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Complementary Deoxyribonucleic Acid Sequence Encoding Bovine Ubiquitin Cross-Reactive Protein

A Comparison with Ubiquitin and a 15-kDa Ubiquitin Homolog

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Pregnancy in the cow depends on secretion of interferon-tau (IFN-τ) by the conceptus (trophoblast and embryo) and the actions of this cytokine on the uterine endometrium. A novel 17-kDa uterine protein that is regulated by IFN-τ during early pregnancy and cross-reacts with ubiquitin antiserum on Western blots, has been named bovine ubiquitin cross-reactive protein (bUCRP). We suspected that bUCRP might be structurally related to ubiquitin, and to a human UCRP (ISG15 product) that has been described in several cell lines to be regulated by Type I IFNs. In this study, immunoscreening of a bovine endometrial cDNA library with ubiquitin antiserum resulted in the isolation of cDNAs encoding bUCRP. Nucleotide sequence of the bUCRP cDNA shared 70% identity with hUCRP and 30% identity with a tandem ubiquitin repeat. Computer translation revealed that bUCRP shared the Leu-Arg-Gly-Gly (LRGG) C-terminal sequence with ubiquitin and hUCRP that has been implicated in the modulation of intracellular proteins. However, some ubiquitin residues known to function in the ligation (Arg-54) to targeted proteins and in the processing of conjugates through the proteasome (His-68), have been lost through mutation in bUCRP. Lys-48, known to function in formation of ubiquitin polymers, was present in hUCRP, but mutated to Arg in bUCRP. Because bUCRP is secreted and retains the LRGG sequence, it may have both intracellular and secreted endocrine roles in maintaining pregnancy. Bovine UCRP also may have very different intracellular roles when compared with ubiquitin and hUCRP because of mutations in residues known to form polymers and to target proteins to degradation.

Key Words: Bovine; cDNA; interferon; ubiquitin; uterus.

Introduction

The bovine conceptus (embryo proper and trophoblast) secretes interferon-tau (IFN-τ) during pre- and peri-implantation stages of pregnancy (i.e., from days 14 to 35) (Thatcher et al., 1995). Interferon-τ shared 70% cDNA sequence identity with bIFN-ω, but was unique enough to warrant subclassification as a novel IFN (Roberts et al., 1992). Interferon-τ interacts with uterine endometrial receptors to attenuate the release of prostaglandin $F_2\alpha$ (PGF) (Bazer et al., 1994; Thatcher et al., 1995). Through this interaction, it indirectly rescues the ovarian corpus luteum from luteolysis, and protects the pregnancy.

Interferon-τ also regulates secretion of several uterine proteins (Rueda et al., 1993; Naivar et al., 1995; Austin et al., 1996). One of these proteins was first identified by our group as a ~16-kDa protein that was secreted in response to both IFN-τ and IFN-α (Naivar et al., 1995). Human ubiquitin cross-reactive protein was of similar size (15-kDa) to this protein and was regulated by Type I IFNs (Haas et al., 1987). Because the 16-kDa uterine protein cross-reacted with ubiquitin and hUCRP antisera on Western blots, we called it bUCRP (Austin et al., 1996).

UCRP was first identified as a protein that was regulated by IFN in mouse Ehrlich ascites tumor cells (Farrel et al., 1979). Later, it was described in human lung carcinoma cells (A-549), human Daudi cells, and in bovine MDBK cells as an IFN-regulated UCRP (Korant et al., 1984; Blomstrom et al., 1986; Haas et al., 1987; Knight and Cordova, 1991; Loeb and Haas, 1992). The appearance of hUCRP following culture with IFN was closely correlated with the acquisition of an antiviral state. Amino acid (Knight et al., 1988; Reich et al., 1987; Loeb et al., 1992), cDNA (Blomstrom et al., 1986) and gene (i.e., interferon-stimulated gene 15 or ISG15; Reich et al., 1987) sequences have been reported for hUCRP. Two domains of hUCRP showed 29–31% sequence identity with ubiquitin. However, the most notable similarity in structure between hUCRP and ubiquitin was conservation of the carboxyl-terminal LRGG sequence that functions in the ligation of ubiquitin (Wilkinson and Audhya, 1981; Ecker et al., 1987) and hUCRP (Loeb et al., 1992; Narasimhan et al., 1996) to targeted intracellular proteins.

