The cAMP Response Elements of the α Subunit Gene Bind Similar Proteins in Trophoblasts and Gonadotropes but Have Distinct Functional Sequence Requirements*

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Analysis of transcriptional elements regulating expression of the human α subunit gene in trophoblast cell lines (BeWo or JEG3) revealed the presence of a composite enhancer located within the first 180 base pairs (bp) of the promoter (5–12). This enhancer confers trophoblast-specific expression and consists of an upstream regulatory element (URE) located between positions −180 and −146 and two juxtaposed cyclic AMP response elements (CREs) located between positions −145 and −111 (Fig. 1A). Additional elements required for optimal expression in trophoblasts include the junctional response element and the CCAAT box (13, 14).

In gonadotropes, expression of the α subunit gene involves a more extensive array of regulatory elements located between −330 and −95 bp of the 5′-flanking region. These include the CREs, a specific sub-region in the URE, as well as the gonadotrope-specific element (GSE), the pituitary glycoprotein hormone basal element (PGBE), and the α basal element (αBE, Fig. 1B) (15–17). Mutational analysis of multiple response elements suggested that interactions occur between proteins binding PGBE, αBE, and the CREs (17). In contrast, the GSE acts independently of other promoter elements.

Although no single element is sufficient for targeting expression of the α subunit gene to trophoblasts or gonadotropes, the CREs, which contain the conserved palindromic sequence TGGAGCTCA, provide the strongest contribution to transcription in both cell types (11, 12, 18–20). In trophoblasts, mutation of any base within this core sequence diminished both the binding of proteins to the element and promoter activity (5, 21). Cross-species comparison of promoter sequences revealed that non-primates contain one variant CRE with a single nucleotide difference in the core of the CRE palindrome (TGGAGCTCA to TGGTGTCA) (22, 23). This disclosed a correlation between the presence of the variant CRE (TGATGTCA) and the inability to express this gene in trophoblasts. The correlation was further substantiated by the observation that changing the variant CRE of the bovine promoter to the palindromic sequence reconstituted activity of this element in a trophoblast cell line (7).

While the CRE contributes strongly to activity of the human α subunit promoter in both trophoblasts and gonadotropes, the transcription factors that transduce this signal in these cells have not been clearly identified. In this study, we describe the proteins binding the CREs in both cell types and use the non-primate variant CRE as a probe for exploring their functional significance when studied in the context of the human α subunit promoter.

The α subunit gene encodes a common subunit shared by all glycoprotein hormones. This single copy gene is expressed in pituitary gonadotropes and thyrotropes of all mammals and in placental trophoblasts of primates and horses. Tandem cAMP response elements (CREs) in the promoter of the human gene are key mediators of this pattern of cell-specific expression. Replacing the palindromic CREs with non-primate variant CREs significantly attenuated activity in trophoblasts but not in gonadotropes. Furthermore, proteins binding the palindromic CRE cross-reacted with antibodies for CREB, CREM, ATF1, ATF2, and c-Jun, while proteins binding the variant CRE cross-reacted only with ATF2 and c-Jun antibodies. The data suggest that ATF2 and c-Jun can activate transcription through the CREs in gonadotropes but not in trophoblasts. Additional analyses indicated that while promoters with either palindromic or variant CREs have similar overall activity in gonadotropes, the variant CREs make a much smaller contribution to promoter activity than their palindromic counterparts. The weaker contribution of the variant CREs is compensated by the activity of two upstream elements present in the promoter. This compensation probably occurs through an indirect mechanism, as the binding affinity of proteins to the CRE is not influenced by the presence of these upstream elements.

The glycoprotein hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (CG) are essential regulators of a variety of biological processes such as reproduction (LH and FSH), growth and metabolism (TSH), and maintenance of pregnancy (CG). Each consists of a common α subunit and a unique β subunit that confers hormone specificity (1). In all species, FSH, LH, and TSH are synthesized in the anterior pituitary, while only primates synthesize CG in the placenta (2–4). A fifth glycoprotein hormone, pregnant mare serum gonadotropin, is produced in the placenta of horses. Synthesis of the complete spectrum of glycoprotein hormones requires accurate expression of the single α subunit gene in gonadotropes (LH and FSH) and thyrotropes (TSH) of the pituitary as well as the trophoblasts of the placenta in primates and horses.

