An Sp1-NF-Y/Progesterone Receptor DNA Binding-dependent Mechanism Regulates Progesterone-induced Transcriptional Activation of the Rabbit RUSH/SMARCA3 Gene*

Received for publication, April 15, 2003, and in revised form, July 29, 2003 Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.M303921200

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Steroids regulate alternative splicing of rabbit RUSH/SMARCA3, an SWI/SNF-related transcription factor. Transactivation was evaluated in 2057 bp of genomic sequence. Truncation analysis identified a minimal 252-bp region with strong basal promoter activity in transient transfection assays. The size of the 5′-untranslated region (233 bp) and the transcription start site were determined by primer extension analysis. The transcription start site mapped to a consensus initiator (Inr) element in a TATA-less region with a downstream promoter element (+29). These elements were authenticated by mutation/deletion analysis. The Inr/downstream promoter element combination is conserved in the putative core promoter (−35/+35) of the human ortholog, suggesting that transcription initiation is similarly conserved. Two Sp1 sites that are also conserved in the putative promoter of human SMARCA3 and a RUSH binding site (−616−611) that is unique to the rabbit promoter repress basal transcription. These sites were variously authenticated by gel shift and chromatin immunoprecipitation assays. Analysis of the proximal promoter showed the −162/−90 region was required for progesterone responsiveness in transient transfection assays. Subsequent mutation/deletion analysis revealed a progesterone receptor half-site mediated induction by progesterone. An overlapping Y-box (in the reverse ATG orientation) repressed basal transcription and progesterone-induced transcriptional activation in the presence of the Sp1 sites. The specificity of progesterone receptor and transcription factor NF-Y binding were authenticated by gel shift assays. Chromatin immunoprecipitation assays confirmed the Y-box effects were mediated in a DNA binding-dependent fashion. This represents a unique regulatory scenario in which ligand-dependent transactivation by the progesterone receptor is subject to Sp1/NF-Y repression.

We demonstrated that a servomechanism operates between progesterone and prolactin and their receptors in the regulation of uterine gene expression (1). Prolactin receptor is regulated by progesterone, and prolactin augments the progesterone-dependent increase in uteroglobin mRNA. The search for factors responsible for this cross-talk at the promoter culminated in the cloning and characterization of RUSH-1α and -β (2). The RUSH acronym identified two alternatively spliced rabbit uteroglobin promoter-binding proteins as SWI/SNF-related helicases/ATPases. Coincidentally, the human (3–5) and mouse (6) orthologs for RUSH-1α were cloned and characterized. According to the newest nomenclature, SMARCA3, the symbol for the human gene, and Smarca3, the symbol for the mouse gene, refer to the common characteristics: SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3. These symbols supersede the original human (HIP116, HLTF, and Zbu1) and mouse (P113) designations. If the human nomenclature system is applied to all species other than mouse, then the rabbit ortholog is designated rabbit SMARCA3 with the retention of RUSH as the trivial name to be used interchangeably with rabbit RUSH/SMARCA3.

SMARCA3 is highly conserved among eukaryotes. For example, the cDNA and amino acid sequences for RUSH-1α and SMARCA3 are 91% identical. A DNA binding domain, a nuclear localization signal, seven DNA-dependent ATPase domains, and a CαHCα RING finger motif (7) characterize both proteins. In contrast, RUSH-1β is truncated after the RING finger such that three of the seven DNA-dependent ATPase domains are deleted from its C terminus. The RING finger, a specialized type of zinc finger, is a putative scaffold for macromolecular assembly (8). This motif in RUSH and its human counterpart are highly conserved (7), i.e. they differ by a single amino acid substitution, and the spacing between the metal-coordinating residues is identical. Although the exact role of the RING domain in RUSH remains unclear, this motif contains a protein interaction site that binds an inner nuclear envelope protein (9).

Despite the highly conserved structure of RUSH and its SMARCA3 relatives, the character of the cognate binding sites is highly variable. Identified by three different research groups as a sequence-specific DNA-binding protein, SMARCA3 binds to the B box of the plasminogen activator inhibitor gene (10), the SPH repeats of the SV40 enhancer and the human immunodeficiency virus 1 promoter (3), and the E box of the myosin light chain 1/3 (MLC1/3) enhancer (5). RUSH binds to a unique site (A/C)(A/T)(A/T/G)(G/T) (MCWTDK) in the uteroglobin promoter (11). This paradox, in which ubiquitous, highly conserved transcription factors bind degenerate target sequences, provides the opportunity for remarkable promiscuity in so-called gene-specific binding.

