Identification of the RUSH Consensus-Binding Site by Cyclic Amplification and Selection of Targets: Demonstration that RUSH Mediates the Ability of Prolactin to Augment Progesterone-Dependent Gene Expression

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RUSH-1α(β) transcription factors were cloned by recognition site screening with an 85-bp region (−170/−85) of the rabbit uteroglobin gene. Deletion analysis showed this region was essential to prolactin (PRL) action, but conclusions were limited by the complexity of the large deletion. Cyclic amplification and selection of targets (CASTing) was used to identify the RUSH-binding site (−126/−121). Endometrial nuclear proteins were incubated with a pool of degenerate oligonucleotides and immunoprecipitated with RUSH-1α(β) antibodies. Bound DNA was amplified by PCR. The consensus motif (MCWTDK) was identified after five rounds of CASTing, authenticated by CASTing with RUSH-1α-specific antibodies and recombinant protein, and refined with EMSA. Dissociation rate constants (Kd = 0.1–1.0 nM; r = 0.99) revealed high-affinity binding. Chromatin immunoprecipitation confirmed in vivo binding of RUSH to the transcriptionally active uteroglobin promoter. CASTing also revealed RUSH-GATA transcription factor interactions. Endometrial GATA-4 expression is progesterone dependent (Northern analysis) and preferentially localized in the epithelium (in situ hybridization). Although physically affiliated with RUSH, uterine forms of GATA-4 were not required for RUSH-DNA binding. Site-directed mutagenesis and transient transfection assays showed the RUSH motif mediates the ability of PRL to augment progesterone-dependent uteroglobin transcription. RUSH is central to the mechanism whereby PRL augments progesterone-dependent gene transcription.

PRL receptor-deficient phenotype, Ihle (5) has tended the opinion that all physiological functions mediated by PRL require Stat5a/b. However, PRL has more than 300 different biological activities, many of which are achieved in concert with steroid hormones (1). Moreover, very few authentic PRL target genes (β-casein) have been identified.

RUSH, a member of the Smarca3 family of SWI/SNF-related chromatin remodeling machines, interacts with an inner nuclear membrane protein (6) and binds to the proximal promoter (−170/−85) of the uteroglobin gene (7). Deletion analysis showed this region is essential to PRL signal transduction (8, 9). However, our conclusions were limited by its size and complexity. In addition to a putative RUSH-binding site, the region is characterized by a 7-bp identical inverted repeat (CAGTTTC) that is found in a long terminal repeat (−171/−148) common to all murine leukemia viruses and proviruses (10), and by two hepatocyte nuclear factor 3 (HNF3)-response elements centered at −130 and −95 (11) that completely overlap two octamer transcription factor 1 (Oct-1)-binding sites. In contrast, the absence of Stat5a/b-binding elements is compelling evidence that the primary sig-
naling mechanism is not via the Jak/Stat pathway. The rationale for defining the sequence-selective interactions of RUSH with DNA is supported by these findings.

Initially, the RUSH target search was reduced from $-170/-85$ to $-160/-110$ (12). Here we report the use of cyclic amplification and selection of targets (CASTing), a PCR-based immunodetection method that is useful for determining the DNA binding specificity of low-abundance proteins (13), to identify the RUSH-binding site. This method, which is powerful enough to identify a DNA-binding site that occurs only once in the mammalian genome (14), required crude nuclear extract as a source of binding factors and a synthetic oligonucleotide with a sufficient number of internal random bases (26-bp) to accommodate the preferential isolation of multicomponent sites (15) based exclusively on the presence of RUSH. Although the immediate goal was to define the DNA sequence recognized by RUSH, we also show there is a physical interaction between RUSH and GATA-4 that is not required to regulate uteroglobin gene expression in the rabbit uterus. The question of whether or not RUSH, a putative helicase, was transiently affiliated with DNA was eliminated because protein–protein interactions that alter transcriptional activity but do not affect DNA binding are not detected by CASTing (13). Ultimately, we used site-directed mutagenesis to show that the RUSH target sequence is used by PRL to augment progesterone-dependent transcriptional activation of the uteroglobin gene. The identification of an alternative to the Jak/Stat pathway has allowed us to consider a broader role for PRL in modulating steroid-dependent gene transcription.

RESULTS AND DISCUSSION

CASTing to Identify the RUSH Motif

In the CASTing method described here, proteins from nuclear extract were immunoprecipitated as putative multiprotein complexes before exposure to the pool of random sequence oligonucleotides (16). This strategy eliminated the potential exposure of the oligonucleotides to nucleases in the extract. The 76-base oligonucleotides with a random group of 26 bases flanked by PCR priming sequences comprised a $4^{26}$ or $4.5 \times 10^{16}$-fold degenerate oligonucleotide pool (15). After synthesis of the complementary strand by priming with the reverse primer, approximately $10^{14}$ unique double-stranded sequences were used in the first round of binding site selection. Discounting the PCR flanking sequence and assuming the average protein-binding site is a hexamer, the 26-bp degenerate core of each double-stranded oligomer contained 20 possible positions for a 6-bp sequence. Thus, in the initial round of CASTing, $20 \times 10^{14}$ unique sites were available for protein binding. The ratio of specific binding sites to random sequences was increased in subsequent rounds of CASTing because the highest-affinity interactions were selected (13). The number of PCR cycles was kept to a minimum, i.e. 15 cycles were routinely required to obtain a visible band of DNA that could be extracted from the gel. Minimal PCR amplification helped to reduce the formation of heteroduplexes that resulted from the reannealing of products that were mismatched in the 26-bp central region.

