This isoform-specific expression was conserved in mouse tissue (not shown). We concluded that multiple SRA isoforms are expressed quantitatively in a tissue- and cell type–specific manner.

SRA Is a Steroid Receptor–Specific Coactivator

To investigate the functional role of SRA, we subcloned the cDNAs into mammalian expression vectors and assayed the effect of SRA on PR-dependent transactivation. HeLa cells were cotransfected with the CMV-hPR

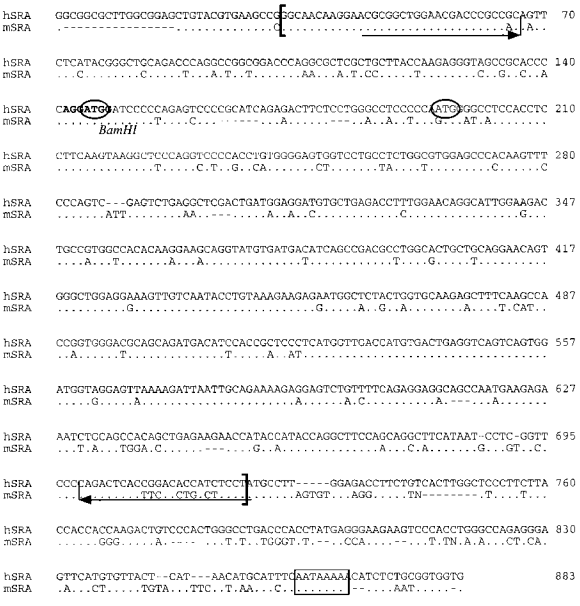

Figure 2. Primary Nucleotide Sequence Alignment of SRA Isoform I cDNA from Human and Mouse

The nucleotides of mouse cDNA are indicated where they differ from the human cDNA sequence. Brackets represent the boundaries of the SRA core sequence. Arrows illustrate the location and orientation of the primer set used for SRA-specific RT-PCR (shown in Figures 6 and 7). The Kozak consensus sequence is marked in bold; circles indicate the putative translation initiation codons (ATG) targeted for mutation analysis (Figure 4); a consensus polyadenylation signal (AATAAAA) is boxed.

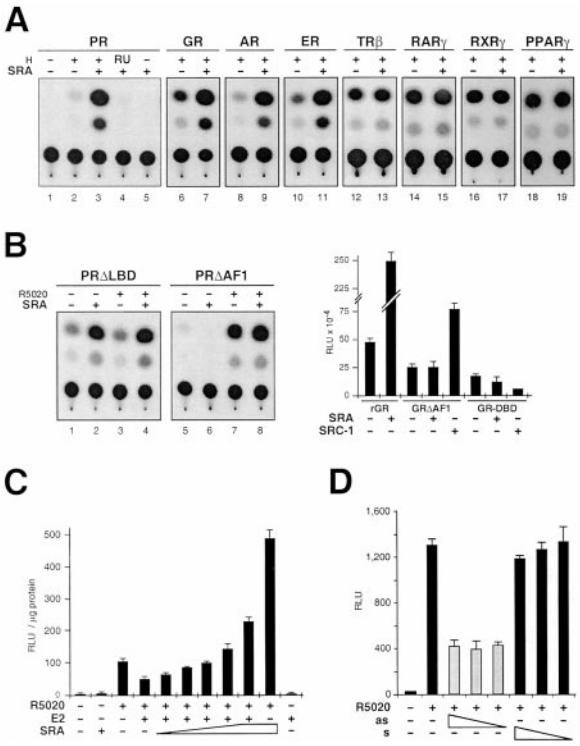

Figure 3. SRA Is a Steroid Hormone Receptor–Specific Coactivator

(A) SRA enhances transcription mediated by steroid receptors. Thin-layer chromatographs showing the chloramphenicol-acetyltransferase (CAT) reporter activity as a response to steroid receptor–mediated transactivation in the presence of SRA (+) or empty vector (−). HeLa cells were transiently transfected with plasmids encoding the human receptors for glucocorticoid (GR), androgen (AR), estrogen (ER), thyroid hormone (TRβ), all-*trans* retinoic acid (RARγ), 9-*cis* retinoic acid (RXRγ), or with peroxisome proliferator–activated receptor (PPARγ) along with their cognate hormone response element coupled to a TATA-CAT reporter gene and induced with their appropriate ligands or the PR antagonist RU486.

(B) SRA enhances transcription via the N-terminal activation domain AF1 of steroid receptors. (Left panel) PR deletion mutants lacking either the ligand-binding domain (PRΔLBD: lanes 1–4) or the amino-terminal activation function (PRΔAF1: lanes 5–8) were assayed as in (A). Similar experiments with rat GR or domains thereof excluded the DNA-binding domain (DBD) as a possible target for SRA (right panel). The GR truncations were fused to the activation domain of GAL4 to monitor transactivation. SRC-1 was used as control. MMTV-driven luciferase gene expression is shown as relative light units (RLU).

(C) Overexpression of SRA reverses squelching of PR by ER in a dose-dependent manner. HeLa cells were transfected with PR and ER expression plasmids (50 ng), MMTV-luciferase reporter (2.5 μg), and different amounts of SRA (0–4 μg) and supplemented with 50 nM receptor-specific ligands R5020 or E2, or both, as indicated. Luciferase activities are shown as RLU per microgram of protein assayed as the mean (±SD) of triplicate values.

(D) PR transactivation is inhibited by digestion of endogenous SRA in an oligonucleotide-dependent manner. HeLa cells were transfected with PR expression plasmid (20 ng) and MMTV-luciferase reporter (2.5 μg) together with different concentrations (200, 100, and 50 nM) of SRA-specific, 2′methoxyethyl-modified antisense deoxyoligonucleotide (as), or sense control (s). Luciferase activities are shown as the mean (±SD) of triplicate values.

and CMV-SRA (CMV, cytomegalovirus) along with $(PRE)_2$-TATA-CAT reporter (CAT, chloramphenicol acetyltransferase) and induced with progestin (R5020). We observed that SRA enhanced PR transactivation (Figure 3A; compare lanes 2 and 3) and that SRA did not alter the activity of PR in the presence of its antagonist RU486 (lane 4). Furthermore, SRA did not significantly elevate the basal activity of the minimal promoter (lane 5). Similar transfection experiments with the human receptors for GR, AR, ER, thyroid hormone (TR), retinoic acid– (RAR and RXR) or peroxisome proliferator–activated receptor (PPAR), and CAT reporters containing cognate hormone response elements revealed that SRA selectively enhanced steroid receptor–mediated transactivation (Figure 3A). SRA did not enhance transactivation induced by other activators such as GAL4, Sp1, E2F, E47, and forskolin-stimulated CREB (data not shown).