Functional diversity is further emphasized by the fact that RUSH orthologs have been implicated in chromatin reorgani-
Rabbit RUSH/SMARCA3 Gene

Cloning of the RUSH Promoter—An amplified rabbit genomic library in the EMBL3 SP6/T7 vector was double-screened with the original 1509-bp RUSH cDNA clone (2) and a cDNA clone (A1) that encodes the first 282 bases of the RUSH cDNA including 170 bp of the 5′-untranslated region (UTR). Nitrocellulose filter replicas were prepared from λgt11 recombinants (3 × 10^6 plaque-forming units/plate) and screened with the random prime-labeled cDNAs (specific activity = 1 × 10^10 cpm/μg). A genomic clone (4.4 kilobases) that was recognized by both probes used in two PCR reactions was used. The clones with PCR reaction contained PCR buffer (1×), magnesium acetate (1.1 mM), T7 DNA polymerase (0.1 units), T7Start antibody (0.01 μg/μl), dNTPs (0.2 mM each), and primers (0.2 μM each). The forward primer or 5′-LD amplimer (5′-CTG CTT CTA ATA GAG CAC ACC GGG CAC GAA CAA ACT GCG CAC CAA C-3′) matched vector sequence. The reverse primer (5′-GGC TAC GCT TCA GCT TCG CAG GTC GTG AC-3′) matched sequence in the cDNA (see Fig. 1). The hot start PCR amplification reaction was performed as follows: 60 s at 95 °C followed by 30 cycles of 95 °C for 15 s, 68 °C for 22 min, and a final extension for 22 min at 68 °C. Samples were rapidly cooled to 15 °C. A single 2098-bp PCR product was cloned into pCRII-TOPO and sequenced in both directions by the dye-deoxy chain termination method.

The second 50-μl PCR reaction contained PCR buffer (1×), TaKaRa ExTaq DNA polymerase (2.5 U), TaqStart antibody (0.05 μg/μl), dNTPs (0.2 μM each), and primers (0.2 μM each). The forward primer (5′-GGCC GAC GAC TCA GGA CAT GCC GTC GCT CAG GTC G-3′) was designed to overlap the SMARCA3-expressed region of the RUSH gene. The reverse primer (5′-CAT CCA GGA CAT GCC GTC GCT CAG GTC G-3′) was designed to match the flag vector sequence. The reverse primer (5′-CAT CCA GGA CAT GCC GTC GCT CAG GTC G-3′) was designed to match the sequence in the cDNA (see Fig. 1). The hot start PCR amplification reaction was performed as follows: 60 s at 95 °C followed by 30 cycles of 95 °C for 15 s, 68 °C for 22 min, and a final extension for 1 min at 68 °C. Samples were rapidly cooled to 4 °C. A single 182-bp PCR product was cloned into pCRII-TOPO and sequenced in both directions by the dye-deoxy chain termination method.

Cloning the RUSH promoter and its contiguous 5′-upstream regulatory sequence showed that RUSH transcription is driven by an Inr/downstream promoter element (DPE)3 that is highly conserved in the putative SMARCA3 promoter. RUSH is negatively regulated by two Sp1 sites, which are also highly conserved in the human counterpart. In contrast, the PRE half-site/overlapping Y-box dependent alternative splicing mechanism (2) in which progesterone-treatment increased the uterine content of RUSH message. Competitive reverse transcription-PCR and high performance liquid chromatography analysis confirmed a steroid-dependent alternative splicing mechanism (2) in which RUSH-1a is the progesterone-dependent splice variant. The next step toward understanding the potential involvement of progesterone in regulating transcription required systematic analysis of the RUSH promoter.

EXPERIMENTAL PROCEDURES

Reagents and Tools—A rabbit genomic library in the EMBL3 SP6/T7 vector, TaqStart antibody, Tth Start antibody, Advantage PCR kit, and Advantage Tth polymerase mix were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). TaKaRa ExTaq enzyme and 10× LA PCR buffer were purchased from PanVera Corp. (Madison, WI). The pCRII-TOPO vector was a component of individual TA cloning kits from Invitrogen. The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Mutagenesis primers and custom oligonucleotides were synthesized at Midland Certified Reagent Co. (Midland, TX). The Primer Extension System-AMV reverse transcriptase was purchased from Promega (Madison, WI). Phenol red-free α-modified minimum essential medium, phenol red-free Opti-MEM 1, and the LipofectAMINE PLUS reagent package were purchased from Invitrogen. Defined fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). TransSignal transcription factor (TF)-TF interaction arrays (MA5010 and MA5011) were purchased from Panomics (Redwood City, CA). Dynabeads with protein G were purchased from Dynal Biotech, Inc. (Lake Success, NY). QIAEX II was purchased from Qiagen, Inc. (Valencia, CA). Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) was the commercial source of nuclear extract from SK-BR-3 cells, consensus and mutant oligonucleotides, and antibodies for Sp1 (sc-59 X), NF-Y (sc-7711 X), also known as the CCAAT binding factor, and progerrome receptor (sc-539 X). Promegestone (R2002) was purchased from PerkinElmer Life Sciences.