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1 The abbreviations used are: bp, base pair(s); CRE, cAMP response element; GSE, gonadotrope-specific element; PGBE, pituitary glycoprotein hormone basal element; αBE, α basal element; PCR, polymerase chain reaction; NRS, nonimmune rabbit serum; EMSA, electrophoretic mobility shift assay.

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Electrophoretic Mobility Shift Analysis and Supershift Analysis—Nuclear extracts were prepared as described elsewhere (25). Electrophoretic mobility shift analysis was performed as described previously, except a 4% polyacrylamide gel was used to resolve the complexes (17). For supershift analysis, the reactions were initially incubated on ice for 5 min in the presence of all reaction components except antibody. After the addition of antibody, samples were incubated at room temperature for 15 min, followed by a brief incubation on ice prior to loading. Electrophoresis was performed for 4.5 h at 240 V. CREB, CREM, c-Jun# (where # indicates different c-Jun antibodies), and c-Fos antibodies were purchased from Upstate Biotechnology, Inc. ATF1, ATF2, ATF3, ATF4, and c-Jun antibodies were purchased from Santa Cruz Biotechnology, Inc. Either 1 μl of antibody for CREB, CREM, c-Fos, c-Jun#, and nonimmune rabbit serum (NRS) or 3 μl of the remaining antibodies was added to the reactions.

RESULTS

The CRE Contributes to Both Trophoblast- and Gonadotropespecific Expression, but the Sequence Requirements for This Element Differ between the Two Cell Types—In both trophoblasts and gonadotropes, full activity of the human α subunit promoter depends on the presence of the tandem CREs. Earlier studies indicated that a single natural variant of the CRE (TGATGTCGA) is incompatible with expression of the α subunit gene in trophoblasts (26). To determine the effect of this variant CRE on both trophoblast- and gonadotrope-specific expression, we replaced the two palindromic CREs of the human α subunit promoter with variant CREs by making a single C to T mutation in the palindromic core. The variant CREs diminished promoter activity to the same extent as a complete block mutation through the CREs when analyzed by transient transfection in BeWo cells (Fig. 2A). In contrast, the same mutation had a minimal effect on promoter activity when analyzed in αT3 cells (Fig. 2B). Thus, even though the CREs are important for promoter activity in both gonadotropes and trophoblasts, the variant CRE can interchange functionally with the palindromic element only in gonadotropes. This indicates that trophoblasts and gonadotropes have distinct sequence requirements for the CREs.

Differences in the Proteins Binding the Palindromic and Variant CREs Can Be Accounted for by Variations in Binding Affinities—The observation that the sequence requirements of the CRE are different for trophoblasts and gonadotropes suggested that different proteins may function at this site. To address this possibility, we performed electrophoretic mobility shift assays (EMSA) using both the palindromic and variant CREs as probes and nuclear extracts from either αT3 or BeWo cells. In the presence of αT3 nuclear extracts, the palindromic CRE probe was incubated with BeWo nuclear extracts. In contrast, the variant CRE (Var) probe appears to bind only one of the two complexes that bind the palindromic CRE (Band 2, Fig. 3). This complex is detected in both αT3 and BeWo extracts, although the signal is less intense with BeWo extracts. In short, the variant CRE appears to bind only one of the two protein complexes bound by the palindromic CRE. This pattern holds for both cell types.

