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The Presence and Activation of Two Essential Transcription Factors (cAMP Response Element-Binding Protein and cAMP-Dependent Transcription Factor ATF1) in the Two-Cell Mouse Embryo[1]

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ABSTRACT

The expression of two members of an important family of transcription factors, cAMP response element-binding protein (CREB) and cAMP-dependent transcription factor ATF1 (ATF1), is essential for normal preimplantation development. There is a high degree of functional similarity between these two transcription factors, and they can both homodimerize and heterodimerize with each other to form active transcription factors. CREB is present in all stages of mouse preimplantation embryo, and we show here that ATF1 is localized to the nucleus in all preimplantation stages. Activation of these transcription factors requires their phosphorylation, and this was only observed to occur for both transcription factors (serine 133 phosphorylation of CREB and serine 63 phosphorylation of ATF1) at the two-cell stage. Nuclear localization and phosphorylation of ATF1 were constitutive. The nuclear localization and phosphorylation of CREB showed a constitutive component that was further induced by the autocrine embryotropin Paf (1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). Activation of CREB by Paf was independent of cAMP but was dependent on calcium, calmodulin, and calmodulin-dependent kinase activity. ATF1 nuclear localization was unaffected by inhibition of the calcium/calmodulin pathway. A complex pattern of expression of calmodulin-dependent kinases was observed throughout preimplantation development. At the two-cell stage, only mRNAs coding for calmodulin-dependent protein kinase kinase beta, calmodulin-dependent protein kinase II gamma, and calmodulin-dependent protein kinase IV were detected. A selective antagonist for calmodulin-dependent protein kinase kinase (STO-609) and calmodulin-dependent protein kinases I, II, and IV (KN-62) blocked the Paf-induced phosphorylation of CREB. The study demonstrates a role for trophic signaling and constitutive activation of two essential transcription factors at the time of zygotic genome activation.

calcium, calmodulin, calmodulin-dependent kinase, early development, embryo, gene regulation, signal transduction, transcription

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INTRODUCTION

The expression of two members of an important family of transcription factors, cAMP response element-binding protein (CREB) and cAMP-dependent transcription factor ATF1 (ATF1), is essential for normal preimplantation development [1]. There is a high degree of functional similarity between these two transcription factors [2]. The expression of either one allows normal development, yet deletion of both results in the death of the preimplantation embryo by the late blastocyst stage [1].

These transcription factors possess DNA-binding motifs that recognize the same elements within promoters—CRE elements (full-CRE palindrome, TGACGTCA, or half-CRE TGACG/CGTCA) [3]. CREB and ATF1 can homodimerize and heterodimerize [4], and the resulting dimers are the functional transcription factor. This dimerization may provide a basis for the functional compensation or redundancy between CREB and ATF1 action in the early embryo. A CRE-reporter construct could be expressed in the two-cell embryo but not oocytes [5], yet its normal mechanism of activation has not been defined.

CREB was the first transcription factor to be identified as phosphorylation-state dependent, and this applies to all members of the family (for review, see Shaywitz and Greenberg [3]). In their unphosphorylated state, the members of this family are ineffective as transcription factors [6]. Serine 133 is an important phosphorylation site on CREB (Ser133 pCREB) [7], and serine 63 ATF1 (Ser63 pATF1) is thought to be its functional equivalent. Phosphorylation promotes recruitment of the CREB-binding protein (CBP) and other cofactors to the target promoter elements [8]. CBP functions as a ''scaffolding'' protein capable of recruiting the transcriptional machinery [7]. Thus, assessment of the phosphorylation of these transcription factors is critical to understanding their activation state.

Creb1 mRNA and CREB protein are detected throughout the preimplantation phase of embryo development [9]. However, most protein is localized to the cytoplasm at all developmental stages, except at the midcycle two-cell and compacted eight-cell stages of development [9]. An antibody that recognizes Ser133 pCREB showed a pattern similar to CREB staining, except that it only showed enhanced nuclear staining in the mid-to-late two-cell stage [9]. This nuclear localization and phosphorylation of CREB were calcium, but not cAMP, dependent [9].

The appearance of nuclear CREB at the mid-two-cell stage is interesting because this is the stage of development when definitive transcription from the zygotic genome occurs in the early embryo [10, 11]. The mechanisms that induce and control this activation of transcription have yet to be fully defined. It has been proposed that one important mechanism may be the regulated expression and recruitment of transcription factors at this time [12]. It has been found that some transcription factors are continuously present and constitutively active—for example, transcription factor SP1 [12–14]—whereas others are recruited at the time of zygotic genome activation, such as TEA DNA-binding domain 2 (TEAD2) [12]. Yet, to date, a detailed analysis of the transcription factors recruited at the time of zygotic genome activation has not been undertaken. The extensive transcriptome capable of being mobilized by the CREB family of transcription factors [7, 15] and the essential role of CREB and ATF1 in normal embryo development [1] make them interesting targets for further analysis in this context.

This study examined the presence, nuclear localization, and phosphorylation of CREB and ATF1 in the mouse embryo. The activated (phosphorylated) forms ATF1 and CREB were evident only at the two-cell stage. Activation of CREB but not ATF1 could be further induced by ligand-activated calcium/calmodulin/calmodulin-dependent kinase activity. The results support the essential role of these transcription factors in early embryo development and provide evidence for their recruitment to the nucleus during the two-cell stage of development at the time of definitive transcription from the zygote genome.

MATERIALS AND METHODS

Animals

The use of animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Institutional Animal Care and Ethics Committee. C57BL/6j (B6), hybrid (B6 × CBA/He), and *Ptafr*$^{-/-}$ [16] mice were used in experiments. Animals were housed and bred in the Gore Hill Research Laboratory (St. Leonards, Australia). All animals were under 12L:12D cycle and had access to food and water ad libitum. Females 6 wk old were superovulated by intraperitoneal injection of 5 IU of equine chorionic gonadotrophin (Folligon; Intervet International, Boxmeer, The Netherlands), followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Chorulon; Intervet). Females were paired with males of proven fertility. Pregnancy was confirmed by the presence of a copulation plug the following morning (Day 1).

Mouse Embryo Collection and Culture

Embryos were collected from the reproductive tract in Hepes-buffered modified human tubal fluid medium (Hepes-mHTF) at the times indicated in experiments, and then they were cultured in mHTF [17]. All components of the media were tissue culture grade (Sigma) and contained 3 mg of bovine serum albumin per milliliter unless otherwise stated (CSL Ltd., Melbourne, Australia). Embryos were cultured individually in 10-μl volumes in 60-well culture plates (LUX 5260; Nunc, Naperville, IL) overlaid by approximately 2 mm of heavy paraffin oil (Sigma). Culture was at 37°C in 5% CO_2 for the periods indicated in individual experiments. mHTF is a minimal essential medium for the mouse preimplantation embryo [17]. Culture under these conditions results in reduced release of autocrine embryotrophins, including Paf (1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), and when accompanied by culture in limiting dilution (single embryos in 10 μl) results in the depletion of trophic signaling [17, 18]. These culture conditions provide for a partial ablation model suitable for studying the action of autocrine trophic ligands [18].

