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EGF-induced *jun B*-expression in transfected P19 embryonal carcinoma cells expressing EGF-receptors is dependent on Jun D

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ABSTRACT

The TPA-inducible transcription factor AP-1, consisting of homo- or hetero-dimers of members of the Jun- and Fos-families, regulates transcription of a wide variety of genes containing the TPA response element (TRE). In P19 embryonal carcinoma (EC) cells, Jun D is the only component of AP-1 expressed, while in these cells until now none of the members of the *jun*- and *fos*-families have been found to be inducable by external stimuli. Here we demonstrate that Jun B is the only member of the Jun- and Fos-families that is induced by Epidermal Growth Factor (EGF) in transfected murine P19 EC cells, expressing functional human EGF receptors (hEGF-Rs). Induction of *jun B* can be mimicked in wild type P19 EC cells by the synergistic action of the phorbol ester TPA and the calcium ionophore A23187, through activation of signal transduction pathways, that are activated simultaneously by EGF. The EGF induced *jun B* expression in the hEGF-R expressing P19 EC cells is mediated by an inverted repeat (IR) sequence in the *jun B* promoter, previously shown to be responsive to both PKC and PKA signal transduction. Transactivation of the IR sequence by EGF can be blocked completely by prior expression of antisense Jun D, but not by antisense c-Jun. These studies therefore implicate Jun D in the regulation of immediate early gene expression by external stimuli.

INTRODUCTION

Transcription factor AP-1, consisting of homo- and heterodimers of members of the Jun and Fos families, mediates gene expression in response to serum, growth factors and phorbol esters through binding to the *cis*-active 12-O-tetradecanoyl-phorbol-13-acetate (TPA) response element (TRE) (reviewed in references 1–3). The Jun-family presently consists of three members, indicated as c-Jun, Jun B and Jun D, which exhibit different expression patterns in response to external stimuli (4–6). In addition, the individual Jun gene products have different transcription regulatory properties, with c-Jun and Jun B being a potent transactivator and transrepressor of the TRE, respectively (7).

Undifferentiated embryonal carcinoma (EC) cells resemble the pluripotent stem cells of the inner cell mass of pre-implantation embryos (8,9). These cells can be induced to differentiate *in vitro* in response to chemical agents such as retinoic acid (RA) (10,11). c-*Jun* and *jun B* are differentially expressed during EC cell differentiation, while *jun D* is expressed constitutively at high levels. Both basal level expression and inducibility of c-*jun* are upregulated during RA-induced EC cell differentiation, while *jun B* becomes inducible by extracellular stimuli upon differentiation (12–15).

A number of receptor protein tyrosine kinases are also differentially expressed in EC cells. The epidermal growth factor receptor (EGF-R) is absent in undifferentiated EC cells, while upon RA-induced differentiation EGF-R's can be detected within 3–5 days of RA treatment (16,17).

In order to study tyrosine kinase signaling in undifferentiated EC cells, we stably transfected the pluripotent P19 EC cell line (10) with an expression vector, containing the complete human EGF-R cDNA (18). The plasmamembrane induced signals elicited by EGF (i.e. elevation of inositol-phospates concentration, intracellular free Ca^{2+}-concentration and intracellular pH) in the hEGF-R expressing P19 EC cell line, P19 8–39, are similar to the EGF induced responses in other EGF-R bearing cells (19). However, only *jun B* is induced by EGF in the EGF receptor expressing P19 EC cells (18), as opposed to most other EGF-R bearing cells, expressing multiple members of the *fos*- and *jun*-families in response to EGF (5, 20–23).

In the present study, we investigate the mechanism of *jun B* regulation in the hEGF-R expressing P19 EC cells. We show that *jun B*-induction in response to EGF can be mimicked by the synergistic action of TPA and the calcium ionophore A23187. Furthermore, we demonstrate that the observed *jun B*-induction by EGF is mediated by an inverted repeat sequence, present in the *jun B*-promoter, previously shown to confer responsiveness

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to both protein kinase A and protein kinase C signaling. Here we provide evidence that transactivation of the inverted repeat sequence is dependent on the presence of Jun D. Implications for the role of jun D in regulation of immediate early gene expression will be discussed.

