Transcriptional control of c-*jun* by retinoic acid

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ABSTRACT

The proto-oncogene c-*jun*, a major component of transcription factor AP-1, is expressed at very low levels in undifferentiated embryonal carcinoma (EC) end embryonic stem (ES) cells. Retinoic acid (RA) induced differentiation causes a strong increase in the levels of c-*jun* mRNA. In this paper we report the cloning and characterization of the mouse c-*jun* promoter. Our results show that RA treatment causes a strong enhancement in c-*jun* promoter activity, an effect probably mediated by the RA-receptor β (RARβ). Sequences located between −329 and −293 are responsible for the observed RA effect, and bind at least five different protein complexes, of which three are decreased upon RA treatment. These protein binding sites do not resemble RA-responsive elements (RARE's) found in the promoters of retinoic acid receptor β (RARβ) and laminin B1. Furthermore, we could not detect a direct interaction of RARα and RARβ to these sequences, indicating that RA-induced c-*jun* expression is an indirect effect of RAR action.

INTRODUCTION

The c-*jun* proto-oncogene is the cellular homolog of the transforming gene of avian sarcoma virus 17 v-*jun* (1), and encodes the TPA-inducible transcription factor AP1 (2−4). AP1 is a sequence specific DNA binding protein that binds to the TPA responsive element, thereby regulating the expression of a number of TPA-inducible genes (5−11). Two other c-*jun* related genes, *jun* B and *jun* D, have been cloned from a cDNA library from growth factor stimulated mouse fibroblasts (12−14). Both c-*jun* and *jun* B are rapidly induced by agents such as growth factors and phorbol esters (12, 15−17), whereas *jun* D is relatively unresponsive (13−14). All three Jun proteins can form homodimers or heterodimers with the products of the *fos* gene family (c-*fos*, *fos* B and *fra*-1), thereby increasing their affinity for the TRE as well as their trans-activating potential (11, 18−24). Recent work however indicates that jun B might rather be a trans-repressing component of transcription factor AP1 (25).

Previously we have shown, that the *jun* genes are differentially regulated during retinoic acid (RA) induced differentiation of mouse P19 embryonal carcinoma (EC) cells (26). c-*jun* expression and TRE binding activity were very low in undifferentiated P19 EC cells and were strongly enhanced by RA treatment, while the level of *jun* D mRNA remained constant. By contrast, *jun* B was neither expressed in undifferentiated nor in RA-differentiated EC cells. However, *jun* B expression was strongly and transiently induced by TPA and growth factors in RA treated P19 cells. Furthermore, we have shown that ectopic expression of c-*jun* leads to differentiation of P19 EC cells in the absence of RA (37), suggesting that c-*jun* induction is likely to be of great importance for RA-induced EC cell differentiation.

RA is a strong inducer of differentiation of a variety of mouse and human EC and ES (embryonic stem) cells. In addition, it acts as a morphogen during vertebrate development (for a review see ref. 27). RA effects are mediated by at least three nuclear retinoic acid receptors (RAR) α, β, and γ (28−32). RARα is constitutively expressed in mouse F9 and P19 EC cells, whereas RARβ expression is rapidly induced following RA treatment of these cells (33). The induction of RARβ is probably resulting from direct binding of the RARα to an RA-responsive element (RARE) present in the RARβ promoter (34). Laminin B1, a gene that is induced after RA treatment of F9 EC cells, also contains a RARE in its 5′ flanking region (35). The induction of RARβ is thought to be crucial for RA-induced differentiation of EC cells, since expression of mutant RARα constructs inhibits RA-induced differentiation of F9 EC cells (36). Moreover, expression of a functional RARβ in P19-RAC65, a RA-resistant clone of P19 EC that fails to express RARβ upon RA treatment, induces morphological and biochemical differentiation in these cells (v/d Saag et al., in preparation).

To further study the molecular mechanisms underlying the RA-induced expression of c-*jun*, we have cloned the murine c-*jun* promoter from a Balb-c liver genomic DNA library. In this paper we show, that sequences located between −329 and −293 in the c-*jun* promoter mediate its expression in response to RA. In addition, we provide evidence that RA induced expression of c-*jun* is probably mediated by an indirect effect of the retinoic acid receptor β (RARβ).

MATERIALS AND METHODS

Isolation of genomic c-*jun* fragments

The mouse balb-c liver genomic library containing Sau3A partially digested fragments cloned into lambda EMBL-3 was a kind gift of Gerard Grosveld. c-*jun* positive fragments were

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cloned into pGEM 3 plasmids (Promega) and were sequenced using the T7 polymerase sequencing kit (Promega).