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The carboxy terminus of ubiquitin and hUCRP (i.e., terminal Gly) links to primary amines on targeted proteins through a posttranslational modification. Conjugation of ubiquitin to proteins requires three enzymatic reactions (reviewed in Finley and Chau, 1991; Hershko and Ciechanover, 1992). The most studied enzymes have been named E1, E2, and E3. E1 activates the C-terminal Gly of ubiquitin to generate a covalent El-ubiquitin thiol ester. El-ubiquitin is then transacetylaled to a cysteine residue on E2. The E2 family of carrier proteins forms isopeptide bonds between the carboxyl-terminal Gly of ubiquitin and the ε-amino groups of lysine residues on targeted proteins. The final isopeptide bond can be generated in pathways that are either dependent on or independent of E3 (ubiquitin:protein ligase). Recently, Haas and coworkers (Narasimhan et al., 1996) described a ligation pathway for hUCRP that was distinct, but parallel with the pathway described for ubiquitin.

Human UCRP may be secreted in response to Type I IFN to regulate secretion of IFN-γ by T- and B-lymphocytes (Recht et al., 1991). It also has been suggested that hUCRP, by inducing secretion of IFN-γ from T-cells, augments natural killer (NK) cell proliferation, and activates monocytes and macrophages (D'Cunha et al., 1996). Other than these two experiments and the description of conjugation to intracellular proteins, little else is known regarding the function of hUCRP and potential interactions with neighboring cells.

The present experiments were designed to isolate, sequence, and characterize the cDNA encoding the bovine uterine UCRP. It was expected that inferred amino acid sequence could be compared with ubiquitin and hUCRP to determine putative functional residues. Complementary DNA sequence identity confirms that the ~16-kDa uterine protein belongs to the ubiquitin family of proteins. Significant amino acids have been retained (i.e., LRGG), yet discrete differences in other functionally described amino acids may play significant roles in substrate specificity, ability to form poly-UCRP chains, and secreted/targeted endocrine effects.

Results

Isolation of bUCRP and Ubiquitin cDNAs

Immunoscreening of the endometrial cDNA library with ubiquitin antiserum resulted in isolation of 220 positive plaques out of 90,000 total plaques screened. After excising 20 SK plasmids from phage, three clones with insert sizes of ~600 bp were further characterized. DNA sequencing revealed that these cDNAs (clones: KA-6, KA-16, and KA-18) encoded bUCRP and were identical.

Comparison of bUCRP and hUCRP cDNA Sequences

Shown in Fig. 1 is the nucleotide sequence for bUCRP (clone KA16) and alignment with the sequence encoding the gene for hUCRP (i.e., ISG15; Reich et al., 1987). The bUCRP cDNA contains an open reading frame of 154 codons starting at the ATG codon at position 106 and ending in the termination codon, TAG at position 591. The initiation codon, ATG, is immediately preceded by the sequence CGGCC, which is typical of the consensus sequence (CCA/GCC) for the initiation of translation in eukaryotes (Kozak, 1986). The hexanucleotide AATAAA (bases 642–647) appears to be the signal for polyadenylation (Proudfoot and Brownlee, 1976).

Comparison of aligned cDNA sequences revealed that bUCRP shared 70% sequence identity with hUCRP. The highest sequence identity (74%) was shared between coding regions. The greatest divergence in sequence identity was in 5' (58%) and 3' (53%) nontranslated regions. A major difference in cDNA sequence was the deletion in bUCRP of seven nucleotides at base position 345, and two nucleotides at base position 349. These deletions resulted in removal of three codons, but retention of reading frame. Another major difference in cDNA sequence was the presence of a termination codon (TAG) immediately following sequences encoding LRGG in the bovine sequence. In the hUCRP sequence, the termination codon is 23 bp downstream from nucleotides encoding these amino acids. Also, bUCRP had longer 3' and 5' nontranslated regions when compared with hUCRP.