MATERIALS AND METHODS

Cells and plasmids

Wild type P19 EC cells (10) were cultured in bicarbonate buffered DF-medium containing 7.5% fetal calf serum as described elsewhere (18,24). Previously we described the isolation of a stably transfected P19 EC cell line, P19 8−39, obtained by cotransfection of pSV2neo, conferring resistance to geneticin, and pSV2HERc, an expression vector for the human EGF-R (18). These cells express approximately 30,000 cell membrane localized hEGF-Rs per cell and were cultured exactly like wild type P19 EC cells.

As probes for hybridization studies, a 1.0 kb PstI mouse c-*jun* genomic fragment (15), a 1.5 kb EcoRI fragment of *jun B* (5), a 1.7 kb EcoRI cDNA fragment of *jun D* (6), a 0.8 kb PstI fragment of v-*fos* (25) and a 1.4 kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (26) were used. pSG5-based expression vectors for c-*jun* and *jun B* have been previously described (15). A eucaryotic expression vector for *jun D* was constructed by insertion into pSG5 (27) of a 1.7 kb EcoRI fragment, containing the complete *jun D* cDNA. The *jun B* promoter deletion constructs, pJB4-6, pIR and pIRM have been described elsewhere (28).

Northern blotting analysis

RNA was isolated, using a phenol extraction method (29). Fifteen μg of total RNA was fractionated on a 0.8% formaldehyde-agarose gel and subsequently transferred to nitrocellulose filters as described previously (30). Hybridization of the Northern blots to ^{32}P-labeled probes was performed as previously described (18).

Transient transfections and CAT assays

P19 EC and P19 8−39 cells were transfected, using a calcium phosphate precipitation method, as previously described (15). Twenty-four hours after plating, the calcium-phosphate/DNA (10−20 μg of plasmid DNA)-precipitate was added directly to the cell culture medium. Twenty-four hours later, the cells received fresh medium with or without EGF (50 ng/ml). Sixteen hours after stimulation the cells were harvested and the CAT-activity was determined as described by Gorman et al. (31). The results were quantitated and processed, using the PhosphorImager and the ImageQuant software (Molecular Dynamics), respectively.

Cell labeling, lysis and immunoprecipitation

Cells grown in 6 well plates were labeled with ^{35}S-translabel (containing ^{35}S-methionine and ^{35}S-cysteine; ICN) (100 μCi/ml) for 4 hours. Subsequent cell lysis under denaturing conditions and immunoprecipitation was done exactly as described by Kovary and Bravo (32). The cells were lysed in 400 μl denaturing buffer (50 mM Tris-HCl [pH 7.5]; 0.5% SDS; 70 mM β-mercaptoethanol), boiled for 10 min. and then diluted with 1.6 ml RIPA buffer without SDS. After preclearance of the cell lysates with normal rabbit serum, the lysates were incubated with anti-Jun D antibodies (33) (kind gift of Dr. R.Bravo) for 1 hr on ice followed by 20 μl of protein A-Sepharose CL-4B (1:1 slurry) (Pharmacia) for 2 hr at 4°C with rotation. After extensive washing the protein A-Sepharose beads were resuspended in 50 μl Laemmli sample buffer, boiled and fractionated on a 12.5% polyacrylamide gel.

RESULTS

Jun B-induction in response to EGF can be mimicked by TPA and A23187

It has been shown previously, that the immediate early response genes c-*fos*, c-*jun*, and *jun B* are induced in response to EGF in a variety of EGF-R bearing cells (5, 20−23). In P19 8−39 however, only *jun B* is induced in response to EGF as shown by Northern blotting analysis (18, Figure 1A). Recently, we have demonstrated that in P19 8−39, like in other EGF-R bearing cells, EGF not only activates PKC, but also leads to a rise in intracellular free [Ca^{2+}]-concentrations (19,34,35). Neither stimulation of wild type P19 EC cells with TPA nor with a calcium ionophore (A23187) altered the expression of the *jun* or c-*fos* genes (Figure 1B, lanes T and A). However, simultaneous activation of PKC and elevation of the cytoplasmic free [Ca^{2+}]-concentrations led to a rise in *jun B* mRNA (Figure 1B, lanes T+A). The kinetics of the *jun B* induction of both the hEGF-R expressing and the wild type P19 EC cells is delayed as compared to the *jun B* induction of the RA-differentiated P19 cells in response to EGF. Analogous to the EGF-response of the hEGF-R expressing P19 EC cells, c-*jun*, *jun D* and c-*fos* are refractile to the synergistic activity of TPA and A23187 in wild type P19 EC cells. Apparently both activation of PKC as well as elevation of cytoplasmic free [Ca^{2+}]-levels are sufficient for the induction of *jun B*. These results indicate that, although *jun B* is not induced by most stimuli in undifferentiated P19 EC cells, *jun B* expression is not blocked.

