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Combinatorial Roles of Protein Kinase A, Ets2, and 3′,5′-Cyclic-Adenosine Monophosphate Response Element-Binding Protein-Binding Protein/p300 in the Transcriptional Control of Interferon-τ Expression in a Trophoblast Cell Line

Padmalaya Das, Toshihiko Ezashi, Rangan Gupta, and R. Michael Roberts

Division of Animal Sciences (P.D., T.E., R.M.R.) and Veterinary Pathobiology (R.G., R.M.R.) and Biochemistry (R.M.R.), University of Missouri-Columbia, Columbia, Missouri 65211

In ruminants, conceptus interferon-τ (IFNT) production is necessary for maintenance of pregnancy. We examined the role of protein kinase A (PKA) in regulating *IFNT* expression through the activation of Ets2 in JAr choriocarcinoma cells. Although overexpression of the catalytic subunit of PKA or the addition of 8-bromo-cAMP had little ability to up-regulate *boIFNT1* reporter constructs on their own, coexpression with Ets2 led to a large increase in gene expression. Progressive truncation of reporter constructs indicated that the site of PKA/Ets2 responsiveness lay in a region of the promoter between −126 and −67, which lacks a cAMP response element but contains the functional Ets2-binding site and an activator protein 1 (AP1) site. Specific mutation of the former reduced the PKA/Ets2 effects by more than 98%, whereas mutation of an AP1-binding site adjacent to the Ets2 site or pharmacological inhibition of MAPK kinase 2 led to a doubling of the combined Ets2/PKA effects, suggesting there is antagonism between the Ras/MAPK pathway and the PKA signal transduction pathway. Although Ets2 is not a substrate for PKA, lowering the effective concentrations of the coactivators, cAMP response element-binding protein-binding protein (CBP)/p300, known PKA targets, reduced the ability of PKA to synergize with Ets2, suggesting that PKA effects on *IFNT* regulation might be mediated through CBP/p300 coactivation, particularly as CBP and Ets2 occupy the proximal promoter region of *IFNT* in bovine trophoblast CT-1 cells. The up-regulation of IFNT in the elongating bovine conceptus is likely due to the combinatorial effects of PKA, Ets2, and CBP/p300 and triggered via growth factors released from maternal endometrium. (*Molecular Endocrinology* 22: 331–343, 2008)

INTERFERON-τ (IFNT) IS regarded as a crucial signal from the conceptus that triggers early maternal responses to pregnancy in ruminants (1). One role of IFNT is to prolong the lifespan of the corpus luteum (CL) by suppressing the pulsatile release of the luteolysin, prostaglandin $F_{2\alpha}$ from the uterine endometrium, thereby preventing a return to ovarian cyclicity (2–5). The production of large amounts of IFNT by the still unattached, early elongating, conceptus is believed to be critical to the establishment of pregnancy by modulating the uterine output of the luteolytic factor, prostaglandin $F_{2\alpha}$ and adjusting gene expression locally in the endometrium to accommodate the growing conceptus (6, 7).

The genes encoding the IFNT proteins (*IFNT*) differ most markedly from other type I IFN in their lack of inducibility in response to virus, their localized transcription in trophectoderm before firm attachment of the conceptus to the uterine wall, and the high rate and persistence of their expression over a critical period of pregnancy when progesterone production by the CL must be maintained if the conceptus is to survive (1–5). IFNT is the major secretory product of conceptus between d 13 and d 21 of pregnancy in sheep (8, 9) and between d 14 and d 24 in cattle (10, 11), but a low rate of production can be measured as the blastocyst forms (12, 13). However, there is a marked increase in IFNT synthesis coinciding with the morphological transition of the blastocyst from a spherical to a filamentous form, rather than a strict correlation with day of pregnancy (14, 15).

Hormones and growth factors secreted by the maternal endometrium may be required for the optimal production of IFNT, because supplementation of cul-

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Abbreviations: AP1, Activator protein 1; CBP, cAMP response element-binding protein (CREB)-binding protein; ChIP, chromatin immunoprecipitation; CL, corpus luteum; CMV, cytomegalovirus; CRE, cAMP response element; CREB, CRE-binding protein; IFNT, interferon-τ; hCG, human chorionic gonadotropin; MEK, MAPK kinase; PKA, protein kinase A; PKI, inhibitor of PKA; RSV, Rous sarcoma virus; siRNA, short interfering RNA.

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ture medium with uterine flushing from ewes in the late luteal phase of estrous cycles increases the production of IFNT by cultured, *in vitro*-produced blastocysts (13, 16, 17). Moreover, when endometrial glands fail to form in ewes, conceptuses release lower amounts of IFNT (18), suggesting that maternal secretions produced by the glands might be responsible for the up-regulation of production. When uterine secretions in bred ewes are stimulated after mating by progesterone administration, conceptus IFNT production is increased more than 50-fold at d 12 of pregnancy compared with the controls (19). Similarly, early progesterone supplementation promotes IFNT production by conceptuses in the uterine tract of the cow (20). We (16) and others (21–23) have suggested that factors present in maternal glandular secretions trigger these events by binding their cognate receptors on trophectoderm and activating key intracellular signaling pathways For example, the growth factor, colony-stimulating factor-1, which operates through the Ras/MAPK pathway, is able to up-regulate *IFNT* promoter activity (16). On the other hand, uterine secretions probably contain a complex mixture of growth factors and hormones, and the increase in IFNT production during the period in which the trophoblast elongates is massive and sustained, raising the possibility that more than a single hormone and intracellular signaling pathway are involved.

Considerable evidence has accumulated to suggest that the transcription factor, Ets2, through its interaction with the proximal promoter region of *IFNT*, is the key transcription factor governing *IFNT* expression in trophectoderm (24, 25). It is, for example, a target for the Ras/MAPK pathway discussed above and, as such, probably mediates the action of hormones, such as colony-stimulating factor-1, on *IFNT* gene transcription (16). In the case of ovine trophoblast Kunitz domain protein-1, which has an almost identical expression pattern to IFNT during ovine conceptus development, Ets2 interacts with a second transcription factor, CCAAT enhancer binding protein-β to transactivate the trophoblast Kunitz domain protein-1 gene (26). Ets2 is essential for full placental development in the mouse, with deletion of the *ets2* gene leading to embryonic mortality before d 8.5 of embryonic development (27). Ets factors, including Ets2, can up-regulate the promoters of several genes known to be expressed in trophoblast of species other than cattle and sheep, including CGA (*hCG*α) (28) and CGB5 (hCGβ) (29, 30), *CYP11A1* (31), *MMP1* (32), *MMP3* (33–35), urokinase-type plasminogen activator (*PLAU*) (36, 37), and *MMP9* (38), rat placental lactogen II (39), prolactin related protein (40), and porcine pregnancy associated glycoprotein 2 (*PAG2*) (41), suggesting that Ets2, and probably other key transcription factors, play dual roles. One is in the regulation of genes encoding specific trophoblast products; the other is ensuring proper functional differentiation of the trophoblast (42, 43).

The cAMP signaling system is another key regulator of trophoblast differentiation and function (44). Mononuclear cytotrophoblast cells aggregate and fuse to form multinucleated synctiotrophoblasts in the human placenta, and this behavior may be controlled by cAMP (45). Single cytotrophoblast-like cells of the human choriocarcinoma (BeWo) cell line fuse and undergo extensive morphological differentiation to yield syncytia in the presence of the cAMP analog, forskolin, (45, 46). Finally, although only about 5% of JAr cells normally produce measurable human chorionic gonadotropin (hCG), the addition of 8-bromo-cAMP increases the number of hCG-producing cells several fold, as well as stimulating hCG synthesis (47, 48). These data suggest that the expression of the *IFNT,* like many other genes associated with trophoblast function, might be regulated by the cAMP/protein kinase A (PKA) signal transduction pathway and hormones and growth factors that activate that pathway.

RESULTS

Synergistic Effects of Ets2 and PKA on *IFNT* Promoter Expression

First we investigated the effect of the constitutively active, α catalytic subunit of PKA on the expression of the bovine *IFNT1* promoter in JAr choriocarcinoma cells. The bovine -126 *IFNT1* promoter, which includes the proximal enhancer sequence (-91 to -61), is sufficient to drive basal expression (49), as well as Ets2-mediated up-regulation of luciferase *(luc)* reporter activity (25). This promoter-reporter construct was modestly up-regulated (usually between 2- to 4-fold) by ectopic expression of PKA (Fig. 1A). Under the same transfection conditions, overexpression of Ets2 increased *luc* reporter activity from the -126 promoter approximately 58-fold, although this value can vary according to the plasmid preparation and cell culture parameters (data not shown). Cotransfection of the Ets2 expression construct with the activated PKA construct up-regulated the -126 *IFNT* reporter by about 500-fold (Fig. 1A). A qualitatively similar, but lower up-regulation of reporter activity was observed when JAr cells were exposed to 0.5 mM 8-bromo-cAMP, rather than to ectopically expressed PKA (Fig. 1B). When coexpressed in a second choriocarcinoma cell line, JEG3, Ets2 and PKA provided a dramatic, synergistic increase on reporter activity of even larger magnitude ($\sim$2000-fold) than that noted in JAr cells (Fig. 1C). Curiously, PKA alone had a much larger effect in JEG3 than in the JAr cells ($\sim$200-fold *vs.* 2-fold). The reason for this difference is not clear but may relate to the relative basal activities of the PKA signal transduction pathway in the cells.