We next found that SRA enhanced transactivation through the N-terminal AF1 portion of steroid receptors. Truncation of the A/B domain of the PR (PRΔAF1) significantly reduced coactivation by SRA (Figure 3B, lanes 7 and 8), whereas transcription activation by PR lacking the LBD (PRΔLBD) was fully responsive to SRA (lanes 1–4). In order to exclude the DNA-binding domain as a mediator for SRA coactivation, we tested different domains of rat GR as fusion proteins with the activation domain of GAL4 (Figure 3B, right panel). As expected, neither the amino-terminally truncated GRΔAF1 nor the DNA-binding domain of rat GR (GR-DBD) responded to SRA to enhance luciferase reporter activity. As a control, GRΔAF1 enhanced reporter activity in the presence of SRC-1. These results indicated that SRA mediates transcriptional activation of steroid receptors by a mechanism involving the AF1 of the receptors. We next tested the possibility that intramolecular "crosstalk" between

the AF1 and AF2 of the receptor is mediated by SRA and SRC-1 but found that coexpression of both coactivators had only an additive effect on the coactivation of PR-mediated transactivation (not shown). To better determine the coactivation potential of SRA in vivo, we transfected different SRA clones into T-47D cells and tested the ability of SRA to enhance the activity of endogenous PR. All three isoforms of SRA cDNA, in addition to a portion of the human genomic SRA, enhanced transactivation mediated by the endogenous PR by 8- to 12-fold, and the core domain of SRA was found to be necessary and sufficient for this coactivation (not shown).

Another criterion for classification as a coactivator is the ability of a factor to reverse interference (squelching) by transcriptional activators with common coregulators. To ask if SRA is a limiting factor that can be sequestered by an excess of another receptor, we used a PR-regulated gene reporter assay in the presence of ER (Figure 3C): while ligand-activated ER reduced the transcription activity of PR by 50%, full PR transactivation was reestablished by addition of SRA, confirming that SRA regulates the transactivation of both PR and ER in a dose-dependent manner and indicating that SRA is a limiting cellular factor for steroid receptors.

In order to assess the effect of abrogation of endogenous SRA on PR-mediated transcription, we developed an assay in which SRA transcripts were selectively degraded. By administration of stabilized antisense deoxyoligonucleotides, we attempted to digest SRA by endogenous RNase H, an endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA/DNA hybrids. Figure 3D shows that PR–target gene expression was reduced by ~70% by cotransfection of an SRA antisense 2′methoxyethyl oligonucleotide, whereas the corresponding sense construct had no effect on PR transactivation. Similar experiments were performed with three other SRA-specific deoxyoligonucleotides, all of which significantly reduced PR target gene expression. In addition, the antisense oligonucleotides were capable of reducing reporter gene expression mediated by endogenous receptor (not shown). These results imply that endogenous SRA has a direct impact on steroid-mediated transcription in vivo. Together with the ability of SRA to enhance transactivation on minimal and natural promoters without altering basal transcription, these results clearly characterized SRA as a bona fide coactivator, specific for the AF1 domain of steroid receptors.

SRA Does Not Exhibit Characteristics of a Protein

Sequence analysis of the SRA clones indicated an ORF terminated at the 3′ end of the core sequence (see Figure 1A; a detailed ORF map is shown in Figure 4A: this ORF is denoted ORF1). A second ORF, ORF2, contains a consensus Kozak sequence in the 5′ portion of the SRA cDNA. This ORF2 corresponds to the presumed receptor-interacting reading frame of the yeast hybrid clones. However, an in-frame stop codon terminated GAL/SRA fusion products prematurely at the 5′ end of the core sequence. We concluded that the interaction of the original yeast two-hybrid SRA clones with the AF1 of PR was unlikely to have been mediated by a protein product encoded by ORF2 of SRA.

We next attempted to characterize the presumptive

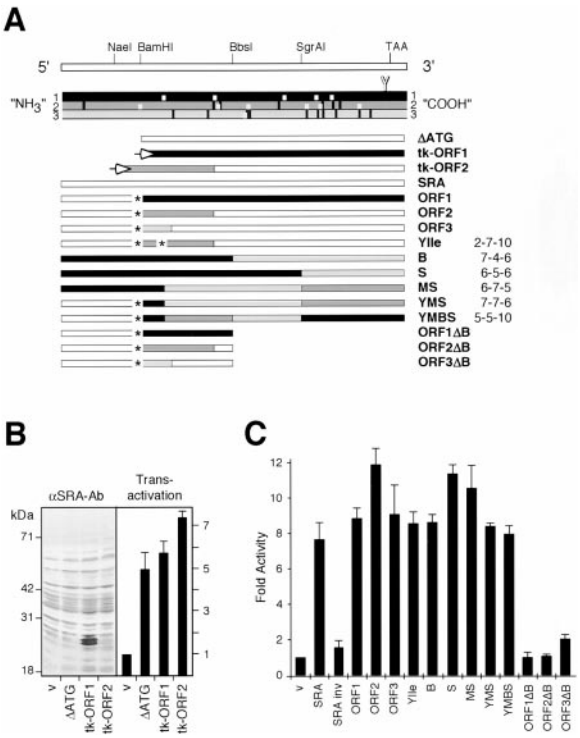

Figure 4. Mutated SRA Constructs Enhance PR Transactivation

(A) Schematic presentation of SRA mutants. Top: SRA core cDNA and deduced ORF map; selected restriction sites and presumptive termination codon for ORF1 (TAA) are indicated; "Y," location of peptide sequence used to generate SRA-mAb; vertical lines in ORF map, stop codons; white, putative initiation codons. Bottom: SRA mutants used in the transactivation studies presented and described in (B) and (C). Triple numbers indicate total of stop codons in each ORF; asterisks, point mutation(s); arrows, translation initiation region of the thymidine kinase promoter (tk); the shade of gray indicates reading frames of presumptive translation products: white, unconstrained; black, ORF1 (recognized by mAb); gray, ORF2; light gray, ORF3.