Functional Jun B protein is expressed in response to EGF

Chiu et al. (7) have shown that Jun B is acting as a transrepressor for a single TRE, but is a transactivator of multimerized TRE's. c-Jun on the other hand is a potent transactivator of both single and multiple TRE's (7). Since P19 8−39 cells express only *jun B* mRNA in response to EGF, we set out to identify functional Jun B protein, using its transrepressing and activating properties on single and multiple TRE's. Unfortunately, due to the toxicity of A23187 upon prolonged exposure (longer than 4hr), we could not perform these assays with wild type P19 EC cells stimulated with TPA and A23187. Three reporter constructs, all containing the chloramphenicol-acetyl-transferase (CAT) reporter gene under transcriptional control of the HSV tk-promoter containing either no (tk), one (T1) or three (T3) copies of the collagenase TRE were used (Figure 2A). As shown in Figure 2B, Jun B does not transactivate T1 and represses c-Jun mediated transactivation of T1. Furthermore, Jun B transactivates T3, while the effects of c-Jun and Jun B on T3 are additive. Cotransfection of an expression vector for Jun D together with the TRE-tk-CAT constructs demonstrates that Jun D, like c-Jun, is a potent activator of T1 and T3 (Figure 2B). In addition, transactivation of T1 by Jun D is repressed by Jun B, while the effects of Jun D and Jun B on T3 are additive.

Transactivation of T1 and T3 is not altered by EGF in wild type P19 EC cells, due to the lack of EGF-Rs (Figure 2C). In P19 8−39, EGF causes a slight but significant reduction in transactivation of T1, while transactivation from T3 is enhanced

approximately 3-fold. These results strongly suggest that functional Jun B protein is expressed in P19 8−39 cells in response to EGF.

Jun B-induction in response to EGF is mediated by the IR

The *jun B*-promoter sequences, mediating the EGF-response in P19 8−39 cells were identified, using a variety of *jun B* promoter-CAT fusion constructs. pJB4 contains *jun B* promoter sequences from −196 to +240. Previously we have shown that this construct is responsive to external stimuli that enhance *jun B* expression (28). As shown in figure 3, EGF causes an increase in *jun B* promoter activity of approximately 5-fold. The deletion construct pJB5, lacking the Zif 268 binding site (36) is equally responsive to EGF, while a further deletion of 47 bp, as present in pJB6, results in a complete loss of EGF-responsiveness of the *jun B*-promoter (Figure 3). This indicates that the EGF-response is mediated by *jun B* promoter sequences present between positions −91 and −44 bp. Previous studies have shown that