Comparison of bUCRP, hUCRP and Ubiquitin Amino Acid Sequences

Shown in Fig. 2 is the amino acid sequence after computer translation of the cDNA encoding bUCRP. Also shown are amino acid sequences encoding hUCRP (Loeb and Haas, 1992) and a bovine di-ubiquitin repeat (Schlesinger et al., 1975). The inferred bUCRP amino acid sequence had 68% identity with hUCRP and 31% identity with ubiquitin. The most notable conservation in sequence was retention of the C-terminal LRGG amino acids in bUCRP, hUCRP, and ubiquitin. Other shared extended amino acid sequences between bUCRP and ubiquitin were not clearly evident. However, in many cases, sequences spanning three to 11 amino acids were identical between bUCRP and hUCRP (*see* Fig. 2, conserved amino acids shown in upper case letters).

Several potentially significant structural differences in amino acid sequence between bUCRP and hUCRP were observed. First, bUCRP had three cysteines (Cys-78, Cys-109, Cys-143), whereas hUCRP had only one (Cys-78). Human UCRP had an insertion of three amino acids (Asp-Glu-Pro) after Cys-78, and an extended C-terminal amino acid sequence (Gly-Thr-Glu-Pro-Gly-Gly-Arg-Ser; not found in bUCRP) that has been suggested to be cleaved to yield a mature protein terminating in Gly-157 (Feltham et al., 1989). Bovine UCRP has a predicted Mr of 17,300 and a pI of 7.2.

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bUCRP: 1 GGAATTCGGC CCCCGGGCTG CAGGAATTCG CGGCCGCGTC GACAGCAGAG AGGCAGCCGC
hUCRP:     AATagGGC CggtGtGCTG C-GGAAgcCG CGGCtGaG-a GgCAGC-GA- ActCAtCgcC
                        ↓               ↓                              ↓
                        c               g                              tt
                                                    1
                                                    Met Gly Gly Asp Leu Thr
61  AGCCAACCAG TGTCTGCAGA GAGCCTGGCA CCAGAACCCA CGGCC ATG GGC GGG GAC CTG ACG
    AgtacAggAG ctcgTGCcGt GgcCCacagc CCAcAgCCCA CaGCC ATG GGC tGG GAC CTG ACG

    7                                                                      25
    Val Lys Met Leu Gly Gly Gln Glu Ile Leu Val Pro Leu Arg Asp Ser Met Thr Val
124 GTG AAG ATG CTA GGG GGC CAG GAG ATC CTG GTG CCT CTG AGG GAC TCC ATG ACG GTA
    GTG AAG ATG CTg GcG GGC aAc GAa tTC CaG GTG tCc CTG AGc agC TCC ATG tCG GTg

    26                                                                     44
    Ser Glu Leu Lys Gln Phe Ile Ala Gln Lys Ile Asn Val Pro Ala Phe Gln Gln Arg
183 TCC GAG CTG AAG CAG TTC ATC GCC CAG AAG ATC AAT GTG CCT GCT TTC CAG CAG CGC
    TCa GAG CTG AAG gcG cag ATC aCC CAG AAc ATt ggc GTG Cac GCc TTC CAG CAG CGt

    45                                                                     63
    Leu Ala His Leu Asp Ser Arg Glu Val Leu Gln Glu Gly Val Pro Leu Val Leu Gln
242 CTG GCC CAC CTT GAC AGC AGG GAG GTG CTG CAG GAA GGG GTG CCC CTT GTC CTC CAG
    CTG GCt gtC Cac ccg AGC gGt GtG GcG CTG CAG GAc aGG GTc CCC CTT GcG agC CAG