Cloning of the RUSH Promoter—An amplified rabbit genomic library

1 The abbreviations used are: DPE, downstream promoter element; UTR, untranslated region; TF, transcription factor; Sp, specific protein; NF-Y, nuclear factor Y; PRE, progerrome response element.
unit/ml), streptomycin (100 μg/ml), amphotericin B (250 μg/ml), and t-glutamine (20 μM) in a humidified atmosphere of 95% air, 5% CO₂ at 33 °C. As verified by RIA, charcoal stripping reduced estradiol levels to <0.6 pg/ml. When the effects of progesterone were tested, cultures were treated with R5020 (10⁻¹⁰ M) in dimethyl sulfoxide (0.1% v/v) for 20 h. Controls were treated with vehicle alone for the same amount of time.

Cells were seeded in 24-well tissue culture plates and transfected at a ratio of 500:1 in the absence of serum at 50–60% confluency with 100 ng of pRUSH-LUC DNA and 200 pg of pRL-TK-LUC (transfection efficiency control) in 5 μl of PLUS reagent with 2 μl of LipofectAMINE reagent. After 3 h of incubation, the volume of medium was increased to normal (0.5 ml/well), and the final concentration of serum was restored to 4%. Transfections were performed in quadruplicate, and pRUSH-LUC expression was normalized to pRL-TK-LUC activity. Multiwell data were ranked (17), and the ranks among the groups were analyzed by analysis of variance, followed by Student-Newman-Keuls multiple comparison.

**Primer Extension and Sequence Analysis**—Total RNA was isolated from the endometrium of a progesterone-treated rabbit by the cold precipitation method of Han et al. (18). Poly(A)+ RNA was isolated by chromatography on oligo(dT) cellulose (19). An end-labeled oligonucleotide (5'-GCC CGG GTC TAA TCA GCA GTC GTC GCG GAG ACT GTG ACG-3') complementary to the most 3'-most end of the RNA sequence was hybridized to 1 μg of poly(A)+ RNA. This primer hybridized less than 100 bases downstream from the 5'-end of the RNA and did not self-anneal. The size of the single primer extension product was determined by resolution on denaturing 8% polyacrylamide gels (7 M urea) together with [3H]labeled OX174 Hinfil DNA markers and products from positive (87 bp) and negative (no RNA) control reactions. The product from the primer extension reaction was also resolved by sequencing with the same primer.

**Chromatin Immunoprecipitation and PCR**—All studies with New Zealand White rabbits were conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals as reviewed and approved by the Animal Care and Use Committee at Texas Tech University Health Sciences Center. For chromatin immunoprecipitation one virgin, adult female rabbit received subcutaneous injections of ovine progesterone (3 mg/kg/day) for 5 days. Nuclei were isolated from the endometrium as described by Kleis-SanFrancisco et al. (20).

Chromatin immunoprecipitation was performed according to Hewetson et al. (11). Briefly, nuclei (1 x 10⁶/experiment) were collected by centrifugation (1000 x g for 5 min at 4 °C) and resuspended in buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.5 mM dithiothreitol) that contained the following protease inhibitors: leupeptin (1 μg/ml), antipain (2 μg/ml), benzamidine (10 μg/ml), chymostatin (10 μg/ml), pepstatin (10 μg/ml), phenylmethylsulfonyl fluoride (2 mM). Chromatin was digested in situ with 250 units/ml Sau3A1 for 30 min at 37 °C. Nuclei were collected by centrifugation (750 x g for 2 min at 4 °C), resuspended in 1 ml of buffer (12 μM Tris, pH 7.5, 3 mM EDTA plus the mixture of protease inhibitors described above), and mixed (tumbled) at 4 °C for 30–60 min. Nuclei debris was collected by centrifugation (750 x g for 2 min at 4 °C). The supernatant containing soluble chromatin was transferred to a new tube. Sodium chloride and bovine serum albumin were added to final concentrations of 200 mM NaCl and 1 mg/ml, respectively, and the tube was incubated for 5 min at 4 °C. Residual nuclear debris was collected by centrifugation (12,000 x g for 5 min at 4 °C).

The supernatant was preabsorbed with protein A/G PLUS-Sepharose beads for 60 min at 4 °C. Beads were collected by centrifugation (1000 x g for 5 min at 4 °C). The supernatant was divided into two aliquots which were incubated plus 2 μg of the relevant antibodies, i.e., RUSH-1α/β, progesterone receptor, or NF-Y, with continuous mixing (tumbling) for 60 min at 4 °C. Protein A/G PLUS-Sepharose was added with continuous mixing (tumbling) for 60 min at 4 °C. Beads were collected by centrifugation (3000 x g for 30 s) and washed 3 times with phosphate-buffered saline containing 1 mM MgCl₂ and 1 mM CaCl₂. Beads were resuspended in buffer (50 μM Tris, pH 8.0, 1% SDS, 100 mM NaCl containing Pronase (1 mg/ml) and incubated for 1 h at 50 °C. The DNA was extracted with phenol/chloroform and ethanol precipitated with ethanol. DNA (50 ng) aliquots were amplified in 50-μl PCR reactions containing LA PCR buffer (1×), TaKaRa Ex Taq DNA polymerase (2.5 units/50 μl), TaqStart antibody (0.55 μg/50 μl), dNTPs (0.2 μM each), and primers (0.2 μM each). The forward (5'-GCA ACC GGG ACC CCC ACT G-3') and reverse (5'-GAC CGA CGG CCT GTC TG-3') primers flank a 136-bp region near the putative PRE half-site/overlapping Y-box. A 5-step PCR reaction was performed with the following conditions: 30 s at 94 °C followed by 5 cycles of 94 °C for 5 s, 63 °C for 5 s, 65 °C for 4 s, 64 °C for 5 s, 60 °C for 5 s, 60 °C for 5 s, 65 °C for 5 s, 70 °C for 5 s, 75 °C for 5 s, 60 °C for 5 s, 65 °C for 5 s, 60 °C for 5 s, 68 °C for 5 s, 65 °C for 5 s, 60 °C for 5 s, 70 °C for 5 s, 60 °C for 5 s, 69 °C for 5 s, 60 °C for 5 s, 65 °C for 5 s, 60 °C for 5 s, 69 °C for 5 s, 70 °C for 5 s, 60 °C for 5 s, 69 °C for 5 s, 70 °C for 5 s, 60 °C for 5 s, 70 °C for 5 s, 60 °C for 5 s.