The RUSH consensus-binding site was determined by CASTing (Table 1). A hexameric core sequence of $C/\text{ACT}/\text{ATNT}/G$ (MCWNTK), which is at times palindromic (ACATGT), was identified in 38.5% of the sequences after five cycles of selection. Because the fraction of random sequences appeared to be significant, probably because RUSH is a low-abundance protein and because there was some nonspecific binding of the antibody, the number of CASTing cycles was extended to 20. Sequences were cloned and examined at regular intervals to show that 38.5% of a total of 96 sequences contained the consensus-binding site. Despite the inability to enrich beyond this level, the probability $[P = 20 \div 128 (6!)/2]$ of such a match occurring in either orientation in sequences with 20 potential hexameric binding sites is $1$ in $2304$. This $1:2304$ probability would occur less than once in 96 sequences, making the presence of this binding site in the examined sequences highly significant.

RUSH proteins are products of alternatively spliced messages (7). RUSH-1$\alpha$ is the predominant protein expressed in the progesterone-dominated rabbit endometrium. RUSH-1$\beta$ is negligible. The proteins have the same DNA-binding domain, and both proteins are recognized by the RUSH-1$\alpha$($\beta$) antibodies. The use of these antibodies should have resulted in optimal immunoprecipitation of RUSH–DNA complexes. Nevertheless, antibodies against specific epitopes can disrupt critical protein–DNA or protein–protein interactions. To eliminate this potential problem, CASTing was repeated with RUSH-1$\alpha$ antibodies that selectively bind the predominant RUSH-1$\alpha$ protein. After five cycles of CASTing, the hexameric consensus sequence was present in 60% of the sequences.

Although CASTing with nuclear extract allowed for the identification of multicomponent interactions, it was also important to determine whether RUSH alone had the ability to specifically bind to the consensus sequence. The hexameric consensus sequence was present in 47% of the sequences after CASTing (five cycles) with recombinant RUSH. As a result of these additional experiments, the consensus shown in Table 2 was derived from the analysis of 59 sequences derived from five cycles of CASTing. However, the frequency of C in position 5 of the RUSH consensus was less than 10%. The fact that the frequency of occurrence was less than 25%, the frequency expected for a random base, was justification for the exclusion of this base from the consensus. Moreover, binding was negligible when sequences with a C in this position were tested in gel
shift assays with nuclear extract proteins. Thus, the optimal consensus binding sequence is C/ACT/ATA/T/GT/G (MCWTDK).

**Binding Analysis of Individual Selected Sites**

Representatives of the eight different groups (Table 1) that contribute to the consensus binding sequence were tested in competition gel shift assays. Scatchard (17) analysis of specific binding data revealed the affinity of RUSH-DNA binding varied more than 1 order of magnitude [dissociation constant ($K_d$) $= 0.1 - 1.0 \text{ nM}$; $r = 0.99$]. For example, RUSH has 8 times the residence time ($K_d = 0.15 \text{ nM}$) on sequence 39 (Table 1) compared with sequence 5 ($K_d = 1.18 \text{ nM}$). The expression of various target genes could be differentially regulated by the duration of RUSH-DNA residence. In addition, binding of group 6 sequences (CCTTNG) to RUSH was negligible.

Table 1. Alignment of RUSH-Binding Sites Obtained from CASTing with Nuclear Extract

| Group | EMSA | RUSH Site |
|-------|------|-----------|
| ACTTN | +    | TAGTTG    | GAGTCACATACGAGTT |
|       | +    | ACTTGTTCTCATTTGT | ACTTGTTCTCATTTGT |
|       | +    | ACTGTTCTCATTTGT | ACTGTTCTCATTTGT |
|       | +    | CAGCATGGGA | ACTTGTTCTCATTTGT |
|       | +    | TACTAGAGC | ACTTATGAGAGAAG |
|       | +    | AGTAGC | ACTTATGAGAGAAG |
|       | +    | TTATCTTTTAAAAACTCA | ACTTATGAGAGAAG |
| ACTTTG |      | GCTTTACCACCATATTATA | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
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|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
|       | +    | ACTTTG | ACTTTGTTCTCATTTGT |
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Sequences from the eight core groups were tested by EMSA. Thirteen putative GATA sites are underlined. Putative Oct-1 (lines 13 and 39) and HNF3b (line 17) sites are in bold italics. Asterisks show the RUSH site as found in the rabbit uteroglobin promoter (*) and the only sequence with GG in the last two positions (**). N designates A, T, G, or C, and W designates A or T.
Six of 59 sequences, five from CASTing with nuclear extract and RUSH-1α(β) antibodies and one from CASTing with nuclear extract and the α-specific antibody, contained two or three RUSH-binding sites. The low frequency of expression of multiple binding sites, coupled with the fact that binding affinity was within the range for single sites, suggests that RUSH binding is noncooperative. Negative controls for the gel shift assays included oligonucleotides with flanking sequence plus three or four consecutive elements of the RUSH-binding site. Probe 13, a representative of group 3 (ACATWT Table 1) was used to further confirm the specificity of RUSH binding. The experiment depicted in Fig. 1 shows that RUSH-specific binding was eliminated when immunodepleted nuclear extract, as verified by Western analysis, was used as the protein source. RUSH-specific binding was eliminated by competition with a 100-fold molar excess of unlabeled probe 13, but not eliminated by competition with a negative control probe that had two partial RUSH sites (four of six bases at each site). The supershift assay with antibody to RUSH-1α(β) confirmed RUSH-specific binding. Its diffuse appearance supports the suggestion that RUSH is a member of a multiprotein complex.

