Upstream Stimulatory Factor (USF) Is Recruited into a Steroid Hormone-triggered Regulatory Circuit by the Estrogen-inducible Transcription Factor δEF1*

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In the past decade, investigation into steroid hormone signaling has focused on the mechanisms of steroid hormone receptors as they act as signaling molecules and transcription factors in cells. However, the majority of hormone-responsive genes are not directly regulated by hormone receptors. These genes are termed secondary response genes. To explore the molecular mechanisms by which the steroid hormone estrogen regulates secondary response genes, the ovalbumin (Ov) gene was analyzed. Three protein-protein complexes (Chirp-I, -II, -III), which do not contain the estrogen receptor, are induced by estrogen to bind to the 5′-flanking region of the Ov gene. The Chirp-III DNA binding site, which is required for estrogen induction, binds a complex of proteins that contains the estrogen-inducible transcription factor δEF1. Experiments undertaken to identify proteins complexed with δEF1 led to the elucidation of a novel mechanism of action of upstream stimulatory factor-1 (USF-1), which involves its tethering to the Ov gene 5′-flanking region by δEF1. Gel mobility shift assays and co-immunoprecipitation experiments identify USF-1 as a component of Chirp-III. However, USF-1 is not able to bind to the Chirp-III site independently. In addition, USF-1 overexpression is able to induce Ov gene promoter activity in transfection experiments. USF-1 can also potentiate the induction of the Ov gene by the transcription factor δEF1. Moreover, mutating the δEF1 binding sites in the 5′-flanking region of the Ov gene abrogates induction of the gene by USF-1. These data begin to establish a molecular mechanism by which hormone-inducible transcription factors and ubiquitous transcription factors cooperate to regulate estrogen-induced secondary response gene expression.

Estrogen is a lipophilic molecule that diffuses into cells and binds to the estrogen receptor. The estrogen-estrogen receptor complex then binds to and regulates genes called primary response genes (for reviews, see Refs. 1–5). In some cases, a primary response gene encodes a transcription factor that is capable of regulating downstream genes, or secondary response genes. In the last decade, uncovering the mechanism of action of the estrogen receptor as a signaling molecule and as a regulator of transcription has been a priority. However, the majority of genes regulated by estrogen are not primary response genes but secondary response genes. To investigate the mechanisms of transcriptional activation of secondary response genes, the induction of the ovalbumin (Ov)† gene by estrogen was analyzed. Ov gene expression is up-regulated 200-fold upon estrogen administration in vivo (6). This is due to a 20-fold increase of transcription of the Ov gene (7) coupled with a 10-fold increase in mRNA stability (8). The Ov gene is classified as a secondary response gene because the estrogen receptor does not directly bind to it and because there is a requirement for concomitant protein synthesis for transcriptional activation (9). Estrogen induction of the Ov gene requires two cis-acting regulatory elements in the 5′-flanking region (7), the steroid dependent regulatory element (SDRE), which spans from −892 to −793, and the negative regulatory element (NRE), which spans from −308 to −88 (Fig. 1). Ten-base pair linker scanner mutations or deletion mutations spanning the SDRE abrogate Ov gene induction by estrogen (10). Furthermore, in vivo genomic footprinting has identified three protein-protein complexes that bind to the SDRE upon estrogen administration (10). These are called the chicken inducible regulatory proteins-I, -II, and -III (Chirp-I, -II, -III) (for reviews, see Refs. 11 and 12). The NRE binds proteins that are positive and/or negative regulators of the Ov gene (13).

Chirp-III includes the estrogen-inducible transcription factor δEF1 (14). However, analysis by in vivo footprinting and gel mobility shift assays (GMSAs) indicates that an additional protein(s) is present in the Chirp-III complex (10). Based on studies that demonstrate that estrogen induces USF to bind to regulatory regions of genes (15, 16), experiments were conducted to test whether USF is a component of the Chirp-III complex.

USF-1 and USF-2 are ubiquitous basic helix-loop-helix proteins (17) that were first identified as activators of the adenovirus major late promoter (18). USF-1 and USF-2 are capable of homodimerization or heterodimerization and typically bind to E-box DNA sequences (21). USF can be recruited to bind E-boxes in the 5′-flanking region of target genes by transcription factors responding to a specific signal (15, 19–22). For example, the USF heterodimer cooperates with Stat1 to bind to and to induce the class II transactivator (CIITA) regulatory region (19). The USF heterodimer is also recruited to bind to the cathepsin D promoter in an estrogen-dependent manner.