    64                                                                     82
    Gly Leu Arg Ala Gly Ser Thr Val Leu Leu Val Val Gln Asn Cys Ile Ser Ile Leu
301 GGC CTG AGA GCT GGC AGC ACC GTC CTG CTG GTG GTG CAG AAC TGC ATC TCC ATC CTG
    GGC CTG gGc cCT GGC AGC ACg GTC CTG CTG GTG GTG gAc AAa TGC cTC TgC ATC CTG
                                                          ↓     ↓
                                                       gacgaac  ga
    83                                                                     101
    Val Arg Asn Asp Lys Gly Arg Ser Ser Pro Tyr Glu Val Gln Leu Lys Gln Thr Val
360 GTG AGG AAC GAC AAG GGT CGC AGC AGC CCC TAT GAG GTC CAG CTG AAG CAG ACT GTG
    GTG AGG AAt aAC AAG GGc CGC AGC AGC aCC TAc GAG GTg CgG CTG AcG CAG ACc GTG

    102                                                                    120
    Ala Glu Leu Lys Gln Gln Val Cys Gln Lys Glu Arg Val Gln Ala Asp Gln Phe Trp
419 GCT GAG CTC AAG CAG CAG GTG TGC CAA AAG GAG CGT GTA CAA GCA GAC CAG TTC TGG
    GCc cAc CTg AAG CAG CAa GTG aGC ggg ctg GAG gGT GTg CAg Gac GAC CtG TTC TGG

    121                                                                    139
    Leu Ser Phe Glu Gly Arg Pro Met Asp Asp Glu His Pro Leu Glu Glu Tyr Gly Leu
478 CTG TCT TTT GAA GGG AGG CCC ATG GAT GAT GAG CAC CCG CTG GAG GAA TAC GGC CTC
    CTG aCc TTc GAg GGG AaG CCC cTG GAg GAc cAG CtC CCG CTG GgG GAg TAC GGC CTC

    140                                                 154
    Met Lys Gly Cys Thr Val Phe Met Asn Leu Arg Leu Arg Gly Gly Stop
546 ATG AAG GGG TGC ACC GTG TTC ATG AAT CTA CGT CTT CGG GGT GGG TAG GGAAGGGCCA
    AaG ccc ctG aGC ACC GTG TTC ATG AAT CTg CGc CTg CGG GG- aGG cgG cacAGaGCCt


605 GGAGGGCCTT AGGGAGGGCT CCCCATGCAG CGCAGTGAAT AAAGTTGTAG CAAAGCC
    GGcGGGCgga gctaAGGGCT CCaCcaGCAt CcgAGaGgAT cAAG-gGccG gAA
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Fig. 1. Alignment of complementary DNA sequences encoding bUCRP and hUCRP. Nucleotides are numbered to the left based on the bUCRP cDNA sequence. Identity in nucleotide sequence is shown in upper case letters. Nucleotides that differ in sequence are shown in lower case letters. Nucleotides present in hUCRP, but absent in bUCRP, are designated with an ↓ at the position immediately preceding the insertion. Inserted nucleotides are described in lower case beneath the ↓. Amino acid sequence is numbered from Met-1 to Gly-154. Cys residues are shown in bold. The LRGG sequence is shown in bold. The putative polyadenylation signal is underlined. Nucleotides absent in hUCRP, but present in bUCRP are designated with –.

In Vitro Translation:
Evaluation of Signal Peptide

Shown in Fig. 3 is a representative autoradiograph of ^{35}S-labeled proteins resulting from in vitro translation of β-lactamase mRNA or bUCRP mRNA in the absence or presence of microsomes. Translation in the presence of microsomes caused a reduction in Mr indicative of processing pre-β-lactamase (31.5 kDa) to β-lactamase (28.9 kDa). Presence of microsomes had no effect on size of bUCRP mRNA translation products.

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bUCRP   1-MGGDLTVKML GGQEILVPLR DSMTVSELKQ FIAQKINVPA FQQRLAHLDS REVLQEGVPL
hUCRP   1-MGwDLTVKML aGnEfqVsLs sSMsVSELKa qItQKIgVhA FQQRLAvhpS gvaLQdrVPL
bUBIQ   1-M--qifVKtL tGktItleve pSdTienvKa kIqdKegiPp dQQRL-ifag k-qLedGrtL