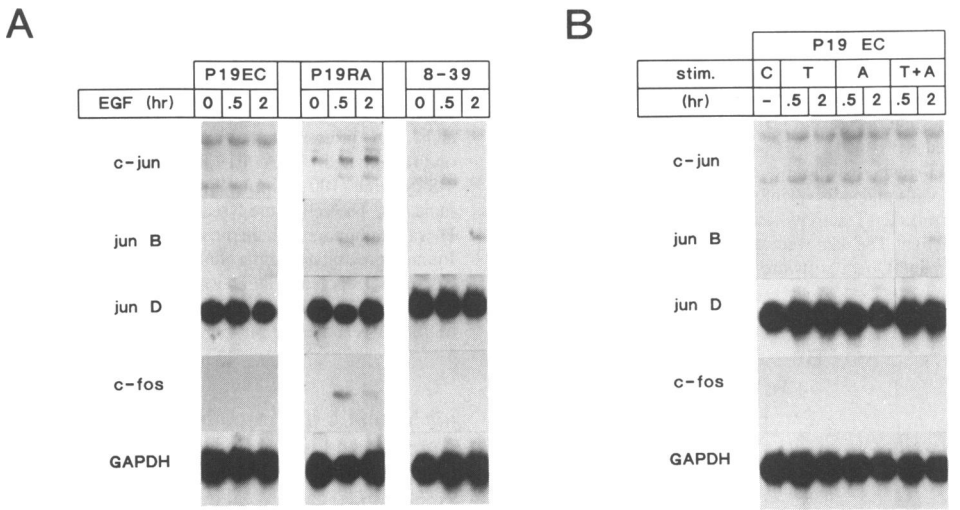

Figure 1. Induction of *jun*- and *fos*-genes in response to external stimuli. (A) Wild type P19 cells (P19 EC), RA-differentiated P19 cells (10^{-6} M for 5 days; P19RA) or hEGF-R transfected P19 EC cells (8−39) were treated with EGF (50 ng/ml) for the indicated times (hours) followed by RNA extraction. 15 μg of total RNA was loaded per lane on a formaldehyde/ agarose gel. After electrophoresis the gel was blotted and the filters were sequentially hybridized with ^{32}P-labeled c-jun, jun B, jun D, c-fos and GAPDH probes (see methods section). (B) Wild type P19 EC cells were treated with TPA (lanes T; 100ng/ml), A23187 (lanes A; 2.5μM) or a combination of TPA and A23187 (lanes A + T) for the indicated times (hours) followed by RNA extraction and Northern blotting, as described under (A).

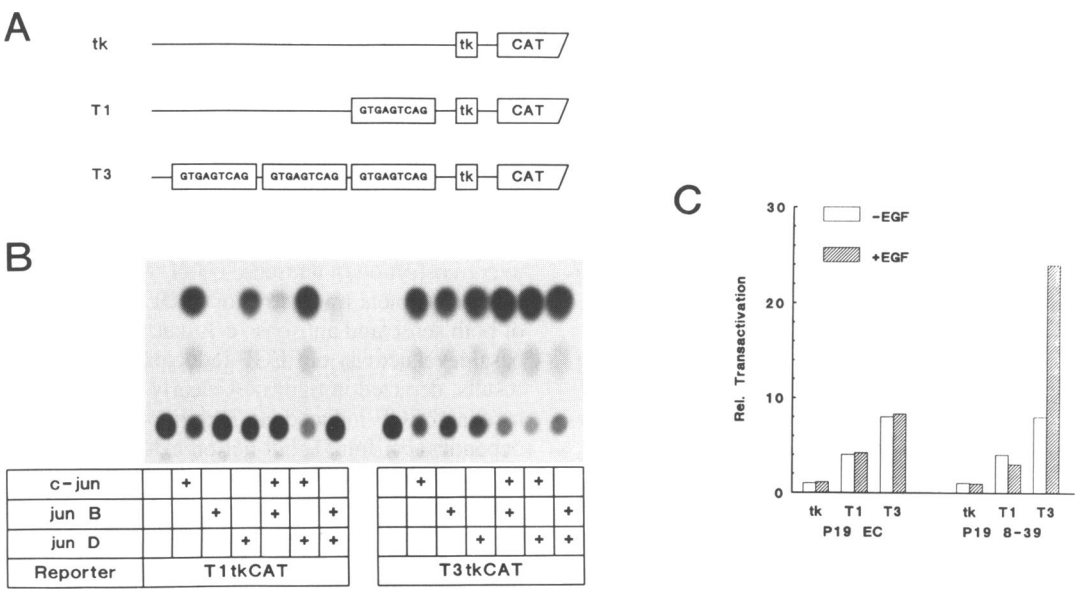

Figure 2. Transactivation of TRE-containing CAT-constructs. (A) The three CAT-constructs all contain the CAT gene under transcriptional control of the HSV-tk promoter. The construct tk contains no TRE-sequences, while T1 and T3 contain one or three copies of the TRE from the human collagenase promoter, respectively, fused to the tk-promoter. (B) pSG5-based expression vectors for c-Jun, Jun B and Jun D were cotransfected with T1 and T3 and subsequently the CAT-activity was determined as described in the materials and methods section. The total amount of transfected plasmid DNA was normalized by the addition of empty expression vector, pSG5. (C) Wild type P19 EC and transfected hEGF-R expressing P19 EC cells (8−39) were transfected transiently with the three CAT-constructs as depicted in (A). After stimulation with EGF (50 ng/ml) for 16 hours the cells were harvested and the CAT-activity was determined. All CAT-assays, depicted in this figure, have been performed three to five times with similar results. The results were quantified, using the Phosphor Imager and Image Quant software.