Pharmacological Agents and Treatments

Paf (Sigma) was prepared as described previously [19]. BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid tetrakis (acetoxymethyl ester); Sigma), STO-609 (1,8-naphthoylene benzimidazole-3-carboxylic acid; Calbiochem, Sydney, Australia), and KN-62 (1-[N,O-*bis*-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; Calbiochem) were prepared as 2000-fold concentrated stock in dimethyl sulfoxide and on day of use diluted to working concentrations of 10–50 μM BAPTA-AM and 160–240 nM STO-609 with mHTF. Other agents were prepared at working concentrations by dissolving directly in mHTF on day of use: Rp-cAMP (Rp-cyclic 3,5-hydrogen phosphorothioate adenosine triethylammonium salt; Sigma); W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl; Calbiochem), and Apafant (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)triazolo(4,3-a)(1,4)diazepine-2-yl)-1-(4-morpholinyl)-1-propanone apafant triazolodiazepine; Boehringer Ingelheim).

To assess the regulation of pCREB, two-cell embryos were collected 40 h after hCG. Embryos were recovered in minimal volume and assigned individually to treatments in 10 μl of medium. Treatments were: 1) control media alone; 2) 37 nM Paf for 20 min; 3) 50 μM BAPTA-AM; 4) 7 μM W-7; 5) 22 μM Apafant; 6) 0.1 μM Rp-cAMP; 7) 1–8 μM KN-62; or 8) 160–240 nM STO-609. Inhibitors were applied either alone or in conjunction with Paf treatment. Dose responsiveness was assessed for each agent except for Rp-cAMP [20] and Apafant [21], which are characterized.

Reverse Transcriptase PCR

Reverse transcription PCR was performed as described previously [22]. Mouse oocytes and embryos were collected from the reproductive tract and washed in cold PBS three times to remove the Hepes-mHTF and were transferred in minimal volume as a group of 10–20. RNA was extracted by three repeats of freezing in liquid nitrogen and thawing with vortex. The RNA was purified with RQ1 RNase-Free DNase Kit (Promega, Alexandria, Australia). RNA from 10 embryos was subjected to reverse transcription with 1 μM allele-specific reverse primer. Negative controls were either reactions without reverse transcriptase or with the RNA sample replaced by diethyl pyrocarbonate-treated MilliQ water (to test for any RNA or DNA contamination). An internal positive control was to test for the presence of mRNA for *Actb*. Amplification of cDNA used sequence specific primers as follows: *Actb*, 5′-CGTGGGCCGCCCTAGGCACCA, 3′-TTGGCCTTAGGGTTCAGGGGG (predicted transcript size, 243 bp); *Camkk1*, 5′-ATTTGGTGTTCGACCTCCTG, 3′-TGGACAGCTGAGCATCATTC, 246 bp; *Camkk2*, 5′-TCACTCGGATGTTGGACAAA, 3′-GGCTGGGAATGTGTTTGACT, 174 bp; *Camk1*, 5′-ACGACATCTCTGACTCTGCC, 3′-TCTTGATCTGCTCGCTCAC, 159 bp; *Camk2a*, 5′-CCTCTACTTTCTCTCCTCCAC, 3′-TGGTTCAAAGGCTGTCATTC, 259 bp; *Camk2b*, 5′-CAGACAAACAGCACCAAAAAC, 3′-TCTTGATGATTTCCTGCTTCC, 187 bp; *Camk2g*, 5′-CGACGACTACCAGCTTTTC, 3′-TCTTCAAACAACTCCCCTCC, 270 bp; *Camk2d*, 5′-AGAAGTTCAAGGCGACCAG, 3′-TCCCACCAGCAAGATGTAG, 145 bp; *Camk4*, 5′-GAAACCCCCACAGAAATCAG, 3′-GACAATCCCATTTCATGCAG, 159 bp. Samples of each transcript were sequenced to confirm identity (SUPAMAC, Redfern, Australia).

Immunofluorescence

Immunofluorescence was as described previously [23]. After fixation and blocking, embryos were incubated overnight at 4°C with primary antibodies: 4 μg/ml anti-CREB (rabbit anti-CREB polyclonal immunoglobulin G [IgG]; Santa Cruz Biotechnology, Santa Cruz, CA); 2 μg/ml anti-ATF1 (mouse anti-ATF1 monoclonal IgA; Santa Cruz Biotechnology); 0.25 μg/ml anti-Ser133 pCREB/Ser63 pATF1 (rabbit anti-pCREB polyclonal IgG; Upstate Bioscientific, Lake Placid, NY); or an equivalent concentration of isotype control immunoglobulin (negative control). As a further negative control, anti-Ser133 pCREB/anti-Ser63 pATF1 antibody was preabsorbed 10 times excess by weight pCREB-immunizing peptide (KRREILSRRP9(pS)YRK; 12–378; Upstate Bioscientific) or vehicle for 30 min at room temperature with mixing. The preparation was then centrifuged at 4°C for 15 min at 15 000 × *g* to pellet immune complexes. The supernatant was used for staining of embryos by immunofluorescence and compared to control antibody.

Primary antibodies were detected by secondary antibodies coupled to fluorescein isothiocyanate (FITC; goat anti-rabbit FITC conjugated IgG; Sigma) or Texas red (goat anti-mouse Texas red-conjugated IgA; Santa Cruz Biotechnology) for 1 h at room temperature. Optical sectioning was performed with a Bio-Rad Radiance Confocal microscope using a Nikon Plan Apo 60×/1.4 oil immmersion objective. Images were captured using Lasersharp 2000, Version 4.0 (Bio-Rad). Confocal images were equatorial optical sections. Whole-section imaging was performed with mercury lamp ultraviolet (UV) illumination and epifluorescence on a Nikon Optiphot microscope with an Olympus DPlan Apo 40 UV objective. These images were subjected to deconvolution using Image-Pro plus (Sharpstack; Media Cybernetics Inc., Silver Spring, MD). All conversions were performed within Image-Pro Plus (version 6.3; Media Cybernetics). Embryos were counterstained with 0.1 μg of propidium iodide per milliliter or 4 μg of Hoechst per milliliter, and the images of FITC and propidium iodide or Texas red and Hoechst were merged using Image-Pro Plus.

Quantitative analysis of immunostaining in the region of the nucleus was performed using the Histogram function within Image-Pro Plus. The area of the nucleus in each embryo was outlined using the Area of Interest tool. The sum of staining in the area of interest was recorded.

For each experiment, embryos from each treatment were processed at the same time and in parallel. All treatments were exposed to the same preparations and dilutions of all reagents, including primary and secondary antibodies. Similarly, all preparations from an experiment were examined microscopically within the same session and used identical microscope and camera settings. All image analysis was performed in an identical manner for all embryos within an experiment. All preparations were performed by the same experienced operator throughout the study.