                                *                                  *
bUCRP  61-VLQGLRAGST VLLVVQNC     IS ILVRNDKGRS SPYEVQLKQT VAELKQQVCQ
hUCRP  61-asQGLgpGST VLLVVdkCDEP  lS ILVRNnKGRS StYEVrLtQT VAhLKQQVsg
bUBIQ  57-sdyniqkeST lhLVlrlrgg 1-mq IfVktltGkt itlEVepsdT ienvKakiqd

                                          *
bUCRP 111-KERVQADQFW LSFEGRPMDD EHPLEEYGLM KGCTVFMNLR LRGG-154
hUCRP 114-lEgVgdDlFW LtFEGkPleD qlPLgEYGLk plsTVFMNLR LRGGlgte pggrs-165
bUBIQ  33-KEgippDQqr LiFaGkqleD grtLsdYniq KesTlhlvLR LRGG-76
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Fig. 2. Alignment of amino acids encoding bUCRP, hUCRP, and a diubiquitin repeat (bUBIQ). Shared amino acid residues when compared with bUCRP are shown in upper case letters. Amino acids that differ are shown in lower case letters. The LRGG sequence is shown in bold. An insertion of three amino acids in the bUCRP sequence is shown in shadowed letters. The second ubiquitin repeat starts with 1-mq. Lys-48 in both ubiquitin repeats is underlined twice.

Discussion

A uterine endometrial protein was first described by our group to be secreted in response to IFN-τ by examining ^{3}H-Leu-labeled proteins using ID- and 2D- PAGE, and fluorography (Naivar et al., 1995). Because this protein was similar in size with hUCRP, we suspected that it was related. Western blots with ubiquitin and hUCRP antisera confirmed this suspicion. The 16-kDa endometrial protein cross-reacted with both antisera (Austin et al., 1996). Thus, the uterine protein was named bovine ubiquitin cross-reactive protein, or bUCRP.

Western blots and densitometry revealed that bUCRP was not secreted by endometrium from nonpregnant cows (Austin et al., 1996). In pregnant cows, it first appeared in cultured endometrium collected on day 15. Amounts increased to highest levels in endometrium collected on day 18, a time during which bIFN-τ is secreted in maximal amounts by the conceptus (reviewed in Roberts et al., 1992; Thatcher et al., 1995).

Besides having an endocrine role, bUCRP also may have exocrine functions since it was detected in uterine flushings from day 18 pregnant cows (Austin et al., 1996). Bovine UCRP is regulated by IFN-τ as well as IFN-α as interpreted by dose-response studies (Naivar et al., 1995; Austin et al., 1996).

The most compelling evidence of the identity of bUCRP are the present experiments in which we report cDNA (70%) and inferred amino acid sequence (68%) identity with hUCRP. Bovine UCRP retained the LRGG sequence that has been implicated in both hUCRP (Loeb et al., 1992; Narasimhan et al., 1996) and ubiquitin (Ecker et al., 1987; Wilkinson and Audhya, 1981) in ligating to and regulating intracellular proteins.

Ubiquitin conjugates to proteins through a posttranslational modification in which the carboxyl glycine of ubiquitin is linked through an isopeptide bond to primary amines on target proteins (reviewed in Finley and Chau, 1991; Hershko and Ciechanover, 1992). The resulting conjugates are processed and degraded through an ATP-dependent 26 S multicatalytic proteasome pathway (Discoll and Goldberg, 1990), or through a newly described pathway in yeast that is equivalent to lysosomes in eukaryotic cells (Kicke and Riezman, 1996).

Formation of packed multiubiquitin chains provides a tertiary structure with targeted proteins that is probably unique and aids in intracellular trafficking of the complex (Baboshina and Haas, 1996). The conjugation of ubiquitin and hUCRP to targeted cellular proteins proceeds through enzymatic pathways that are parallel, but distinct (Narasimhan et al., 1996). Thus, there may be specific targeted proteins that are regulated by UCRP, but not ubiquitin.