**RESULTS**

A single positive clone (4.4 kilobases) was isolated from an amplified rabbit genomic library by duplicate screening of 7.2 x 10⁸ plaques with the original RUSH-1 (5–A) cDNA (2) and clone A1, which encodes the first 282 bases of RUSH cDNA including 170 bp of the 5' UTR. Toward the functional characterization of the core promoter and relevant enhancer elements, two smaller overlapping genomic clones were obtained by analysis of variance, followed by Student-Newman-Keuls multiple comparison.

**Nuclear Extracts and Gel Shift Assays**—Nuclear extract proteins were prepared from the endometrium as described by Kleis-SanFrancisco et al. (20). Nuclear extract proteins were isolated from HRE-H9 cells as follows. Cells in 100-mm culture dishes were washed with calcium- and magnesium-free Hanks' balanced salt solution and resuspended into the same solution (3 ml/dish). Cells were collected by centrifugation (1875 χ g for 5 min at 4 °C), and the packed cell volume was estimated. Cells were resuspended (5χ packed cell volume) in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.5, 3 mM magnesium acetate, 10 mM KCl and 1 mM dithiothreitol) that contained the protease inhibitors leupeptin (1 μg/ml), antipain (2 μg/ml), benzamidine (10 μg/ml), chymostatin (10 μg/ml), pepstatin (10 μg/ml), phenylmethylsulfonyl fluoride (2 mM). Cells were again collected by centrifugation at 4 °C and resuspended in ice-cold hypotonic buffer containing 0.4% Nonidet P-40. An equal volume of ice-cold hypotonic buffer was added to dilute the detergent to 0.2%. The cell suspension was mixed, and cells were collected by centrifugation at 4 °C. Cells were resuspended in ice-cold hypotonic buffer and homogenized (10 strokes) with a Dounce tissue homogenizer (pestle A). Isolated nuclei were resuspended in nuclear extract buffer (0.4 mM KCl, 20 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 5 mM MgCl₂, and 5 mM dithiothreitol) and placed on ice for 10 min. Nuclei were separated from extract by centrifugation (1500 χ g) for 10 min at 4 °C. Extract was made 15% glycerol (VN) and stored in 20 χ aliquots at –80 °C.

**Tf-TF Interaction Arrays**—One virgin adult female rabbit received subcutaneous injections of progesterone (3 mg/kg/day) for 5 days. Nuclear proteins were extracted from nuclei isolated from the endometrium according to Kleis-SanFrancisco et al. (20). The TransSignal TF-TF Interaction array was processed according to the manufacturer's instructions. Briefly, 50 μg of nuclear extract protein was incubated with biotin-labeled double-stranded oligonucleotide probes, i.e. a library of cis elements. RUSH plus affiliated transcription factors were immunoprecipitated with a mixture (1 μg) of affinity-purified anti-RUSH-1α/β (2) and anti-RUSH-1α (11) antibodies and magnetic protein G beads. For the negative control, the antibodies were replaced with normal IgG. Free cis elements and nonspecific binding proteins were washed away. RUSH-affiliated biotin-labeled probes were eluted from the magnetic beads and hybridized to TransSignal Protein/DNA array membranes. This approach produced an interaction profile for RUSH and 150 unique transcription factors.
by PCR using the larger phage clone as template. A total of 2057 bp of contiguous genomic sequence (GenBank™ accession number AF481732) is shown in Fig. 1. Analysis with FIND-PATTERNS (Genetics Computer Group) and MatInspector Professional 4.2 (Genomatix) revealed several putative binding sites (underlined), including a consensus site or Y-box. In addition this genomic DNA was characterized by multiple GAGA binding elements and alternating pyrimidine-purine elements (CA), which are anisotropically flexible and known to promote DNA bending.