Western Blot

Western blot analysis was performed as described previously [24]. Fresh or treated two-cell embryos were collected and washed three times in cold PBS and then transferred to extraction buffer containing Triton X-100 (Bio-Rad), 24 mM deoxycholic acid, 0.2% (w/v) SDS, 20 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM phenylmethanesulphonylfluoride, 3.08 mM aprotinin, 42 mM leupeptin, and 2.91 mM pepstatin A (all from Sigma) in PBS. Embryos were lysed by three cycles of freezing in liquid nitrogen and thawing (with vortexing). MBL5 cells were cultured in embryonic stem cell medium containing knockout Dulbecco modified Eagle medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with 100 μM 2-mercaptoethanol (Sigma-Aldrich), 10% fetal bovine serum (Invitrogen), and 1000 U/ml leukemia inhibitory factor (ESGRO; Chemicon International) [25] and T47D cells (HTB-133; American Type Culture Collection) in DMEM. At 90% confluence, cells were homogenized in extraction buffer (1 ml for 0.5×10^7 to 5×10^7 cells). Cell extracts were centrifuged at $8000 \times g$ for 10 min at 4°C, and the supernatant subjected to Western blot analysis.

The samples were diluted with Laemmli loading buffer and separated on 20% homogenous SDS-polyacrylamide gels (Amersham Pharmacia Biotech) using a PhastSystem apparatus (PhastSystem separation and control unit; Pharmacia). The proteins were transferred onto polyvinylidene fluoride transfer membrane (Hybond-P; Amersham) with transfer buffer containing 12 mM Tris (Sigma), 96 mM glycine (BDH, Sydney, Australia), and 20% (v/v) methanol (BDH) by a semidry PhastTransfer system (Amersham Pharmacia Biotech). The membrane was incubated in 10 ml of blocking buffer containing 2.5% (w/v) skim milk powder (Diploma) and then stained with 0.4 μg/ml rabbit anti-CREB IgG or 0.4 μg/ml rabbit anti-Ser133 pCREB/Ser63 pATF1 IgG in blocking buffer at 4°C overnight on shaker. Primary antibody was detected with 1:5000 horseradish peroxidase-conjugated secondary antibody (Sigma) and detected using chemiluminescence. The membrane was incubated in Super Signal West Femto (Pierce, Rockford, IL) diluted 1:4. The membrane was stripped by incubation in 200 mM NaOH (Sigma) for 30 min at room temperature and reprobed with 1:1000 anti-actin, cytoplasmic 1 (Actin) antibody (Sigma) for 1 h at room temperature. The bands were quantitatively analyzed using Labworks software Ver 4.5 (UVP Inc., Upland, CA). Integrated optical density (IOD) of each band was measured. Relative IOD was the ratio of IOD of target band compared to the IOD of actin.

Statistical Analysis

The immunofluorescent staining (intensity), optical density for micrographs, or relative IOD for Western blot was quantitatively measured and analyzed by univariate analysis of variance. In the model, staining intensity was set as the dependent variable and test compound concentrations as the independent variable. The replicates were incorporated in the model as a covariate. Tests of main factor effects and interaction effects were performed. Difference between individual test concentrations was assessed by the least significance difference test. The development rate of embryos to the blastocyst stage was assessed by binary logistical regression analysis.

RESULTS

Atf1 mRNA was detected by RT-PCR at all stages of preimplantation embryo development (Fig. 1A). Immunodetectable ATF1 was also observed in each stage of development (Fig. 1B). Antigen was predominantly detected within the nucleus at each stage of development (both pronuclei in

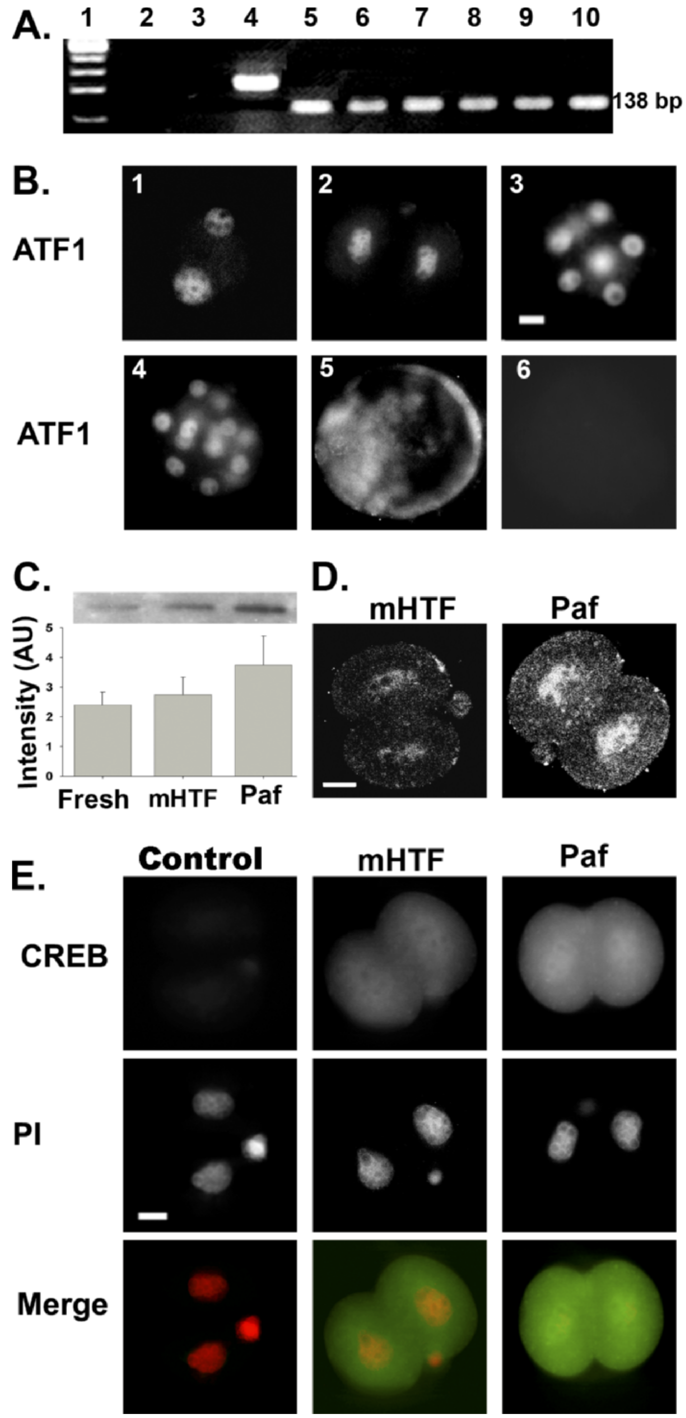

FIG. 1. ATF1 and CREB in the mouse embryos. **A**) The RT-RCR detected *Atf1* mRNA. **1**) Molecular weight ladder. **2**) No RNA. **3**) No reverse transcriptase (in zygotes as an example). **4**) Expression of *Actb* transcript as a positive control in all stages of embryo (presented in zygotes as an example). **5**) Brain. **6**) Oocytes. **7**) Zygotes. **8**) Two-cell embryos. **9**) Eight-cell embryos. **10**) Blastocysts. Expected size: *Actb*, 238 bp; *Atf1*, 138 bp. Results represent at least three replicates, each band with one oocyte or embryo. **B**) Immunofluorescence detection of ATF1 expression in (**1**) zygote of 20 h after hCG; (**2**) two-cell embryo; (**3**) eight-cell embryo; (**4**) morula; (**5**) blastocyst; and (**6**) Nonimmune IgA control (in the zygote of 20 h after hCG as an example). Images are representative of three replicates with at least five embryos at each stage of development per replicate. **C**) CREB expression in two-cell embryos assessed by Western blot analysis. Embryos were freshly collected or cultured in mHTF for 20 min supplemented with or without 37 nM Paf. A representative Western blot is shown, as are the mean and SEM of the optical density of bands (arbitrary units [AU]) of five replicates, 30 embryos each lane. **D**) Immunolocalization of CREB expression in cultured embryos in mHTF and Paf-supplemented medium. Images are single equatorial optical sections through each embryo generated by confocal microscopy. **E**) Whole-section immunolocalization of CREB expression counterstained with propidium iodide (PI), with merged images shown. Control is nonimmune IgG and propidium iodide. Bar = 10 μm (**B**, **D**, and **E**).