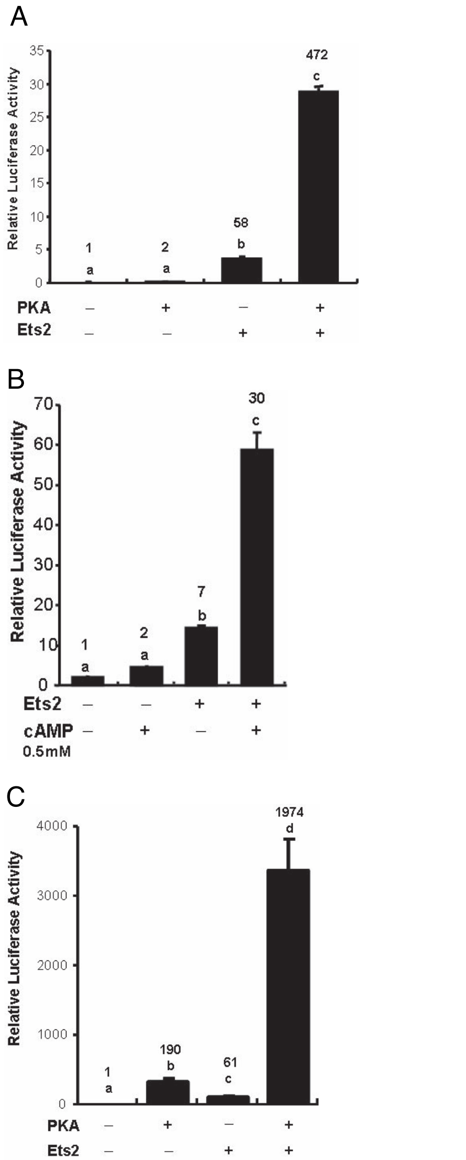

Fig. 1. Coexpression of Ets2 and PKA Synergistically Up-Regulates the *IFNT* Promoter in Choriocarcinoma Cells

A, The −126*luc* promoter was cotransfected with expression plasmids for Ets2 and constitutively active catalytic subunit of PKA alone (PKA) or in combination into JAr cells. B, The −126*luc* promoter was cotransfected with the Ets2 expression plasmid into JAr cells. After 24 h, cells were treated with 0.5 mM 8-bromo-cAMP for 36 h before collection of cell lysates. C, The −126*luc* promoter was cotransfected with expression plasmids for Ets2 and PKA alone or in combination into JEG3 cells. In all experiments, the *luc* activity was normalized relative to β-galactosidase activity from the reference reporter, pRSVLTR-βgal, and the normalized *luc* activities are presented relative to the activity of −126*luc* from nontreated cells (means ± SEM; n = 3), with fold activation shown *above each bar*. If *letters above bars* are different, there was a significant effect of treatment ($P < 0.05$).

Deletion and Mutational Analysis of the *IFNT* Promoter

To define which region of the *IFNT* promoter was responsible for the ability of PKA and cAMP to act synergistically with Ets2 and provide the large up-regulation of *luc* reporter activity noted in Fig. 1, we progressively truncated the −126 *IFNT* promoter and cotransfected these shorter constructs either with the

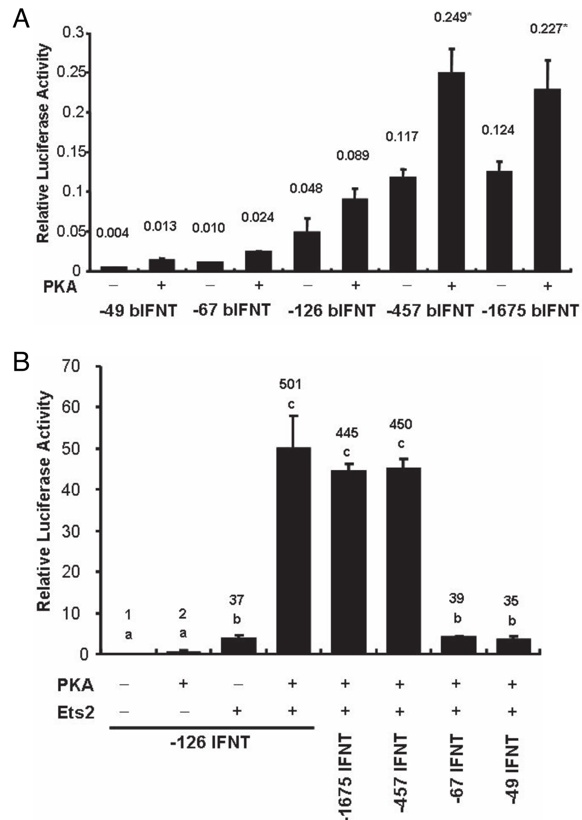

Fig. 2. A Truncation Analysis of the *IFNT* Promoter to Define the Gene Control Region Responsible for the Activation of the *IFNT* Promoter by PKA

A, JAr cells were cotransfected with the −49, −67, −126, −457, and −1675*luc* promoters and either empty vector or the expression plasmid for the constitutively active catalytic subunit of PKA, and relative *luc* activities were measured (*left scale* and values *above each bar*). Values with *asterisks* indicate where the treatment (ectopic expression of PKA) had a significant effect on reporter expression from a particular promoter construct. A significant effect was only noted with the −457 and −1675 promoter constructs. B, JAr cells were transfected with −126, −457, −1675, −67, and −49*luc* promoters with and without the expression plasmids for Ets2 and PKA. In Fig. 2A, *luc* activities are compared with average *luc* activity of −49*luc* from cells nontreated with PKA. In Fig. 2B, *luc* activities are compared with averaged *luc* activity of −126*luc* from cells nontreated with Ets2 and PKA (means ± SEM; n = 3). Fold activation over the activity of −126*luc* from nontreated cells is shown *above each bar*. If *letters above bars* are different, there was a significant effect of treatment ($P < 0.05$). bIFNT, bovine IFNT.

PKA expression plasmid (Fig. 2A) or with the combination of PKA/Ets2 (Fig. 2B) expression plasmids into JAr cells. In addition, we examined a series of longer promoter constructs to determine whether regions upstream of −126 were influenced by Ets2/PKA overexpression (Fig. 2, A and B). Basal *luc* activity from the *boIFNT1* −457*luc* and −1675*luc* promoter constructs was up-regulated approximately 2-fold when coexpressed with the constitutively active catalytic subunit of PKA (Fig. 2A). Reporter activity from the three shorter constructs was quite low and varied between experiments. Although there was a tendency for ectopic PKA expression to increase reporter expression with all three promoter constructs, the differences observed were not significant ($P > 0.05$). It would appear that the entire IFNT upstream region lacks a strong PKA target sequence. The slight positive effects are possibly the results of nonspecific effects on cell metabolism rather than direct effects on the promoter. Cotransfection of Ets2 and PKA transactivated the −1675*luc*, −457*luc*, and −126*luc* promoters to similar extents, namely 445- and 450- and 501-fold, respectively (Fig. 2B). By contrast, the −67*luc* and −49*luc* promoters, which lack the Ets binding site, were much less responsive (a 93% decrease compared with −126*luc*) when Ets2 and PKA were coexpressed. This experiment suggests that the proximal enhancer region between −126 and −67 bp of the *IFNT* gene is involved in mediating PKA responsiveness, but only when Ets2 is overexpressed.

Dependency of Ets2/PKA Synergism on the Kinase Activity of PKA

We next examined whether the *IFNT* promoter was up-regulated when Ets2 was coexpressed with an inactive form of PKA, which has a methionine substitution for a lysine in the ATP-binding region. As also observed in Figs. 1 and 2, the overexpression of Ets2 and PKA synergistically up-regulated the *IFNT* promoter (Fig. 3A). *Luc* reporter activity was reduced approximately 93% when the mutant form of PKA was substituted for the wild-type enzyme. We also examined the effects of coexpressing an inhibitor of PKA, PKI. In its presence, the synergistic effect of Ets2 and PKA was markedly reduced (Fig. 3A). Together, these experiments suggest that the PKA-mediated enhancement of Ets2 effects require a catalytically active kinase.

As a further test of whether the PKA signal transduction pathway was involved in the transactivation of the *IFNT* promoter by Ets2, we tested a pharmacological inhibitor (H-89) of the pathway. PD98059, an inhibitor of the Ras/MAPK signal transduction pathway, which is also known to target Ets2 at Thr72 (16, 25, 50), was used as a control. Ets2 and PKA caused a major (469-fold) up-regulation of reporter activity (Fig. 3B). This stimulatory effect was inhibited approximately 93% by H-89, which binds to the ATP-binding site of the catalytic subunit of PKA. Surprisingly, the addition of MAPK kinase (MEK) inhibitor, PD98059, rather than acting as an inhibitor, almost doubled reporter gene activity (from 469-fold to more than 900-fold) (Fig. 3B). Again, it is clear that there is a requirement for PKA catalytic activity in the synergistic interaction of PKA and Ets2. In addition, it would appear that the Ras/MAPK pathway antagonizes the PKA-induced stimulatory effects.