Two minor complexes were also observed. One complex binds predominantly the palindromic CRE and migrates to a position between Bands 1 and 2 in both αT3 and BeWo cells. The second minor complex migrates just above the nonspecific doublet and was observed only in αT3 cells. This second band was detected only in a small number of nuclear preparations, suggesting that it may be a degradation product.
Fig. 2. The variant CRE is functional only in αT3 cells. The CREs of the human α subunit promoter were replaced with either a full block replacement mutation (μCRE) or with a variant form of the CRE that contains a C to T mutation in the core of each CRE (Var, TGAGCTCA to TGATGTCA). These were inserted into 1500 bp of the human α subunit promoter and used to drive expression of the luciferase reporter gene. The luciferase constructs were cotransfected with Rous sarcoma virus-luciferase constructs were cotransfected into either BeWo (A) or αT3 (B) cells. The luciferase activity of each construct was normalized to β-galactosidase activity of each construct normalized to the luciferase-β-galactosidase activity of the wild type construct (Pal). Transfections were done a minimum of three times, and the error bars represent standard error of the mean.

Fig. 3. Two major shifted complexes bind the palindromic CRE while one shifted complex binds the variant CRE. Radiolabeled probes corresponding to either the palindromic CRE (Pal, 5′-GATCAAAATTGACGTCATGGTAAA-3′) or the variant CRE (Var, 5′-GATCAAATTGAGCTCAATGGTAAA-3′) were used in electrophoretic mobility shift assay with either αT3 or BeWo nuclear extracts. 40 fmol of radiolabeled probe was incubated with 7 μg of nuclear proteins and resolved on a 4% nondenaturing polyacrylamide gel as described under “Experimental Procedures.” When indicated, unlabeled competitors were added at a 200 × molar excess prior to the addition of probe. NS, nonspecific competitor.

To further characterize the proteins binding the palindromic and variant CREs, a competition profile was established using the palindromic CRE (Pal) as a probe and nuclear extracts from αT3 cells. A 50-fold molar excess of homologous competitor (Pal) effectively eliminated the signal from Band 2, whereas effective competition of the proteins in Band 1 required a 250-fold excess of unlabeled Pal (Fig. 4). When the variant CRE (Var) was used as a competitor, a 100-fold excess was required to compete the signal from Band 2, while a 1000-fold excess was required for competition of Band 1. Even at this level, complete elimination of the signal from this band was not observed.

The above data indicate that the variant CRE binds the same proteins as the palindromic CRE but with lower affinity. The proteins in Band 2 have an estimated 2- to 5-fold greater affinity for the palindromic CRE than for the variant CRE, while the Band 1 proteins have at least a 200-fold greater affinity for the palindromic CRE than they do for the variant CRE. This may explain why the variant CRE appears to bind only proteins in Band 2 when used as a probe under standard EMSA conditions and suggests that Band 1 proteins do not have a high enough affinity for the variant CRE to allow complex formation under the conditions of the assay, while Band 2 proteins do have sufficiently high affinity to permit complex formation.

Fig. 4. The variant CRE binds the same proteins as the palindromic CRE but with lower affinity. EMSA was performed as described in the legend of Fig. 3 using radiolabeled palindromic CRE as probe and αT3 nuclear extracts. Increasing concentrations of unlabeled homologous (Pal) or variant (Var) competitors were added to the reactions prior to the addition of probe in the fold molar excess indicated. Also included as a competitor was a mutant oligonucleotide (μ6–11, 5′-CTAGAAAATTCTCTCTGGATGGTAAA-3′) that has seven of the eight nucleotides (underlined) mutated in the palindrome core.

Multiple Proteins in the bZIP Family Bind to the Palindromic CRE Whereas Only c-Jun and ATF2 Appear to Bind the Variant CRE—Several members of the bZIP family of transcription factors are known to bind to CREs (27, 28). To identify proteins binding the α subunit CRE, a variety of antibodies generated against members of this family were tested for their ability to cross-react with the protein complexes observed by EMSA. When antibodies were added to EMSA reactions containing αT3 nuclear extracts and a palindromic CRE probe, cross-reactivity with Band 1 proteins occurred with antibodies against CREB, CREM, and ATF1 (Fig. 5A). In the presence of these antibodies, there is a loss in proteins bound in Band 1 and a concomitant appearance of a supershifted complex (indicated by an asterisk). No change in intensity for Band 2 proteins was observed in these reactions, indicating that they do not cross-react with these antibodies. However, proteins in Band 2 did cross-react with antibodies against c-Jun and ATF2 as indicated by a loss in proteins bound in this band. As observed with many antibodies, the ATF2 antibody does not form a super-
shifted complex. The c-Jun and ATF2 antibodies did not cross-react with proteins in Band 1. Furthermore, antibodies specific for c-Fos, ATF3, and ATF4 as well as NRS did not cross-react with any of the proteins binding the CRE. It is important to note that antibodies from serum preparations (CREB, CREM, JUN#) contain a nonspecific complex that migrates in the gel just above Band 2. This band was also detected in the absence of added nuclear extracts and in NRS.