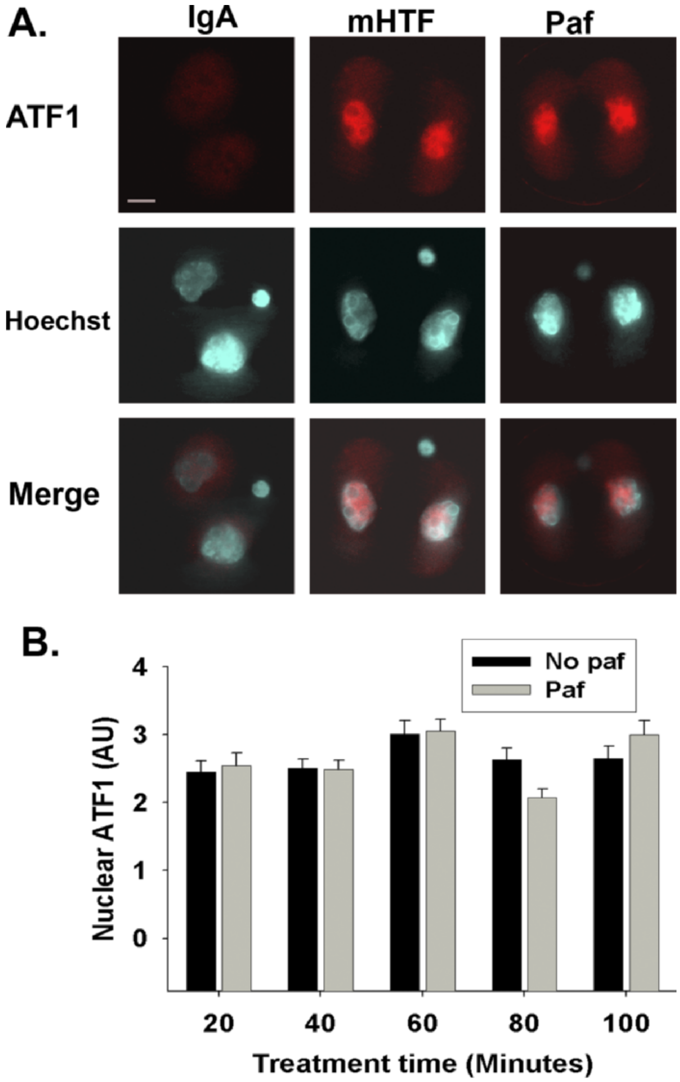

FIG. 2. Nuclear staining of ATF1 in the two-cell embryo. **A**) Immunostaining of ATF1 expression counterstained with Hoechst, with the merged images shown. Control is nonimmune IgA and Hoechst. The data are representative results of three independent replicates, and each had at least five embryos in each treatment or control. Bar = 10 μm. **B**) The effect of Paf on ATF1 in hybrid two-cell embryos. Embryos were treated with Paf or control media and assessed for nuclear ATF1 staining intensity at intervals for up to 100 min of treatment (mean ± SEM of nuclear ATF1 intensity of three independent replicates, each with at least six embryos in each treatment per replicate, $P > 0.05$ for effect of Paf).

zygotes; Fig. 1B1). This pattern of staining for ATF1 differed from that previously detected for CREB, where nuclear accumulation of staining was restricted to the two-cell stage (and, to a lesser extent, the eight-cell stage) [9]. We confirm here that CREB was detected in the two-cell embryo (Fig. 1, C–E) and that it accumulated in the nucleus (Fig. 1, D and E). It was previously shown that the nuclear accumulation of CREB was dependent on intracellular calcium [9]. Paf is an autocrine embryotrophin that induces discrete pulsatile increases in intracellular calcium during the two-cell stage [21]. Treatment of two-cell embryos with Paf induced increased nuclear localization of CREB (Fig. 1, D and E) compared with untreated controls. We show that Paf had no effect on the nuclear localization of ATF1 during a 100-min period of treatment (Fig. 2).

CREB and ATF1 are transcription factors, but their activity depends on their phosphorylation state. Thus, nuclear localization itself does not confirm activation of the transcription factors. We previously [9] used an antibody that detects both CREB phosphorylated at serine 133 (Ser133 pCREB) and ATF1 phosphorylated at serine 63 (Ser63 pATF1) and found that marked nuclear accumulation of staining was only observed at the two-cell stage. This shows that the nuclear staining of ATF1 at the other stages of preimplantation development was of its unphosphorylated form.

Because the activation state-specific antibody recognizes the phosphorylated form of both CREB and ATF1, and both of these proteins were detected within the nuclei of the two-cell-stage embryo, further analysis was required to assess the relative contribution of each. Western blot analysis showed both transcription factors to be phosphorylated in the two-cell embryo (two bands of 37.75 ± 0.17 kDa and 43.5 ± 0.1 kDa [mean ± SEM]; Fig. 3A). Brief treatment of the embryo with Paf induced a significant increase ($P < 0.05$) in the Ser133 pCREB signal but did not induce a change in Ser63 pATF1 staining (Fig. 3B). Two significantly larger bands were also present in two-cell embryos (but not control cell lines MBL and T47D; Fig. 3A). Both CREB and ATF1 are commonly subjected to sumoylation, and it is likely that the larger bands (57.5 ± 0.40 kDa and 65.5 ± 0.25 kDa [mean ± SEM]) reflect this covalent modification, although this requires further verification. The enhanced nuclear staining detected by the activation-state-specific antibody is confirmed by confocal (Fig. 3C) and whole-section (Fig. 3D) immunofluorescence microscopy. The specificity of the antibody was further assessed by preabsorption with excess antigen, and this caused a marked reduction in staining (Fig. 3E).

Paf is known to induce signaling events in the early embryo via a defined G-protein-coupled membrane receptor [21]. Exposure of embryos to Paf induced an increase in the level of Ser133 pCREB staining in wild-type embryos but not in those lacking the Paf receptor (*Ptafr*$^{-/-}$; Fig. 4, A and B). The selective Paf receptor antagonists (Apafant; 22 μM) also blocked the Paf-induced increase in phosphorylation and nuclear localization (Fig. 4C).