The Effect of Mutating Thr 72 of Ets2 on the Synergistic Interaction of Ets2 and PKA

Thr72 represents a well-defined site for phosphorylation on Ets2 and is a downstream target for the Ras/MAPK signal transduction pathway (16, 25, 50). Here, we tested whether a form of Ets2 with Thr72 replaced with Ala (Ets2A), was as effective as wild-type Ets2 (Ets2T) in transactivating the −126*luc* promoter in the presence of PKA. The up-regulation of the promoter in the presence of the ectopically expressed mutant form of Ets2A was not significantly different from that observed with overexpressed control protein (510-fold *vs.* 600-fold) (Fig. 3C). Furthermore, the MEK inhibitor PD98059 increased transcription from the mutated Ets2A promoter as effectively as it did from the wild-type Ets2T promoter (Fig. 3D). This experiment demonstrates that Thr72 is not essential for the enhancement of Ets2 effects by PKA and, second, that this amino acid residue is probably not a target for PKA-catalyzed phosphorylation.

Role of the Activator Protein 1 (AP1) Site Adjacent to the Ets2-Binding Site in Ets2/PKA Synergistic Regulation of the IFNT Promoter

Transcription factors of the AP1 family have been implicated as downstream mediators for several signal transduction pathways, including the Ras/MAPK (51, 52) and the PKA pathways (53, 54). Accordingly, we examined the effects of mutating the AP1-binding site that lies adjacent to the Ets2-binding site in the −126*luc* promoter (Fig. 4A). As expected, the combination of Ets2 and PKA provided the usual large up-regulation (490-fold) of promoter activity compared with either factor on its own (43-fold and 5-fold, respectively) (Fig. 4B). Mutation of the Ets-binding site again caused a major drop in reporter activity (>98% compared with the wild-type construct). Unexpectedly, a mutation designed to disrupt the AP1-binding site doubled (to nearly 900 fold) reporter activity resulting from Ets2/PKA overexpression compared with the wild-type promoter (Fig. 4B). This effect was almost identical to that observed with PD98059 (Fig. 3, B and D). These data suggest that the presence of a factor, presumably an AP1 family member, normally occupying this site and targeted by the Ras/MAPK pathway, may have an inhibitory effect on the PKA-signaling pathway.

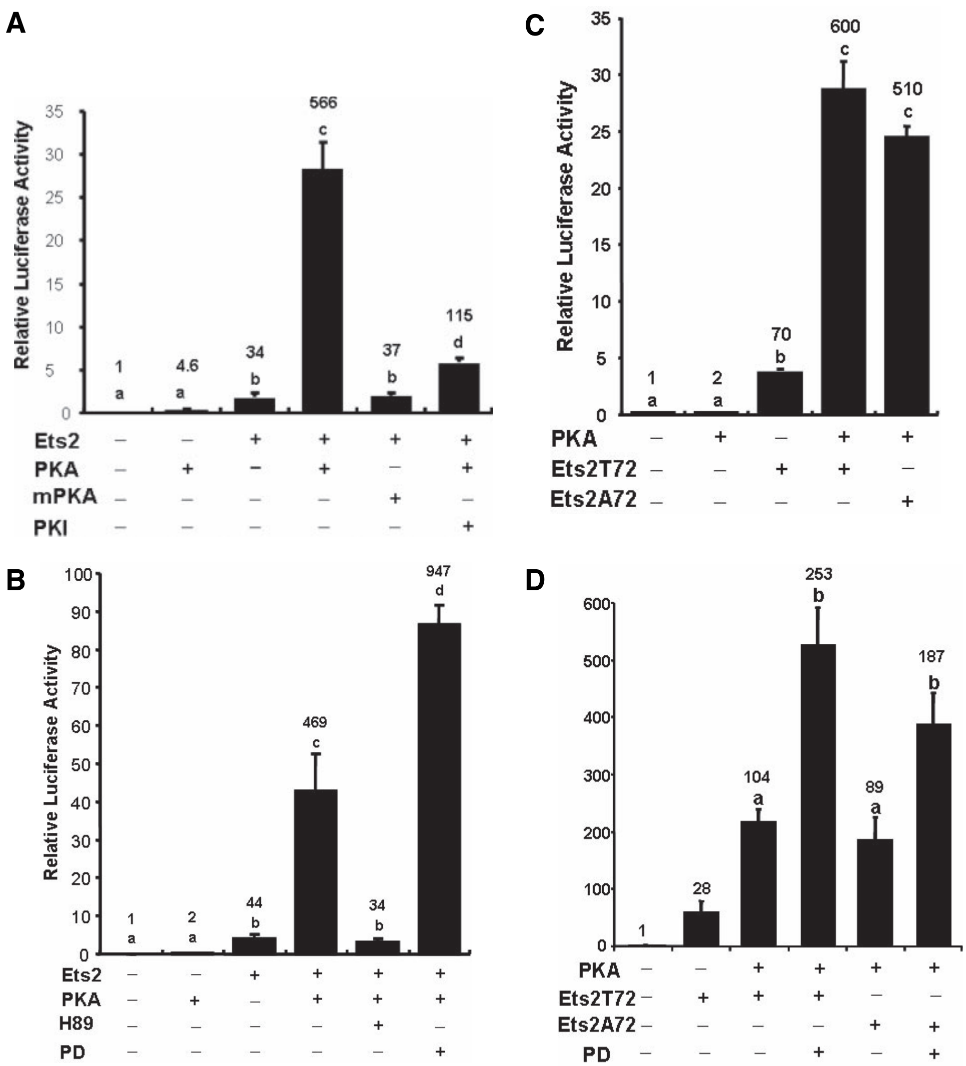

Fig. 3. Cooperative Transactivation of the *IFNT* Promoter by Ets2 and Activated PKA in JAr Cells is Dependent on the Kinase Activity of PKA but Not on the ERK1/ERK2 MAPK Pathway and the Presence of the Phosphorylatable Thr 72 (T72) of Ets2

A, The −126*luc* promoter was cotransfected with combinations of expression plasmids for Ets2, PKA, mutated PKA (mPKA), and a specific inhibitor of PKA (PKI). B, The −126*luc* promoter was cotransfected with expression plasmids for Ets2 and PKA in the presence and absence of the PKA inhibitor H89 and the MEK1/MEK2 inhibitor PD 98059 (PD). C, The −126*luc* promoter was cotransfected with expression plasmids for Ets2T72, Ets2A72, and PKA alone or in combination. D, The −126*luc* promoter was cotransfected with expression plasmids for Ets2T72, Ets2A72, and PKA in the presence and absence of the MEK1/2 inhibitor PD 98059 (PD). In all three experiments, the normalized *luc* activities are presented relative to control values (means ± SEM; n = 3), with fold activation shown *above each bar*. If *letters above bars* are different, there was a significant effect of treatment ($P < 0.05$).

Ets2 Is Not a Direct Target of PKA Phosphorylation

The consensus target sites for PKA are RXS and RXXS (55). The Ets2 amino acid sequence provides two such potential PKA phosphorylation sites (RLS^{245} and RVPS^{310}). We therefore tested whether truncated Ets2 constructs, some carrying these sites, others lacking them, were substrates for PKA in an *in vitro* assay. As a positive control, we employed ovine IFNT11, which had been engineered to carry a PKA target site at its COOH terminus (56). Products of the reactions were separated by SDS-PAGE and detected by autoradiography (supplemental Fig. 1, A and B, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

In the initial experiment, we compared similar amounts of full-length Ets2 and IFNT11 as substrates for PKA in the *in vitro* reaction and employed a 15-min exposure to film to detect the radioactive bands. Whereas IFNT11 (Mr~18,000) was clearly a substrate for PKA, the incorporation of ^{32}P into full-length Ets2 was very low (supplemental Fig. 1A), with a faint doublet (Mr ~58,000) visible on the gel. As expected, there was no incorporation of ^{32}P in the absence of PKA. These data suggest that if Ets2 is a substrate for PKA, it is a very poor one.

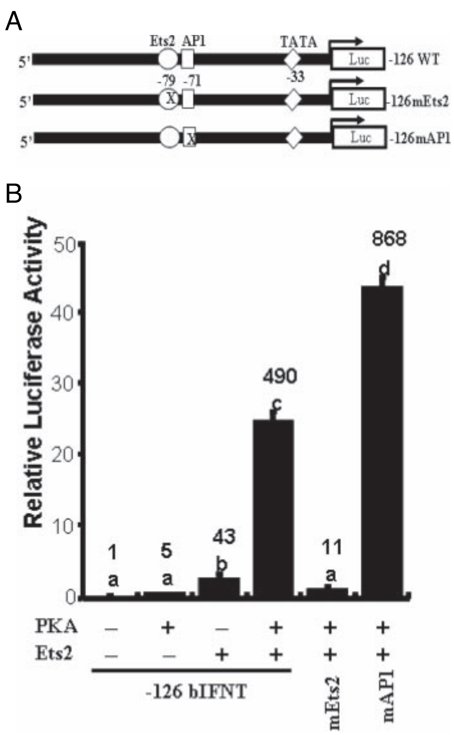

Fig. 4. The Effect of Mutating Transcription Factor Binding Sites within the *IFNT* Proximal Promoter on Ets2/PKA Cooperative Effects on Reporter Gene Expression in JAr Cells

A, A diagrammatic illustration of the −126*luc* reporter containing the −126/+50 regulatory region. The positions of the binding sites for Ets2 and AP1 are marked. B, The −126*luc* promoter construct (−126WT) and its two mutated forms (−126mEts2 and −126mAP1) were cotransfected with expression plasmids for Ets2 and PKA alone or in combination. In both experiments, the normalized *luc* activities are presented relative to control values (means ± SEM; n = 3), with fold activation shown *above each bar*. If *letters above bars* are different, there was a significant effect of treatment ($P < 0.05$). bIFNT, Bovine IFNT; mAP1, mutant AP1; WT, wild type.