Cells and plasmids

P19-EC and P19-RAC65 cells were cultured in DF-Bic containing 7.5% fetal calf serum as described earlier (37). Mouse embryonic stem cells ES-5 were cultured in MEM containing 20% FCS and 64% conditioned medium of buffalo rat liver (BRL) cells as described (38).

As probes for hybridization studies, a 1.0 kb PstI cDNA fragment of c-*jun* (de Groot, unpublished) and a 1.4 kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ref. 39) were used. RARα and β expression vectors (complete cDNAs cloned into pSG5, ref. 40) and c-*jun*, *jun*B and c-*fos* expression vectors are described elsewhere (26).

c-*jun* promoter CAT constructs were made as follows: pcJ1 consists of a 1.8 kb HindIII-EcoRI (−1250 to +551) c-*jun* promoter fragment cloned into the SmaI site of the promoterless CAT vector pKT; pcJ2 is a 0.8 kb (−439 to +150) BamHI fragment cloned in the SmaI site of pKT; pcJ3 consists of a 0.7 kb (−271 to +551) SmaI-EcoRI fragment cloned in the SmaI site of pKT; pcJ4 is a 0.23 kb (−133 to +97) StyI-SstII fragment cloned in the SmaI site of pKT; pcJ5 consists of a 168 bp (−439 to −271) BamHI-SmaI fragment cloned into the SmaI site of pBLCAT2 (41). pcJ6 and pcJ7 contain a 77 bp BamHI-BglI (−439 to −362) and a 91 bp BamHI-BglI (−362 to −271) fragment cloned into pBLCAL2, respectively. pcJ8 and pcJ9 contain oligonucleotides spanning regions from −398 to −382 and −329 to −293 cloned into pBLCAT2, respectively.

RNA isolation and Northern blotting

Total cellular RNA was isolated by the guanidine isothiocyanate/caesium chloride method of Chirgwin et al. (42). 15 μg of total RNA transferred to nitrocellulose and hybridized as described previously (26, 37).

DNA transfection and transient expression assays

P19 EC and P19-RAC65 cells were transfected as described previously (37). Two hours before transfection, the dishes received fresh medium. Cells were incubated for 16−20 hrs with calcium phosphate precipitated DNA's (10−20 μg plasmid per 50 mm dish), followed by addition of fresh medium with or without RA (10^{-6} M, Sigma). 16−24 hours later, the cells were harvested followed by measuring CAT activity. For RA time course experiments, the cells were treated with RA 1 (3 days RA) or 3 days (5 days RA) prior to transfection. CAT activity was determined as described by Gorman et al. (43), and was quantitated by liquid scintillation counting of TLC plate ^{14}C spots.

Gel mobility shift assay

End-labeling of oligonucleotides, isolation of nuclear extracts and gel mobility shift assays were performed as described previously (26, 37).

DNase I Footprinting

DNase I footprinting reactions were performed as described by Jones et al. (45) with some modifications using nuclear extracts of P19 EC or P19 RAC65 cells. In short, DNA fragments were end-labeled by filling in with Klenow fragment of DNA polymerase I. Fragments were incubated in a total volume of 50 μl containing 5% v/v glycerol, 20 mM Hepes-KOH pH 7.5, 10 mM $MgCl_2$, 75 mM NaCl, 1 mM DTT, 0.18% v/v NP40 and 1 μg poly(d[I-C]) with 10−20 μg of nuclear extract. After incubation for 30−45 min. at room temperature, 4 μl of freshly prepared DNase I diluted in 50 mM $MgCl_2$ was added. Digestion was allowed for 1.5 min. at room temperature, after which 3.5 μl stop buffer (0.2 M EDTA, 2% w/v SDS) was added. Reactions were analyzed on 6% polyacrylamide/7.5M urea gels after extraction with phenol:chloroform and precipitation with isopropanol.