CREB phosphorylation in somatic cells is commonly induced by the calcium and/or cAMP secondary messenger pathways. Inhibition of cAMP with Rp-cAMP had no effect on CREB phosphorylation or nuclear localization ($P > 0.05$) in the two-cell embryo (Fig. 5, A and B). By contrast, buffering intracellular calcium by treatment of embryos with BAPTA-AM prevented Paf-induced CREB phosphorylation and nuclear localization ($P < 0.001$; Fig. 5, A and C). Treatment of embryos with a calmodulin antagonist (W-7) also blocked the increase in phosphorylation (Fig. 5, A and D), and we confirm that W-7 induced a dose-dependent block ($P < 0.001$) of normal embryo development (Fig. 5E). This inhibition of development by W-7 could be partially reversed ($P < 0.02$) by exogenous Paf (Fig. 5E).

Calcium/calmodulin activates a range of kinases that in turn activate CREB by its serine 133 phosphorylation. We therefore undertook a screen for the mRNA that code for many of the calmodulin-dependent protein kinases in the embryo (Fig. 6A). RNA for two forms of calmodulin-dependent protein kinase kinase (*Camkk1* and *Camkk2*) were detected in oocytes, but only mRNA for *Camkk2* persisted until the zygote and two-cell stages. This RNA was lost after the two-cell stage. RNA for calmodulin-dependent protein kinase IIγ (*Camk2g*) and calmodulin-dependent protein kinase IV (*Camk4*) showed the same pattern as *Camkk2*. By contrast, calmodulin-dependent protein kinase IIδ (*Camk2d*) was only detected in the eight-cell to blastocyst stages. We did not detect genes encoding calmodulin-dependent protein kinase I (*Camk1*), calmodulin-dependent protein kinase IIα (*Camk2a*), or calmodulin-dependent protein kinase IIβ (*Camk2b*) at any stage of development. This complex pattern of mRNA coding for the calcium/calmodulin-dependent kinases during ontogeny of the early embryo provides for a rich diversity of cellular control at each developmental stage.

Camkk2, *Camk4*, and *Camk2g* may be potential candidates for mediators of ATF1/CREB phosphorylation. A selective antagonist for calmodulin-dependent protein kinase kinases (STO-609) blocked the Paf-induced phosphorylation of CREB (Fig. 6B) but did not change baseline phosphorylation levels. KN-62 is an inhibitor of calmodulin-dependent protein kinase 1, calmodulin-dependent protein kinase II, and calmodulin-dependent protein kinase IV. This inhibitor also blocked the Paf-induced phosphorylation of CREB across the concentrations of 1–8 μM (Fig. 6C) without changing baseline levels. This study implicates a range of calmodulin-dependent protein kinases in the Paf-induced phosphorylation and activation of CREB in the two-cell embryo, but not in the constitutive phosphorylation of ATF1/CREB. The study demonstrates a role for trophic signaling in the preimplantation embryo in the activation of an essential transcription factor via the calcium/calmodulin/calmodulin-dependent kinase signal transduction pathway.

DISCUSSION

This study shows the nuclear accumulation of ATF1 at each stage of preimplantation development and confirms the presence of CREB in the mouse two-cell embryo. In unstimulated two-cell embryos, a proportion of CREB staining occurred outside the nucleus. This was less obvious for ATF1 staining, with most staining occurring predominantly in the nucleus. Brief exposure of embryos to Paf induced a marked increase in the accumulation of nuclear CREB, but this was not the case for ATF1. Both CREB and ATF1 are important transcription factors, and their mutual expression is essential for normal preimplantation development [1]. Their nuclear localization and activation in the two-cell embryo at a time when definitive transcription from the zygotic genome occurs may be indicative of a role for these transcription factors in this process.

Activation of these transcription factors requires their phosphorylation. This phosphorylation does not primarily determine their capacity to bind to target DNA elements, but rather promotes recruitment of CBP and other cofactors to target promoter elements [8]. Phosphorylation results in the exposure of a kinase-inducible domain (KID) in CREB [7], which is recognized by a CREB-binding domain in cofactors,