Testing a series of 5'-deletion mutants in two rabbit uterine epithelial cell lines led to the identification of the proximal promoter (~162/+90). Luciferase activity was negligible in both cell types when transfected with the pGL3-Basic vector alone. Maximum transcriptional activation of the RUSH gene was achieved by the ~162/+90 construct versus the ~1812/+90 construct in HRE-H9 (p < 0.05) and RBE-7 (p < 0.001) cells (Fig. 2). Transcriptional repressors from HRE-H9 cells likely bound sequences between ~1812 and ~162. Thus, repression was persistent across 1650 nucleotides in this SV40-transformed rabbit uterine epithelial cell line derived from a human chorionic gonadotropin-primed rabbit. In contrast, repression was relieved (p < 0.05) when the ~1262/+90 construct was tested in RBE-7 cells. Transcriptional activation for ~1262/+90 and ~162/+90 constructs was comparable (p > 0.05) in this SV40-transformed rabbit uterine epithelial cell line derived from a progesterone-primed rabbit (16). Thus, the hormone treatments appear to have altered the expression of one or more factors that mediate transcriptional activation of the RUSH gene.

A Genomatix Promoter Inspector search found a good promoter match (~244/~31) with two putative consensus Sp1 sites centered at ~128 and ~58 (Fig. 1). The sites were authenticated with competition gel shift assays and antibody supershift assays (data not shown). When these sites were evaluated in transient transfection assays, the elimination of individual sites had little or no effect on transcriptional activation of the RUSH promoter. However, dual ablation of these sites produced an increase (p < 0.01) in promoter activity regardless of the cellular background (Fig. 3).
controls. No smaller extension products resulting from secondary RNA structure or nonspecific priming were detected. No alternate initiation sites were identified. The \textit{RUSH}-specific product was analyzed against a sequencing reaction with the same primer used for the primer extension reaction. The amount of RNA and the film exposure time were adjusted to compensate for the low abundance of \textit{RUSH} message. A single 90-bp primer extension product was identified by electrophoresis through polyacrylamide gels under denaturing conditions (Fig. 4). The extension product mapped to the A residue in a putative initiator (Inr) shown in Fig. 1. The original report of the cDNA sequence (GenBank™ accession number U66564) contained 170 bp of 5-UTR. The elimination of individual Sp1 sites had little or no effect on transcriptional activation of the \textit{RUSH} promoter. However, abolition of both sites resulted in a dramatic increase in promoter activity regardless of the cellular background. Assays were performed 3–16 times in quadruplicate. Mean ± S.E. values are based on as few as 12 and as many as 64 data entries.

![Image](240x580 to 563x738)  

\textbf{FIG. 3. The effects of the Sp1 sites on basal transcription.} The elimination of individual Sp1 sites had little or no effect on transcriptional activation of the \textit{RUSH} promoter. No alternate initiation sites were identified. The \textit{RUSH}-specific product was analyzed against a sequencing reaction with the same primer used for the primer extension reaction. The amount of RNA and the film exposure time were adjusted to compensate for the low abundance of \textit{RUSH} message. A single 90-bp primer extension product was identified by electrophoresis through polyacrylamide gels under denaturing conditions (Fig. 4). The extension product mapped to the A residue in a putative initiator (Inr) shown in Fig. 1. The original report of the cDNA sequence (GenBank™ accession number U66564) contained 170 bp of 5-UTR.

A putative DPE is located 29 nucleotides from the putative transcription start site (Fig. 1). Because an authentic DPE is functionally analogous to the TATA box in some eukaryotic promoters, both the Inr and the DPE were authenticated in transient transfection assays with the template in which the Sp1 sites were silent. Mutation of the Inr reduced (\( p < 0.001 \)) basal transcription levels (Fig. 5) in both cell types and confirmed it is the RNA start site. Removal of the DPE by mutation demonstrated it is required for Inr function. Removal of both elements by mutagenesis had no additional deleterious effect (\( p > 0.05 \)) in either cell type. A Blast search against the human genome revealed the \textit{RUSH} proximal promoter (∼184/+54) is 78% identical to the putative promoter of \textit{SMARCA3} (GenBank™ accession number NT_005616) located in a working draft of chromosome 3. Sequences for the two Sp1 sites, the Inr and the DPE, are highly conserved (Fig. 6). Although the putative DPE differs by a single nucleotide in position +30 (T → A), the consensus G(AC)/CG sequence motif is preserved.

The putative PRE half-site/overlapping Y-box (−38/−26), which is not well conserved between the rabbit and human promoters (Fig. 6), was authenticated in competition gel shift assays with nuclear extract from SK-BR-3 cells and with antibodies in supershift assays (Fig. 7). Chromatin immunoprecipitation confirmed the specificity of protein-DNA binding \textit{in vivo} (Fig. 8). The restriction enzyme \textit{Sau}3AI was used to digest chromatin in intact nuclei. Accessible transcriptionally active chromatin was digested \textit{in situ}, and low salt elution conditions optimized the maintenance of protein-DNA complexes during immunoprecipitation. No protein-DNA fragments were immunoprecipitated from antibody-negative controls. A 136-bp region (∼98/+38) of the \textit{RUSH} promoter was isolated by PCR from the total available NF-Y-DNA binding sites (13 of 13 positive clones examined). The same 136-bp region (∼98/+38) of the \textit{RUSH} promoter was isolated by PCR from the total available progesterone receptor-DNA binding sites (13 of 13 positive clones examined). These results confirm the simultaneous binding of NF-Y and progesterone receptor proteins to this unique element in the transcriptionally active \textit{RUSH} promoter.