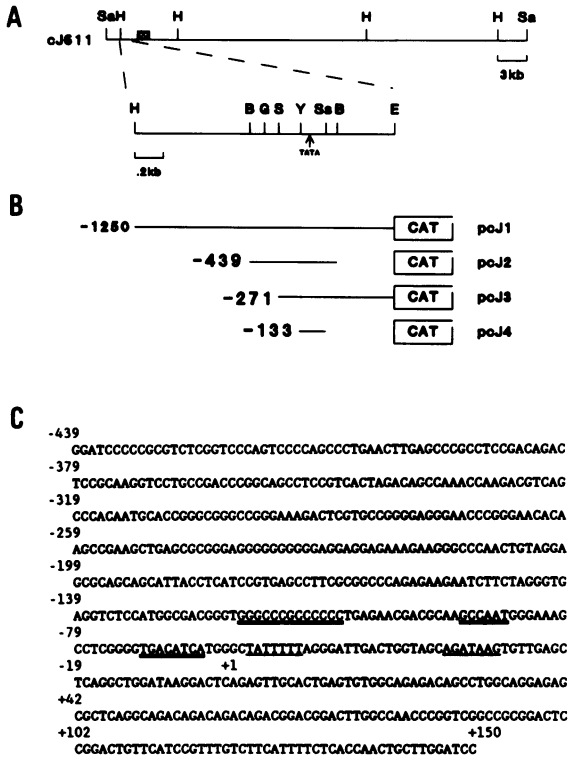

Figure 1. Organization and sequence of the mouse c-*jun* promoter. **A**—A clone (λ611) containing an 19kb SauIIIa fragment was isolated form a mouse BALBc liver genomic library using a human c-*jun* cDNA fragment as a probe. After subcloning and partial sequence analysis, an 1800 bp HindIII-EcoRI fragment was isolated that contained about 1250 bp 5′ flanking sequences. A restriction map of this fragment is shown. The TATA-box homology is indicated. H, HindIII; B, BamHI; G, BglI; S, SmaI; Ss, SstII; Y, StyI. **B**—Structure of three progressive deletions of the c-*jun* promoter coupled to the CAT gene. pcJ1 contains the 1.8 kb HindIII-EcoRI fragment (−1250 to +551), pcJ2 CAT contains the 0.6 kb BamHI fragment (−439 to +150), pcJ3 the 0.8 kb SmaI-EcoRI fragment (−271 to +551) and pcJ4 the 0.23 kb StyI-SstII fragment (−133 to +97). **C**−Nucleotide sequence of the c-*jun* promoter region. The 600 bp BamHI fragment was subcloned and sequenced using the dideoxy chain termination method. The two TATA-like sequences are underlined. The TRE (TGACATCA), an NF-I binding site (GCCAAT) and a sequence homologous to an SP-1 recognition site (GGGCCCGCCCCCC) are double underlined.

RESULTS

Cloning of the mouse c-*jun* gene

RA treatment of mouse P19 EC cells causes a strong increase in c-*jun* expression within 24−48 hours (26). To study the molecular mechanism underlying this increase, clones containing the genomic c-*jun* gene were isolated from a mouse BALBc liver genomic library using a human c-*jun* cDNA fragment as a probe. As depicted in Fig. 1A, one clone (λcJ611) contained the