**In Vivo RUSH-Target Sequence Confirmed**

Chromatin immunoprecipitation confirmed the specificity of RUSH-DNA binding in vivo. The restriction enzyme Sau3A I was used to digest chromatin in intact nuclei isolated from the endometrium of a rabbit treated sequentially with ovine PRL (oPRL) + progesterone. 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 110-bp fragment (−180/−70) of the uteroglobin promoter was isolated by PCR from the total available RUSH-DNA-binding sites (10 of 10 positive clones examined). These results confirm that endogenous RUSH binds to its consensus-binding site in the uteroglobin promoter when the gene is transcriptionally active. The next challenge will be to identify biologically relevant RUSH-binding sites in other target genes.

**Characterization of the RUSH Motif as a Subelement of HNF3 and Oct-1**

As shown in Fig. 2A, the RUSH-binding site (−126/−121) is a subelement of the HNF3- and Oct-1-binding sites. Analysis with MatInspector V2.2 (Ref. 18; available at http://www.gsf.de/cgi-bin/matssearch2.pl) identified the consensus match for HNF3 as a putative binding site for HNF3β. However, Braun and Suske
used transfection assays to show that both HNF3/\textit{H9251} and HNF3/\textit{H9252} enhanced transcriptional activation of the rabbit uteroglobin promoter. Thus it was important to determine whether HNF3- or Oct-1-binding sites were preferentially isolated with RUSH-binding sites. If confirmed, these results would support the idea that nucleotides outside of the consensus sequence influence the relative binding of RUSH proteins, or that RUSH binding to the \textit{H11002/11002136/121} region of the promoter is affected by the presence of additional proteins such as HNF3 and/or Oct-1. Evidence that RUSH does not require HNF3 or Oct-1 binding is found in Table 1. CASTing for multi-protein complexes from nuclear extract, a source of multiple factors, revealed a very low frequency of occurrence of the other binding sites with RUSH. One of 59 sequences had a putative HNF3/\textit{H9252}-binding site, and 2 of 59 sequences had putative Oct-1-binding sites.

The possible involvement of GATA transcription factors in a DNA-binding complex with RUSH was suggested by CASTing experiments with nuclear extract from uterine endometrium. Table 1 shows the sequence of 39 clones obtained after CASTing with RUSH-specific probes. Lane 1, labeled probe (no. 17; Table 1) that contains putative HNF3/\textit{H9252} and GATA-binding sites plus endometrial nuclear extract (NE, 5 \(\mu\)g) from the endometrium of a progestosterone-treated rabbit; lane 2, labeled probe (no. 13; Table 1) that contains a putative Oct-1-binding site + NE. The arrow on the left indicates the single, slow migrating RUSH-specific complex.

**RUSH-GATA Interaction**

The possible involvement of GATA transcription factors in a DNA-binding complex with RUSH was suggested by CASTing experiments with nuclear extract from uterine endometrium. Table 1 shows the sequence of 39 clones obtained after CASTing with RUSH-specific probes. Lane 1, labeled probe (no. 17; Table 1) that contains putative HNF3/\textit{H9252} and GATA-binding sites plus endometrial nuclear extract (NE, 5 \(\mu\)g) from the endometrium of a progestosterone-treated rabbit; lane 2, labeled probe (no. 13; Table 1) that contains a putative Oct-1-binding site + NE. The arrow on the left indicates the single, slow migrating RUSH-specific complex.
77–80% identical with rabbit GATA-4. Therefore, the same RT-PCR strategy that was used to obtain the conserved zinc-finger DNA-binding domain of GATA-1 in the mouse testis (21) was used to obtain a 217-bp amplicon from endometrium. A computer search of sequence databases with the BLASTN 2.2.2 program at the National Center for Biotechnology Information revealed that 167 bp of sequence (GenBank accession no. AF467985) that was not forced by PCR priming was 83% identical with the partial coding sequence of GATA-4 (GenBank accession no. 8648976) from rabbit heart, which is 94% identical with the coding sequence of human GATA-4 (GenBank accession no. 50483).

Northern analysis with the 217-bp amplicon was used to evaluate mRNA expression in rabbit endometrium. Figure 3 shows two endometrial transcripts with molecular sizes of 3.2-kDa and 2.5-kDa were detectable in poly(A⁺) RNA from estrous animals. In a physiologically relevant comparison, steady state levels of both transcripts were increased 10-fold in progesterone-dominated, 5-d pseudopregnant rabbits. The hormone dependency of message expression was further confirmed when transcript levels were elevated 3-fold by the treatment of estrous rabbits with progesterone, and decreased 16-fold by the sequential treatment of estrous animals with progesterone and estradiol benzoate. *In situ* hybridization (Fig. 4) showed the spatial expression of putative uterine GATA-4 compares favorably with the Northern analysis. Message was abundantly expressed in the endometrium of 5-d pseudopregnant rabbits compared with estrous rabbits.