To determine whether Ser245, Ser310, or both are targets for PKA, a series of truncations were employed (supplemental Fig. 1B). In the analysis of the reaction products, the gels were exposed to film for 1 h rather than 15 min to provide a more complete identification of reaction products. Several bands of radioactivity of similar molecular weight were detected in each lane, even though the substrate proteins, Ets2 truncations, encompassed a range of sizes. The bands in some lanes, *e.g*. for the amino acids 225–469 substrate, were much more prominent than in others. The most likely explanation for these data is that contaminating bacterial proteins present in trace quantities are the substrates for PKA rather than Ets2 fusion proteins. This likelihood is reinforced by the observation that one truncated form (Ets2 322–469), which lacks both the putative PKA phosphorylation sites, provided the same radioactive products as the other truncations. The variation between lanes probably reflects the relative amount of contaminating bacterial protein present. These observations strongly suggest that Ets2 is not a direct target for PKA phosphorylation.

CBP/p300 Acts in Association with Ets2 to Mediate the Effect of PKA

Here we examined the role of the coactivators cAMP response element (CRE)-binding protein (CREB)-binding protein (CBP)/p300 and the transcription factor CREB in the Ets2-mediated activation of *IFNT* gene by PKA. Contrary to a recent report (57), which indicated that CBP acted as a direct coactivator in the regulation of *IFNT* gene transcription, in our hands CBP suppressed both basal and Ets2-mediated up-regulation of the *IFNT* promoter (Fig. 5A). Overexpression of CREB had a similar suppressive action. Conceivably, CBP (and its homolog p300), as well as CREB, are not rate-limiting in JAr cells and, when overexpressed, have a slight squelching effect on transcription. Therefore, instead of overexpressing these potential activators of Ets2-mediated transcription, we attempted to reduce their effective concentrations in the cells.

To overcome the problem of excess endogenous CBP, we first made use of the adenoviral E1A 12S protein, which inhibits CBP/p300 interaction by binding to the same region of CBP/p300 as transcription factor IIB (58). When E1A 12S was coexpressed with the Ets2 and PKA expression vectors, *luc* activity from the −126 *boIFNT1* promoter was reduced by approximately 50% (Fig. 5B). This reduction was not observed when a mutated form of E1A 12S possessing a deletion (Δ2–36), which removed the CBP/p300-binding region, was substituted for the wild-type protein. Similar results to those obtained in JAr cells were observed in JEG3 cells (Fig. 5C). Again CBP/p300 reduced *luc* reporter expression slightly, whereas E1A 12S had a significant inhibitory effect, presumably by reducing the effective basal concentration of CBP/p300 in the cells.

A second tactic to examine whether suppression of basal CBP/p300 would influence Ets2/PKA effects on the promoter was to use an RNA interference approach with short interfering RNA (siRNA) duplexes directed against CBP and p300 (59) designed to knock down endogenous concentration of endogenous CBP and p300 transcripts and hence protein (Fig. 6A). Western blot analyses indicated that the lowest concentration of siRNA (25 nM) directed against CBP was highly effective in reducing the amount of CBP in the cells, whereas the control siRNA (200 nM) had no effect. In the case of p300, the optimal concentration of the specific siRNA was rather higher (100 nM), although an effect was still observed with 25 nM (Fig. 6A). When they were transfected with the expression constructs for Ets2 and PKA, the siRNAs directed against CBP and p300 reduced reporter gene expression from the −126*luc* by about 50% (Fig. 6B). When both siRNAs were included in the transfection mixture, the ability of Ets2 and PKA to transactivate the promoter was reduced by more than 75%, suggesting

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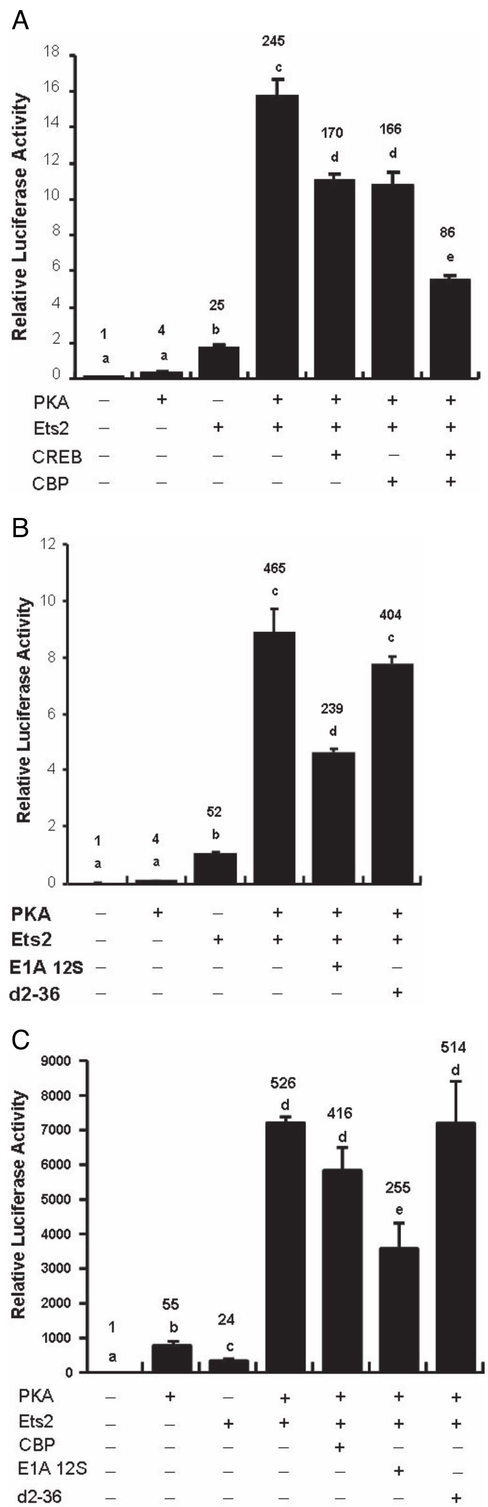

Fig. 5. The Coactivator CBP/p300 Influences the Ets2/PKA-Mediated Transactivation of the *IFNT* Promoter in Choricarcinoma Cells

A, The −126*luc* promoter was transfected into JAr cells with combinations of expression plasmids for Ets2, PKA, CBP, and CREB. Note that overexpression of CBP and CREB significantly suppressed the cooperative transactivation of the −126*luc* promoter by Ets2 and PKA. B, The −126*luc* promoter was transfected into JAr cells with combinations of the expression plasmids for Ets2, PKA, CBP, E1A 12S, and E1A 12S (Δ2–36). C, The −126*luc* promoter was transfected into JEG3 cells with combinations of expression plasmids for Ets2, PKA, CBP, E1A 12S, and E1A 12S (Δ2–36). In all three experiments, the normalized *luc* activities are presented relative to the activity of −126*luc* from nontreated cells (means ± SEM; n = 3), with fold activation shown *above each bar*. If *letters above bars* are different, there was a significant effect of treatment ($P < 0.05$).

that both coactivators were capable of interacting either directly or indirectly with Ets2. In contrast, no such effect was observed when the control siRNA duplex was transfected.

The siRNA targeted against CREB mRNA had a modest but specific ability to reduce the concentration of CREB protein in JAr cells (supplemental Fig. 2A), but had no effect on the ability of Ets2 and PKA to transactivate the −126 *IFNT* promoter (supplemental Fig. 2B). These data are consistent with the possibility that CREB, which has no consensus binding site on the *IFNT* promoter, does not play a direct role in PKA/Ets2 up-regulation of *IFNT*.

Association of CBP/p300 and Ets2 with the *IFNT* Proximal Promoter Region in CT-1 Cells

We then sought to determine whether CBP/p300 was associated with Ets2 on the proximal (−188 to +3 region) of actively transcribed *IFNT* by classical chromatin immunoprecipitation (ChIP) assays. Sheared chromatin was prepared from bovine CT-1 cells, which actively secrete IFNT into the culture medium. DNA collected in immunocomplexes after addition of affinity-purified rabbit anti-p300 and anti-Ets2 immunoglobulin, respectively, was subjected to PCR analysis with specific primers (Fig. 7). Both antibodies provided DNA that contained the *IFNT* proximal regulatory region, whereas a nonspecific immunoglobulin was unable to do so. These data strongly suggest that CBP/p300 is associated with transcription factor complexes, including Ets2, bound to the *IFNT* promoter.