(B) Immunodetection and coactivation of transfected SRA mutants indicate that SRA coactivates PR transactivation in an ORF-independent manner. SRA mutants along with MMTV-Luc reporter and PR expression plasmid were transfected into COS cells and analyzed for immunoreactivity to SRA antibody raised against a peptide sequence deduced from the C terminus of ORF1 (left panel) and for coactivation (right panel). Mutants are as follows: (1) N-terminal truncation at the intrinsic Kozak sequence (ΔATG) and (2) fusion with translation initiation region of the tk in two different open reading frames (tk-ORF1 and tk-ORF2). The constructs are illustrated in (A). Protein size markers are indicated on the left. Fold coactivation in relation to PR transcription as the mean (±SD) of triplicate values is indicated on the right.

(C) Enhancement of PR-mediated transactivation of the MMTV-Luc reporter by various mutated SRA constructs shown schematically in (A). Fold coactivation is indicated relative to expression of empty vector (v) and shown as the mean (±SD) of triplicate values.

SRA, wild-type SRA; SRA inv, cDNA of SRA expressed in 3′–5′ orientation; ORF1, ORF2, ORF3, nonsense mutations at the BamHI site obliterating the Kozak sequence of two reading frames and permitting only one putative translation product; YIle, mutant ORF2 with an additional point mutation altering an ATG (Figure 2) and generating an MfeI site; B, frameshift mutation at the BbsI site of SRA; S, frameshift mutation at the SgrAI site; MS, frameshift mutations at the MfeI and SgrAI sites; YMS, mutant MS with additional frameshift mutation at the BamHI site; YMBS, mutant ORF2 with additional frameshift mutations at MfeI, BbsI, and SgrAI; ORF1ΔB/ORF2ΔB/ORF3ΔB, 3′ deletion at BbsI of the mutants ORF1, ORF2, and ORF3, respectively.

SRA protein product. Surprisingly, all our efforts to generate SRA-encoded protein were unsuccessful. In vitro translation of different SRA cDNAs did not result in detectable levels of protein, whereas carboxy-terminal fusions of SRA with GAL4 or GST produced the expected translation products (not shown). In addition, GAL/SRA fusion constructs failed to activate the UAS heterologous promoter, indicating that SRA did not possess an intrinsic activation function. We then generated a monoclonal antibody (mAb) against the peptide sequence encoded by the 3′ end of the SRA core (ORF1). In Western analysis, only ORF1 fusion constructs of GAL/SRA and GST/SRA were immunoreactive (not shown), whereas no proteins generated by expressed SRA cDNAs were detectable in cell extracts. We concluded that the SRA cDNA sequence did not encode a viable translation product.

We then correlated the coactivation function of SRA with its expression. Mutated SRA constructs were transfected into cultured cells and analyzed in a side-by-side comparison for SRA "immunoreactivity" and for coactivation of PR-mediated transactivation. The constructs tested were (1) a 5′ truncation at the BamHI site (Figure 4A), eradicating the consensus Kozak sequence, and (2) a fusion of this truncated cDNA to the HSV-thymidine kinase initiation sequence (tk) in two distinct reading frames, producing tk-ORF1 and tk-ORF2. All three SRA mutants enhanced PR-mediated transactivation (Figure 4B, right panel), whereas only one construct—the reading frame of which corresponded to ORF1—was recognized by the mAb (4B, left panel). No endogenous SRA protein was detected that corresponded to the constrained translation of tk-ORF1. Importantly, screening of a panel of tissue culture cell lines by matrix-bound SRA-mAb confirmed the absence of endogenous SRA protein in these lines (not shown). Taken together, these results suggested to us that coactivation by SRA was unlikely to be mediated by its presumptive protein product.

In order to substantiate these results, we generated various SRA mutants and tested them in cell culture for their ability to coactivate PR-dependent transcription. Figure 4A shows the sequence of the SRA mutants relative to the original SRA clone. Several of the mutants lacked the ATGs in ORF1 and ORF2; others contained mutations within the Kozak sequence, allowing a presumptive translation of only one given reading frame (see legend to Figure 4A for details). Other constructs contained single or multiple frameshift mutations along the core sequence, resulting in a "mosaic" organization of reading frames, each containing approximately six translational stop codons on average. A representative assay of in vivo–expressed SRA mutants (Figure 4C) clearly demonstrates that most of the SRA mutants retained the ability to enhance PR transcription by 8- to 12-fold. Only SRA expressed in 3′–5′ orientation (SRA inv) or 3′ half-truncated versions of the ORF exclusion mutations were inactive. Similar results were obtained with mutants of other SRA isoforms (not shown). These results further suggested to us that the coactivation exerted by SRA on steroid receptor transcription was unlikely to be mediated by a translation product of the *SRA* gene.

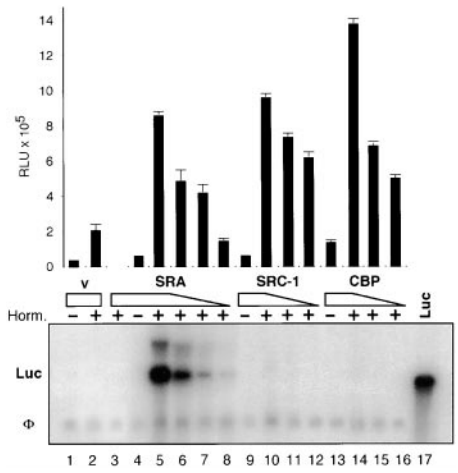

Figure 5. SRA Is an RNA Coactivator

SRA does not require protein synthesis to enhance endogenous GR-mediated transactivation. Two separate groups of HeLa cells were transiently transfected with reporter MMTV-Luc (2 μg) along with different amounts of CMV-driven expression plasmids for SRA (3, 2, 1, 0.5 μg), SRC-1 and CBP (3, 2, 1 μg), or empty vector (v; 3 μg) and treated with EtOH control (−) or dexamethasone (+). One set of transfected cells was assayed for luciferase protein expression (upper panel), the other set of cells incubated in medium containing 50 μM cycloheximide and subjected to RNA isolation and DNaseI digestion, followed by Northern analysis for luciferase RNA expression (lower panel). The Northern blot was hybridized with probes specific for cyclophilin (Φ) and luciferase RNA (Luc). Lane numbers (bottom) are common to both assays. Dilution (10,000-fold) of RNA from cotransfected CMV-luciferase plasmid expression is shown as control in the Northern analysis (lane 17). A longer exposure of the blot revealed low levels of luciferase transcripts in all samples that were treated with dexamethasone.