When the variant CRE was used as a probe with αT3 nuclear extracts, c-Jun and ATF2 antibodies cross-reacted with proteins in the Band 2 complex (Fig. 5B) as indicated by the loss of complex proteins in the presence of these antibodies. In addition, a supershifted complex was observed with JUN# antibody but not with the JUN antibody. Since a supershifted complex was observed with the palindromic CRE, the lack of a distinct supershifted complex with the JUN antibody on the variant CRE suggests that this antigen-antibody complex is distinct from that formed on the palindromic CRE or that lower binding affinity, which diminishes the band intensity, obscures visualization of the supershifted complex. The nonspecific bands from the serum antibody preparations (CREB, CREM, NRS, and JUN# lanes) are located just above the Band 2 complex.

To compare this with the proteins that bind to the CREs in trophoblasts, a similar antibody analysis was performed with BeWo nuclear extracts and the palindromic CRE probe. The same proteins that bind the CRE in αT3 cells bind to this element in BeWo cells (Fig. 5C). That is, the proteins in Band 1 cross-reacted with the CREB, CREM, and ATF1 antibodies, while the proteins in Band 2 cross-reacted with c-Jun and ATF2 antibodies. As in αT3 cells, the variant CRE was found to bind to c-Jun and ATF2 (data not shown).

An α Subunit Promoter with Variant CREs Depends More Heavily on the Upstream Elements, PGBE and aBE, Than a Promoter with Palindromic CREs—The data described above revealed that the palindromic CRE binds CREB, CREM, ATF1, ATF2, and c-Jun, while the variant CRE binds predominantly ATF2 and c-Jun. Furthermore, the variant CRE was shown to have a lower binding affinity for these proteins than the palindromic CRE (Fig. 4). In a previous study, we determined that the upstream promoter elements, PGBE and aBE, contributed more to the activity of the human α subunit promoter in the absence of the CRE binding proteins than in their presence (17). This suggested that in the company of the “weaker” variant CRE, the upstream promoter elements might play a more significant role in regulating gene transcription. To address this possibility, mutations in the upstream elements were placed in promoters carrying either the variant or palindromic CREs, and their activities were compared using transient transfection analysis in αT3 cells.

When assayed in the presence of the palindromic CREs, mutations in the PGBE and aBE elements decreased promoter activity by approximately 3-fold when compared with the wild type control (Fig. 6A). In contrast, when assayed in the context of a variant CRE, mutations in either the PGBE or αBE diminished promoter activity between 10- and 15-fold (Fig. 6B). Since the activity of palindromic- and variant-containing α subunit promoters are nearly equivalent (Fig. 2), these data strongly suggest that the PGBE and aBE elements provide a greater contribution to promoter activity to compensate for the weaker variant CRE.

To further address whether the compensation observed with the PGBE and aBE was unique for this pair of elements, we analyzed activity of palindromic- and variant-CRE containing α subunit promoters with mutations in the GSE. Our previous studies indicated that this element contributes independently to a subunit promoter activity (17). A mutation in the GSE had approximately the same impact on promoter activity whether assayed in a subunit promoters having palindromic or variant CREs.