DISCUSSION

IFNT is known only to be expressed in a single tissue, trophectoderm, and for a limited period during early pregnancy when its production, whether assessed either on a per cell basis or as total amount produced by the conceptus (1, 11, 12, 60), rises dramatically in the period before the conceptus makes firm attachment to the uterine wall to form the definitive placenta. Unlike other type I IFN, the *IFNT* genes are not responsive to virus and do not contain a conserved viral response element, even though some sequence similarities with the virally inducible *IFNW* and *IFNA* remain evident (61). Conversely, the virally responsive *IFNW* and *IFNA* are not up-regulated

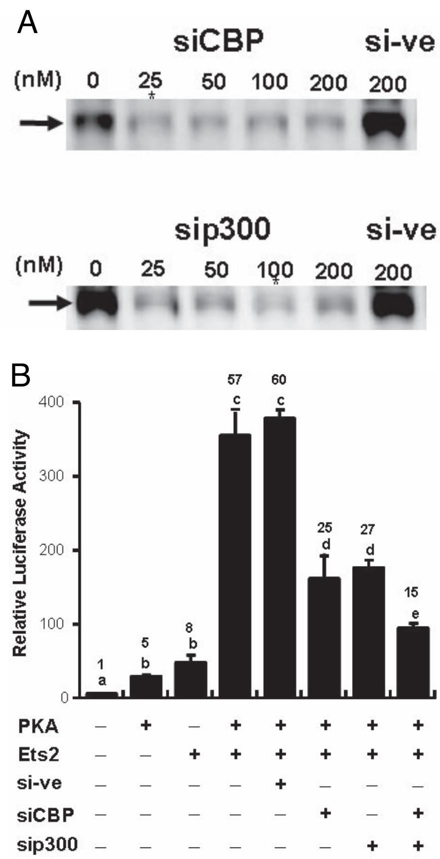

Fig. 6. Effects of CBP and p300 Expression on the Ability of Ets2 to Transactivate the *IFNT* Promoter in JAr Cells

A, Cells were either mock transfected (lane 1) or transfected with a control siRNA (si-ve) and increasing concentrations of siRNA directed against the CBP (*upper panel*) and p300 (*lower panel*) mRNA. Whole-cell lysates were analyzed by SDS-PAGE, and relative levels of CBP and p300 protein were determined by SDS-PAGE and Western blotting. *Asterisks* indicate the concentration of siRNA used to knock down expression of CBP and p300 (25 nM and 100 nM, respectively) in panel B. B, The −126*luc* promoter was cotransfected with combinations of expression plasmids for Ets2 and PKA, siRNAs directed against mRNAs for CBP and p300 (25 nM and 100 nM, respectively), and a negative siRNA control (si-ve, 200 nM) that is not known to target any known human gene. In panel B, the normalized *luc* activities are presented relative to the activity of −126*luc* from nontreated cells (means ± SEM; n = 3), with fold activation shown *above each bar*. If *letters above each bar* are different, there was a significant effect of treatment ($P < 0.05$). siCBP, Short interfering CBP; sip300, short interfering p300.

during trophoblast differentiation in cattle (62). Therefore, the up-regulation of the *IFNT* reflects unique features of the *IFNT*-regulatory region, a permissive combination of transcription factors in cells of trophectoderm, and input from the external environment that prompts superinduction of expression. One of the key transcription factors is Ets2, which appears to play a central command role in regulating the *IFNT* (25, 63) and other trophoblast-associated genes in ruminants (26).

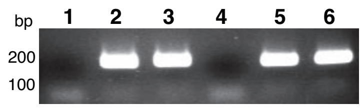

Fig. 7. Association of CBP/p300 and Ets2 with the Regulatory Region Actively Transcribed *IFNT* from Bovine CT-1 Cells

Sheared chromatin prepared from formaldehyde-fixed CT-1 cells was exposed to no antibody (lane 1), anti-p300 (lane 2), anti-Ets2 (lane 3), and purified rabbit IgG (lane 4). The DNA recovered from each immunocomplex (lanes 1–4), 10% of the total in-put chromatin (lane **5**), and 0.6 ng of plasmid DNA containing the −457*IFNT* promoter (lane 6) were analyzed by PCR with primers specific for a region (−188 to +3) containing the Ets2/AP1 enhancer element within the *IFNT* promoter.

The *IFNT* is only expressed weakly in trophectoderm of cattle at the blastocyst stage of development (11, 13, 60, 64). The increase that follows is correlated with the initial rapid elongation of the conceptus and the rise in progesterone accompanying the full maturation of the CL of the mother (14, 15). As argued in the introduction to this paper, the rise in IFNT production is probably dependent upon maternal factors released into the immediate environment of the conceptus, which provide a means of coordinating the growth and activity of the conceptus with the hormonal state of the mother. The data presented here suggest that the PKA signal transduction pathway, presumably activated by receptors in response to binding with exocrine factors of maternal origin, participates in the up-regulation of the IFNT in the preimplantation conceptus. Clearly, a robust luteal phase in the mother will be required if conceptus IFNT is produced in time and in sufficient quantities to rescue the CL of pregnancy. What has also become clear is that the timing of the increase in progesterone in the early luteal phase (d 4 to d 5) may be a key factor in setting the stage for these subsequent events because it is necessary to program the secretory activity of the reproductive tract (86). Management strategies, including nutrition, that optimize this early rise in progesterone in the mother are likely, therefore, to be crucial for ensuring an adequate IFNT response by the conceptus.

Ets2 can be activated directly by the Ras/MAPK signal transduction pathway in many different kinds of cells (37, 50, 65, 66). Ets2 is not, however, a well established target of the cAMP/PKA signal transduction pathway, although PKA and Ets2 have been implicated in the regulation of the *CGB5* subunit gene (30), which, like the *IFNT*, lacks a classical CRE (67). Our laboratory has shown that 8-bromo-cAMP and overexpressed PKA up-regulated *CGB5* promoter activity primarily through a previously unrecognized proximal Ets2 enhancer on the promoter (28). The Ets2-binding sites on the promoter for the *CGB5* partner gene, *CGA*, are also crucial for mediating cAMP/PKA effects on *CGA* gene expression (29, 30). Mutation of the Ets2-binding sites, for example, virtually abolishes cAMP responsiveness of the *CGA* subunit gene. Similarly, inactivation of the CREs by either truncation or point mutation, abrogates any ability of Ets2 to up-regulate the promoter (29). These were the first reports that implicated Ets2 as a target for the cAMP/PKA signal transduction pathway, a connection that has been further strengthened by the results presented here for the regulation of the *IFNT* genes.

Evidence in favor of Ets2 being the downstream target for PKA includes the following observations: 1) Coexpression of Ets2 and PKA greatly up-regulated the *IFNT* promoter in a synergistic manner (Figs. 1 and 2); 2) Either a deletion of the Ets2-binding sequence (Fig. 2B) or a point mutation within its core (Fig. 4B) largely eliminated the PKA effects; 3) Inhibitors of the PKA signal transduction pathway prevented the synergistic up-regulation of the promoter by the combination Ets2 and PKA (Fig. 3, A and B). Finally PKA catalytic activity is required to observe any effect (Fig. 3A). Ets2 is not, however, a substrate for PKA, at least *in vitro* (supplemental Fig. 1), suggesting that some other transcription factor closely linked to the action of Ets2 is the direct target.

Our data argue against a role for CREB itself in promoting the synergistic action of PKA and Ets2. The *IFNT* promoter lacks an obvious binding site for CREB, and overexpression of CREB depresses the Ets2/PKA combinatorial effect, possibly because it provides an alternative substrate for PKA and may divert PKA activity from the pathway that transactivates the *IFNT* promoter. In addition, silencing of CREB by using an RNA interference approach had little effect (supplemental Fig. 2).

Our experiments are consistent with the possibility that CBP or its close relative p300 mediate the action of PKA on the transactivation of *IFNT*. CBP (CREB-binding protein) and p300 are two homologous, conserved, nuclear phosphoproteins that function as transcriptional coactivators by bridging a very large number of DNA-bound transcription factors with the basal transcription complex to activate transcription of genes (68, 69). CBP can serve as a link between the basal transcription machinery and many DNA-binding factors, including Ets1 and Ets2 (35). Although p300 and CBP had a negative effect on the −126 *IFNT* promoter when they were ectopically expressed in JAr cells, this unexpected phenomenon could be due to the fact that both proteins were already present in optimal concentrations and that overexpression, rather than promoting increased transcription, had an, as yet, ill-defined squelching effect. Although others have described positive effects of CBP/p300 on *IFNT* promoters in choriocarcinoma cells (57), such tumor-derived cell lines may not maintain a constant phenotype. In our experiments, the evidence for a positive involvement of p300/CBP arose from three observations. The first was that ectopic expression of the adenoviral E1A 12S protein, which inhibits CBP/p300 interaction with transcription factor IIB and components of the transcriptional machinery (70), also blocks reporter expression. The second was that the siRNA approach was effective in inhibiting the ability of PKA to promote Ets2-based transactivation of the *IFNT* promoter. The third was that CBP, along with Ets2, occupies the crucial enhancer region of actively transcribed IFNT in CT-1 cells, a bovine trophoblast cell line that produces IFNT. Finally, a search of EST databases reveals the presence of transcripts of PKA subunits, CBP/p300 and CREB in bovine trophoblast (data not shown). Although not proving a role for CBP/p300 in regulating *IFNT* expression, these data are quite compelling.