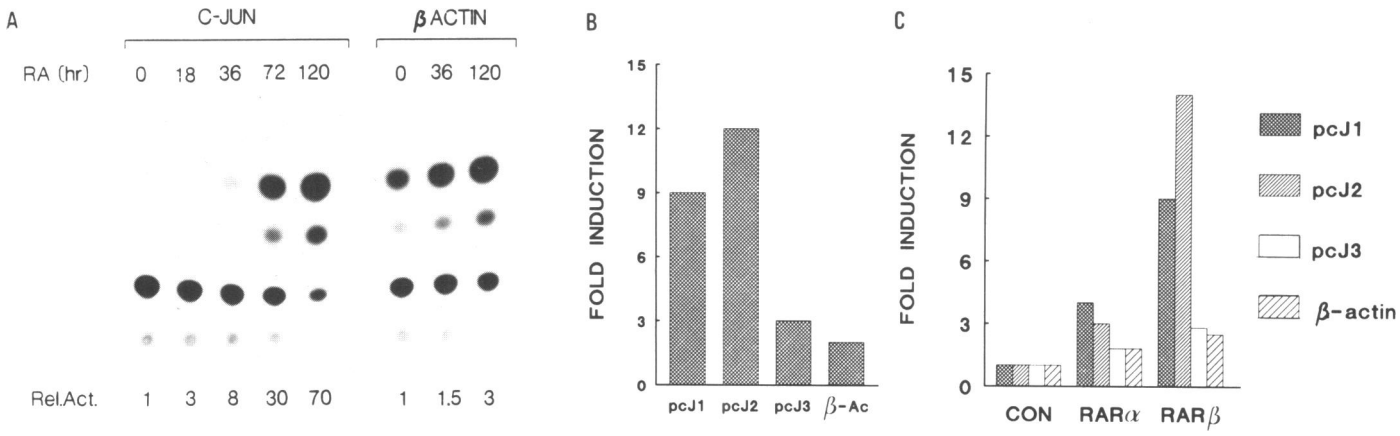

Figure 2. Activation of the c-*jun* promoter by RA. **A**—The 1800 bp HindIII-EcoRI fragment was coupled to the bacterial CAT gene (pcJ1, see 1B) and transfected in P19 EC cells as described in Materials and Methods. Cells were stimulated for different periods with RA (10^{-6}M) and assayed for CAT enzyme activity. As a control, the β-actin-CAT construct was used. % acetylation is the mean of three independent experiments. **B**—Three c-*jun* CAT constructs (pcJ1-3, see 1B) were transfected into P19 EC cells and treated for 50 hours with RA (10^{-6}M) or with carrier alone. CAT activity was quantitated by liquid scintillation counting of ^{14}C-TLC spots. Fold induction represents RA treated samples relative to controls, and is the mean of at least three independent experiments. **C**—RARβ trans-activates the c-*jun* promoter. P19-RAC65 cells were cotransfected with the c-*jun* CAT constructs pcJ1-3 in combination with either RARα, RARβ or the empty expression vector pSG5 (con). Cells were treated with or without RA (10^{-6}M) for 48 hours, and CAT activity was determined. Fold induction represents CAT activity relative to untreated cells, and is the mean of at least three independent experiments.

complete c-*jun* gene including about 2.5 kb 5′ and 15 kb 3′ flanking sequences. Like the human c-*jun* gene (46), the mouse c-*jun* gene lacks intervening sequences as determined by partial sequence analysis (not shown). An 1800 bp HindIII EcoRI fragment containing the putative c-*jun* promoter was subcloned and sequenced. This fragment contains sequences from −1250 to + 551 relative to the major transcription start site as determined by Hattori et al. (46). Part of this sequence (BamHI-BamHI −439 to +150) is shown in Fig. 1C, and is highly homologous to the human c-*jun* promotor (46), containing a TRE (TGACATCA), a CTF/NF1 recognition site (GCCAAT) and a potential binding site for SP1 (GGGCCCGCCCCCC).

Induction of the c-*jun* promoter by RA

To determine whether the cloned mouse c-*jun* promoter fragment was inducible by RA, we coupled this fragment (−1250 to +551) to the bacterial CAT gene (pcJ1, see figure 1B) and studied its inducibility in transient transfection assays in P19 EC cells. As shown in Fig. 2A, RA treatment for 36 hours causes a strong (8-fold) increase in CAT activity, while this effect was further enhanced up to 70-fold after 5 days of RA treatment. These results indicate, that sequences located in the −1250 to +551 fragment mediate RA-induced c-*jun* expression. To further pinpoint the potential retinoic acid responsive element (RARE) in the c-*jun* promoter, two progressive deletions were constructed and tested for RA-inducibility. A fragment containing c-*jun* sequences from −439 to +150 (pcJ2, fig. 1C) was found to be fully inducible by RA, while sequences from −271 to +551 (pcJ3) were induced to a much smaller extent by RA treatment for 60 hours (Fig. 2B), indicating that the putative RARE(S) are probably located between −439 to −271. Construct pcJ3 contains a functional TRE, as determined by TPA induction and cotransfection with c-*jun* and c-*fos* expression vectors (not shown). The slight induction of this construct by RA is therefore likely to be caused by induction of endogenous c-*jun* activating the promoter through the TRE, a situation analogous to the human c-jun promoter (48).

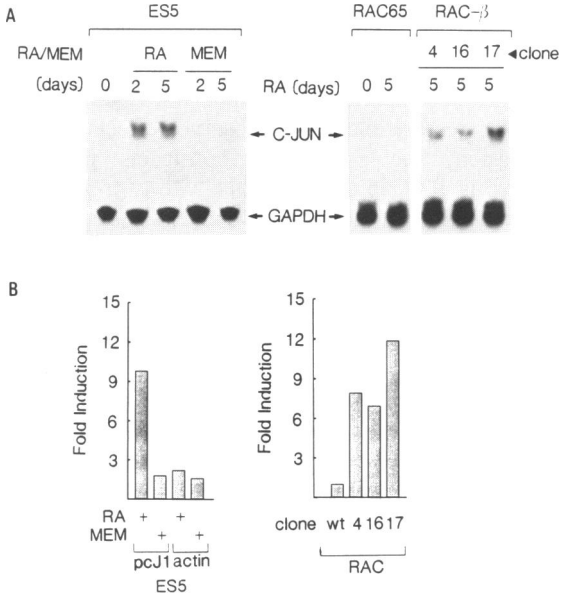

Figure 3. Induction of c-*jun* in ES 5 and P19-RAC65 cells. **A**—Mouse embryonic stem cells (ES 5) were grown in MEM containing 20% FCS and 64% conditioned medium of buffalo rat liver (BRL) cells. ES5 cells were either differentiated by treatment with RA (10^{-6}M) for 2 and 5 days or by omitting the BRL conditioned medium for the same times. Wild type P19-RAC 65 cells were treated with RA or with carrier alone for 5 days, and RAC 65 cells stably transfected with human RARβ (clones 4, 16 and 17) were treated for 5 days with RA. RNA was isolated and analyzed for c-*jun* transcripts by Northern blotting. GAPDH serves as a control for the amount of RNA loaded in each lane. **B**—ES-5 cells were transfected with pcJ1 and cultured for 50 hours in medium containing RA (10^{-6}M) or carrier alone (CON) or in medium lacking DIA-LIF (MEM). RAC-65 cells and the RARβ transfected clones were transfected with pcJ1, and treated with RA or carrier alone. As control, a β-actin CAT construct was transfected. CAT activity was determined as described in the legend of figure 2. Fold induction is relative to unstimulated ES or RAC65 cells, and is the mean of at least three independent experiments.