Structure-function studies using site-directed mutagenesis of ubiquitin revealed that position Arg-72 of ubiquitin, retained as Arg-153 in hUCRP and as Arg-150 in bUCRP, is involved with binding to E1 and required for ordered addition of substrate in a three-step ligation pathway (Burch and Haas, 1994). Arg-54 has been implicated in ubiquitin as an additional binding site during enzyme-mediated conjugation (Burch and Haas, 1994). Arg-54 in the second repeat of ubiquitin has been lost through mutation to the corresponding Leu-135 in hUCRP and His-132 in bUCRP. His-68 also is essential for function of ubiquitin since

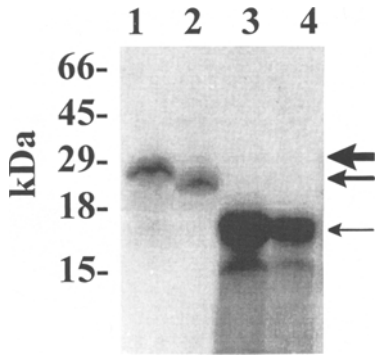

Fig. 3. In vitro translation of β-lactamase (lanes 1 and 2) and bUCRP (lanes 3 and 4) transcripts in the presence (even lanes) or absence (odd lanes) of microsomal membranes. The largest arrow identifies pre-β-lactamase. The medium sized arrow identifies β-lactamase. The smallest arrow identifies bUCRP.

mutation impairs the ability to support ATP-dependent degradation through the 26 S proteasome (Ecker et al., 1987). His-68 has been lost through mutation to the corresponding Phe-149 in hUCRP and Phe-146 in bUCRP. Together, these changes in amino acid sequence can be interpreted to mean that bUCRP and hUCRP share the general ability of ubiquitin to ligate to proteins, but may differ in substrate specificity and interaction with ligating enzymes. The absence of His-68 in bUCRP may result in the lack of processing through the 26 S proteasome and degradation. Other pathways of degradation (i.e., lysosome) and/or stabilization/modulation could conceivably exist for proteins conjugated to UCRP.

Cellular proteins are targeted for degradation by the formation of multiubiquitin chains in which ubiquitin is linked by isopeptide bonds between its carboxyl termini and Lys-48 residues of successive monomers (Finley et al., 1994). Subunit 5 of the 26 S proteasome binds to Lys-48-linked chains of ubiquitin and commits the conjugated targeted protein to degradation with associated release of free ubiquitin monomers following disassembly.

Currently, in addition to Lys-48, only Lys-63-linked chains of ubiquitin have been described in vivo (Spence et al., 1995). However, Lys-63-linked chains have been associated with regulatory function, whereas Lys-48-linked chains represent the principle mechanism of targeting protein degradation (Spence et al., 1995; Baboshina and Haas, 1996). Recent in vitro conjugation experiments described multiubiquitin chain linkages at Lys-6 and Lys-11 (Baboshina and Haas, 1996). Only Lys-6 was retained by ubiquitin, hUCRP and bUCRP. Lys-48 was retained in ubiquitin and hUCRP, but was absent in bUCRP. Since Lys-48 is the only residue known to form polymers associated with proteolysis in vivo, this mutation in bUCRP may impair its ability to target proteins to degradation.

Lys-11 and Lys-63 of ubiquitin were absent in hUCRP and bUCRP in the first repeat. Likewise, Lys-6 and Lys-11 of ubiquitin were absent in hUCRP and bUCRP corresponding to the second ubiquitin repeat. Oddly, Lys-48 of ubiquitin was retained in hUCRP (Lys-129), and Lys-63 of ubiquitin was retained in bUCRP (Lys-141), corresponding to the second ubiquitin repeat. The shared and distinct Lys residues between ubiquitin, hUCRP, and bUCRP may significantly affect generation of polymers, processing, and intracellular trafficking of targeted and conjugated proteins.