Ets2 is believed to interact with AP1 family members to mediate some of the downstream effects of the MAPK pathway on the *IFNT* and other Ets2-responsive promoters (71), so that a positive effect of mutating the AP1 site adjacent to the Ets2-binding site was unexpected (Fig. 4). Because the MEK-specific inhibitor, PD98059, also doubled Ets2/PKA stimulation of *luc* reporter activity (Fig. 3, B and D), it would appear that an operational Ras/MAPK pathway is antagonistic to activation of *IFNT* genes by the PKA signal transduction pathway. Cross talk is known to exist between these two signaling pathways (72, 73). Sometimes, the PKA pathway stimulates ERK signaling, whereas on other occasions it inhibits. There is at least one report where the inhibitor, PD98059, has been reported to up-regulate CRE-dependent gene activity (74). In addition, activation of PKA can lower MAPK activity (75) whereas its inhibition can increase it (76). One explanation for this phenomenon may be the ability of the cAMP pathway to target, and presumably inactivate, c-Raf, a component of Ras/MAPK signaling (73). Alternatively, the two pathways may converge on the transcription factors that bind at the Ets2/ AP1-like site and interfere with each other's ability to drive promoter expression. What adaptive significance, if any, such antagonism might have is unclear, because factors that activate the Ras/MAPK pathway, *e.g*. CSF1 (77), and the cAMP/PKA pathway, *e.g*. FGF2 (23), are likely present simultaneously in maternal uterine secretions (19, 78–80), with the possibility that one can partially counteract the other in terms of their control of IFNT expression. This antagonistic relationship between PKA and MAPK and the precise role played by CBP/p300 in the context of *IFNT* regulation is of interest, but clearly complex. Although our experiments, for example, suggest that the effects of PKA are independent of Ets2 phosphorylation at Thr72 (Fig. 3, C and D), there is at least one reported example in which MAPK-mediated Ets2 phosphorylation at Thr72 augments the interaction of Ets2 with CBP/p300 (81). Further experiments are needed to understand the phenomenon. Nevertheless, we conclude that the most likely explanation for the steep increase in IFNT production at the time of conceptus elongation is an increase in intracellular cAMP, activation of PKA, and subsequent downstream effects on transcription factors already driving transcription of the *IFNT* at a low rate.

MATERIALS AND METHODS

Reporter Gene Constructs

Bovine *IFNT1* promoters-*luc* gene reporter constructs −49*luc*, −126*luc*, −457*luc*, and −1675*luc*, containing the gene control regions −49 to +66 bp, −126 to +50 bp, −457 to +66 bp, and −1675 to +66 bp, respectively, and mutated sequences have been described previously (24, 25). Control region −67 to +66 bp of the *boIFNT1* gene was generated by *Xba*I digestion and after self-ligation of the mutated AP1 binding site at −71 on the reporter (−126μAP1) (16). Fidelity of all constructs was verified by DNA sequencing.

Expression Vectors

The expression plasmids for Ets2 and its mutant form (pCGNEts2T72 and pCGNEts2A72), PKA expression plasmids, constitutively active catalytic subunit [Rous sarcoma virus (RSV)-PKA], and an expression vector of the specific inhibitor of PKA (RSV-PKI), have been described previously (25, 29, 82). A mutant form of PKA expression plasmid with a lysine replaced by methionine in the ATP-binding region, which results in an inactive catalytic subunit was gift from Dr. Richard Maurer (Oregon Health and Science University, Portland, OR) (82). The expression vectors for CBP and p300 were provided by Dr. Tony Kouzarides (University of Cambridge, Cambridge, UK) (83). The expression plasmid for human CREB (84) was obtained from Dr. T. F. Osborne (University of California-Irvine), and the CREB coding sequence was cloned into *Bam*HI and *Not*I sites of pCDNA3.0 vector (Invitrogen, Carlsbad, CA). Either the β-galactosidase gene driven by the Rous sarcoma virus long-terminal repeat (pRSVLTR-βgal) or the *Renilla luc* gene driven by the cytomegalovirus (CMV) promoter (pRL-CMV; Promega, Madison, WI) was used as an internal control in all transfection experiments.

Cell Cultures and Transfections

JAr and JEG3 cells (HTB-144 and HTB-36; American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and MEM supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 10% fetal bovine serum, respectively. The cells were transfected either by the calcium phosphate method as described previously (25) or using Lipofectamine Plus (Invitrogen) as per manufacturer's instructions. JAr or JEG3 cells were plated in six-well plates (1 $\times$ 10^5 cells per well) overnight and transfected with 0.5 μg of reporter gene constructs and 1.5 μg of expression vector DNA per well in the presence of 25 ng of pRSVLTR-βgal or 5 ng of pRL-CMV of internal control plasmid. Total amount of transfected DNA was kept constant by including the insert-free parental vectors.

After 36 h exposure to the transfection agent, cells were washed twice with PBS, (Invitrogen) and lysed with Passive Lysis Buffer (Promega). Luciferase activities were measured by injecting luciferase assay reagent (Promega) into cell extracts and recording chemiluminescence (a 10-sec light output) in a 20/20^n Luminometer (Turner Biosystems, Mountain View, CA). β-Galactosidase activities were measured by using Tropix Galacto-Light substrate (Applied Biosystems, Foster City, CA) added to extracts after they had been heated at 48 C for 50 min to inactivate endogenous eukaryotic β-galactosidase. The *Renilla* luciferase activity as internal control was assayed with the Dual Luciferase Reporter Assay System (Promega). The transcriptional activity of each promoter-*luc* reporter construct was normalized with either the β-galactosidase or *Renilla* luciferase activity (25).

siRNA Transfections

siRNA duplexes against human CBP and p300 (59), human CREB (85), and human Ets2 (23) were purchased from Dharmacon (Chicago, IL). JAr cells were plated on six-well plates at a density of 1 $\times$ 10^5 per well and transfected in triplicate in OptiMEM by using Lipofectamine2000 (Invitrogen). Cells were transfected with 2 μg of plasmid DNA either alone or with duplex siRNA together with 25 ng of β-galactosidase as an internal control. A siCONTROL RISC-Free siRNA (Dharmacon) was used as negative control. Luciferase and β-galactosidase activities were measured as mentioned in the previous section.

For biochemical analyses, 1 $\times$ 10^5 JAr cells per well were transfected with 0, 25, 50, 100, and 200 nM CBP-siRNA and p300-siRNA and CREB-siRNA, respectively, or 100 nM siCONTROL RISC-Free siRNA in Opti-MEM. Cells were lysed 36 h later in Passive Lysis Buffer (Promega), and 30 μg of each lysate was separated on either 8% or 10% SDS-PAGE gels (for CBP or CREB, respectively). Western blot procedures have been described previously (28). Detection was performed with either rabbit polyclonal CBP antibody (sc-583) or CREB antibody (Cell Signaling Technology, Beverly, MA) in a blocking solution (25 mM Tris-HCl, pH 7.3; 150 mM NaCl; 0.1% Tween 20; and 5% nonfat dry milk). After overnight incubation with primary antibody at 4 C, the blot was incubated with antirabbit IgG coupled to horseradish peroxidase (Cell Signaling Technology) at 1:1000 dilution in blocking solution. Membranes were developed with the Phototype-horseradish peroxidase Western Blot Detection System (Cell Signaling Technology), and images were acquired with the Fuji LAS 3000 Imaging System (Fujifilm Medical Systems, Stamford, CT).

In Vitro PKA Phosphorylation Assay

Full-length human Ets2 (469 amino acids) and truncations, Ets2 (1–261 amino acids), Ets2 (225–469), Ets2 (295–469), and Ets2 (322–469), were expressed as GST fusion proteins in DH5α *Escherichia coli* and purified after thrombin cleavage to remove a putative PKA phosphorylation site at the junction of the Ets2 and GST polypeptides. Purified proteins (0.5 μg) were tested for their ability to serve as PKA substrates by incubating in the presence of the catalytic subunit of PKA from bovine heart (Sigma Chemical Co., St. Louis, MO) (15 U) and 10 μCi/μl [γ-^{32}P]ATP in a buffer (20 mM Tris-HCl, pH 7.5; 100 mM NaCl; 12 mM $MgCl_2$; and 1 mM dithiothreitol) for 1 h at 30 C. Ovine IFNT11, with an introduced PKA phosphorylation site at its carboxyl terminus (56), served as a positive control. At the end of the incubation, the reaction was terminated by the addition of equal volume of 2$\times$ SDS-PAGE loading buffer to the sample. The proteins were resolved in a 10% SDS-PAGE gel and dried, and ^{32}P-labeled proteins were detected by autoradiography on BioMax MS (maximum sensitivity) x-ray film (Eastman Kodak Co., Rochester, NY).

