Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8670–8674, November 1990
Cell Biology

Differentiation of F9 embryonal carcinoma cells induced by the c-*jun* and activated c-Ha-*ras* oncogenes

(transcription factor AP1/gene regulation/retinoic acid/endoderm)

YUKO YAMAGUCHI-IWAI*, MASANOBU SATAKE*, YOTA MURAKAMI*, MASAHARU SAKAI†‡, MASAMI MURAMATSU†, AND YOSHIAKI ITO*§

*Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606, Japan; †Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan; and ‡Department of Biochemistry, Hokkaido University School of Medicine, Kita-ku, Sapporo 060, Japan

Communicated by Peter K. Vogt, August 6, 1990

ABSTRACT **The activated c-Ha-*ras* oncogene induced AP1-site DNA-binding activity in F9 cells. This induction appeared to be due, at least in part, to the induction of c-*jun* transcription. Both activated c-Ha-*ras* and c-*jun* induced the differentiation of F9 cells to endoderm-like cells. Thus, AP1 appears to play a key role in the initial stage of F9 cell differentiation.**

The nuclear protooncogene c-*jun* was originally identified as a cellular homologue of the transforming gene of an avian retrovirus, avian sarcoma virus 17 (1). The product of the gene, Jun protein, forms a heterodimer with Fos, the product of the c-*fos* nuclear protooncogene and its family, to become a transcriptional activator protein, AP1, which recognizes and binds to an AP1 consensus sequence (reviewed in refs. 2 and 3). When quiescent cells are stimulated by serum growth factors, very rapid and transient expression of c-*fos* and c-*jun* is observed. AP1 is also known to respond to a tumor promoter, phorbol 12-myristate 13-acetate (PMA), and to enhance gene expression (4, 5). Therefore, AP1 is considered to play a key role in switching on the gene expression that ultimately leads to DNA replication and cell division. More recently, AP1 has been implicated as a regulator of DNA replication (6). Here we present evidence that AP1 is also involved in the initial stage of cell differentiation.

The murine embryonal carcinoma cell line F9 has been used widely to study the mechanism of gene regulation in the process of cell differentiation (7), since F9 cells can be induced to differentiate to endoderm-like cells by retinoic acid and cAMP (8). AP1 is undetectable in F9 cells, whereas it is easily detectable after differentiation of the cells (9). The expression of both c-*jun* and c-*fos* is very low in F9 cells (10). An interesting question is whether AP1 becomes detectable as a consequence of differentiation or whether AP1 is required for cell differentiation. Müller and Wagner reported in 1984 (11) that the overexpression of c-*fos* in F9 cells induced F9 cell differentiation. However, only a small fraction of the cells expressing c-*fos* underwent differentiation, regardless of the levels of c-*fos* expression (12). Moreover, the phenotype of the differentiated cells was somewhat different from that of the cells induced to differentiate by retinoic acid (11).

The activated Ha-*ras* oncogene [Ha-*ras*(Val-12)] and PMA stimulate the DNA binding of murine AP1, PEBP1 (13), or PEA1 (14) in lymphoid cells and in fibroblasts (15, 16). PMA superinduces the transcriptional activating function of AP1 in the presence of protein synthesis inhibitors by posttranslational modification of the proteins (17). On the other hand, the mechanism of the induction of AP1 by Ha-*ras*(Val-12) has not been studied in detail.

Here we show that Ha-*ras*(Val-12) induces c-*jun* expression and the AP1 DNA-binding activity in F9 cells. This finding allowed us to analyze whether AP1 would induce F9 cell differentiation.

MATERIALS AND METHODS

Cell Culture. F9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum at 37°C in humidified 10% CO_2/90% air.

Plasmids. The chloramphenicol acetyltransferase (CAT) gene constructs used were $pA_{10}CAT_2$ derivatives, $p(WA)_2$-CAT and $p(M1A)_2$-CAT through $p(M4A)_2$-CAT, in which the CAT gene is regulated by the simian virus 40 (SV40) early promoter under the control of dimers of the polyomavirus A element or a mutated A element (16). pT24c3 is a recombinant plasmid harboring Ha-*ras*(Val-12) (18). pHβARJ101, in which rat c-*jun* derived from pRJ101 (19) is regulated by the human β-actin enhancer and promoter, was constructed by inserting a 2010-base-pair (bp) *Eco*RI fragment of rat c-*jun* cDNA into the *Hin*dIII–*Bam*HI sites of the mammalian expression vector pHβAPr-1 (20). pHβARJ101(tr) is a frameshift mutant of pHβARJ101 in which 2 bp were inserted at the *Acc* I site in the c-*jun* coding region by restriction enzyme digestion at this site, filling the sticky ends with appropriate nucleotides, and religation. This insertion resulted in the truncation of the c-*jun* product. pSV2neo contains the neomycin-resistance gene driven by the SV40 enhancer and promoter (21).