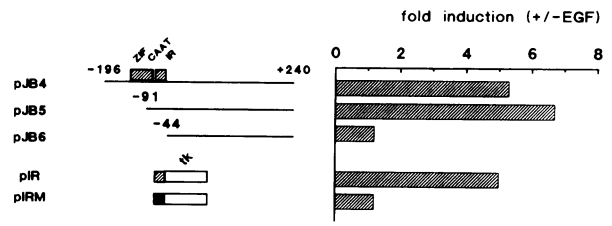

Figure 3. Mapping of the EGF-effect on the *jun B* promoter. *Jun B* promoter sequences were fused to the CAT-gene and progressive deletions of these constructs were made. The location of the consensus Zif 268- and CAAT-binding sites are indicated. pJB4 is the largest construct and contains *jun B* promoter sequences from −196 bp upstream of the transcription initiation site, while pJB6 is the smallest *jun B* promoter deletion-construct, from −44 bp. pIR and pIRM are two constructs containing the inverted repeat (IR; 5'AGTGCACT3') from the *jun B* promoter and a mutated IR (IRM; 5'AGTGCTCT3'), respectively, fused to a tk-promoter driven CAT-gene. All these constructs were transfected into hEGF-R expressing P19 EC cells and subsequently the cells were stimulated for 16 hours with EGF (50 ng/ml). CAT-activity was determined as described in the materials and methods section. The data were quantified and processed, using the Phosphor Imager and Image Quant software respectively.

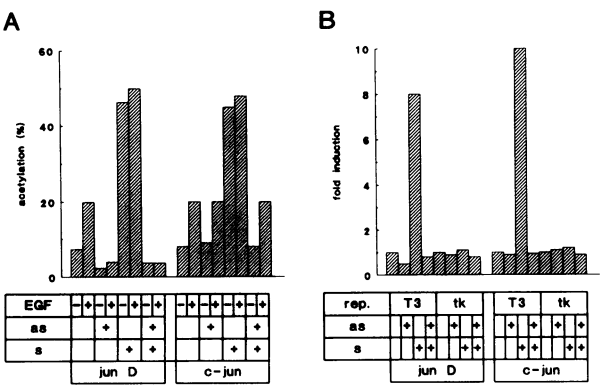

Figure 4. EGF-induced transactivation of the *jun B* promoter is dependent on Jun D. pSG5-based expression vectors for c-Jun and Jun D in both sense (0.5 μg of plasmid DNA/ transfection) and antisense (5.0 μg of plasmid DNA/ transfection) orientations were cotransfected with CAT-constructs (5.0 μg plasmid DNA/ transfection), pIRCAT in hEGF-R expressing P19 EC cells (figure A) or with T3tkCAT and tkCAT in wild type P19 EC cells (figure B). All cells were harvested 48 hours after transfection and the hEGF-R expressing P19 cells were stimulated with EGF (50 ng/ml) 16 hours prior to harvesting and subsequently CAT-activity was determined. The data obtained were quantified, using the Phosphor Imager and Image Quant software.

an inverted repeat sequence (IR; 5'AGTGCACT3'), present in this region, confers responsiveness to both PKC-and PKA-signaling (28). The involvement of IR in the EGF-induced *jun B*-upregulation was investigated using CAT-constructs containing either the IR or a mutated IR (IRM; 5'AGTGCTCT3') fused to the tk-promoter. Transfection of these constructs into P19 8−39 cells shows that IR but not IRM is responsive to EGF, in that the activity of pIR unlike pIRM is enhanced approximately 5-fold in response to EGF (Figure 3). These results demonstrate that the observed upregulation of *jun B* mRNA in response to EGF is mediated by the IR present in the *jun B* promoter.