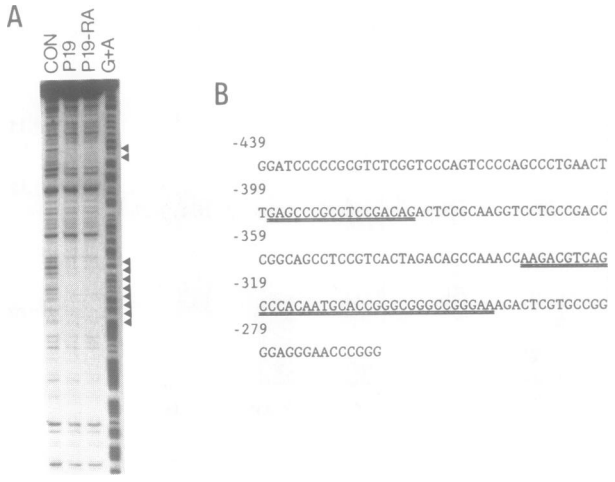

Figure 4. Two protein binding sites in the −439 to −271 fragment. **A**—The BamHI−SmaI fragment was end-labeled at the SmaI site (or the BamHI site, not shown). The probes were incubated with 20 μg BSA (CON) or with 20 μg of nuclear extract from P19 EC cells (P19) or P19 cells differentiated for 5 days with RA (P19-RA), and subjected to DNase I footprinting reactions. As a marker, a G+A ladder of the probe was loaded onto the gel. **B**—Schematic representation of the sequences of the protected sites (double underlined).

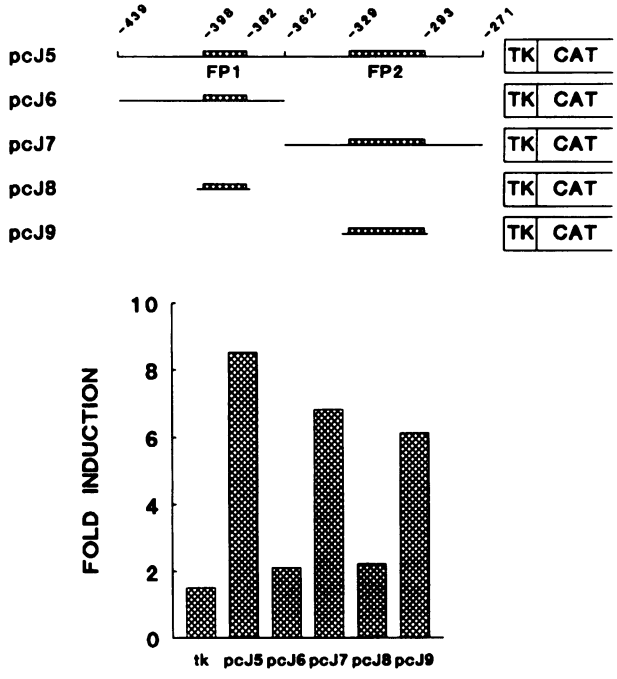

Figure 5. RA effects are located between −329 and −293. P19 EC cells were transfected with the tk-CAT constructs containing fragments spanning from −439 to −271 (pcJ5), −439 to −362 (pcJ6), −362 to −271 (pcJ7), −398 to −382 (pcJ8) or −329 to −293 (pcJ9), and treated for 48 hours with RA or carrier alone. Fold induction is as in Figure 2B.

RA effects are mediated by at least three different RARs, α-γ (28−32). RARα and RARγ are constitutively expressed in F9 and P19 EC cells, while RARβ is rapidly induced by RA in these cells (33). By contrast, P19-RAC65 cells, an RA-resistant variant cell line, does not express RARβ upon RA treatment. To test which RAR is responsible for the observed effects of RA on c-jun expression, the c-*jun*-CAT constructs pcJ1-3 were cotransfected in RAC65 cells together with expression vectors containing the human RARα or RARβ cDNA's (cloned in pSG5, ref. 40). As shown in figure 2C, RARα only slightly stimulates CAT expression of all three c-*jun*-CAT constructs. By contrast, cotransfection of RARβ causes a strong (10−15 fold) increase in activity of pcJ1 and 2, and only a moderate increase in the activity of pcJ3. The β-actin promoter, which is active in both undifferentiated as well as differentiated EC cells, is only slightly activated by both RARα and RARβ. These results are in agreement with the RA-induction of these constructs in P19 EC cells (Fig. 2B), and strongly suggest that RA-induced c-*jun* expression in P19 EC cells is caused by transactivation of c-*jun* 5′ flanking sequences located between −439 and −271 by RARβ.