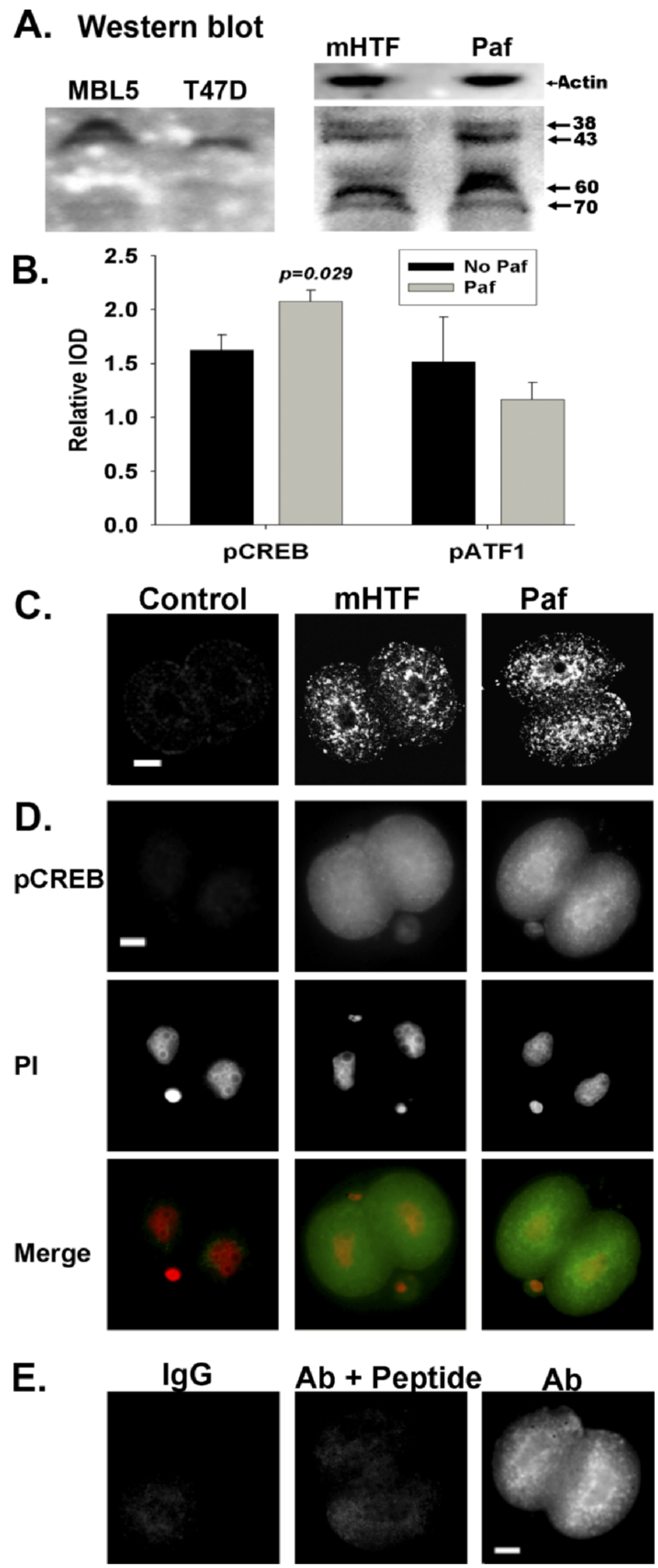

FIG. 3. Ser133 pCREB and Ser63 pATF1 expression in two-cell embryos. **A**) Western blot analysis with anti-Ser133 pCREB/anti-Ser63 pATF1 antibody. Two-cell embryos were cultured for 20 min in mHTF supplemented with (Paf) or without Paf (mHTF). Control cells were MBL5 and T47D. A representative blot is shown. Molecular mass for four bands was identified with Labworks software by comparing them to the protein standard (kDa; SM0671; Fermentas). The top blot is the blot stripped and reprobed with anti-Actin. **B**) The relative optical density (IOD) for Ser133 pCREB (pCREB) and Ser63 pATF1 (pATF1) was measured in three independent replicates. Each treatment represents 90 two-cell embryos per lane. **C**) Confocal microscopy with anti-Ser133 pCREB/anti-Ser63 pATF1 antibody. **D**) Epifluorescent microscopy with dual staining of anti-pCREB antibody and PI. Negative control (Control) was stained with nonimmune IgG instead of primary antibody in Paf-treated embryo. Shown are representative images of three independent replicates, and each treatment had at least five embryos. **E**) Effect of preabsorbing the activation-state-specific antibody with excess antigen on staining of Paf-treated two-cell embryos. Images were immunostained with nonimmune, preabsorbed antibody (Ab + Peptide) and standard antibody staining (Ab). Shown are representative images of three independent replicates, and each treatment had at least five embryos per replicate. Bar = 10 μm.

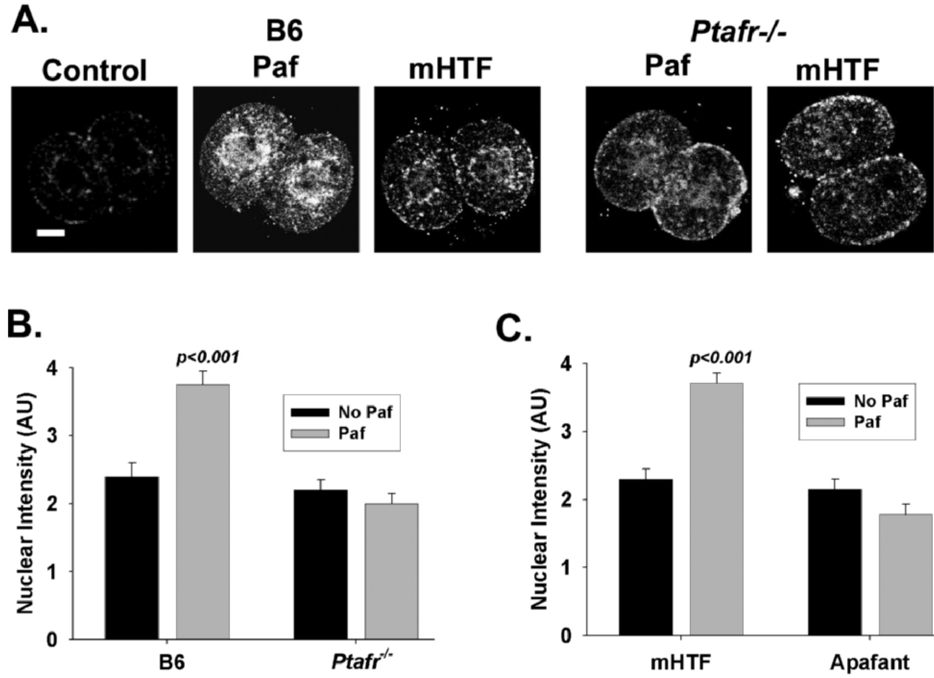

FIG. 4. The dependence of Paf-induced CREB nuclear localization and phosphorylation on the Paf receptor. **A**) Confocal microscopy of Ser133 pCREB expression in two-cell embryos in wild-type (B6) embryos or those lacking the Paf receptor (*Ptafr*$^{-/-}$) after culture in media supplemented with (Paf) or without Paf (mHTF). Negative control (Control) was nonimmune IgG in Paf-treated B6 two-cell embryos. Images are representative of at least five embryos for each group. Bar = 10 µm. **B**) Relative intensity of nuclear pCREB expression in mouse B6 and *Ptafr*$^{-/-}$ two-cell embryos. The values were the mean ± SEM of three independent replicates, each with at least five embryos in each treatment per replicate. AU, arbitrary units. $P < 0.001$, compared to the corresponding conditions without Paf or with *Ptafr*$^{-/-}$. **C**) The effect of Apafant on the expression of nuclear pCREB. The data were the mean ± SEM of three independent replicates, each with at least five embryos in each treatment. $P < 0.001$, compared to the corresponding conditions without Paf or with Apafant.