SRA Is an RNA Coactivator

We next focused our attention on the transcription products of the cDNA encoding SRA. We designed an assay for RNA-mediated transactivation by targeting endogenous GR in HeLa cells cultured in the presence of the de novo protein synthesis inhibitor cycloheximide and asked if SRA retained the ability to coactivate GR-mediated transcription. As controls, we used the coregulators SRC-1 and CBP, both of which interact with nuclear receptors as proteins. Two separate pools of HeLa cells were transiently transfected with an identical mixture of MMTV-luciferase reporter (MMTV, mouse mammary tumor virus LTR) along with CMV-driven expression plasmids for SRA, SRC-1, CBP, or empty vector and treated with carrier or dexamethasone. One set of transfected cells was subjected to a conventional luciferase protein assay for GR-mediated transactivation. The second set of cells was first incubated in medium containing cycloheximide from 3 hr prior to transfection until harvesting and then subjected to Northern analysis for luciferase RNA expression. Figure 5 shows a representative side-by-side comparison of luciferase expression as protein (upper half) and RNA (lower half). As expected, we observed a hormone- and dose-dependent enhancement of transactivation by all coregulators in the absence of cycloheximide (upper panel). The relatively low coactivity for all coactivators resulted from lower protein expression levels due to the necessarily shorter incubation time for cycloheximide-treated cells. In contrast,

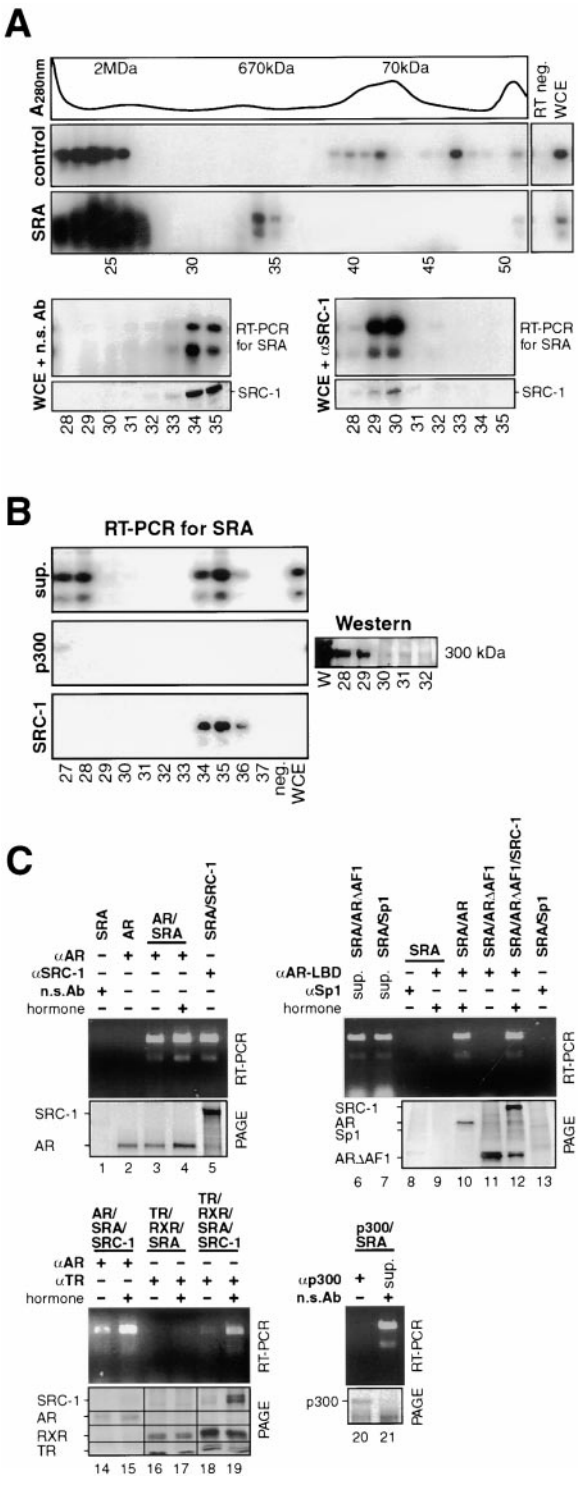

Figure 6. SRA Is Present in an SRC-1 Complex

(A) Copurification of SRA and SRC-1 complexes by gel filtration chromatography. Upper panels: T-47D lysates (~400 μg) were fractionated on a Superose 6 column and analyzed for total protein elution ($A_{280\ nm}$, top), SRC-1-specific RT-PCR (control), and SRA-specific RT-PCR (SRA). RT-PCR analysis of ~20 μg input whole-cell extract in the presence (WCE) or absence of reverse transcriptase (RT neg.) are shown to the right. Numbers indicate fractions. Elution peaks of molecular size markers are given for mammalian SWI/SNF complex (~2 MDa) and thyroglobulin (670 kDa); the void volume (4 MDa for globular proteins) was determined at fraction 20 by silver staining (not shown). Lower panels: SRA-specific RT-PCR and parallel immunoblots with SRC-1-specific antibody of fractionated T-47D cells after preincubation of the lysate with either nonspecific antibody (WCE + n.s. Ab) or SRC-1 antibody (WCE + SRC-1 Ab). In addition to SRA, fractions 34 and 35 contained the AF2 coactivators TIF2 and SRC-3 (McKenna et al., 1998 and not shown). Since SRC-3 was reported to be an exclusively nuclear protein (Suen et al., 1998), it indicates that our lysis conditions extract complexes both of nuclear and cytoplasmic origin.

(B) Coimmunoprecipitations of SRA in fractionated cells. T-47D lysates were fractionated as in (A), subsequently immunoprecipitated with antibodies against p300 (middle) and SRC-1 (bottom), and analyzed for SRA by RT-PCR (left panels) or by parallel Western analysis for precipitation of p300 (right). Numbers indicate fractions. sup., combined supernatant of both precipitation reactions; neg., RT-PCR omitting reverse transcriptase; WCE, input lysate (note that the conditions for immunoprecipitation of fractionated extracts did not coprecipitate SRA in WCE).