RARβ trans-activates the c-*jun* promoter

In the mouse embryonic stem cell line ES5, RARβ is also induced upon RA treatment (38, our unpublished results). However, differentiation of ES5 cells by omission of DIA/LIF is not accompanied by RARβ induction (our unpublished results). As shown in Fig 3A, RA induced differentiation of ES5 cells causes a strong enhancement of c-*jun* mRNA, while this effect is not caused by omission of DIA/LIF. These results suggest that RA-induced c-*jun* expression is probably not a side-effect of the differentiation process, but rather indicate RARβ as a potential trans-activator of c-*jun*. In addition, RA treatment of P19-RAC65, a variant clone that fails to induce RARβ upon RA addition, does not lead to enhanced levels of c-*jun* mRNA (Fig. 3A). However, high levels of c-*jun* mRNA are found in RAC65 cells stably transfected with an SV40 driven RARβ expression vector (Fig. 3A), but not in cells transfected with an empty expression vector pSG5 (not shown). To test whether the induction of c-*jun* mRNA by RA in ES-5 cells and by RARβ in RAC-65 cells resulted from enhanced c-*jun* promoter activity, we transfected these cells with pcJ1. As shown in figure 3B, RA treatment for 50 hours of ES-5 cells causes a strong (10-fold) activation of the c-*jun* promoter, while differentiation in MEM only slightly enhances CAT activity. In P19-RAC65 cells, RA failed to activate the c-*jun* promoter, while in the RARβ expressing clones, but not in the pSG5 control clone (not shown), CAT activity was enhanced significantly (Fig. 3B). These results support the hypothesis that RARβ is involved in RA-induced c-*jun* expression in EC and ES cells.

Identification of RARβ responsive elements

In order to identify regulatory sequences in the −439 to −271 region, we performed a footprinting analysis on this c-*jun* promoter fragment. As shown in figure 4A, two protected regions were found in the region between −439 and −271. No differences were observed between c-*jun* expressing (P19+RA) and non-expressing (P19) cells (Fig. 4A). Closer examination of the protected regions shows that both are non-homologous to the two described RARE's from the RARβ and laminin promoters (Fig. 4B, refs. 34−35).

In order to examine whether these footprinted regions are involved in the observed effects of RA on c-*jun* expression, a number of constructs spanning the −439 to −271 region were made (Fig. 5). P19 EC cells were transfected with these construct and treated for 48 hours with RA or carrier alone. As shown in figure 5, RA treatment causes a strong (±9-fold) activation of pcJ5, a constuct containing the complete −439 to −271 region, while tk-CAT was only slightly induced. Moreover, pcJ7