Bovine UCRP has some additional unique components to its amino acid sequence. First, it appears to be processed differently than hUCRP. For example, the stop codon (TAG) in bUCRP immediately follows the DNA sequence encoding LRGG yielding a mature protein that is 17.3 kDa in size. Human UCRP has a stop codon that is downstream from this sequence (Blomstrom et al., 1986; Reich et al., 1987). It has been suggested that pre-hUCRP (17-kDa) undergoes a posttranslational modification to remove the C-terminal amino acids to yield the mature protein (15-kDa) that terminates in LRGG (Knight et al., 1988; Feltham et al., 1989). The bovine protein does not go through this modification. Another major difference in amino acid sequence was the presence of three cysteines in the bovine sequence, compared with only one cysteine in the human sequence. If disulfide bridges are formed in the bovine protein, this could greatly affect the three dimensional structure and function, when compared with hUCRP. The presence of a free sulfhydryl group on the odd Cys in bUCRP and on the single Cys in hUCRP, also may interact with targeted proteins or form homologous dimers. Finally, a deletion of three amino acids was noted in the bovine sequence when compared with the human sequence. Since this deletion occurs in what might be a "hinge" region between two ubiquitin-like domains, its functional significance may be minimal.

Human UCRP does not have a signal peptide (Feltham et al., 1989). However, the N-terminal Met and a C-terminal extension are removed post-translationally to yield bioactive hUCRP (Feltham et al., 1989; Knight et al., 1988). In vitro translation of bUCRP transcripts in the presence and absence of microsomes resulted in a major translation product of ~17-kDa that did not change in size. The broad 17-kDa and the minor 16-kDa bUCRP bands were probably a result of initiation of translation at one of three codons for Met (nucleotide positions 106, 130, and 173). The quality of translation products can also be affected by the fidelity and amount of the mRNA/transcript (Jackson, 1991). The in vitro translation approach used was optimized for the positive control: β-lactamase. Translation of β-lactamase transcript yielded a primary translation product of ~31-kDa that was processed through removal of a signal peptide in the presence of microsomes to produce a ~29-kDa product.

Presence of a signal peptide was also analyzed by using von Heijne rules. On first inspection, applying the "(-3,-1) rule" (von Heijne; 1983, 1984) for acceptable amino acids near a signal sequence cleavage site, a potential cleavage site exists between Ser-26 and Glu-27 of bUCRP. However, on closer inspection of this site, the following objec-

tions become apparent. First, the site is one amino acid too far downstream from the initial hydrophobic tetrapeptide of the von Heijne algorithm. Second, the resulting peptide, although within the range of known signal sequences, is extreme in length. Eukaryotic signal peptides have been described to be 13–36 residues long, but typically are within the 18–20 amino acid range. Finally, the hydrophobic "core" of the putative signal sequence is too short, being interrupted by Gln, Glu and Gly. Whereas there are isolated examples of signal sequences containing a Gln or Gly, there are no examples in von Heijne's data base that contain a Glu within the hydrophobic core (von Heijne, 1984). This analysis coupled with the aforementioned inability of microsomes to cleave bUCRP in vitro, indicates it is unlikely that there is a signal peptide in bUCRP.

The function of bUCRP is currently being examined by our group during early pregnancy in the cow. Because it can be found in uterine flush and in endometrial culture medium, a role as a secreted protein has been proposed (Austin et al., 1996). Bovine UCRP shares sequence identity with ubiquitin and hUCRP. Thus, we suspect that through the LRGG residues and through Lys and His residues implicated in the function of ubiquitin, it can conjugate to and regulate intracellular proteins. We speculate that bUCRP in response to IFN-τ ligates to and regulates proteins (i.e., receptors: oxytocin; enzymes: prostaglandin synthetase) involved with release of prostaglandin $F_2\alpha$. Future experiments by our group will test these hypotheses.

Materials and Methods

Bovine Endometrial cDNA Library

Endometrium was collected from the uterine horn of a day 12 nonpregnant cow, minced and digested in Earl's balanced salt solution containing 150 U/mL each of collagenase and hyaluronidase for 2 h at 38°C (Betts and Hansen, 1992). Digested endometrium was centrifuged at 500*g*, resuspended in Earl's salt solution, and filtered through four layers of gauze and a 30 μm Spectramesh nylon filter. Retained epithelial cells were collected and cultured in 45% Ham's F12, 45% Dulbecco's Minimal Essential Media (MEM), and 10% fetal bovine serum (Sigma, St. Louis, MO) under 95% air/5% CO_2. Cells in monolayer were cultured in serum-free MEM in the presence of 25 n*M* rbIFN-τ (3×10^6 IU/mg; Klemann et al., 1990) for 18 h.