Mutant constructs were then used to characterize the elements in this domain that are important for promoter activity. Removal of the PRE from either the intact template (−162/+90) or the same template with mutated Sp1 sites drastically repressed (\( p < 0.001 \)) basal transcription of the \textit{RUSH} promoter in HRE-H9 cells (Fig. 9). Comparable results indicating the intact PRE is a powerful activator of basal transcription were obtained for RBE-7 cells (Fig. 9). Transcriptional repression at the Y-box was also demonstrated with this mutagenesis strategy. When the Y-box was functionally altered, transcriptional activation of the \textit{RUSH} gene was increased (\( p < 0.001 \)) in the presence of the Sp1 sites (Fig. 10). In fact transcriptional activation was increased (\( p < 0.01 \)) more dramatically by removal of the Y-box than by removal of the Sp1 sites. However, in the absence of the Sp1 sites removal of the Y-box failed (\( p > 0.05 \)) to augment transcriptional activation.

Mutant constructs were next used to show that the progesterone receptor half-site mediates progesterone signaling. Treatment of HRE-H9 cells with promegestone (R5020) induced transcriptional activation in the presence (\( p < 0.05 \)) and absence (\( p < 0.001 \)) of the Sp1 sites (Fig. 11). When the Y-box was functionally altered, progesterone-dependent transcriptional activation of the \textit{RUSH} gene was increased (\( p < 0.001 \)) in the presence of Sp1 sites (Fig. 11). However, in the absence of the Sp1 sites, removal of the Y-box failed (\( p > 0.05 \)) to

![Image](40181)  

\textbf{FIG. 4. Transcription start site mapping and sequence of the \textit{RUSH} promoter.} Polyadenylated RNA from the endometrium of a progesterone-treated rabbit was reverse-transcribed with a primer that annealed to the 27-most 5′ nucleotides of the published cDNA sequence (shown in blue in Fig. 1). The single, 90-bp primer extension product aligned with an A residue in the sequencing ladder that was generated with the same primer.
Augment progesterone-dependent transcriptional activation.

A search of the TFMatrix transcription factor binding site profile database (version 1.3) was performed with the RUSH genomic sequence as query. This search revealed the putative RUSH site (H11002) shared a score of 89% (100% identity; threshold 85%) with an Ets domain (GGA(A/T)) that partially overlapped on the complementary strand. Chromatin immunoprecipitation confirmed the specificity of RUSH-DNA binding in vivo (Fig. 8). Accessible, transcriptionally active chromatin was digested in situ, and low salt elution conditions optimized the maintenance of RUSH-DNA complexes during

**Fig. 5.** The DPE functions cooperatively with the Inr to direct transcription. The Inr was identified in affiliation with the transcription start site in Fig. 4. The DPE was subsequently identified 29 nucleotides downstream. The DPE adheres to the strict spacing requirement. Mutation analysis shows it is required for basal promoter activity. Assays were performed 3–6 times in quadruplicate. Mean ± S.E. values are based on as few as 12 and as many as 36 data entries.

**Fig. 6.** Sequence alignment of the rabbit RUSH promoter with the putative human promoter (version BLASTN 2.2.3). Note the highly conserved Inr/DPE combination as well as the Sp1 sites. In contrast, it unlikely that NF-Y will bind to the PRE/Y-box combination in the putative human promoter because of the absolute requirement of NF-Y for the CCAAT pentanucleotide.

**Fig. 7.** NF-Y and progesterone receptor binding (PRE) sites were authenticated by competition gel shift assays. Nuclear extract from SK-BR-3 cells was used as a commercially available source of both proteins. Panel A, gel shift assays confirm the specificity of NF-Y binding in nuclear extract from SK-BR-3 cells (left arrow). Lane 1, labeled PRE-Y-box (45/15) probe alone; lane 2, labeled PRE-Y-box probe plus nuclear extract (5 µg); lane 3, same as lane 2 plus a 100-fold molar excess of unlabeled consensus Y-box probe; lane 4, same as lane 2 plus affinity-purified goat anti-human NF-Y subunit A (10 µg). The supershift assay (*) is always diffuse. Panel B, progesterone receptor binds the PRE in the RUSH promoter (right arrow). Lane 1, labeled PRE-Y-box probe with the Y-box binding site mutated; lane 2, labeled mutated probe plus nuclear extract; lane 3, same as lane 2 plus a 100-fold molar excess of unlabeled PRE probe; lane 4, same as lane 2 plus affinity-purified rabbit anti-human progesterone receptor. The supershift assay (*) is always diffuse.