Jun B-induction in response to EGF is dependent on Jun D

In search of mechanisms, involved in *jun B*-regulation, we investigated the influence of c-Jun and Jun D on *jun B* promoter activity. Expression vectors, containing the complete cDNA for

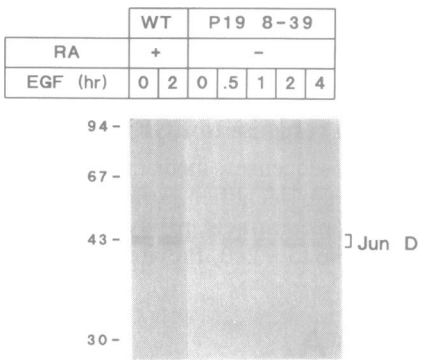

Figure 5. Constitutive expression of Jun D protein in EGF-stimulated P19 8−39 cells. RA-differentiated wild type P19 (treated with RA for 5 days) and undifferentiated (−RA) P19 8−39 cells were labeled with ^{35}S-methionine and ^{35}S-cysteine (100 μCi/ml, 4hr) and treated with EGF (50 ng/ml) for the times indicated. The cells were lysed under denaturing conditions and subsequently Jun D was immunoprecipitated as described in the materials and methods section. Immunoprecipitates and a molecular weight marker were electrophoresed on a 12.5% SDS-polyacrylamide gel. Autoradiography was for 5 days. Molecular weights of the marker are indicated in kilodaltons on the left and the position of Jun D is indicated on the right.

jun D and c-*jun* in both sense and antisense orientations were cotransfected with pIR into P19 8−39. Control experiments were performed in wild type P19 EC cells with T3 and tk. Both Jun D and c-Jun are potent transactivators of T3 but not of tk, lacking TRE-sequences (Figure 3B, 4B). Cotransfection of antisense Jun D as well as c-Jun does not alter the basal level activity from T3 and tk, although antisense Jun D reduces T3 basal activity to some extent (Figure 4B). Cotransfection of both sense and antisense Jun D and c-Jun, with the antisense constructs in a 10-fold excess, completely blocks the transactivation of T3 by Jun D and c-Jun, indicating that the antisense constructs are functional (Figure 4B). As shown in figure 4A, cotransfection of antisense jun D with pIR completely blocks both basal level activity as well as EGF-inducibility from the IR. On the other hand, Jun D is a potent transactivator of IR, since cotransfection of sense jun D activates IR 5-fold (Figure 4A). IR activation by EGF is completely abolished by cotransfection with antisense Jun D, which reduces IR-activity to levels, comparable to cotransfection with antisense Jun D alone. IR-activity is refractile to cotransfection of antisense c-Jun, while on the other hand sense c-Jun is a potent transactivator of IR (Figure 4A). Cotransfection of both sense and antisense c-Jun at a 1 to 10 ratio does not alter basal level activity nor EGF-inducibility of pIR (Figure 4A). The results, depicted in figure 4A clearly demonstrate, that both basal level and EGF-induced *jun B*-promoter activity in P19 8−39 are dependent on Jun D, but not on c-Jun. This finding is probably related to the absence of c-*jun* mRNA expression in these cells, since c-Jun, like Jun D is able to transactivate the *jun B* promoter.

Jun D-mediated *jun B*-induction is not caused by enhanced Jun D protein levels

A possible mechanism underlying the Jun D-mediated upregulation of *jun B*-promoter activity might be that Jun D protein levels are enhanced in response to EGF in P19 8−39 cells. *Jun D* is not upregulated by EGF at the transcriptional level, since no enhanced *jun D* mRNA levels could be detected (Figure 1A). However, Jun D protein levels might be regulated by EGF post-transcriptionally. To investigate whether Jun D

protein levels are influenced by EGF in P19 8−39 cells, we immunoprecipitated Jun D from ^{35}S-methionine/cysteine labeled cells, stimulated with EGF for different periods of time. Immunoprecipitations were performed as described in the materials and methods section, using anti-Jun D-antibodies, generated by Kovary and Bravo (32,33). These antibodies are highly specific for Jun D and do not show any cross-reactivity with Jun B or c-Jun protein (32,33). As shown in figure 5, Jun D protein levels in P19 8−39 cells remain constant during EGF-stimulation. In addition, Jun D protein levels are not upregulated by EGF in RA-differentiated wild type P19 cells (figure 5). These data clearly demonstrate that Jun D protein is constitutively expressed in these cells, as expected from Northern blotting analysis (figure 1). Therefore we conclude that the Jun D-mediated *jun B*-induction is not caused by enhanced Jun D-protein expression in response to EGF.

DISCUSSION

The three Jun proteins identified to date, c-Jun, Jun B and Jun D, are components of the transcription factor AP-1 (1−3). The transactivating properties as well as the expression patterns in response to external stimuli differ between the three Jun genes (7). Here we demonstrate that only *jun B* is induced in response to EGF in transfected P19 EC cells, expressing functional hEGF-Rs. In addition, this upregulation of *jun B*-expression is mediated by the inverted repeat, present in its promoter and is dependent on Jun D.