Gel shift assays were used to examine the binding pattern of endometrial nuclear extract proteins from a progesterone-treated rabbit to a GATA consensus oligonucleotide (Fig. 5A). The specificity of endometrial GATA-DNA binding was confirmed by gel shift competition assay. A 100-fold molar excess of unlabeled GATA oligonucleotide completely abrogated GATA binding, whereas a 100-fold molar excess of a mutant probe with two “GA” to “GT” substitutions in the binding region (22) did not (Fig. 5A). In companion experiments, competition with the GATA probes failed to have any affect on RUSH binding to the uteroglobin promoter (Fig. 5B). These results support the previous conclusion that RUSH does not require additional transcription factors for DNA binding, and they are particularly meaningful to experiments in which mutagenesis of the RUSH-binding site also altered the GATA site.

A physical interaction between RUSH and GATA proteins either directly or via a protein intermediate was confirmed when immunodepletion of nuclear extract with RUSH-1α(β) antibodies (Fig. 5C) also eliminated GATA-specific binding. Companion experiments with nuclear extract that was immunodepleted with GATA antipeptide antibodies were unsuccessful because neither of the commercially available antibodies for GATA-1 or GATA-4 appeared to recognize epitopes in the rabbit proteins (Western data not shown). Thus the conclusive identification of uterine GATA as GATA-4 requires additional experimentation.

Fine mapping of the −160/−110 region of the uteroglobin promoter revealed that the AT-rich region −132/−123 was critical for transcriptional activation (12). It is now clear that this region (−136/−115) is a composite regulatory element (Fig. 2A) with overlapping binding sites for three proteins capable of initiating chromatin-opening events. Both HNF3 and GATA-4 can bind nucleosome arrays and open compacted chromatin in the absence of ATP-dependent enzymes (23). RUSH proteins are SWI/SNF-related ATPases (24, 25). This site appears uniquely designed to invite chromatin restructuring within the context of many different cell types.

### The RUSH Motif Mediates the PRL Effect

To show that the RUSH-binding site mediates the action of PRL, control and hormone-dependent transcriptional activities were measured for the full-length uteroglobin gene, pUG3.1-LUC, and the RUSH mutant, pUG3.1M-LUC. HRE-H9 cells were pretreated with estradiol (10−7 M) and oPRL (10−8 M) for 48 h as previously described (8, 26). After transfection, cells were used as untreated controls, treated with progesterone (10−7 M) alone, or treated with oPRL (10−8 M) + progesterone. As shown in Fig. 6, oPRL alone failed to stimulate transcription above control levels. Progesterone alone increased (P < 0.05) the transcriptional activity of pUG3.1-LUC. Transcription was further increased (P < 0.05) when cells were treated with oPRL + progesterone. Progesterone alone also increased (P < 0.05) the transcriptional activity of pUG3.1M-LUC. However, mutation of the RUSH-binding site, tested here with pUG3.1M-LUC, was highly deleterious to the PRL effect (Fig. 6). Using this mutational approach, it was possible to eliminate (P > 0.05) the PRL-mediated increase in uteroglobin gene transcription while retaining a progesterone-dependent increase (P < 0.05) in transcription.

![Fig. 3. Northern Analysis Reveals GATA Expression Is Progesterone Dependent in the Endometrium](image-url)
Proposed Mechanism Whereby PRL Acts via RUSH to Augment Progesterone-Dependent Gene Expression

A schematic representation of the classic Jak/Stat and putative RUSH signal transduction pathways initiated by activation of the PRL receptor are shown in Fig. 7. In the Jak/Stat pathway, members of the Stat protein family are the central transducer molecules (1, 2). Stats, which share five conserved regions that include a DNA-binding domain, are phosphorylated by the receptor-associated tyrosine kinase known as Jak2. Phosphorylated Stats hetero- or homodimerize and translocate to the nucleus where they initiate gene transcription via a γ-interferon-activated sequence element. RUSH nucleophosphoproteins, which share domains typical of ATPases and DNA helicases, a putative nuclear localization signal, a DNA-binding domain, and a RING finger motif, coimmunoprecipitate with Jak2 (Fig. 8), suggesting they too are phosphorylated by the receptor-associated mechanism. In the nucleus, RUSH interacts via its RING finger with an atypical type IV P-type ATPase in the inner nuclear membrane (6) and augments gene transcription in a sequence-selective manner. Unlike Stat proteins that activate otherwise silent targets, RUSH transcription factors enhance progesterone-dependent transcriptional activation of its targets. This sequence-selective interaction of RUSH with the uteroglobin promoter is believed to be the prototypical mechanism whereby PRL augments the

Fig. 4. Localization of GATA mRNA in Rabbit Endometrium by in Situ Hybridization

A, B, E, and F, Bright- and dark-field photomicrographs of sections probed with antisense GATA riboprobes. C, D, G, and H, Bright- and dark-field photomicrographs of sections probed with sense GATA riboprobes as negative controls. A and B, Sections of endometrium from 5-d pseudopregnant rabbits reveal abundant expression of GATA mRNA in glandular and luminal epithelium with modest expression in the stroma. C and D, Negative controls for A and B, respectively. E and F, Sections of endometrium from estrous rabbits reveal minimal expression of GATA mRNA in the epithelium and negligible expression in the stroma. G and H, Negative controls for E and F, respectively. Bar, 145 μm.