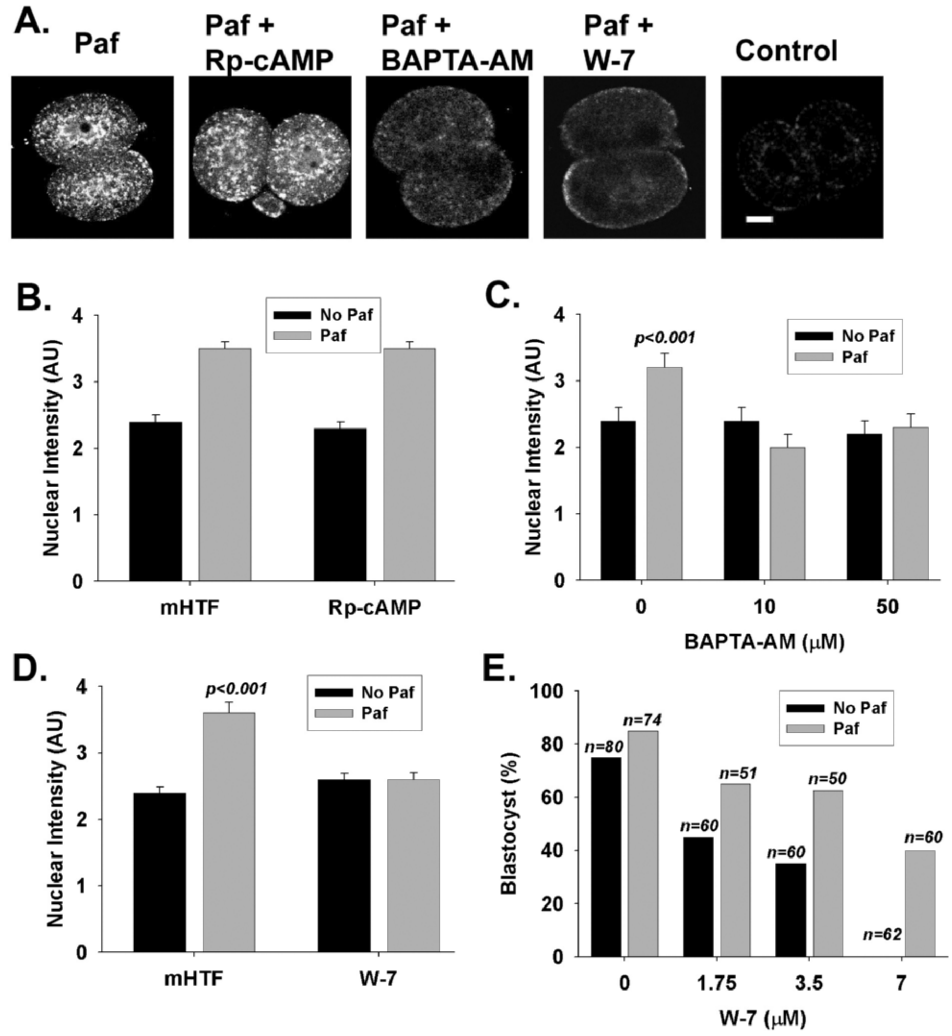

FIG. 5. Immunofluorescence assessed the effects of cAMP and Ca^{++}/calmodulin on CREB phosphorylation in mouse hybrid two-cell embryos. **A**) Confocal fluorescent microscopy assessed the pCREB expression in the two-cell embryos that were treated with Paf, Paf plus Rp-cAMP, Paf plus BAPTA-AM, or Paf plus W-7. Negative control (Control) was the staining of nonimmune IgG in Paf-treated embryo. The images are representative results of three independent replicates, with at least five embryos for each group per replicate. Bar = 10 µm. The effect of (**B**) Rp-cAMP, (**C**) BAPTA-AM, and (**D**) W-7 on Paf-induced nuclear pCREB staining. The data in each figure showed the mean ± SEM of three independent replicates of the experiment, each with at least five embryos in each treatment. Paf significantly increased the nuclear intensity ($P < 0.001$). This was not affected by Rp-cAMP ($P > 0.05$), but was inhibited by BAPTA-AM ($P < 0.001$) and W-7 ($P < 0.001$). **E**) The effect of W-7 on the proportion of blastocysts formed during the 96-h culture of zygotes (total of three independent replicates with least 15 embryos per treatment). W-7 was shown to significantly affect the rate of blastocyst formation ($P < 0.001$), which was partially reversed by exogenous Paf ($P < 0.02$).

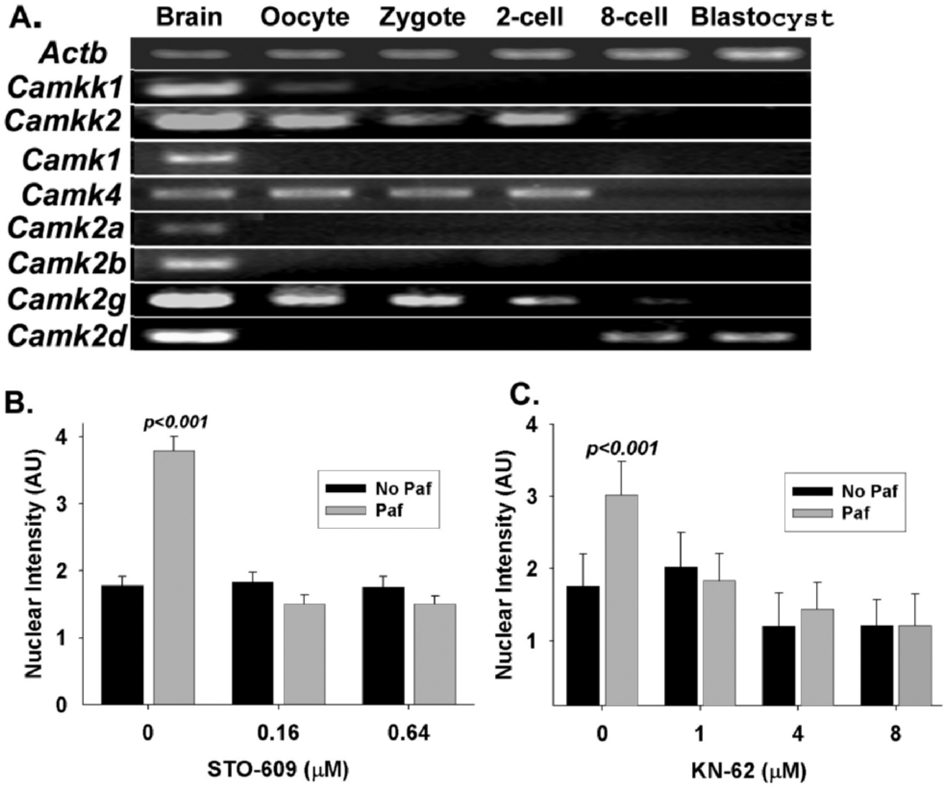

FIG. 6. The effect of protein kinases on pCREB. **A**) Analysis by RT-PCR of mRNA for *Camkk* and *Camk* in mouse oocytes and preimplantation embryos. Brain tissue was used as a positive control for all transcripts, and *Actb* transcripts were used as a positive control for all stages of development in the oocyte and preimplantation embryos. RNA for each gene was tested with one to two oocytes or embryos in at least three independent replicates. The negative controls were reactions without RNA or without RT (data not shown). **B** and **C**) Nuclear pCREB intensity was measured in the two-cell embryos treated with (**B**) STO-609 or (**C**) KN-62, with or without Paf. Each inhibitor was studied in at least three independent replicates, and each treatment had at least eight embryos per replicate (mean ± SEM) Paf caused a significant increase in nuclear intensity ($P < 0.001$), which was antagonized by STO-609 ($P < 0.001$) and KN-62 ($P < 0.01$).

such as CBP [3]. Upon binding, CBP functions as a ''scaffolding'' protein capable of recruiting the transcriptional machinery [7]. Western blot analysis showed that a proportion of both CREB and ATF1 is present in their phosphorylated forms (Ser133 pCREB and Ser63 pATF1) in two-cell embryos. The exposure of embryos to Paf caused a marked increase in the amount of Ser133 pCREB but no detectable increase in Ser63 pATF1. The increase in CREB phosphorylation was accompanied by an increase in its nuclear localization. The results indicate that there is a level of constitutive activation of both transcription factors in the two-cell embryo, and that CREB phosphorylation is further inducible by the trophic ligand, Paf.

The regulation of the constitutive phosphorylation of ATF1 and CREB is not defined by this study. The observation that treatment of embryos with BAPTA-AM, W-7, or calmodulin-dependent kinase inhibitors did not reduce the level of nuclear phosphorylation below the level of untreated controls shows that the calcium/calmodulin/calmodulin-dependent kinase pathways are not primarily involved in this constitutive level of phosphorylation. The Paf-inducible phosphorylated component was primarily CREB, and phosphorylation of this component appears to be under the regulation of calcium/calmodulin/calmodulin-dependent kinase.

Throughout the preimplantation phase of development, nuclear localization and phosphorylation of CREB were restricted to the mid-to-late two-cell stage [9]. Artificial induction of transient increases in intracellular calcium concentration $[Ca^{2+}]_i$ in two-cell embryos induced the nuclear accumulation and phosphorylation of CREB, yet transient elevation of cAMP did not [9]. Paf is a naturally produced ether phospholipid [26] that is synthesized de novo soon after fertilization [27, 28] and is released by the embryo [29]. Upon release, it binds with albumin [30, 31] and acts back in an autocrine fashion to enhance embryo metabolism [32, 33], development [34], and viability [35, 36]. Paf receptor occupancy induces $[Ca^{2+}]_i$ pulses [37] that occur at 60- to 90-min intervals [21]. These first occur in the late zygote, and the pulses had the greatest amplitude at the mid two-cell stage of development [21]. These calcium transients are necessary for normal preimplantation stage development, are mediated by a 1-*o*-phosphatidylinositol-3-kinase [38, 39], and occur as a consequence of the combined release of internal calcium stores and the influx of external calcium through dihydropyridine-sensitive membrane channels [40, 41].