(C) Coimmunoprecipitation of SRA with AR or SRC-1 in *Xenopus* oocyte extracts. Ethidium bromide–stained agarose gel of SRA-specific RT-PCR products from immunoprecipitation reactions (RT-PCR) and parallel SDS-PAGE analysis of precipitated proteins (PAGE). In vitro–transcribed RNAs for SRA, SRC-1, p300, AR and the AR mutant ARΔAF1, TR, RXR, and Sp1 were injected along with L-^{35}S-methionine into *Xenopus laevis* oocytes (as indicated at the top) and the translation products subsequently targeted for immunoprecipitation (antibodies indicated at the left). n.s. Ab, nonspecific antibody; αAR-LBD, polyclonal antibody against the C terminus of androgen receptor (AR); ARΔAF1, AF1-depleted AR; sup., supernatant of immunoprecipitation reaction.

the Northern analysis for luciferase expression of the set of cycloheximide-treated cells (lower panel) revealed that SRA (lanes 5–8), but not SRC-1 (10–12) or CBP (14–16), was able to enhance transcription under these conditions. As a control, we assayed ^{35}S-methionine incorporation in the two groups of cells and found that the amount of cycloheximide used in our assays (50 μM) abolished >99% of the total cellular translation products (not shown). Moreover, a third control set of transfected cells treated with cycloheximide and analyzed for luciferase reporter activity produced relative light units corresponding to basal activity. The fact that only SRA and not SRC-1 and CBP was capable of potentiating GR-mediated transcription in the absence of de novo protein synthesis was clear evidence that the functionality of SRA was not contingent upon translation of the primary SRA transcript.

SRA Is Present in a Distinct Steroid Receptor Coregulator Complex

Given that functional RNAs are known to associate with proteins as ribonucleoprotein complexes, we next asked if SRA might function as a component of similar complexes. To investigate protein–SRA interaction in a steady-state situation in vivo, we fractionated whole-cell extract from human T-47D cells on a Superose 6 column as previously described (McKenna et al., 1998). One-half of the collected fractions were processed for Western analysis using specific antibodies against transcriptional coregulators, and the remainder of each fraction was subjected to RNA isolation followed by SRA transcript–specific RT-PCR and Southern analysis. We validated our RT-PCR method by incubation of cell extract with SRA-specific antisense deoxyoligonucleotides and subsequent digestion with endoribonuclease

RNase H, which destroyed the SRA signal in an oligonucleotide- and dose-dependent manner (not shown). We found that endogenous SRA specifically eluted in complexes of 600–700 kDa (Figure 6A, fractions 34 and 35). We verified that these fractions do not reflect a nonspecific peak of proteins and RNA (Figure 6A, upper panels). Western analysis indicated that SRA copurified with fractions containing SRC-1 (Figure 6A, SRC-1). Interestingly, SRA was not detected in fractions containing p300/CBP (fractions 28–31). The colocalization of SRC-1 and SRA led us to consider that they may be part of a common complex in vivo. Based on this assumption, we attempted to alter the elution pattern of this putative complex by incubating cell lysates with anti-SRC-1 antibody and rabbit anti-mouse IgG prior to fractionation. As predicted, this resulted in a clear shift of both the SRA signal and SRC-1 immunoreactivity from fractions 34–35 to fractions 29–30 (Figure 6A, lower panel). To exclude the possibility that the shifted SRC-1 was due to nonspecific antibody binding, the Western blots were stripped and reprobed with anti-CBP antibody. The elution profile of CBP was the same irrespective of preincubation of cell lysate with SRC-1 antibody (not shown).

To verify our findings that SRA associates—directly or indirectly—with SRC-1, we attempted to coimmunoprecipitate SRA and SRC-1. To quantitatively enrich SRA in the cells subjected to fractionation, we transfected HeLa cells with a plasmid encoding SRA. We then fractionated the lysates and subjected the fractions to immunoprecipitation with antibodies against SRC-1 and p300 prior to RT-PCR analysis. SRA was detected in the anticipated fractions in both the inputs and the SRC-1 precipitates (Figure 6B, upper and lower panels, fractions 34–36), but SRA was not coimmunoprecipitated with p300 (middle panel). Parallel Western analysis indicated that p300 was specifically precipitated by anti-p300 antibody in the anticipated fractions. These results verified our finding that SRA resides in a complex containing SRC-1 but not p300 or CBP. To test the possibility that SRA might have a structural role in the SRC-1 complex, we treated cell extracts with RNase prior to fractionation. These extracts did not produce SRA signals in the RT-PCR analysis, whereas SRC-1 was detectable in fractions 35–36 (not shown), suggesting that SRA does not have a vital structural role in SRC-1 complexes. Taken together, biochemical fractionation experiments indicated that SRA is a component of distinct ribonucleoprotein complexes, one of which contains the nuclear receptor coactivator SRC-1.

Having established that SRA was present in SRC-1-containing complexes, we wished to know whether SRA interacted with steroid receptors as a component of a ribonucleoprotein complex. To address this possibility, we performed coimmunoprecipitation experiments using a previously described expression system in *Xenopus* oocytes (Wong et al., 1995). In vitro–generated RNAs encoding SRA, SRC-1, p300, AR and the AR mutant ARΔAF1, TR, RXR, and the nonnuclear receptor transcription factor Sp1, along with L-^{35}S-methionine, were injected into oocytes and their cell extracts subjected to coimmunoprecipitation with antibodies against the expected protein products. Figure 6C shows the cDNA products generated by SRA-specific RT-PCR of the various immunoprecipitates along with SDS-PAGE analysis. SRA was undetectable after immunoprecipitation using a nonspecific antibody from cell lysates programmed with SRA (lane 1). Similarly, SRA was not detected after immunoprecipitation with an AR antibody from cell lysates injected with RNA encoding AR, although AR was specifically precipitated (lane 2). In contrast, the AR antibody precipitated SRA in a hormone-independent manner in extracts from oocytes injected with RNAs for SRA and AR (lanes 3 and 4). We next investigated the specificity of the interaction of SRA with SRC-1 and AR. An AR mutant lacking the amino-terminal domain (ARΔAF1) did not retain coinjected SRA (lane 11), although the supernatant clearly contained SRA (lane 6). However, in oocytes containing SRC-1 in addition to SRA and ARΔAF1, the antibody against the LBD of AR coprecipitated SRA and SRC-1 (lane 12). In addition, immunoprecipitation using a monoclonal antibody against SRC-1 from oocytes programmed with RNAs for SRA and SRC-1 clearly coprecipitated SRA and SRC-1 (lane 5), verifying that SRA is in a stable association with SRC-1. Similarly, analysis of oocytes programmed with SRA, TR, RXR, and SRC-1 and precipitated with TR antibody indicated SRA only in SRC-1-containing extracts (lane 19), verifying again the selectivity of SRA for steroid receptors and SRC-1. Taken together, we conclude that SRA exists in a ribonucleoprotein complex containing SRC-1 and that this complex is recruited by a steroid receptor.