Fig. 5. GATA Binding Was Characterized by EMSA

A, EMSA confirms specificity of GATA binding in endometrium. Lane 1, Labeled GATA probe alone; lane 2, labeled GATA probe + endometrial nuclear extract (NE, 5 μg) from the endometrium of a progesterone-treated rabbit; lane 3, same as lane 2 + 100-fold molar excess of unlabeled GATA probe; lane 4, same as lane 2 + 100-fold molar excess of mutated GATA probe. B, RUSH binding to the uteroglobin promoter is unaffected by GATA probes. Lane 1, Labeled UG200 (−194/+9), the DNA fragment first used to identify RUSH binding (7) to the uteroglobin promoter; alone; lane 2, labeled UG200 + NE; lane 3, same as lane 2 + 100-fold molar excess of unlabeled GATA probe; lane 4, same as lane 2 + 100-fold molar excess of mutated GATA probe. C, Confirmation of a physical interaction between RUSH and GATA transcription factors. Lane 1, Labeled GATA probe alone; lane 2, labeled GATA probe + NE; lane 3, same as lane 2, with anti-GATA-4 antibodies (3 μg); lane 4, same as lane 2 except that NE was precleared; lane 5, same as lane 2 except that NE was immunodepleted with RUSH-1α(β) antibodies.
transcriptional activation of progesterone-dependent target genes.

In conclusion, CASTing was used to identify the consensus motif (MCWTDK) for the RUSH-binding site. In vivo binding of RUSH to this motif in the transcriptionally active promoter of the uteroglobin gene was confirmed by chromatin immunoprecipitation. RUSH binding was independent of other transcription factors. Site-directed mutagenesis and transient transfection assays showed that the region of the
uteroglobin promoter that mediates the synergy between progesterone and PRL binds RUSH. Collectively, these data support the working model that RUSH mediates the ability of PRL to augment progesterone-dependent uteroglobin gene transcription.

**MATERIALS AND METHODS**

**Reagents, Antibodies, and Tools for PCR**

Protein A-Sepharose CL-4B, poly(dI-dC), horseradish peroxidase-conjugated donkey antirabbit IgG, and all isotopes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). NucleoTrap Nucleic Acid Purification Kit and TagStart antibody were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The GeneAmp RNA PCR Core Kit and AmpliTaq Gold DNA polymerase were purchased from Applera Co. (Foster City, CA). TaKaRa ExTaq enzyme and 10× ExTaq buffer were purchased from PanVera Corp. (Madison, WI). The pCRRII-TOPO vector, a component of the TA Cloning Kit, was purchased from Invitrogen (San Diego, CA). NuSieve GTG agarose was purchased from BioWhittaker, Inc. (Rockland, ME). QIAEX II was purchased from QIAGEN (Santa Clarita, CA). TriReagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). Human choriconic gonadotropin was purchased from Sigma (St. Louis, MO). TNT coupled Reticulocyte Lysate, pG3L-Basic vector, pRL-TK vector, and dual-luciferase reporter assay system were purchased from Promega Corp. (Madison, WI). The QuikChange Site-Directed Mutagenesis Kit that contains Phu Turbo DNA polymerase, 10× reaction buffer, and DpnI endonuclease; and the phagemid pBluescript II SK(−) were purchased from Stratagene (La Jolla, CA). Pronase protease was purchased from Calbiochem (La Jolla, CA). Staff at Midland Certified Reagent Co. (Midland, TX) synthesized the mutagenesis primers. The random sequence oligonucleotide R76 plus the forward and reverse CASTing (PCR) primers were designed according to Pollock and Treisman (15) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Protein A/G PLiUS-Sepharose, GATA gel shift oligonucleotides (consensus and mutant), and goat polyclonal antibodies to GATA-1 (M-20) and GATA-4 (C-20, C-20X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antimouse Jak2 whole antisera was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phenol red-free a-modified MEM, phenol red-free OPTI-MEM 1, and the Li/pofectAMINE PLUS Reagent package were purchased from Life Technologies (Gaithersburg, MD). Defined fetal bovine serum was purchased from HyClone Laboratories, Inc. (Logan, UT). Dr. A. F. Parlow, Scientific Director, National Hormone and Pituitary Program (Torrance, CA), generously provided the oPRL.

RUSH-1α(β) antipeptide antibodies (7, 9) recognize both α and β because they were made to sequences (amino acids 370–387) common to both proteins. For this study, antipeptide antibodies were also made to a unique region of RUSH-1α. The C-terminal portion of RUSH-1α consists of 173 amino acids with seven regions that display strong antigenicity as indicated by the PeptideStructure program (Genetics Computer Group, Madison, WI). As a result, antibodies to amino acids 932–951 were generated in rabbits by staff at Research Genetics, Inc. (Huntsville, AL). The titer of each antibody was determined by ELISA with free MAP-peptide on the solid phase (1 μg/well), goat antirabbit IgG-horseradish peroxidase conjugate as the secondary antibody, and peroxidase dye. To eliminate complications from nonspecific binding, both antibody preparations were purified by affinity chromatography on columns of immobilized antigen. Antibody-RUSH binding was authenticated by Western analysis. For EMSA and coimmunoprecipitations, small aliquots of affinity-purified antibody (600 μl) were dialyzed against two changes × 2 liters each of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at 4°C.