The Upstream Elements Do Not Affect the Binding of Proteins to the CRE—The above data, as well as previously published data, suggested that the upstream elements PGBE and aBE contribute more to promoter activity when there is a loss in proteins binding to the CREs (17). This could occur through a mechanism in which the upstream elements help stabilize or enhance the CRE binding proteins to their element. To test this possibility, we used EMSA and various DNA competitors containing mutations in the upstream elements to determine if these elements influence the ability of promoter fragments to compete and thus bind the CRE binding proteins. Seven different promoter fragments that span from nt 556 to nt 60 of the human α subunit promoter were used (Fig. 7A). In the presence of the palindromic CREs (competitors 1–4), the fragments competed equally for the CRE binding proteins regardless of the presence of mutations through the upstream elements (Fig. 7B). This was confirmed by densitometric analysis, which revealed no significant difference in the intensities from these bands. Fragments containing mutations through the CREs (competitor 5) or through the CREs and the upstream elements.
The CREs play a major role in cell-specific regulation of the human α subunit promoter in both trophoblasts and gonadotropes. In trophoblasts, mutation or deletion of the CREs results in a dramatic loss in promoter activity (5, 12, 17). Furthermore, proteins binding a second element, the URE, cannot activate transcription in the absence of CRE binding proteins (5, 6, 8, 9). Together, the URE and CRE define a composite enhancer sufficient for imparting trophoblast-specific activity to the α subunit promoter. In gonadotropes, mutation of the CREs also has a significant effect on promoter activity. However, instead of the URE, two elements further upstream, PGBE and αBE, act with the CREs to define another composite element that appears responsible for most of the activity of the α subunit promoter in gonadotropes (17).

In this report, we establish yet another significant difference in how the CREs contribute to activity of the α subunit promoter in trophoblasts and gonadotropes. By exchanging the palindromic CREs of the human α subunit promoter with a species variant of the element, we demonstrated that the variant CRE is compatible with expression of the α subunit gene in gonadotropes but not in trophoblasts.

Earlier studies using DNase footprinting or EMSA disclosed that the α subunit CREs are occupied by proteins in both trophoblasts and gonadotropes, but the proteins were not identified (8, 5, 12, 15, 16, 21). Steger et al. (29) showed that purified CREB binds to the human α subunit promoter and, with much lower affinity, to the mouse and equine α subunit promoters. Additional studies suggested that heterodimers of CREB might be involved in activation of the variant CRE in GH3 cells (26). Our studies are the first to clearly identify proteins from cell lines derived from both trophoblasts and gonadotropes that bind the CREs of the α subunit promoter.

The palindromic CRE binds proteins that cross-react with antibodies generated against several members of the bZIP family, including CREB, CREM, ATF1, ATF2, and c-Jun. This indicates that the proteins binding the human α subunit CRE are either similar or identical to these transcription factors that form both homodimeric and heterodimeric complexes. CREB-CREB, CREB-CREM, CREM-CREM, CREB-ATF1, and ATF2-c-Jun dimers are all known to bind to palindromic CREs (28, 30). The precise roles for each of these proteins in the activation and/or regulation of the α subunit gene are not yet known, but the ability to bind so many members of the CREB/ATF family implicates the CRE as a potential target for multiple signaling pathways in the regulation of this gene.

The variant CRE binds the same proteins as the palindromic CRE but with lower affinity. In fact, under standard gel shift conditions, the non-palindromic CRE binds predominantly ATF2 and c-Jun. Interestingly, these two bZIP proteins were previously shown to bind to a similar response element, jun1, on the c-Jun promoter (TGACATCA) (31). ATF2 and c-Jun have approximately two to five times lower affinity for the variant CRE than they have for the palindromic CRE. While CREB, CREM, and ATF1 also bind the variant CRE, they do so with approximately a 200-fold lower affinity, making it unlikely that
the palindromic CRE, where fragment 1 is the wild type promoter
competitor DNAs (17). The corresponding promoter fragments used as
plate DNAs that contain either the wild type sequence or different
CRE that is unoccupied in trophoblasts. An
palindromic CRE (sequence in legend of Fig. 2) as probe and
AtF2 and c-Jun are the principal bZIP proteins that contribute
to activity of α subunit promoter with a variant CRE in gona-
drotropes. Significantly, our data also establish that even
though ATF2 and c-Jun are able to bind the variant CRE, this
complex is unable to interact productively with the α subunit
promoter in trophoblasts.