ChIP Analysis

ChIP analysis on bovine CT-1 cells was conducted essentially as described by Ghosh *et al.* (29). In brief, sheared chromatin prepared from approximately 10^7 CT-1 cells, was precleared with a Protein G-Agarose bead slurry (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Twenty percent of the preparation was saved as “total input” control. The remaining chromatin was either left untreated (“no antibody” control), or treated with 2 μg of p300 antibody (sc-585, Santa Cruz Biotechnology), rabbit anti-Ets2 antibody (raised in our laboratory), and purified nonspecific IgG (Active Motif, Carlsbad, CA), respectively. The immune complexes were collected on Protein G-Agarose beads, eluted, and prepared for PCR analysis (29). A volume of 5 μl of the ChIP DNA was used as template for each PCR. The primers used were: forward, 5′-tga caa acc caa att tta ttg gga aa; reverse, 5′-tct gat gat gat cgt tct aag caa gg, and were designed to amplify a region of the *IFNT* proximal promoter (−188 to +3) containing the Ets2/AP1 enhancer. PCR conditions were as follows: 95 C for 2 min for one cycle, 33 cycles of 95 C for 30 sec, 52 C for 30 sec, 72 C for 2 min, followed by 72 C for 10 min. PCR products were visualized by ethidium bromide staining after electrophoresis in 2% agarose.

Statistical Analyses

Each transfection was carried out in triplicate, and the experiment was repeated either three or four times. Values from individual experiments were log transformed. Statistical analyses (for at least three replicated experiments) were performed by one-way ANOVA, with multiple data set comparisons analyzed by Tukey postcomparison test on Prism analytical software (GraphPad Prism version 4.0; GraphPad Software, Inc., San Diego, CA).

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Address all correspondence and requests for reprints to: R. Michael Roberts, University of Missouri-Columbia, 240b Christopher S. Bond Life Sciences Center, 1201 East Rollins Street, Columbia, Missouri 65211-7310. E-mail: robertsrm@missouri.edu.

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REFERENCES

1. Roberts RM, Leaman DW, Cross JC 1992 Role of interferons in maternal recognition of pregnancy in ruminants. Proc Soc Exp Biol Med 200:7–18
2. Demmers KJ, Derecka K, Flint A 2001 Trophoblast interferon and pregnancy. Reproduction 121:41–49
3. Martal JL, Chene NM, Huynh LP, L'Haridon RM, Reinaud PB, Guillomot MW, Charlier MA, Charpigny SY 1998 IFN-τ: a novel subtype I IFN1. Structural characteristics, non-ubiquitous expression, structure-function relationships, a pregnancy hormonal embryonic signal and cross-species therapeutic potentialities. Biochimie 80: 755–777
4. Roberts RM, Ealy AD, Alexenko AP, Han CS, Ezashi T 1999 Trophoblast interferons. Placenta 20:259–264
5. Spencer TE, Burghardt RC, Johnson GA, Bazer FW 2004 Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 82- 83:537–550
6. Chen Y, Green JA, Antoniou E, Ealy AD, Mathialagan N, Walker AM, Avalle MP, Rosenfeld CS, Hearne LB, Roberts RM 2006 Effect of interferon-τ administration on endometrium of nonpregnant ewes: a comparison with pregnant ewes. Endocrinology 147:2127–2137
7. Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE 2006 Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon τ in the ovine uterus. Biol Reprod 74:383–394
8. Godkin JD, Bazer FW, Moffatt J, Sessions F, Roberts RM 1982 Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13–21. J Reprod Fertil 65:141–150
9. Roberts RM 1991 A role for interferons in early pregnancy. Bioessays 13:121–126
10. Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW 1985 Characterization of proteins produced in vitro by periattachment bovine conceptuses. Biol Reprod 32:681–693
11. Farin CE, Imakawa K, Hansen TR, McDonnell JJ, Murphy CN, Farin PW, Roberts RM 1990 Expression of trophoblastic interferon genes in sheep and cattle. Biol Reprod 43:210–218
12. Kimura K, Spate LD, Green MP, Murphy CN, Seidel Jr GE , Roberts RM 2004 Sexual dimorphism in interferon-τ production by in vivo-derived bovine embryos. Mol Reprod Dev 67:193–199
13. Kubisch HM, Larson MA, Kiesling DO, RM. R 2001 Control of interferon-t secretion by in vitro-derived bovine blastocysts during extended culture and outgrowth formation. Mol Reprod Dev 58:390–397
14. Ashworth CJ, Bazer FW 1989 Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. Biol Reprod 40:425–433
15. Nephew KP, McClure KE, Ott TL, Dubois DH, Bazer FW, Pope WF 1991 Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. Biol Reprod 44:536–539
16. Ezashi T, Roberts RM 2004 Regulation of interferon-τ (IFN-τ) gene promoters by growth factors that target the Ets-2 composite enhancer: a possible model for maternal control of IFN-τ production by the conceptus during early pregnancy. Endocrinology 145:4452–4460
17. Roberts RM, Ezashi T, Rosenfeld CS, Ealy AD, Kubisch HM 2003 Evolution of the interferon τ genes and their promoters, and maternal-trophoblast interactions in control of their expression. Reprod Suppl 61:239–251
18. Gray C, Burghardt R, Johnson G, Bazer F, Spencer T 2002 Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction 124: 289–300
19. Satterfield MC, Bazer FW, Spencer TE 2006 Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. Biol Reprod 75:289–296
20. Mann GE, Fray MD, Lamming GE 2006 Effects of time of progesterone supplementation on embryo development and interferon-τ production in the cow. Vet J 171: 500–503
21. Ocon-Grove OM, Cooke FN, Alvarez IM, Johnson SE, Ott TL, Ealy AD, Ovine endometrial expression of fibroblast growth factor (FGF) 2 and conceptus expression of FGF receptors during early pregnancy. Domest Anim Endocrinol, in press
22. McGuire WJ, Imakawa K, Tamura K, Meka CS, Christenson RK 2002 Regulation of endometrial granulocyte macrophage-colony stimulating factor (GM-CSF) in the ewe. Domest Anim Endocrinol 23:383–396
23. Michael DD, Alvarez IM, Ocon OM, Powell AM, Talbot NC, Johnson SE, Ealy AD 2006 Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-τ production in bovine trophectoderm. Endocrinology 147:3571–3579