Discussion

In this work we describe the isolation and functional characterization of a novel transcriptional coactivator termed SRA. SRA is different from other known coregulators in that it functions as an endogenous RNA transcript. We have defined several different features of this RNA: SRA is (1) a bona fide transcriptional coactivator, (2) selective for the AF1 of steroid receptors, (3) expressed as multiple isoforms in a cell-specific manner, and (4) present in a steady-state coregulator complex with the AF2 coactivator SRC-1.

We have described the isolation of three SRA isoforms deduced from sequencing of different cDNAs and genomic clones from different species. When overexpressed in mammalian cells, recombinant SRA, regardless of isoform or origin, enhanced steroid receptor–mediated transactivation without significantly enhancing the level of basal transcription of minimal or natural promoters. In assays of endogenous PR–mediated transactivation, a typical enhancement of receptor gene activity of $\sim$10-fold was achieved by coexpression of SRA. Antisense deoxyoligonucleotides added to cells reduced steroid receptor–induced transcription by up to 70%. In addition, we have shown that SRA reverses steroid receptor squelching in a dose-dependent manner. Hence, SRA exhibits many characteristics expected of a bona fide coactivator.

Despite certain functional similarities, SRA differs in some important aspects from many other coactivators in that its coactivation is selective and that it is an RNA. We have presented several independent lines of evidence that indicate that SRA selectively enhances steroid receptor–mediated transactivation but does not influence transactivation by type II nuclear receptors or

by other transcription factors. In addition, using in situ hybridization analysis we have obtained evidence for both a selective expression pattern of SRA and a general colocalization in brain tissue with members of the steroid receptor family (data not shown). In our coimmunoprecipitation assays it appears that, unless SRC-1 is coexpressed, the N-terminal domain of steroid receptors is required for binding of SRA. In our reporter gene assay, SRA per se fails to enhance transcriptional potency of the AF2 receptor domain in cultured cells. The exact nature of the interaction of SRA with the AF1 domain of steroid receptors is as yet unclear. SRA was originally isolated in the yeast two-hybrid system, an assay designed to identify protein–protein interactions. In a reconstructed yeast system, SRA associated with the N-terminal domain of PR but not with a control hybrid (not shown). The lack of sequence homology within the amino terminus of steroid receptors suggests that SRA may interact indirectly with the AF1 of the receptors as part of a ribonucleoprotein complex. It is unlikely that protein–protein interactions between the bait construct and the GAL activation domain played any role in the isolation of SRA. Rather, we envision an interaction of SRA with the PR N-terminal bait, thereby recruiting it to the reporter gene site. The SRA-PR N terminus interaction is likely to have been supported by yeast proteins, possibly through a mediator with functional similarity to SRC-1, and such interactions would have favored reporter gene activity and resulted in a positive hit. This somewhat fortuitous isolation of SRA appears less puzzling when it is considered that yeast proteins contribute functionally to transcriptional activation by steroid receptors (Yoshinaga et al., 1992), even though steroid receptors are not expressed in yeast.

Although we do not totally exclude the existence of a translation product of SRA contained in certain cells at specific developmental stages, we have provided evidence to indicate that SRA exists and functions as an RNA transcript. First, we were not successful in our attempts to translate the SRA clones in vitro or in vivo. Second, an affinity column containing a mAb raised against a sequence at the carboxy-terminal end of the putative ORF1 transcript failed to detect endogenous SRA in various cell lines tested. In addition, extensive mutagenesis of SRA, introducing multiple translational stop codons in all reading frames, did not affect the ability of these mutants to enhance PR transactivation. A final functional test was provided by transfection experiments in the presence of cycloheximide, in which SRA retained its ability to coactivate a reporter gene, while other protein coregulators such as SRC-1 and CBP did not.

The ability of RNA molecules to perform many functions that were commonly attributed to proteins has been well documented. RNA molecules perform enzymatic reactions such as *trans*-esterification (Jaeger, 1997) or catalysis of peptide bond formation (Zhang and Cech, 1997) and can regulate gene expression in *trans* by structure (Jones and Peterlin, 1994), by antisense RNA–RNA interaction (Lee et al., 1993; Crespi et al., 1994), or by the association of two genomic-sense RNAs (Sit et al., 1998). To our knowledge, however, SRA is different from eukaryotic transcriptional coactivators in its ability to function as an RNA transcript to selectively regulate the activity of a family of transcriptional activators.

Functional evidence indicates that coregulators associate with nuclear receptors as members of multiprotein complexes (Rachez et al., 1998). It has been shown that hormone-activated receptors can recruit the coactivators of the SRC-1 family, the cointegrators p300 and CBP, histone acetyltransferase activity P/CAF, or chromatin-remodeling factors such as the human homologs of the yeast SWI/SNF proteins (reviewed by McKenna et al., 1999). In addition, multiple coregulators associate with each other (Kamei et al., 1996; Spencer et al., 1997; Torchia et al., 1997), and our laboratory has shown that distinct preformed complexes contain different subclasses of nuclear receptor coactivators in vivo (McKenna et al., 1998). We have provided biochemical evidence here that SRA elutes in a complex that also contains SRC-1 and that SRA was coimmunoprecipitated by SRC-1 and AR but not by p300, TR, RXR, or AF1-truncated AR in the absence of SRC-1. These observations raise the possibility that, through a specific association, SRA might function in part by modulating the activity of a distinct class of nuclear receptor coactivator complexes. Given its evident functional specificity, we favor a model in which SRA confers functional selectivity upon coactivator complexes recruited by liganded receptor, possibly acting as an adaptor molecule for type I receptors.

Taken together, our results have introduced an entirely novel concept, not only in nuclear receptor–regulated transactivation, but in eukaryotic transcription as a whole. An RNA transcript, specifically expressed in steroid target tissues, functions as a component of a large multiprotein complex to selectively enhance transcriptional activation by steroid receptors. Regulation of transcription is a modular process, probably requiring different combinations of coregulators at different stages of transcription at different times. As an RNA transcript, subjected to rapid turnover and regulation, we envisage an important role for SRA in the dynamic process of transcription in which an activated receptor recruits diverse complexes mediating temporally and spatially distinct functions. Future studies will define more clearly the functional and physiological significance of this interesting eukaryotic transcriptional coregulator.