A 50:50 slurry (wt/vol) of Protein A-Sepharose was prepared by equilibration in Dignam’s (27) buffer D [20 mM HEPES (pH 7.9), 20% (vol/vol) glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol] with 0.1% Nonidet P-40. Modified ESB buffer (16) consists of 20 mM HEPES (pH 7.9), 40 mM NaCl, 6 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 0.1% Nonidet P-40, 10% glycerol, 2% Ficoll, BSA (3 mg/ml), and sonicated salmon sperm DNA (50 μg/ml).

**Isolation of Nuclear Proteins**

All studies with virgin, adult 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. Optimal hormone treatments (28) included sc injections of progesterone (3 mg/kg/d), oPRL (2 mg/d), and estradiol benzoate (50 μg/animal/d). Animals were killed 24 h after the last injection.

For CASTing, EMSA, and coimmunoprecipitation, one rabbit received sc injections of progesterone for 5 d. Nuclear extract proteins were prepared from the endometrium as described by Kleis-SanFrancisco et al. (28). To reduce nonspecific binding during immunoprecipitation, small aliquots of nuclear extract (200 μl) were precleared by gentle mixing with protein A-Sepharose slurry (10 μl) for 2 h at room temperature. The mixture was centrifuged (200 × g) for 1 min and the supernatant was stored at 4°C.

For chromatin immunoprecipitation, one rabbit received sc injections of oPRL for 5 d followed by progesterone for 5 d. Nuclei were isolated from the endometrium as described by Kleis-SanFrancisco et al. (28).

For Northern analysis and/or in situ hybridization, six rabbits were divided into two groups. Group 1 animals (n = 3) were used as estrous controls, and group 2 animals (n = 3) were made pseudopregnant by iv injection of 15–20 IU human chorionic gonadotropin and cervical stimulation. Group 2 rabbits were killed on d 5 of pseudopregnancy, and ovulation was confirmed by the presence of corpora lutea at necropsy. In addition, three rabbits were treated with progesterone for 5 d, and two rabbits were treated sequentially with progesterone (5 d) and estradiol benzoate (5 d).

**Recombinant RUSH**

Recombinant protein was obtained from the 1–5A cDNA (amino acids 40–542) in the phagemid pBluescript II SK(−) by coupled transcription/translation (TNT lysate). The predicted...
56-kDa recombinant protein was dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and substituted (0.61 μg/μl reaction) for nuclear extract in binding site selection assays.

Double-Stranded Oligonucleotide Preparation

The random sequence R76 (5′-CAG GTG AGT TCA GCG GAT CCT GTC G-3′) begins with 25 nucleotides that match the forward primer, followed by a stretch of 26 totally random bases, and ends with 25 nucleotides that match the reverse primer. This oligonucleotide was made double stranded by synthesis with the reverse primer (5′-GCT GCA GTT GCA CTG AAT TCG CCT C-3′), the Klengon fragment of DNA polymerase I, and [32P]dCTP. The final specific activity was 5–8 × 106 cpm/μg.

Binding Site Selection

For the initial reaction, 44.2 μg of precleared nuclear extract protein were mixed (tumbled) with 30 μg of dialyzed RUSH-1α(β) antibodies for 1 h at 4°C. Next, 20 μl of the protein A-Sepharose slurry were added. After gentle mixing (tumbling) for 1 h at 4°C, immune complexes were washed three times with 250 μl ice-cold Dignam’s (27) buffer D with acetylated BSA (1 mg/ml) and 0.1% Nonidet P-40. Immune complexes were then washed twice with 250 μl modified ESB. Double-stranded degenerate oligonucleotides were added (50 μl, 171 pmol DNA), and the mixture was incubated for 30 min at room temperature. Protein-DNA complexes were recovered by centrifugation and washed three times with modified ESB (250 μl each wash).

Amplification by PCR

The entire immunoprecipitated DNA sample was amplified in a 50 μl PCR mix containing ExTaq buffer (1×), TaKaRaEx Taq DNA polymerase (2.5U/50 μl), TaqStart antibody (0.55 μg/50 μl), deoxynucleoside triphosphates (dNTPs) (0.25 mM each), and primers (0.2 μm each). The forward primer (5′-CAG GTG AGT TCA GCG GAT CCT GTC G-3′) had a unique BamHI site (bold) and the reverse primer (5′-GCT GCA GTT GCA CTG AAT TCG CCT C-3′) had a unique EcoRI site (bold). A hot start PCR was performed with the following conditions: 30 sec at 94°C, followed by 15 cycles of 94°C, 5 sec; 62°C, 60 sec; and 72°C, 60 sec. Samples were rapidly cooled to 4°C. Reaction products were fractionated by electrophoresis on a 2% NuSieve GTG agarose gel. The 76-bp DNA products were extracted with QIAEX II according to the manufacturer’s instructions. Purified products were either subjected to additional rounds of binding site selection or cloned. For binding site selection, purified products were resuspended in 50 μl modified ESB buffer, and the entire sample was used in the reaction. For cloning, products were ligated to pCRII-TOPO and sequenced in both directions by the dideoxy chain termination method.