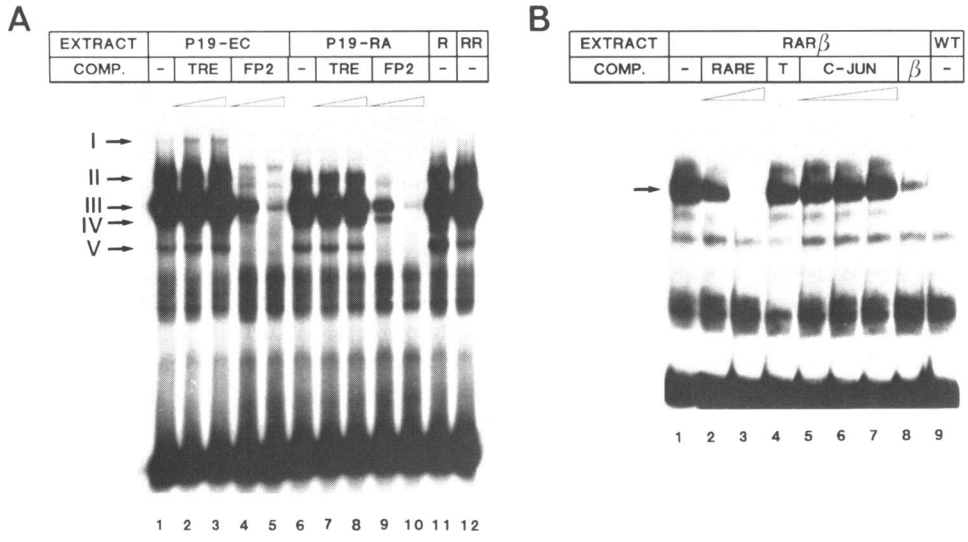

Figure 6. Multiple proteins bind to the c-*jun* promoter. **A**—The −329 to −293 fragment was ^{32}P labeled and used as a probe in a gel retardation assay with nuclear extracts from P19 EC cells, P19 cells differentiated for 2 days with RA (P19-RA), RAC 65 cells (R) or RAC 65 cells treated for 2 days with RA (RR). Protein-DNA complexes were separated on low ionic strength 5% PAA gels, and visualized by autoradiography. For competition experiments, nuclear extracts were preincubated for 5 minutes with a 5−50 fold molar excess of DNA prior to the addition of labeled probe. The following competitor DNA's were used: non-specific—TRE; specific—FP2. Specific complexes are indicated by an arrow. **B**— RARβ does not bind directly to the c-*jun* promoter. An oligonucleotide encompassing the RARE from the human RARβ promoter (34) was ^{32}P labeled and used as a probe in a gel shift assay with nuclear extracts from HeLa cells infected with wild-type vaccinia virus (WT, lane 9) or recombinant virus containing the complete human RARβ cDNA (RARβ, lanes 1−8) (kind gift of dr. H. Stunnenberg). As unlabeled competitor DNA's, the homologous oligonucleotide (RARE 5−50 fold molar excess, lanes 2−3), a heterologous oligonucleotide (collagenase TPA responsive element 250 fold molar excess, lane 4), the −439 to −271 BamHI-SmaI c-*jun* promoter fragment (c-*jun* 5−250 fold molar excess, lanes 5−7) or a 140 bp XbaI-ApaI fragment containing the human RARβ promoter (140, 10-fold molar excess, lane 8) were used. The RARβ specific protein-DNA complex is indicated by an arrow.

and pcJ9 are induced upon RA treatment, while pcJ6 and pcJ8 are only marginally induced. In addition, both pcJ7 and pcJ9 are induced by co-transfection of RARβ in RAC65 cells (not shown). These results show that the most 5′-located footprint (FP2, −329 to −293) is involved in RA induction of c-*jun*.

Multiple proteins bind to the RA-inducible c-*jun* promoter fragment

To determine whether the −329 to −293 element is activated directly by RARβ, or by another RARβ-induced transcription factor, we investigated the nuclear proteins binding to this fragment by gel retardation. This element was ^{32}P labeled with Klenow polymerase, and incubated with nuclear extracts from P19 EC cells, RA differentiated P19 cells, P19-RAC65 cells and RA-treated P19-RAC65 cells. As shown in figure 6A, five different protein-DNA complexes were observed using the FP2 element. Competition experiments indicate that all five complexes (I to V) are specific, since they can be competed by a 50-fold excess of unlabeled homologous (FP2), but not by heterologous (TRE) DNA (Fig. 6A). RA treatment of P19 EC cells causes a 2-fold decrease in the abundance of complex I, II and III. By contrast, this decrease is not observed in RAC65 cells treated with RA (R versus RR in the figure). These results suggest that down-regulation of a repressor might be involved in RA-induced c-jun expression in P19 EC cells.

To investigate whether RARα and/or RARβ bind to the RA-inducible c-*jun* promoter sequences between −439 and −271, we performed gel shift analysis with nuclear extracts from HeLa cells infected with recombinant vaccinia viruses containing human RARα or β cDNA's (a kind gift of dr. H. Stunnenberg, EMBL). Figure 6B shows that, using a 32 bp oligonucleotide encompassing the RARE from the human RARβ promoter as a probe (34), a protein-DNA complex is formed with RARβ containing HeLa extract (lane 1), which is not observed with extracts from HeLa cells infected with wild-type vaccinia virus (WT, lane 9). The formation of this complex can be competed with 5−50 fold molar excess of homologous unlabeled oligonucleotide (lanes 2 and 3) and with a 140 bp fragment of the human RARβ promoter containing the RARE (lane 8), but not with a 250-fold molar excess of non-homologous oligonucleotide (collagenase TPA responsive element (TRE), lane 4), indicating that this complex is specific for the RARE. Competition with 5−250 fold molar excess of c-*jun* promoter fragment (−439 to −271, lanes 5 to 7) shows that RARβ does not bind to these sequences. Similar results were obtained with HeLa extracts containing RARα or γ (not shown). Moreover, using either RARα, β or γ containing extracts from HeLa cells in a DNase I footprinting assay, no protected region could be detected in the −439 to −271 c-*jun* promoter fragment (not shown). These results indicate that RA-induced c-*jun* transcription does not result from direct binding of RARα, β or γ to the c-*jun* promoter, but is rather caused by an indirect effect of either of these two proteins on an as yet unidentified protein, that in turn activates c-*jun* promoter activity.