Experimental Procedures

DNA Library Screening

The coding sequence of the AF1 domain of the human PR_A (amino acids 165–567 of hPR_B) was subcloned into the pAS1 yeast expression plasmid in frame with the amino acid sequence of the GAL4-DBD (1–147). The yeast two-hybrid screen was performed as previously described in Onate et al. (1995). Transformants of a human B-lymphocyte cDNA expression library were tested in the Y190 yeast strain for interaction with progesterone-induced hPR_A. RACE was performed using the Marathon cDNA Amplification Kit (Clontech) with skeletal muscle mRNA (Clontech) and the following primers: − strand 5′-CTGGGGGATCCATCCTGGGGTGCG-3′ (On1), − strand 5′-CCTGCAGCAGTGCCAGGCGTCGG-3′ (On5), and + strand 5′-CGCGGCTGGAACGACCCGCCGC-3′ (On3). SRA clones were isolated by homology screening of human λgt11 cDNA libraries from skeletal muscle, heart, and HeLa S3 cells (Clontech), human genomic library EMBL3 SP6/T7 (Clontech), mouse heart cDNA library λZAP cDNA (Strategene), and 129SVJ mouse genomic library λgtFIX II (Stratagene) using bacteria strains and protocols as provided by

the library manufacturers. Both strands of SRA clones were sequenced using Sequenase (Amersham) or Thermal Cycle DNA Sequencing (New England Biolabs).

Northern and Southern Analysis

A human tissue Northern blot (MTN, Clontech) was hybridized with a probe corresponding to the NaeI–HincII fragment of SRA. Tissue cell blots were prepared by isolation of total RNA using TRIzol Reagent (Life Technologies) and analyzed with a 1.5 kb probe corresponding to SRA isoform III (Figure 1A). The HeLa cell blot (Figure 5) was hybridized with a random labeled fragment of the firefly luciferase cDNA. The blots were stripped and subsequently hybridized with a probe specific for β-actin (MTN and tissue cell blots) or cyclophilin (HeLa cell blot), respectively. RT-PCR products were electrophoresed, blotted, and hybridized with a probe corresponding to isoform I of SRA. Probes were generated using random DNA labeling kit (Life Technologies) and 50 μCi of [α-^{32}P]dCTP, 300 Ci/mmol (ICN) followed by EtOH-precipitation or G-50 (Boehringer Mannheim) column purification.

Plasmids

The reporter constructs $(PRE)_2$-TATA-CAT and $(ERE)_2$-TATA-CAT have been described (Vegeto et al., 1992). The MMTV-Luc (Luc, luciferase) was generated by subcloning the Acc65I–XbaI fragment from pGLBasic3 (Promega) into the blunt-ended EcoRI site of MMTV-KCR (Steve Chua, Baylor College of Medicine). The human CMV-driven mammalian expression vectors pSTC for human PR_B, GR, AR, and ER were generated by fusion of the cDNAs to the HSV-TK leader sequence containing a Kozak consensus sequence (Lanz et al., 1995); rat GR, GRΔAF1, and GR-DBD have been described (Rusconi and Yamamoto, 1987). PRΔAF1 is an N-terminal truncation of pSTC- hPR_B at the AccI site and re-ligation to the blunt-ended BamHI site of the TK leader; PRΔLBD is a C-terminal truncation of pSTC-hPR_B at the DraI site. TRβ, RARα, RARγ, RXRγ, Sp1, E2F, E47D, and CREB and corresponding CAT reporter constructs were from S. A. O. and M.-J. T. and published elsewhere (Cooney et al., 1992; Leng et al., 1994; Onate et al., 1995); PPARγ was a gift from Steven A. Kliewer (Glaxo Research Institute) and CBP from Richard Goodman (Vollum Institute, Oregon Health Sciences University). SRA-containing expression vectors were generated by subcloning the cDNAs into a modified linker of the CMV-driven pSCT-1 (Rusconi et al., 1990). Excision of BamHI fragment of pSCT-SRA and religation generated ΔATG, and the fusion of the BamHI- or NaeI-restricted SRA to the HSV-TK leader sequence generated tk-ORF1 and tk-ORF2, respectively. The reading frame mutations ORF1, ORF2, and ORF3 were generated by PCR using the sense primers 5′-TGGGGGATCCTACCTCAGGTGCGG-3′, 5′-TGGGAGATCTATCCTAGGGTGCGG-3′, and 5′-TGGGGGATCCTACCTAGGGTGCGG-3′, followed by restriction subcloning into pSCT-SRA. YIle used the primer 5′-ATAGCAATTGGGCCTCCACCTCCTTCAAG-3′ to destroy an ATG and to introduce an MfeI site in mutant ORF2. Frameshift mutations were generated by restriction of SRA or mutant ORF2 with selected enzymes, filled in with Klenow DNA polymerase, and re-ligated at the following sites: BbsI (generated mutant B), SgrAI (S), MfeI and SgrAI (MS, YMS), and MfeI, BbsI, and SgrAI (YMBS). 3′ deletions at BbsI of ORF1, ORF2, and ORF3 generated ΔORF1, ΔORF2, and ΔORF3. All the vectors for in vitro transcription (Figure 6C) were generated by subcloning the cDNAs for SRA, SRC-1, AR, and Sp1 into MS2, which is a modified version of pSP64 poly(A) (Promega) containing an additional polylinker 3′ of the poly(A) sequence for linearization of the plasmid. ARΔAF1 was generated by subcloning of the HindIII–XbaI fragment encoding amino acids 605–910 of pAR65 (Jenster et al., 1993) into MS2. pMS2-p300 was from J. W. (unpublished), and pMS2-TR and pMS2-RXR have been described (Wong et al., 1995).