Alternative Approaches To Binding Site Selection

Because antibodies against a specific epitope can disrupt critical protein-protein or protein-DNA interactions, RUSH-1α antibodies were substituted for RUSH-1α(β) antibodies. To determine whether RUSH requires multicomponent interactions, recombinant protein was substituted for nuclear extract. In the initial reactions with either of the described substitutions, the amount of DNA was reduced from 170 pmol to 34 pmol.

Partial Cloning of Rabbit Endometrial GATA-4

Poly(A+)RNA samples from rabbit endometrium and mouse testis (positive control) were reverse transcribed at 42°C for 25 min in a thermal cycler (Mnincycler, MJ Research, Inc., Watertown, MA) with reagents from Applera Co. Maloney murine leukemia virus reverse transcriptase, and random hexamers. After the reaction had been heated to 99°C and cooled to 5°C, 40 μl of a PCR mix containing AmpliTaq DNA polymerase and forward (5′-GAC AGG TCA CTA CCT GTG CAA-3′) and reverse (5′-CAC CTG ATG GAG CCT TTT AAT GAG GCC GC-3′) primers (0.25 μM/each) were added to each tube. A four-step PCR was performed with the following conditions: 30 sec at 95°C, followed by five cycles of 94°C, 5 sec; 62°C, 60 sec; 72°C, 60 sec; five cycles of 94°C, 5 sec; 61.5°C, 60 sec; 72°C, 60 sec; five cycles of 94°C, 5 sec; 61°C, 60 sec; 72°C, 60 sec; 15 cycles of 94°C, 5 sec; 60°C, 60 sec; 72°C, 60 sec; and a final extension for 5 min at 72°C. Samples were rapidly cooled to 4°C. A single 217-bp ampiclon from each sample was cloned into pCRII-TOPO and sequenced in both directions by the dideoxy chain termination method.

Gel Shift Assays

To test the RUSH-binding site within the context of the flanking sequence, DNA fragments (190-bp) were excised from the cloning cassette of pCRII-TOPO with the restriction enzymes XbaI and HindIII. To further test selected RUSH-binding sites in the absence of flanking sequence, DNA fragments (42-bp) were excised from the flanking sequence with the restriction enzymes BamHI and EcoRI. DNA fragments were separated by electrophoresis on 4% polyacrylamide (acrylamide:bis-acrylamide, 30:0.8) gels, in Tris-borate buffer (45 mM Tris; 45 mM boric acid; 1 mM EDTA, pH 8.3), and recovered by electroelution. The 190-bp DNA fragments were radiolabeled on both ends with [α-32P]dATP (5′-end) and [α-32P]dCTP (3′-end) using the Klengon fragment of DNA polymerase I. The 42-bp fragments were radiolabeled on both ends with [α-32P]dGTP (5′-end) and [α-32P]dATP (3′-end). The specific activities for the probes averaged 1 × 107 cpm/μg. GATA oligonucleotides were 32P-labeled with γ-32PdATP using polynucleotide kinase (50,000 cpm/ng). UG200 was 3′-end-labeled on the coding strand at an XbaI site with [α-32P]dCTP using the Klengon fragment of DNA polymerase I. The specific activity for UG200 was 1–2 × 107 cpm/μg. Binding reactions were performed as previously described (28).

Northern Analysis and in Situ Hybridization

Total RNA was isolated from endometrium using TRIzol according to the manufacturer’s instructions. The integrity of each RNA sample was confirmed by electrophoretic fractionation through formaldehyde-containing agarose gels (1.2%). Total RNA was isolated from endometrium using TriReagent according to the manufacturer’s instructions. Purified products were either subjected to additional rounds of binding site selection or cloned. For binding site selection, purified products were resuspended in 50 μl modified ESB buffer, and the entire sample was used in the reaction. For cloning, products were ligated to pCRII-TOPO and sequenced in both directions by the dideoxy chain termination method.

Alternative Approaches To Binding Site Selection

Because antibodies against a specific epitope can disrupt critical protein-protein or protein-DNA interactions, RUSH-1α antibodies were substituted for RUSH-1α(β) antibodies. To determine whether RUSH requires multicomponent interactions, recombinant protein was substituted for nuclear extract. In the initial reactions with either of the described substitutions, the amount of DNA was reduced from 170 pmol to 34 pmol.

Partial Cloning of Rabbit Endometrial GATA-4

Poly(A+)RNA samples from rabbit endometrium and mouse testis (positive control) were reverse transcribed at 42°C for…
DNA polymerase (2.5 U/50 μl), dNTPs (0.2 mM each), and primers (125 ng each). The mutagenesis site is indicated in bold for both the forward (5′-GAG GGA AAT TTA CTT GGC CCA CCA AGT CAA TGC CC-3′) and the reverse (5′-GGG CAT TGA CTT GTT GGG CCA AGT AAA TAT TCC CTT TTC TC-3′) primers. Cycle parameters were as follows: 30 sec at 95 C, followed by 15 cycles of 95 C, 30 sec; 55 C, 60 sec; 68 C, 11 min. Samples were rapidly cooled to 4 C.