Messenger RNA was prepared from 12 flasks (T75) of endometrial cells (Fast Track method; Invitrogen, San Diego, CA) that were cultured as a confluent monolayer in the presence of 25 n*M* (3×10^{-6} IU/mg) IFN-τ. Messenger RNA (15 μg) was ethanol precipitated and sent to Clontech (Palo Alto, CA) where cDNA were prepared using Xho I-(Poly-dT) as the primer. The cDNA were directionally cloned into Lambda Zap II phage using Xho I and EcoR I sites. The amplified cDNA library had a titer of 10^9 pfu/mL, 99% recombinant clones and inserts ranging in size from 0.4–5.0 kb.

Immunoscreening of the cDNA Library

Phage (7500 pfu per 150 mm plate; 90,000 pfu total) were mixed with XL1 Blue cells (O.D. = 2.0; Clontech) in 10 m*M* $MgSO_4$, and allowed to absorb for 30 min at 37°C. Infected cells were added to top agar (10 m*M* $MgSO_4$) and poured over a Luria-Bertani agar plate (Fisher Scientific, Denver, CO). Plates were incubated overnight at 37°C. Fusion proteins were induced on nitrocellulose filters (0.2 μm; MSI Inc., Westborough, MA) that had been previously soaked in 10 m*M* IPTG (isopropyl thiogalacto pyranoside) and dried. Filters were incubated on the plates for 3.5 h at 37°C, washed in TBST (20 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.05% Tween 20), and blocked for 1.5 h in 1% BSA in TBST. Polyclonal rabbit serum containing antibody to ubiquitin (Sigma) was diluted 1:1000 and absorbed with *E. coli* lysate (Promega, Madison, WI) at a final concentration of 1 mg/mL to reduce nonspecific binding. Filters were incubated in this antiserum for 2 h at room temperature with agitation, followed by three washes with TBST. Filters were then incubated in antirabbit alkaline phosphatase conjugate (1:5,000; Promega Corp.) for 30 min at room temperature. Filters were washed three times in TBST. After the last wash, 200 mL of AP buffer (100 m*M* Tris, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$) containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were added. Color development occurred within 30 min, and was stopped with distilled water.

Positive plaques (220 positive plaques out of 90,000 total; frequency =0.24%) were selected and placed in 0.5 mL SM buffer (0.1*M* NaCl, 0.05*M* Tris, pH 7.5, 8 m*M* $MgSO_4$, 0.01% gelatin). Phage were eluted from plaques overnight at 4°C. Phage were amplified, and purified using standard plate lysate procedures. Plasmid (SK–) was excised from phage using R408 helper phage ($\leq 10^7$ pfu/mL), amplified and purified using routine procedures.

Plasmid DNA was digested with Eco RI and Xho I to elaborate cDNA inserts. Three cDNAs (~600 bp; clones named KA-6, KA-16, and KA-18) were sequenced using Sanger dideoxy sequencing methods (Sequenase; Amersham, Arlington Heights, IL). M13 forward and reverse primers as well as several internal primers (based on nucleotide sequence generated from the M13 oligonucleotide-primed reactions) were used to sequence cDNAs from 5' and 3' ends.

In Vitro Translation: Analysis of Signal Peptide

Transcripts encoding bUCRP were synthesized using in vitro transcription (Maxiscript; Ambion, Austin, TX). The bUCRP transcript (Fig. 3) was translated in vitro using nuclease-treated rabbit reticulocyte lysate, amino acids (w/o methionine), and [^{35}S] -methionine (1,200 Ci/mMol; NEN, Boston, MA) in the presence or absence of canine pancreatic microsomal membranes (Promega).

Transcripts encoding pre-β-lactamase also were translated in vitro in the presence or absence of microsomal

membranes. Microsomal membranes in this translation system process pre-β-lactamase (31.5 kDa) to β-lactamase (28.9 kDa) by removing the signal peptide. Radiolabeled translation products were separated by 1D-PAGE and evaluated using autoradiography (Kodak XRP film; Sigma).

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