One plausible explanation for the cell-specific difference be-
tween the palindromic and variant CRE is that concentrations
of ATF2 and c-Jun in trophoblasts are inadequate to generate the
appropriate complexes on the α subunit promoter. In this
regard, the main difference between the CRE binding proteins
in αT3 and BeWo cells is the distribution of the proteins in
Bands 1 and 2 (Fig. 3). In BeWo cells, Band 2, which is com-
prised of complexes containing ATF2 and c-Jun, was noticeably
less intense than in αT3 cells, suggesting that trophoblasts
contain lower concentrations of these proteins. In addition, the
variant CRE binds ATF2 and c-Jun with lower affinity than the
palindromic CRE. These two factors likely result in a variant
CRE that is unoccupied in trophoblasts. An α subunit promoter
containing the variant CRE would be inactive in these cells. Of
course, it is also possible that ATF2 and c-Jun bind sufficiently to
the variant CREs but fail to interact with other essential tran-
scription factors in trophoblasts. Clearly, further studies
are needed to distinguish between these possibilities.

In gonadotropes, the presence of the variant CRE does not
impact negatively on activity of the α subunit promoter nor
markedly alter the transcriptional mechanism. For example,
activities of α subunit promoters with either a palindromic or
variant CRE are nearly identical (Fig. 2). Furthermore, the
same upstream elements, PGBE, αBE, and GSE, are required
for full promoter activity regardless of which CRE is present
(Fig. 6). However, the degree to which promoter activity de-
deps on the upstream elements differs between the variant
and palindromic CREs. In an earlier study (17), we showed
through pairwise mutational analysis that the PGBE and αBE
acted synergistically with each other to contribute to promoter
activity. We also noted that PGBE/αBE interacted with the
tandem CREs. However, in this case the elements appeared to
partially compensate for one another. Consistent with this
finding, we now show that in the presence of the “weaker”
variant CRE, mutations in PGBE and αBE had a four to five
times greater impact on promoter activity than when the mu-
tations were linked to the palindromic CRE. Thus, in the pres-
ence of a “weaker” CRE, more transcriptional demand is placed
on the PGBE/αBE. Significantly, this interaction between
PGBE/αBE and the CREs is specific for that particular combi-
nation as the GSE had the same relative contribution to pro-
ducer activity in the presence of either CRE. The ability of
the GSE binding proteins to activate independently from the CRE
binding proteins was noted earlier as well (17). Thus, it is
apparent from these studies that the proteins binding the up-
stream elements and the CREs can influence each other’s ac-
tivity. However, in the presence of the variant CRE, it is un-
clear if the influence of the upstream elements is greater
because different proteins bind the variant CRE or because this
CRE has a lower affinity for the proteins.

A mechanism by which PGBE/αBE might influence the
CREs is by stabilizing or enhancing the binding of transcrip-
tion factors to this element. By adding different competitor
DNA fragments that contain the CRE as well as the wild type
or mutant upstream elements, we were able to determine that
PGBE and αBE do not influence the ability of these DNA
fragments to bind CRE binding proteins. Since binding is not
influenced, the ability of the upstream elements to compensate
for the weakened variant CRE suggests that a common mech-
anism or pathway is used in activating transcription among the
proteins binding PGBE, αBE, and the CREs. Such a mecha-
nism may involve the use of a common co-activator or member
of the basal transcription apparatus.

In conclusion, the CREs, by interacting with different up-
stream elements, help to assure expression of the α subunit
gene in two distinct cells types. Functional studies with the
palindromic and variant CRE revealed a difference in the se-
quence requirements of the element in trophoblasts and gona-
drotropes. This suggested that interactions between the URE
binding proteins and homo- or heterodimers containing CREM,
CREB, and ATF1 are important for trophoblast-specific expres-
sion, while, at least in non-primates, interactions between
PGBE/αBE binding proteins and Jun and ATF2 are important
for gonadotrope-specific expression. Our goal now is to estab-
lish the relationship between the CRE binding proteins and the
proteins binding the upstream elements.

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