By Northern blotting analysis we were able to demonstrate that *jun B* mRNA expression is upregulated in response to EGF in the hEGF-R expressing P19 EC cells. In addition, making use of the transrepressing properties of Jun B, we identified functional Jun B protein in the hEGF-R expressing P19 EC cells in response to EGF. It is noteworthy that the AP-1 binding activity is enhanced in these cells in response to EGF (data not shown) but further studies are required to analyse the nature of this observation. It is somewhat surprising that only *jun B* is induced in response to EGF in the EGF-R expressing P19 EC cells, since *jun B* and c-*jun* are co-induced by most stimuli (15). In accordance with the Northern blotting analysis, c-*jun* promoter activity, unlike *jun B* promoter activity, is not upregulated by EGF in the EGF-R expressing P19 EC cells in transient assays using c-*jun* promoter CAT-fusion constructs (data not shown). However, since distinct *trans*-acting factors bind to the c-*jun* and *jun B* promoter-regions (28,37) transcriptional regulation of these two *jun*-genes is different, which presumably accounts for the observed differences in expression.

The finding that the EGF-effect in the hEGF-R bearing P19 EC cells can be mimicked in wild type P19 EC cells by TPA together with the calcium ionophore A23187 indicates that activation of PKC and elevation of cytoplasmic free [Ca^{2+}]-levels is sufficient for *jun B*-induction in P19 EC cells. In addition, these data demonstrate that *jun B* expression is not blocked in undifferentiated P19 EC cells. By contrast, c-*jun* is not expressed in response to any of the stimuli, strongly suggesting that c-*jun* expression is repressed in undifferentiated P19 EC cells. Repression of c-*jun* in undifferentiated P19 EC cells would provide these cells with a mechanism to remain undifferentiated, since it has been shown that ectopic expression of c-*jun* in P19 EC cells leads to differentiation (38).

The EGF-effect on *jun B*-mRNA expression was mapped on the *jun B* promoter and we could demonstrate that it is mediated by the IR, which is responsive to PKC- and PKA-signaling (28). Activation of PKC and PKA does not enhance binding of IRBP (IR binding protein) to the IR (28). Similarly, retardation assays, using double stranded IR-oligonucleotides revealed that EGF does not alter binding of the 110 kDa IRBP to the IR (data not shown). The mechanism, by which IRBP-activity is regulated remains to be determined. Possibly post-translational modification of IRBP by for instance phosphorylation or dephosphorylation leads to activation of IRBP and thus to upregulation of *jun B* mRNA expression (28).

Interestingly we found that co-transfection of expression vectors for antisense Jun D together with CAT-constructs containing the IR, fused to a heterologous promoter (tk) completely blocked both basal level activity and EGF-inducibility. The inability of antisense c-Jun constructs to block the IR-activity implicates that at least in P19 EC cells this activity is specific for Jun D. Expression vectors for c-Jun and Jun D can transactivate IR-CAT constructs to a similar extent. In addition, Jun B can transactivate *jun B* promoter CAT constructs albeit to a lesser extent than c-Jun and Jun D (de Groot, unpublished). The mechanism underlying the Jun D-dependent *jun B*-induction remains to be elucidated. *Jun B*-induction is not caused by enhanced Jun D protein levels, since immunoprecipitation of Jun D from EGF-stimulated P19 8−39 cells clearly demonstrates that Jun D protein is expressed constitutively. In addition, *jun B*-induction is not caused by a direct interaction of Jun-proteins with the IR, since IR-binding activity can not be competed by the c-jun-TRE, nor blocked by anti-Fos antibodies (28). A possible mechanism for Jun D-mediated *jun B*-induction might be that Jun D somehow activates IRBP via protein-protein interactions, in analogy to serum response factor (SRF) mediated c-*fos* autoregulation. FOS negatively regulates its expression indirectly via protein-protein interaction with $p67^{SRF}$, bound to the serum response element (SRE) in the c-*fos* promoter (39, 40). Elucidation of the mechanism of Jun D-mediated *jun B* expression will require further characterization of IRBP and attempts to clone IRBP are currently underway.

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