The parental (nonmutated) DNA template was digested for 0.5 h at 37 C with 1 μl of DpnI (10 U/μl). The authenticity of the mutagenized DNA was confirmed by sequencing. Nonmutated (pUG3.1-LUC) and mutagenized (pUG3.1M-LUC) constructs were used in standard transformation reactions.

HRE-H9 cells were grown in α-modified MEM plus 4% charcoal-stripped fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphoterin (Life Technologies) (250 ng/ml) in a humidified atmosphere of 95% air-5% CO2 at 33 C (8, 30). As verified by RIA, charcoal stripping reduced estradiol levels to less than 0.6 pg/ml. Cells were seeded in 24-well tissue culture plates and transfected at 50–60% confluency with 100 pg of pUG-LUC DNA and 200 pg of pRL-TK-LUC (transfection efficiency control) in 50 μl LipofectAMINE reagent. Transfections were performed in quadruplicate, and pUG-LUC activity was measured by luciferase assay. The parental (nonmutated) DNA template was digested for 1.5 h at 37 C (8, 30). As verified by RIA, charcoal-stripped fetal bovine serum, penicillin (100 IU/ml), and phenylmethylsulfonylfluoride (2 mM) were added to the samples. Chromatin was digested in situ with 250 U/ml Sau3A I for 30 min at 37 C. Nuclei were collected by centrifugation (750 g for 5 min at 4 C), resuspended in 1 ml of buffer (12 mM Tris, pH 7.5; 3 mM EDTA) plus the cocktail of protease inhibitors described above, and mixed (tumbled) for 30–60 min. Nuclear debris was collected by centrifugation (750 × g for 2 min at 4 C). The supernatant containing soluble chromatin was transferred to a new tube. NaCl and BSA were added to final concentrations of 100 mM 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 × g for 5 min at 4 C). The chromatin supernatant was preabsorbed with Protein A/G PLUS-Sepharose beads for 60 min at 4 C. Beads were collected by centrifugation (1000 × g for 5 min at 4 C). Supernatant was divided into two aliquots which were incubated with 2 μg RUSH-1α(β) antibodies with continuous mixing (tumbling) for 60 min at 4 C. Protein A/G PLUS-Sepharose beads were added with continuous mixing (tumbling) for 60 min at 4 C. Sepharose was collected by centrifugation (3000 × g for 30 sec) and washed three times with PBS containing 1 mM MgCl2 and 1 mM CaCl2. Sepharose was resuspended in buffer (50 mM Tris, pH 8.0; 1% sodium deoxycholate; 100 mM NaCl) containing Pronase (1 mg/ml) and incubated for 1 h at 50 C. The DNA was extracted with phenol, chloroform (1:1) and precipitated with ethanol. DNA (50 ng) aliquots were amplified in 50-μl PCR containing LA PCR buffer (1X), TaKaRa ExTaq DNA polymerase (2.5 U/50 μl), TaqStart antibody (0.55 μg/50 μl), dNTPs (0.2 mM each), and primers (0.2 μM each). The forward (5′-GGG TGA CTT GAG TTT CAA TAG GG-3′) and reverse (5′-GGG GCC CCA CCA AGT CAA TGC CC-3′) primers flank a 110-bp region (−180 to −70) of the uteroglobin promoter. A five-step PCR was performed with the following conditions: 30 sec at 95 C, followed by five cycles of 94 C, 5 sec; 50 C, 60 sec; five cycles of 94 C, 5 sec; 51.5 C, 60 sec; five cycles of 94 C, 5 sec; 53 C, 60 sec; five cycles of 94 C, 5 sec; 54.5 C, 60 sec; 15 cycles of 94 C, 5 sec; 56 C, 60 sec; and a final extension for 10 min at 68 C. Samples were rapidly cooled to 4 C. Reaction products were fractionated by electrophoresis on a 3% NuSieve GTG agarose gel. The 110-bp amplimers were extracted from a single band with QIAEX II according to the manufacturer’s instructions, ligated to pCRll-TOPO, and sequenced in both directions by the dyeideox chain termination method.

**Coimmunoprecipitation Assays**

Aliquots (30 μg) of precleared nuclear extract protein were incubated overnight at 4 C with affinity-purified, RUSH-1α(β) antibodies, followed by incubation for 2 h at 4 C with a 50% slurry of protein A-Sepharose. Proteins were fractionated by SDS-PAGE on 10% gels and transferred to nitrocellulose membrane. Membranes were processed for Western analysis as previously described (6, 7). Briefly, membranes were blocked in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.6) with 0.1% Tween 20 (TBST) and 2% powdered milk. Membranes were incubated overnight at 4 C in the same buffer containing either rabbit antiantworm Jak2 antibodies (1:5000) or rabbit anti-RUSH-1α(β) antibodies (1:100). Membranes were washed 3 × 30 min in TBST, and incubated in TBST with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000) for 90 min at room temperature. Membranes were subsequently washed 3 × 30 min in TBST. Specific signals were detected by chemiluminescence.

**Acknowledgments**

The authors thank Dr. J.C. Daniel, Jr., Eminent Scholar at Old Dominion University (Norfolk, VA), for stimulating discussions, and Mr. Benny Shaw for artwork.

Received February 8, 2002. Accepted May 13, 2002.

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This work was supported by NIH Grant HD-29457 (to B.S.C.).

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