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† The abbreviations used are: Ov, ovalbumin; USF, upstream stimulatory factor; SDRE, steroid dependent regulatory element; NRE, negative regulatory element; Chirp, chicken inducible regulatory proteins; CAT, chloramphenicol acetyltransferase; GMSA, gel mobility shift assays; oligo, oligonucleotide.
...overexpression of USF-1 and gene transcriptional activity, and activity is potentiated by H9254. Two subdomains bind the transcription factor with 2
nuclear protein extract from estrogen-stimulated chicks was incubated H9254. These data invite us to propose a novel paradigm whereby the ubiquitous transcription factor
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USF-1 is part of the Chirp-III complex that is required for estrogen induction of the Ov gene. Furthermore, overexpression of USF-1 induces Ov gene transcriptional activity, and activity is potentiated by co-overexpression of USF-1 and δEF1. However, mutating the δEF1 binding sites in the 5'-flanking region of the Ov gene abrogates its induction by USF-1. These data invite us to propose a novel paradigm whereby the ubiquitous transcription factor USF is tethered to the Ov gene by the estrogen-inducible transcription factor δEF1.

MATERIALS AND METHODS

Gel Mobility Shift Assays—The DNA oligo C.5, USF consensus oligo, and mutated USF consensus oligo were synthesized and high pressure liquid chromatography-purified by the Microchemical Facility at the University of Minnesota. Oligo C.5 consists of nucleotides 811 to 788, which has historically been called oligo C.5, is necessary for the induction of the Ov gene by estrogen and binds a protein complex called Chirp-III (7, 25). Recently, experiments demonstrated that the estrogen-inducible transcription factor δEF1 binds to the nucleotides from 810 to 806 (14, 27) and that the integrity of this site is required for proper induction of the gene by estrogen or by δEF1 overexpression (14, 23, 27). Furthermore, overexpression of δEF1 induces the Ov promoter in the absence of estrogen (23), which indicates that estrogen is inducing the Ov gene at least in part via δEF1. However, maximal induction cannot be achieved by overexpressing δEF1 (7).

Because USF is recruited to the cathepsin D gene in response to estrogen, we hypothesized that it may also be recruited to the Ov gene. To test whether USF-1 is present in the Chirp-III protein complex in addition to δEF1, a GMSA was performed (Fig. 2A). When chick oviduct nuclear protein extracts are incubated with oligo C.5, one major shifted band is observed (Fig. 2A, band B). There are also several smaller bands that are thought to be degradation products because their intensity decreases with the addition of protease inhibitors during the preparation of the nuclear protein extracts (data not shown). Non-specific DNA does not compete band B (lane 3), whereas unlabeled oligo C.5 competes band B (lane 4), which indicates that proteins bind to oligo C.5 in a sequence-specific manner. δEF1 preimmune serum does not affect protein binding to oligo C.5 (lane 5), yet δEF1 antibody eliminates protein binding (lane 6), indicating that δEF1 is present in band B (lane 14). USF-1 antibody preincubated with a peptide blocker before being added to oviduct nuclear protein extracts does not affect protein binding to oligo C.5 (lane 7). However, USF-1 antibody incubated with oviduct nuclear protein extract results in a supershift of band B (band A, lane 8). Therefore, these data demonstrate that Chirp-III contains both δEF1 and USF-1.

Interestingly, there is no canonical E-box binding site in oligo...
FIG. 2. USF-1 binds to the 5′-flanking region of the Ov gene in a complex with δEF1. In A, lanes 1–8 contain oligo C.5 as a probe. Lanes 2–8 contain 8 μg of chick oviduct nuclear protein extract from estrogen-stimulated chicks. Lane 3 contains 100× unlabeled nonspecific DNA (NS) as a competitor. Lane 4 contains 100× oligo C.5 competitor. Lane 5 contains nuclear protein extract preincubated with δEF1 preimmune serum (PI). Lane 6 contains nuclear protein extract preincubated with δEF1 antibody (δ). Lane 7 contains nuclear protein extract preincubated with USF-1 antibody plus the antibody peptidase blocker (PB). Lane 8 contains nuclear protein extract preincubated with USF-1 antibody (U1). Band B is the band representing the predominant protein-DNA complex. Band A is this protein-DNA complex supershifted with the USF-1 antibody. In B, oligo C.5 was used as the probe in lanes 9, 12, and 13. A consensus USF oligomer (U) was used as probe in lane 10, and a mutant USF oligomer (mU) was used as probe in lane 11. Eight μg of estrogen-stimulated oviduct nuclear extract was used in all reactions. Competition of oligo C.5 with the USF consensus binding oligomer, mutated USF consensus binding oligomer, no competitor is shown in lanes 12, 13, and 9, respectively.