DISCUSSION

The expression of c-*jun*, a major component of transcription factor AP-1, is strongly upregulated during RA-induced differentiation of murine P19 EC cells (26). In this paper we report the isolation of the mouse c-*jun* gene including its 5′ regulatory region. We show that the upregulation of c-*jun* expression by RA is a direct result of enhanced c-*jun* promoter activity, both in P19 EC cells as well as in mouse ES cells.

Expression of RARβ, either in transient assays or in stable transfectants, strongly enhanced both c-*jun* mRNA expression and promoter activity. A region between −329 and -293 was found to be responsible for at least part of the RA-effect. This region binds five different protein complexes, and does not resemble the RARE's from the RARβ and laminin promoters. Furthermore, no direct interaction of either RARα or β with the RA-inducible c-*jun* promoter sequences could be detected.

The transcriptional response to RA is caused by the three nuclear receptors for RA, RARα, β and γ (28−32). Both RARα and γ are constitutively expressed during RA-induced differentiation of F9 and P19 EC cells (although RARγ is only expressed at very low levels), while RARβ is rapidly induced by RA in these cells (33). Our results show that the activation of the c-*jun* promoter by RA is probably mediated indirectly by RARβ, since cotransfection of RARβ strongly enhances c-*jun* promoter activity, while RARα has only a moderate effect. Furthermore in P19-RAC 65 cells, which express normal levels of RARγ but fail to express RARβ upon RA treatment, c-*jun* expression and promoter activity is not enhanced by RA, while in RAC 65 cells stably transfected with RARβ c-*jun* is expressed at high levels after RA treatment. However, since RARβ expression in P19 EC cells can already be detected within 4 hours of RA treatment (our unpublished results), it is unlikely that RA-induced c-*jun* expression results from a direct interaction of RARβ with the c-*jun* promoter. Since we were unable to detect direct binding of either RARβ or α to the c-*jun* promoter in a gel shift assay, it seems more likely that a RARβ activated transcription factor is responsible for the activation of the c-*jun* promoter. The delayed kinetics of c-*jun* expression are however not unprecedented, since RA-induced expression of laminin B1, a gene containing a RARE, is only observed 24−48 hours after RA treatment (35).

Although the major RA-responsive region of the c-*jun* promoter is located between −329 and −293, a construct lacking this region (pcJ3) is still somewhat responsive to RA. These results are in agreement with a recent report showing partial activation by RA of a human c-*jun* promoter construct containing only the TRE (−79 to +170) and a stronger activation of c-*jun* sequences from −1100 to +740 (48). Since our study shows that the mouse c-*jun* promoter, like the human c-*jun* promoter (8), is positively autoregulated by Jun/AP-1 binding to the TRE in the c-*jun* promoter, the induction of pcJ3 is probably the result of activation by RA-induced endogenous Jun/AP-1. This is in agreement with our previous studies showing that the activity of a TRE-tk-CAT construct is enhanced by RA treatment in P19 EC cells (26). RA-induced c-*jun* expression in EC cells is therefore likely to be a two-step process: an initial activation by a RARβ-modulated transcription factor, followed by positive autoregulation by Jun/AP-1. A number of studies suggest that this elevation of Jun/AP1 activity is likely to be of major importance for the differentiation process rather than a secondary effect of differentiation: 1—overexpression of c-*jun* in P19 EC (37) and F9 EC cells (48), or c-*fos* in F9 EC cells (49) leads to differentiation of these cells; 2—introduction of a trans-dominant negative c-*jun* mutant in F9 EC cells leads to resistance to RA-induced differentiation (60); 3—a number of genes controlling differentiation of EC cells contain one or more TRE's in their regulatory region (50−53); 4—differentiation of rat PC 12 cells by nerve growth factor or oncogenic ras is accompanied by a rise in Jun/AP1 (54, 55), and 5—expression of other oncogenes that induce Jun/AP1 (e.g. v-src and E1A) also leads to differentiation of EC cells (56−59).

The region of the c-*jun* promoter between −329 and −293, which is important for RA-induced c-*jun* expression binds at least five different protein complexes. The binding sites of these complexes do not resemble the RARE's found in the promoters of RARβ and laminin B1, and no interaction of RARα and β with these sequences was found. RA treatment caused a slight (2-fold) decrease in three of these complexes in P19 EC cells, but not in RAC65 cells. Therefore, downregulation of a repressor by RARβ might be involved in RA-induced c-jun expression. However, detailed analysis of the proteins binding to the RA-inducible c-*jun* promoter fragment will be needed to elucidate the precise molecular mechanism of RA-induced c-*jun* expression.

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