**Fig. 8.** In vivo binding of Y-box, progesterone receptor, and RUSH proteins to the RUSH promoter was confirmed by PCR amplification of products from individual chromatin immunoprecipitation assays. The left lane shows the dX174/HaeIII size markers. The second and third lanes show the single, 136-bp amplons resulting from immunoprecipitation with antibodies to NF-Y and progesterone receptor, respectively. The fourth lane shows the single, 168-bp amplon resulting from immunoprecipitation with antibodies to RUSH. The fifth and sixth lanes contain negative immunoprecipitation (IgG) and PCR (water blank) controls, respectively.

Augment progesterone-dependent transcriptional activation.

A search of the TFMatrix transcription factor binding site profile database (version 1.3) was performed with the RUSH genomic sequence as query. This search revealed the putative RUSH site (−616/−611) shared a score of 89% (100% identity; threshold = 85%) with an Ets domain (GGAG/V) that partially overlapped on the complementary strand. Chromatin immunoprecipitation confirmed the specificity of RUSH-DNA binding in vivo (Fig. 8). Accessible, transcriptionally active chromatin was digested in situ, and low salt elution conditions optimized the maintenance of RUSH-DNA complexes during
immunoprecipitation with RUSH-specific antibodies. No protein-DNA fragments were immunoprecipitated from antibody-negative controls. A 168-bp region (−712/−545) of the RUSH promoter was isolated by PCR from the total available RUSH-DNA binding sites (9 of 9 positive clones examined). These results confirmed that endogenous RUSH binds this consensus site in its own transcriptionally active promoter. Transient transfection assays with mutant constructs showed that elimination of the RUSH binding site increased \( p < 0.0001 \) transcriptional activation of the promoter (Fig. 12) in HRE-H9 cells. The TranSignal TF-TF interaction array, which profiled the physical interaction of RUSH with 150 unique transcription factors, showed putative interactions with CREB (cAMP-response element-binding protein), AP2, EGR, NFATc, PAX-5, c-Rel, MEF-2, GATA-3, and GATA-4 (data not shown). No physical interactions were identified for Ets transcription factor family members, and none of the proteins with strong RUSH interactions bound in close proximity (3–4 bp) to the RUSH site. These data support the conclusion that RUSH can act alone to repress its own promoter.

**DISCUSSION**

RUSH is the only SMARCA3-related transcription factor known to be regulated by a steroid-dependent alternative splicing mechanism (2). Recent studies on the coordinate regulation of transcription and pre-mRNA processing suggest promoter architecture is integral to this process (21–23). The first step toward understanding the potential involvement of steroids in orchestrating transcription with alternative splicing of the RUSH gene required the systematic analysis of its promoter. Individual RUSH/luciferase constructs plus control plasmid expressing the Renilla gene were transfected into two cell lines that were developed from normal differentiated rabbit uterine epithelium by transformation with a temperature sensitive (ts) SV40, tsA209 virus. The cell line derived from a human chorionic gonadotropin-primed rabbit was called HRE-H9 (15), and the cell line derived from a progesterone-primed rabbit was called RBE-7 (16). The strength of individual constructs normalized for transfection efficiency was expressed as a ratio of luciferase to Renilla reporter activities. Negative control values were negligible. Putative cis elements were variously authenticated by gel shift assays, competition shift assays, supershift assays, and chromatin immunoprecipitation assays. Mutation analysis was used to characterize the elements in transient transfection assays.

Dramatic differences attributable to cellular background were identified when truncation mutants were tested. Significant repression occurred in the −1812/−162 region of RUSH in HRE-H9 cells compared with RBE-7 cells. By comparison, little difference was noted between the cell types when elements in the −162/+90 region were tested. The initial goal was to evaluate cell type-specific regulation of RUSH in two similarly derived cell lines. This goal was realized when different DNA-protein complexes, presumably resulting from the hormone treatments, contributed to the complexity of RUSH regulation. RUSH does not have a TATA motif in the vicinity of the transcription start site, as mapped by primer extension. A unique Inr-DPE combination directs accurate initiation of RUSH transcription. This combination is strictly maintained within the SMARCA3 core promoter (−35/−35). Although DPE elements are as common as the TATA-box in the promoters of *Drosophila* genes (24), they are rarely found in mammalian promoters. Functional DPE elements have been identified in only three human promoters, i.e. the IRF-1 (25), CD30 receptor (26), and TAF(II)55 (27) genes. The highly conserved nature of the Inr-DPE cassette in the RUSH and SMARCA3 genes suggests some aspects of transcriptional activation are also conserved. This concept is underscored by the conservation of two GC-rich sequences that bind the ubiquitous transcription factor, Sp1. Although Sp1 can exert either positive or negative effects on transcription (28), it exerts a negative effect on basal transcription of the RUSH promoter. This finding was somewhat surprising because Sp1 tends to be a strong activator of
Steroid-dependent genes in the endometrium (29). For example, Sp1 mediates estrogen induction of uteroglobin in uterine epithelium (30).