Therefore, a consensus USF binding site was used as a probe in a GMSA for comparison with oligo C.5 (Fig. 2B). The USF consensus oligomer binds a protein from oviduct nuclear protein extracts with a greater mobility than band B (Fig. 2B, band C, lane 10), which indicates that USF must be complexed with other proteins when binding oligo C.5. Furthermore, the USF consensus and mutant oligos show little competition for binding of nuclear proteins to oligo C.5 (band B), suggesting that USF is not binding to oligo C.5 in the same manner as to the USF consensus oligo. The competition of USF is hindered by other proteins that sequester or tether it to oligo C.5.

δEF1 and USF-1 Are Co-immunoprecipitated—To test whether the interaction of δEF1 and USF-1 is DNA-dependent, a co-immunoprecipitation assay was performed (Fig. 3). In the top panel of Fig. 3, preciptitated proteins were subjected to Western blotting with anti-δEF1. In the bottom panel, precipitated proteins were blotted with anti-USF-1. The USF-1 antibody precipitates the USF-1 protein and the δEF1 protein (lane 2). However, preincubation with a USF-1 peptide blocker abolished precipitation of USF-1 and δEF1 (lane 1). Similarly, the δEF1 antibody precipitates both δEF1 and USF from oviduct nuclear extracts (lane 3), whereas the δEF1 preimmune serum does not (lane 4). Therefore, USF-1 and δEF1 can be co-immunoprecipitated from oviduct nuclear protein extracts, supporting the hypothesis that the two proteins are part of the same complex. Furthermore, these data suggest that USF-1 and δEF1 are capable of interacting in the absence of DNA, which may explain why the USF consensus oligo was unable to compete for USF in Fig. 2B.

The δEF1 antibody also precipitates a protein slightly larger than USF-1 (43 kDa) that is recognized by the USF-1 antibody. As human USF-2 is 44 kDa, the most likely explanation is that δEF1 is interacting with the USF-1/USF-2 heterodimer as well as with the USF-1 homodimer. Although the antibody is thought to be specific to human USF-1, it is feasible that it also recognizes chick USF-2 as the human proteins are 70% identical in the C-terminal half of the protein.

USF-1 Induces the Ov Promoter—To test whether the interaction of USF-1 with the 5′-flanking region of the Ov gene is functionally relevant, a human USF-1 expression vector was co-transfected into primary oviduct cells with pOVcat.900 (Fig. 4), which contains all the sequences necessary for proper induction of the Ov gene by estrogen and corticosterone (9). When pCMV-USF-1 is co-expressed with the pOVcat.900 reporter construct, the Ov promoter is induced 5-fold in the absence of steroids. This expression is dose-dependent as transcriptional activity in the absence of estrogen and corticosterone increases proportionally to the amount of the USF-1 expression vector co-transfected. USF-1 has no effect when steroids are present, presumably because endogenous USF is sufficient for maximal transcriptional activation.

δEF1 and USF-1 Cooperate to Activate Ov Gene Transcription—Co-immunoprecipitation and GMSA experiments indicate that δEF1 and USF-1 are part of the same complex, and transfection data demonstrate that the Ov gene can be induced by USF-1 (Fig. 1) as well as by δEF1 (23). To assay whether δEF1 and USF-1 cooperate in cells to activate transcription of the Ov gene, they were co-expressed with the reporter pOVcat.900 (Fig. 5). When δEF1 is overexpressed, there is a 3–5-fold induction of the Ov gene in the absence of hormones (lane 2), which confirms previous data (23). When USF-1 is added, there is an increase in reporter gene expression (lanes 3 and 4). Similarly, when USF-1 is overexpressed, there is a 5-fold induction of the Ov gene in the absence of hormones (lane 5), and when δEF1 is added, there is a further increase of reporter gene expression (lanes 6 and 7). Therefore, Ov gene transcription is potentiated by the combination of δEF1 and USF-1, lending further support to the model that they are functioning together in a complex. These data imply that the induction of the Ov gene by estrogen is largely, if not exclusively, the consequence of the induction of δEF1 and the subsequent recruitment of USF to the Ov gene.

The Mass of USF-1 Is Not Regulated by Estrogen—Overexpression of USF-1 and/or δEF1 alleviates the estrogen requirement for transcriptional activation of the Ov gene. Because δEF1 is induced by estrogen to bind to the Ov gene and to activate Ov gene transcription (23), a Western blot was performed to determine whether USF-1 was induced by estrogen in a manner similar to δEF1. Oviduct nuclear extracts were...
made from estrogen-stimulated chicks and from chicks withdrawn from estrogen for 4 days. These extracts were blotted with a USF-1 or a δEF1 antibody. There is no change in USF-1 or USF-2 protein levels when comparing the stimulated and withdrawn oviduct nuclear protein extracts (Fig. 6). However, more δEF1 protein is present in nuclear extracts from estrogen-stimulated extracts than from estrogen-withdrawn extracts (Fig. 6) (see also Ref. 23). Thus, the data indicate that estrogen does not increase the amount of USF-1 protein expressed and support the contention that USF-1 is recruited to the Ov gene 5'-flanking region to activate transcription.