To date, Paf is the only known embryotrophin to induce $[Ca^{2+}]_i$ transients in the two-cell embryo at the time of zygotic genome activation. The ability of exogenous Paf to induce nuclear localization and phosphorylation of CREB, and the failure of Ser133 pCREB induction in *Ptafr*$^{-/-}$ embryos or wild-type embryos exposed to a Paf receptor antagonist (Apafant), implicates the autocrine actions of Paf in inducing CREB activation. The absence of the Paf receptor reduces embryo viability in vitro [38], and it is generally thought that Paf and other autocrine embryotrophins may have an important role as survival factors for the early embryo [18, 42]. The CREB family of transcription factors has important roles in survival signaling in a number of cell types [43–45]. Given the early embryonic lethality of *Creb*/*Atf1*-null embryos [1], it seems likely that the CREB family of transcription factors mediates part of the survival signaling mechanisms of the early embryo.

Calcium is a universal secondary messenger. Several defined calcium signaling events occur during early embryo development. For instance: 1) calcium transients occur in the mouse zygote as a consequence of fertilization, and these enhance the viability of embryos [46, 47]; 2) embryo-derived Paf causes periodic $[Ca^{2+}]_i$ transients in the late zygote and mid two-cell stage, and these transients are also necessary for normal survival of embryos in vitro [21, 38]; 3) calcitonin-induced $[Ca^{2+}]_i$ transients in four-cell to blastocyst-stage embryos; and 4) lysophosphatidic acid induced $[Ca^{2+}]_i$

transients in blastocysts, which results in transient accumulation of heparin-binding epidermal growth factor-like growth factor on the blastocyst surface [48].

Calcium commonly exerts its actions by binding to calmodulin, and this combination can, in turn, activate a wide range of downstream targets. Notable targets of calcium/calmodulin are the calmodulin-dependent kinases. This study showed that there is a complex pattern of expression of members of this kinase class in the preimplantation embryo. At the two-cell stage, mRNA from genes encoding calmodulin-dependent kinase kinase β, calmodulin-dependent kinase kinase IV, and calmodulin-dependent kinase IIγ were detected. Inhibition of calmodulin blocked the nuclear localization of phosphorylated CREB by both Paf and ionomycin-induced $[Ca^{2+}]_i$ transients. Calmodulin-dependent kinase IIγ and calmodulin-dependent kinase kinase β are directly activated by Ca^{2+}/calmodulin [49–51], and activated calmodulin-dependent kinase kinase β phosphorylates calmodulin-dependent kinase IV [52]. CREB is a direct target for calmodulin-dependent kinase II and calmodulin-dependent kinase kinase IV [53, 54]. The inhibition of CREB phosphorylation by the calmodulin-dependent kinase kinase inhibitor (STO-609) [55] implicates both calmodulin-dependent kinase kinase β and calmodulin-dependent kinase IV in CREB phosphorylation. KN-62 is an inhibitor of calmodulin-dependent kinases. KN-62 is reported to inhibit calmodulin-dependent kinase II at low concentration (~0.9 μM) [56] but is effective against calmodulin-dependent kinase IV only at higher concentrations of 3 μM [49]. The inhibition of Paf-induced CREB phosphorylation by KN-62 across the concentration range 1–8 μM implicates calmodulin-dependent kinase II and does not exclude a role for calmodulin-dependent kinase IV. CREB is known to be a target for many other kinases, and our demonstration of a role for calmodulin-dependent kinases does not exclude a potential role for other signaling pathways in its activation.

The failure of cAMP to induce CREB nuclear localization or phosphorylation [9] and the inability of a cAMP inhibitor to block Paf-induced CREB activation in two-cell embryos are consistent with an earlier finding that cAMP could not induce the expression of a CRE reporter construct in the two-cell embryo [5]. By contrast, treatment with the phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate, did induce CRE reporter expression in the two-cell embryo (but not oocytes) [5]. Canonically, the phorbol esters are considered activators of protein kinase C. However, it is well recognized that there is much cross-talk between the protein kinase C and calcium/calmodulin pathways, and further analysis of this intersection and its role in CRE-activation in the two-cell embryo is warranted.

CRE elements can be activated by both ATF1 and CREB acting as either homodimers or heterodimers. CREB is capable of occupying approximately 4000 promoter sites within the genome, depending on the epigenetic status of the CRE site [15]. Hence, the activation and nuclear localization of these transcription factors at the time of zygotic genome activation, as well as the relative epigenetic unsilencing of much of the genome at the two-cell stage [57], provides for a mechanism of profound alterations in the expressed embryonic transcriptome. The lethality of *Creb*$^{-/-}$*Atf*$^{-/-}$ preimplantation embryos [1] is consistent with a critical role for the activation of these transcription factors. It is yet to be defined whether the actions of both transcription factors create a different transcriptome from the actions of either acting independently. Yet, the viability of embryos lacking only *Creb1* or *Atf1* indicates that any differences in their roles are not essential. Thus, there appears to be a significant redundancy or overlapping action of ATF1 and CREB transcription factors in the early embryo. Relatively high levels of cytoplasmic staining of CREB (but not ATF1) were observed. There is no current compelling evidence for a role for CREB within the cytoplasm, although it has been shown to drive mitochondrial gene expression [58]. CREBα is an alternatively spliced variant that does not possess the nuclear translocation sequence, and thus accumulates within the nucleus [59]. The antibody used in this study does not recognize this variant, and Western blot analysis showed only a single band corresponding to the full-length form. Nuclear translocation of CREB occurs in an importin-β-dependent and Ran-dependent manner [60]. The presence of CREB within the cytoplasm may indicate that nuclear translocation is limiting within the early embryo.

Other proteins that were labeled by the anti-Ser133 pCREB/Ser63 pATF after Western blot analysis were approximately 20 kDa larger than the native protein. Both CREB and ATF1 are known targets for sumoylation. This process results in the covalent addition of a small ubiquitin-related modifier (SUMO1; 20 kDa). Sumoylation can alter the transcriptional activity of CREB, and it is suggested that it can stabilize and promote nuclear localization of CREB [61]. The characteristics of these larger bands require further analysis.

The activation of transcription from the zygotic genome is an essential event in the normal development of the embryo. It has been hypothesized that activation of transcription from the zygotic genome in the two-cell mouse embryo is a consequence of the combined actions of constitutively expressed transcription factors with those newly recruited or activated [12]. The actions of ATF1 and CREB seem to fit this paradigm of action. It is likely that CREB and ATF1 alone are not likely to be responsible for this first definitive round of transcription. Indeed, other transcription factors (e.g., transcription factor Sp1 [13, 62] and TEA DNA-binding domain 2 [TEAD2] [12]) are shown to be active at this time. However, given the very diverse transcriptome under CRE-element regulation, our demonstration of the activation of CREB/ATF1 at the time of genome activation makes this family an important candidate for continued investigation. Their activation provides a basis for the requirement of the calcium/calmodulin signaling pathway for genome activation.

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