24. Ezashi T, Ghosh D, Roberts RM 2001 Repression of Ets-2-induced transactivation of the τ interferon promoter by Oct-4. Mol Cell Biol 21:7883–7891
25. Ezashi T, Ealy AD, Ostrowski MC, Roberts RM 1998 Control of interferon-τ gene expression by Ets-2. Proc Natl Acad Sci USA 95:7882–7887
26. Chakrabarty A, Roberts MR 2007 Ets-2 and C/EBP-β are important mediators of ovine trophoblast Kunitz domain protein-1 gene expression in trophoblast. BMC Mol Biol 8:14
27. Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z, Oshima RG 1998 Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev 12:1315–1326
28. Ghosh D, Ezashi T, Ostrowski MC, Roberts RM 2003 A central role for Ets-2 in the transcriptional regulation and cyclic adenosine 5′-monophosphate responsiveness of the human chorionic gonadotropin-β subunit gene. Mol Endocrinol 17:11–26
29. Ghosh D, Sachdev S, Hannink M, Roberts RM 2005 Coordinate regulation of basal and cyclic 5′-adenosine monophosphate (cAMP)-activated expression of human chorionic gonadotropin-α by Ets-2 and cAMP-responsive element binding protein. Mol Endocrinol 19: 1049–1066
30. Johnson W, Jameson JL 2000 Role of Ets2 in cyclic AMP regulation of the human chorionic gonadotropin β promoter. Mol Cell Endocrinol 165:17–24
31. Pestell RG, Albanese C, Watanabe G, Lee RJ, Lastowiecki P, Zon L, Ostrowski M, Jameson JL 1996 Stimulation of the P-450 side chain cleavage enzyme (CYP11A1) promoter through ras- and Ets-2-signaling pathways. Mol Endocrinol 10:1084–1094
32. Buttice G, Duterque-Coquillaud M, Basuyaux J, Carrere S, Kurkinen M, Stehelin D 1996 Erg, an Ets-family member, differentially regulates human collagenase1 (MMP1) and stromelysin1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. Oncogene 13: 2297–2306
33. Buttice G, Kurkinen M 1993 A polyomavirus enhancer A-binding protein-3 site and Ets-2 protein have a major role in the 12-O-tetradecanoylphorbol-13-acetate response of the human stromelysin gene. J Biol Chem 268:7196–7204
34. Dittmer J 2003 The biology of the Ets1 proto-oncogene. Mol Cancer 2:29
35. Jayaraman G, Srinivas R, Duggan C, Ferreira E, Swaminathan S, Somasundaram K, Williams J, Hauser C, Kurkinen M, Dhar R, Weitzman S, Buttice G, Thimmapaya B 1999 p300/cAMP-responsive element-binding protein interactions with ets-1 and ets-2 in the transcriptional activation of the human stromelysin promoter. J Biol Chem 274:17342–17352
36. Stacey KJ, Fowles LF, Colman MS, Ostrowski MC, Hume DA 1995 Regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor. Mol Cell Biol 15:3430–3441
37. Yordy J, Muise-Helmericks R 2000 Signal transduction and the Ets family of transcription factors. Oncogene 19:6503–6513
38. Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, Sato H, Seiki M, Ishii S, Fujinaga K 1998 The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. Int J Cancer 77: 128–137
39. Sun Y, Duckworth ML 1999 Identification of a placental-specific enhancer in the rat placental lactogen II gene that contains binding sites for members of the Ets and AP-1 (activator protein 1) families of transcription factors. Mol Endocrinol 13:385–399
40. Orwig KE, Dai G, Rasmussen CA, Soares MJ 1997 Decidual/trophoblast prolactin-related protein: characterization of gene structure and cell-specific expression. Endocrinology 138:2491–2500
41. Szafranska B, Miura R, Ghosh D, Ezashi T, Xie S, Roberts RM, Green JA 2001 Gene for porcine pregnancy-associated glycoprotein 2 (poPAG2): its structural organization and analysis of its promoter. Mol Reprod Dev 60: 137–146
42. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JC 2003 Genes, development and evolution of the placenta. Placenta 24:123–130
43. Roberts RM, Ezashi T, Das P 2004 Trophoblast gene expression: transcription factors in the specification of early trophoblast. Reprod Biol Endocrinol 2:47
44. Strauss III JF, Kido S, Sayegh R, Sakuragi N, Gafvels ME 1992 The cAMP signalling system and human trophoblast function. Placenta 13:389–403
45. Feinman MA, Kliman HJ, Caltabiano S, Strauss III JF 1986 8-Bromo-3′,5′-adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. J Clin Endocrinol Metab 63:1211–1217
46. Wice B, Menton D, Geuze H, Schwartz AL 1990 Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. Exp Cell Res 186:306–316
47. Martell RE, Ruddon RW 1990 Patterns of human chorionic gonadotropin expression in untreated and 8-bromoadenosine-treated JAR choriocarcinoma cells. Endocrinology 126:2757–2764
48. Sibley CP, Hochberg A, Boime I 1991 Bromo-adenosine stimulates choriogonadotropin production in JAr and cytotrophoblast cells: evidence for effects on two stages of differentiation. Mol Endocrinol 5:582–586
49. Leaman DW, Cross JC, Roberts RM 1994 Multiple regulatory elements are required to direct trophoblast interferon gene expression in choriocarcinoma cells and trophectoderm. Mol Endocrinol 8:456–468
50. Yang BS, Hauser CA, Henkel G, Colman MS, Van Beveren C, Stacey KJ, Hume DA, Maki RA, Ostrowski MC 1996 Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. Mol Cell Biol 16:538–547
51. Solis-Herruzo JA, Rippe RA, Schrum LW, de La Torre P, Garcia I, Jeffrey JJ, Munoz-Yague T, Brenner DA 1999 Interleukin-6 increases rat metalloproteinase-13 gene expression through stimulation of activator protein 1 transcription factor in cultured fibroblasts. J Biol Chem 274:30919–30926
52. Tong L, Smyth D, Kerr C, Catterall J, Richards CD 2004 Mitogen-activated protein kinases Erk1/2 and p38 are required for maximal regulation of TIMP-1 by oncostatin M in murine fibroblasts. Cell Signal 16:1123–1132
53. Piech-Dumas KM, Best JA, Chen Y, Nagamoto-Combs K, Osterhout CA, Tank AW 2001 The cAMP responsive element and CREB partially mediate the response of the tyrosine hydroxylase gene to phorbol ester. J Neurochem 76:1376–1385
54. Swanson DJ, Zellmer E, Lewis EJ 1998 AP1 proteins mediate the cAMP response of the dopamine β-hydroxylase gene. J Biol Chem 273:24065–24074
55. Kennelly PJ, Krebs EG 1991 Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem 266: 15555–15558
56. Alexenko AP LJ, Mathialagan N, Izotova L, Mariano TM, Pestka S, Roberts RM 1995 Interaction of bovine interferon-τ with the type I interferon receptor on Daudi cells. J Interferon Cytokine Res 15:S97
57. Xu N, Takahashi Y, Matsuda F, Sakai S, Christenson RK, Imakawa K 2003 Coactivator CBP in the regulation of conceptus IFNτ gene transcription. Mol Reprod Dev 65:23–29
58. Goldberg MJ, Moses MA, Tsang PC 1996 Identification of matrix metalloproteinases and metalloproteinase in-

hibitors in bovine corpora lutea and their variation during the estrous cycle. J Anim Sci 74:849–857
59. Dohda T, Kaneoka H, Inayoshi Y, Kamihira M, Miyake K, Iijima S 2004 Transcriptional coactivators CBP and p300 cooperatively enhance HNF-1α-mediated expression of the albumin gene in hepatocytes. J Biochem (Tokyo) 136:313–319
60. Farin CE, Imakawa K, Roberts RM 1989 *In situ* localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. Mol Endocrinol 3:1099–1107
61. Roberts RM, Liu L, Alexenko A 1997 New and atypical families of type I interferons in mammals: comparative functions, structures, and evolutionary relationships. Prog Nucleic Acid Res Mol Biol 56:287–325
62. Cross JC, Roberts RM 1991 Constitutive and trophoblast-specific expression of a class of bovine interferon genes. Proc Natl Acad Sci USA 88:3817–3821
63. Roberts R, Yong H, Smith S 2006 What drives the formation of trophectoderm during early embryonic development? J Reprod Dev 52:S87–S97
64. Hernandez-Ledezma JJ, Sikes JD, Murphy CN, Watson AJ, Schultz GA, Roberts RM 1992 Expression of bovine trophoblast interferon in conceptuses derived by in vitro techniques. Biol Reprod 47:374–380
65. Fowles LF, Martin ML, Nelsen L, Stacey KJ, Redd D, Clark YM, Nagamine Y, McMahon M, Hume DA, Ostrowski MC 1998 Persistent activation of mitogen-activated protein kinases p42 and p44 and ets-2 phosphorylation in response to colony-stimulating factor 1/c-fms signaling. Mol Cell Biol 18:5148–5156
66. Sharrocks A 2001 The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2:827–837
67. Albanese C, Kay TW, Troccoli NM, Jameson JL 1991 Novel cyclic adenosine 3′,5′-monophosphate response element in the human chorionic gonadotropin β-subunit gene. Mol Endocrinol 5:693–702
68. Goldman PS, Tran VK, Goodman RH 1997 The multifunctional role of the co-activator CBP in transcriptional regulation. Recent Prog Horm Res 52:103–119
69. Janknecht R, Monte D, Baert JL, de Launoit Y 1996 The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. Oncogene 13:1745–1754
70. Goodman RH, Smolik S 2000 CBP/p300 in cell growth, transformation, and development. Genes Dev 14:1553–1577
71. Verger A, Duterque-Coquillaud M 2002 When Ets transcription factors meet their partners. Bioessays 24:362–370
72. Stork PJS, Schmitt JM 2002 Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12:258
73. Dumaz N, Marais R 2005 Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways: based on The Anniversary Prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. FEBS J 272:3491–3504
74. Constantinescu A, Wu M, Asher O, Diamond I 2004 cAMP-dependent protein kinase type I regulates ethanol-induced cAMP response element-mediated gene expression via activation of CREB-binding protein and inhibition of MAPK. J Biol Chem 279:43321–43329
75. Pursiheimo J, Keiksi A, Jalkanen M, Salmivirta M 2002 Protein kinase A balances the growth factor-induced Ras/ERK signaling. FEBS Lett 521:157–164
76. Sevetson BR, Kong X, Lawrence Jr JC 1993 Increasing cAMP attenuates activation of mitogen-activated protein kinase. Proc Natl Acad Sci USA 90:10305–10309
77. Ezashi T, Das P, Roberts RM 2005 Low O_2 tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci USA 102:4783–4788
78. Johnson ML, Redmer DA, Reynolds LP 1997 Uterine growth, cell proliferation, and c-fos proto-oncogene expression throughout the estrous cycle in ewes. Biol Reprod 56:393–401
79. Lee RS, Li N, Ledgard AM, Pollard JW 2003 Dynamic regulation of expression of colony-stimulating factor 1 in the reproductive tract of cattle during the estrous cycle and in pregnancy. Biol Reprod 69:518–528
80. Michael DD, Wagner SK, Ocon OM, Talbot NC, Rooke JA, Ealy AD 2006 Granulocyte-macrophage colony-stimulating-factor increases interferon-τ protein secretion in bovine trophectoderm cells. Am J Reprod Immunol 56:63–67
81. Foulds CE, Nelson ML, Blaszczak AG, Graves BJ 2004 Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. Mol Cell Biol 24:10954–10964
82. Maurer RA 1989 Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. J Biol Chem 264:6870–6873
83. Bannister AJ, Kouzarides T 1995 CBP-induced stimulation of c-Fos activity is abrogated by E1A. EMBO J 14:4758–4762
84. Dooley KA, Bennett MK, Osborne TF 1999 A critical role for cAMP response element-binding protein (CREB) as a co-activator in sterol-regulated transcription of 3-hydroxy-3-methylglutaryl coenzyme A synthase promoter. J Biol Chem 274:5285–5291
85. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, Rao NP, Landaw EM, Sakamoto KM 2005 The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell 7:351–362
86. Wathes DC, Taylor VJ, Cheng Z, Mann GE 2003 Follicle corpus luteum function and their effects on embryo development in postpartum dairy cows. Reprod Suppl 61:219–237

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