Cell Culture and Transient Transfection Assays

Cell lines were routinely maintained at 37°C/5% CO_2 in Dulbecco's modified Eagles' medium (HeLa, COS) or RPMI medium 1640 (T-47D) supplemented with 5%–10% charcoal-stripped fetal calf serum. 10^5 cells were plated out per well in 12-well dishes for luciferase assays, 5×10^5 cells per well in 6-well dishes for CAT assays, and 10^6 cells per 10 cm dish for assays that involved cell culture in the presence of cycloheximide. Medium was replaced 3 hr prior to transfection with medium containing 50 μM cycloheximide and maintained until cell harvesting. Cells were transfected with the indicated DNAs using lipofectin (Life Technologies) or SuperFect (QIAGEN) and treated according to the manufacturer guidelines. In all transfection experiments, reporter plasmids were abundant (2.5 μg per 10^6 cells), whereas nuclear receptors were transfected in limiting amounts (20–100 ng per 10^6 cells). 2′-O-methoxyethyl ribose/2′-deoxyribose-stabilized oligonucleotides were generated by F. Bennett at ISIS Inc., Carlsbad, CA. Fifty to two hundred nanomoles of the antisense 5′-GGAACCGAGGATTATGAA-3′ and corresponding sense control were cotransfected and treated as described for plasmid DNA. Upon DNA addition, cells were cultured for 36–42 hr for CAT assays, 20–24 hr for luciferase assays, and 11–14 hr in the presence of cycloheximide. Ligand stimulation involved incubation of cells with progesterone (10 nM), RU486 (50 nM), dexamethasone (50 nM), R1881 (10 nM), or estradiol E2 (10 nM) for 6–8 hr prior to cell harvesting. Cell lysates were assayed for CAT activity with 100 μCi of [^{14}C]chloramphenicol and 5 mM acetyl coenzyme A (Sigma) as substrate and separated by thin-layer chromatography. Luciferase activity was determined using the luciferase assay system (Promega). Values were corrected for protein concentration. Data are presented as the mean (±SD) of triplicate values obtained from a representative experiment that was independently repeated at least three times.

Antibodies and Western Analysis

The mAbs against SRA, SRC-1, and hAR were prepared at the University of Colorado Health Science Center in collaboration with D. P. Edwards. SRA-mAb was raised against the peptide sequence TAEKNHTIPGFQQAS corresponding to the C terminus of the presumptive ORF1 of human SRA. The mAb was purified from hybridoma culture supernatants using a mAb TRAP GII column (Pharmacia). SRC-1-mAb was described previously (Spencer et al., 1997); AR-mAb recognizes the residues 299–315 of hAR and was a gift from N. Weigel (Baylor College of Medicine); the polyclonal Ab for AR-LBD (SP066; Kuiper et al., 1993) was a gift from J. Trapman and A. Brinkmann, Erasmus University, Rotterdam, The Netherlands; p300-Ab (Eckner et al., 1994) was a gift from R. Eckner, Molecular Biology, Zurich, and Sp1-Ab was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Immunoblotting was performed as described (Elashry-Stowers et al., 1988; Hanstein et al., 1996).

Gel Filtration

Biochemical fractionation of cell lysate on a Superose 6 gel filtration column (Pharmacia) was carried out as described (McKenna et al., 1998) except that cell lysates contained 1–2 U/μl RNasin ribonuclease inhibitor (Promega) and the columns were preequilibrated with RNasin. For antibody shift experiments, clarified lysates were preincubated at 4°C with 2 μg of SRC-1 mAb and a 4-fold excess of rabbit anti-mouse IgG (Zymed). Half of each column fraction (400 μl) was processed for RNA isolation and RT-PCR analysis, while the other portion was precipitated with BSA/trichloracetic acid, separated on 7.5% polyacrylamide gels, and transferred overnight to nitrocellulose membrane (BioRad) at 0°C–4°C for Western analysis.

SRA-Specific RT-PCR

Cell extracts (20–30 μl) or column fractions (200 μl) were supplemented with 5 mM $MgSO_4$ and incubated for 25 min at 37°C with 20–40 U RNase-free DNaseI (Boehringer Mannheim) and 1–2 U/μl RNasin ribonuclease inhibitor (Promega). Total RNA was extracted using 1 ml TRIzol Reagent and processed according to the manufacturer's protocol (Life Technologies). EtOH-washed RNA was resuspended in 12 μl 2 pmol SRA-specific primers (On3: 5′-CGCGGCTGGAACGACCCGCCGC-3′ and On8: 5′-CAGACTCACCGGACACCATCTCCTA-3′; see Figure 2). First-strand cDNA synthesis was generated using Moloney reverse transcriptase and reagents supplied with the SuperScript II Kit (Life Technologies). Twenty percent (4 μl) of the reaction was used in a 50 μl PCR amplification using 5 U of Taq-DNA polymerase (Promega), 2 mM $MgCl_2$, 150 μM dNTPs, 1 μM of primers (On3/On8). PCR was performed as follows: 3 min at 95°C, 25–40 cycles of 30 s at 95°C, 45 s at 58°C, 40 s at 71°C, and 5 min at 72°C. Alternatively, RNA from immunoprecipitations was processed by using the SuperScript One-Step RT-PCR System

(Life Technologies) and incubated at 50°C for 30 min prior to cDNA amplification. PCR products were visualized on 1.2% agarose/TAE gels and blotted to Zeta-Probe GT membrane (BioRad) by alkaline transfer for Southern analysis.

Immunoprecipitation in *Xenopus laevis* Oocytes

pP(A)LiSK-cDNA constructs were transcribed in vitro with SP6 RNA polymerase and the mMessage mMachine kit (Ambion) to generate 200–400 μg/μl specific mRNA. *Xenopus laevis* oocytes were injected with 27.6 nl specific mRNA and L-^{35}S-methionine and cultured for 12–16 hr at 18°C in MBSH [10 mM HEPES {pH 7.6}, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$]. Oocytes were lysed in extract buffer (20 mM HEPES [pH 7.6], 70 mM KCl, 2 mM DTT, 0.1% NP-40, 8% Glycerol, 1 mM PMSF, and 1 U/μl RNasin) in a ratio of 10 μl extract buffer per oocyte. Clear lysates were incubated with 4 μg of SRC-1-mAb, 2 μg of AR-mAb, 8 μl of SP066 (AR-LBD Ab), 4 μg of rabbit polyclonal Sp1-Ab, or 4 μg of rabbit polyclonal TR-Ab together with a 4-fold excess of rabbit anti-mouse IgG (Zymed) for 35 min at 4°C followed by 30 min incubation at 4°C with protein A–Sepharose (Pharmacia) that was washed and equilibrated in extract buffer. Subsequently, beads were washed five times with 5 vol of extract buffer, and bound material was analyzed by RT-PCR and by SDS-PAGE.

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GenBank Accession Numbers

The GenBank accession numbers for the human SRA and mouse SRA reported in this paper are AF092038 and AF092039, respectively.