Basal transcription of RUSH is regulated by multiprotein Sp1/PRE/NF-Y interactions in the –131/–26 region of the promoter. Data in Fig. 3 show that the Sp1 sites bind repressors of RUSH transcription. However, the bipartite PRE/Y-box element, which is not conserved in the putative promoter of SMARCA3, provides a site for either activation or repression (Figs. 9 and 10). The PRE half-site promotes basal transcription in cells that are progesterone receptor-positive (16, 31, 32). It also mediates progesterone-dependent transcription (Fig. 11) in HRE-H9 cells, the most thoroughly characterized in terms of steroid responsiveness (31, 32). The other component part of this site is the Y-box or CCAAT sequence, which is found in the reverse orientation (ATTGG), a common occurrence in higher eukaryotes (33). NF-Y, a heteromeric transcription factor, also known as CCAAT binding factor (CBF), is responsible for repression of RUSH. Fine-tuning the magnitude of progesterone-induced RUSH expression appears to depend on the availability of progesterone receptor and NF-Y proteins, which are capable of simultaneous, independent binding to the bipartite PRE/Y-box complex. The demonstration that transcription factor NF-Y antagonizes progesterone-induced RUSH expression in a DNA binding-dependent fashion is the first of its kind. Other reports of NF-Y-mediated steroid action are DNA binding-independent. For example, NF-Y repression of estrogen receptor-α-mediated trans-activation of the FXII promoter does not involve competition between the two factors at their overlapping binding sites (34) because NF-Y antagonism does not require binding to the CCAAT elements. Induction of the murine multidrug resistance gene mdr1b by progesterone occurs via a Y-box and does not require a progesterone response element (35). And finally, transcriptional activation of the E2F2 gene by estrogen is regulated by NF-Y/Sp1/estrogen receptor interactions that do not include an estrogen response element (36).

Sp1 and NF-Y may also regulate basal transcription by inducing conformational changes in chromatin. Ultimately they could be responsible for the recruitment of transcription machinery through DNA-protein and/or protein-protein interactions. A Y-box in the reverse CCAAT motif is located in the upstream uteroglobin gene enhancer. Found in close proximity to the progesterone receptor response elements, this binding site is associated with chromatin remodeling (30). Autorepression by RUSH could also be achieved by this mechanism. The rationale for this scenario is based on the fact that the RUSH binding site in the uteroglobin promoter (11) is part of a composite regulatory element with overlapping binding sites for three proteins capable of initiating chromatin-opening events. The element is uniquely designed to invite chromatin restructuring within the context of many different cell types. RUSH may also conduct long distance interactions with DNA-bound transcription factors. Potential candidates were identified with the TransSignal TF-TF interaction array. This experiment confirms a previously identified physical affiliation between RUSH and GATA-4 (11). Perhaps, as Wolfgang et al. (37) suggested, autorepression of promoter activity is an effective strategy to achieve transient expression of an inducible gene. This appears to be true for c-fos (38), ICER (39), c-myc (40), and c-rel (41).

In conclusion, this initial characterization of the structural and functional organization of the promoter and 5-flanking region of the RUSH gene provides the first step toward understanding the molecular mechanisms that regulate RUSH expression. We have now established that combinatorial regulation of RUSH includes strong transcriptional activation by an Inr-DPE core promoter that can be preferentially activated or repressed by a PRE/Y-box at its very 3’ edge. Sp1 and RUSH

**Fig. 11.** The PRE half-site mediates progesterone-dependent RUSH expression. Progesterone (R5020) induced transcriptional activation of the RUSH promoter in the presence ($p < 0.05$) and absence ($p < 0.001$) of the Sp1 sites. The selective elimination of the Y-box in the presence of the Sp1 sites produced a significant increase ($p < 0.001$) in progesterone-one-induced RUSH transcription. Assays were performed 2–9 times in quadruplicate. As a result, mean ± S.E. values are based on as few as 8 and as many as 36 data entries.

**Fig. 12.** Site-directed mutagenesis of the RUSH site. The selective elimination of the RUSH site leaving all other elements intact produced an increase ($p < 0.0001$) in transcriptional activation. Assays were performed 9 times in quadruplicate. Mean ± S.E. values are based on 36 data entries.
sites bind repressors of transcription. Thus, negative regulation of RUSH by Sp1, NF-Y, and its own gene product indicates that suppression of transcription is the major mechanism controlling basal expression. DNA-bound Sp1 and NF-Y proteins also play a central role in antagonizing the effects of progesterone after direct binding of the receptor to a PRE half-site motif. Although progesterone-dependent transactivation appears unique to RUSH, transcriptional control at the core promoter is likely to be conserved in the human SMARCA3 gene.

Acknowledgment—We thank Dr. J. C. Daniel, Jr., Eminent Scholar at Old Dominion University (Norfolk, VA), for stimulating discussions.

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