**Fig. 6.** USF-1 is not induced by estrogen. Oviduct nuclear extracts were harvested either from chicks stimulated with diethylstilbestosterone pellets for 2 weeks (+E) or from chicks stimulated with diethylstilbestosterone pellets for 2 weeks after which the pellets were withdrawn for 4 days (−E). Extracts were run on a denaturing polyacrylamide gel and blotted with either a USF-1 antibody or a δEF1 antibody. The USF (USF-1 and USF-2) and δEF1 proteins are denoted.

**Fig. 7.** USF induction of the Ov gene is abolished when δEF1 binding sites in the 5'-flanking region of the Ov gene are mutated. Oviduct cells were transfected and cultured as described previously. The fold induction due to transfected pCMV-USF-1 of each reporter construct in the absence of steroids is plotted. pOvCAT.900 is not induced by the empty expression vector (pCMV-empty), and this CAT activity is set to 1. The fold induction of CAT activity from either pOvCAT.900 or LS-810-150 co-transfected with 450 ng/ml pCMV-USF-1 is shown. Data were pooled from three experiments. *Error bars denote the standard error of the mean.*
regulatory element by another transcription factor without actually binding to its cognate E-box site.

**DISCUSSION**

Altogether, these data indicate that the Ov gene is induced via a unique molecular mechanism involving the tethering of USF to DNA by δEF1. GMSA and co-immunoprecipitation experiments clearly demonstrate that USF-1, and probably USF-2, is part of the Chirp-III complex containing δEF1 (Figs. 2 and 3). However, whether the interaction between δEF1 and USF-1 is direct or whether the interaction is mediated by another protein(s) present in the nuclear protein extracts is yet to be determined. Future co-immunoprecipitation experiments with purified proteins should address this issue. Unfortunately, these experiments must await the cloning of the chicken USF-1 and USF-2 homologs. In the meantime, it is tempting to speculate that the interaction involves the δEF1 Pit, Oct, UNC (POU) homeodomain, which is postulated to be a protein-protein interaction domain.

The interaction between δEF1 and USF is functionally relevant. Overexpression of USF-1 induces the Ov gene in the absence of estrogen (Fig. 4), presumably through the small but detectable amount of δEF1 in oviduct cells in the absence of estrogen (Fig. 6 and 26). The observation that USF-1 does not induce Ov gene transcription in the presence of steroids is likely due to endogenous USF. More importantly, Ov gene transcription is potentiated by the combination of overexpressed δEF1 and USF-1 (Fig. 5), further substantiating the concept that they form a functionally relevant complex.

Most data indicate that the specificity of gene activation by USF is directed by a binding partner or signaling protein. For example, CCAAT/enhancer-binding protein α (C/EBPα) stimulates USF to bind to an E-box in the Ov promoter's 5'-flanking region in an autoregulatory circuit (22). USF cooperatively binds with Stat1 to contiguous DNA elements to induce the class II trans-activator promoter (19). Interestingly, USF is also targeted to promoters in estrogen signaling cascades. In vivo UV cross-linking has shown estrogen-dependent loading of USF to an E-box in the 5’ regulatory region of the cathepsin D gene (15). USF is also involved in the regulation of other estrogen-responsive genes such as the chicken vitellogenin II gene (27) and the mouse efp gene (16) via binding to E-box sites. The data herein demonstrate that USF contributes to Ov gene activation as well. More importantly, the mechanism of USF induction of the Ov gene apparently does not include a direct interaction with DNA (Fig. 2). Instead, it is dependent on intact δEF1 binding sites (Fig. 7). Furthermore, USF-1 is not regulated by estrogen (Fig. 6). This suggests that the estrogen-inducible transcription factor δEF1 tethers USF to the 5’ regulatory region of the Ov gene. Additionally, this concept is made plausible by the GMSA (Fig. 2) and co-immunoprecipitation (Fig. 3) experiments, which demonstrate that USF cannot be competed from the Chirp-III binding site with a USF consensus oligomer and that δEF1 and USF-1 can interact in the presence or absence of DNA. This supports a novel mechanism for steroid hormone signaling whereby USF is recruited to the 5’-flanking region of the Ov gene via tethering to the estrogen-inducible transcription factor δEF1 in the absence of a bona fide USF binding site. These data also extend the emerging paradigm in which the ubiquitous USF transcription factor is recruited by a signal-specific transcription factor to enhance activation of a target gene.

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