DNA Transfection and CAT Assays. F9 cells were plated at 10^6 per 100-mm dish 5 hr before transfection. Each CAT construct (2.5 μg) was cotransfected into cells with 17.5 μg of pT24c3 or pBR322 by calcium phosphate coprecipitation. Twelve hours later, cells were treated with 15% (vol/vol) glycerol in DMEM for 1 min and harvested 36 hr after transfection. CAT assays were performed as described (16). Transfections were performed at least three times with two different preparations of plasmid DNA.

Preparation of Whole Cell Extract and Mobility-Shift Assay. F9 cells were transfected with pT24c3 (20 μg) and pSV2neo (1 μg). Selection with G418 (400 μg/ml) was started 24 hr later and cells were cultured for 4 days. Surviving cells were collected and a whole cell extract was prepared (22). The polyomavirus A element cloned into the *Bam*HI site of the pUC13 polylinker was excised by cleavage at *Eco*RI and *Hin*dIII sites and the 5′ ends were labeled with ^{32}P. The binding reaction of the probe with the cell extract and subsequent polyacrylamide gel electrophoresis were performed as described (13).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase.

§To whom reprint requests should be addressed.

Phase-Contrast Microscopy. Plasmid DNA (20 μg) of pHβARJ101, pHβARJ101(tr), or pT24c3 was cotransfected with 1 μg of pSV2neo into F9 cells. Cells were incubated with G418 and photographs were taken at a magnification of 100 after 6 days of selection.

Immunofluorescent Staining. F9 cells cultured on glass coverslips were cotransfected with pHβARJ101, pHβARJ101(tr), or pT24c3 together with pSV2neo, and G418-resistant cells were selected. Surviving cells were fixed with 3.7% (vol/vol) formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. In the case of staining of intracellular antigens, the cells were permeabilized with 0.1% (vol/vol) Nonidet P-40 in PBS for 10 min after fixation. The cells were incubated for 40 min at 37°C with the first antibody (mouse monoclonal anti-SSEA-1 IgM, rabbit anti-mouse laminin IgG fraction, or rat monoclonal anti-Hsp47 IgG) and then incubated with rhodamine-conjugated goat antibody against mouse IgM, rabbit IgG, or rat IgG (Cooper–Cappel). The coverslips were mounted on glass slides and photomicrographs were taken with a ×40 objective.

Plasminogen Activator Assay. F9 cells were cotransfected with pHβARJ101, pHβARJ101(tr), or pT24c3 together with pSV2neo in 35-mm dishes. After 48 hr of incubation with G418, cells were washed twice with PBS and covered with 1.5 ml of prewarmed (45°C) DMEM containing 0.75% Bacto purified agar (Difco), 2.5% skim milk (Difco), and 0.2 unit of bovine plasminogen (Daiichi Chemical, Tokyo). The plates were incubated for 24 hr at 37°C and photographed.

RNA Preparation and Northern Blot Analysis. Total RNA was prepared (16) from F9 cells 20 hr after transfection. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose column chromatography. RNA was electrophoresed in 1% agarose gels with 0.66 M formaldehyde and transferred onto nitrocellulose or Zeta-Probe (Bio-Rad) filters in 20× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7). Hybridization was carried out for 18 hr at 42°C in 50% (vol/vol) formamide/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/5× SSC/50 mM Tris·HCl, pH 7.4/0.1% SDS containing sheared salmon sperm DNA at 100 μg/ml (for nitrocellulose filters) or in 0.5 M $NaHPO_4$, pH 7.2/1 mM EDTA/7% SDS (for Zeta-Probe). To probe the RNA derived from the c-Ha-*ras*, c-*jun*, or β-actin gene, a 6.5-kbp *Bam*HI fragment of pT24c3 (18), a 2.0-kbp *Eco*RI fragment of pRJ101 (19), or a 0.8-kbp *Pst* I–*Kpn* I fragment of pAL41 that harbors mouse skeletal muscle β-actin cDNA (23), respectively, was used. Hybridized filters were washed twice in 2× SSC/0.5% SDS for 30 min at room temperature and twice in 0.1× SSC/0.5% SDS for 30 min at 43°C.

RESULTS

Induction of the AP1-Site DNA-Binding Activity by Ha-*ras*(Val-12). The DNA-binding activity of murine AP1, PEBP1 (13), or PEA1 (14) can be enhanced by transiently expressed Ha-*ras*(Val-12) (13, 15). To analyze this enhancement in detail, Ha-*ras*(Val-12) was transfected into F9 cells in which AP1/PEBP1 was undetectable (9). The AP1/PEBP1 DNA-binding activity was monitored using the A element of the polyomavirus enhancer as a probe (Fig. 1). The A element represents one of the two cores of the enhancer and contains the three sequence motifs recognized by PEBP1 (PEA1), PEBP2/3 (PEA2), and PEBP5 (PEA3) (16). Since there is no need to distinguish PEBP1 (PEA1) from AP1 for the discussion in the present study, the term AP1 will be used throughout the rest of this paper to indicate the factor(s) that binds to the AP1 consensus sequence, including PEBP1 (PEA1). When the CAT construct that was under the control of the A-element dimer was transfected, the CAT activity became detectable only after cotransfection with Ha-*ras*(Val-12) (Fig. 1, lanes 1 and 2). Introduction of pairwise mutations in the

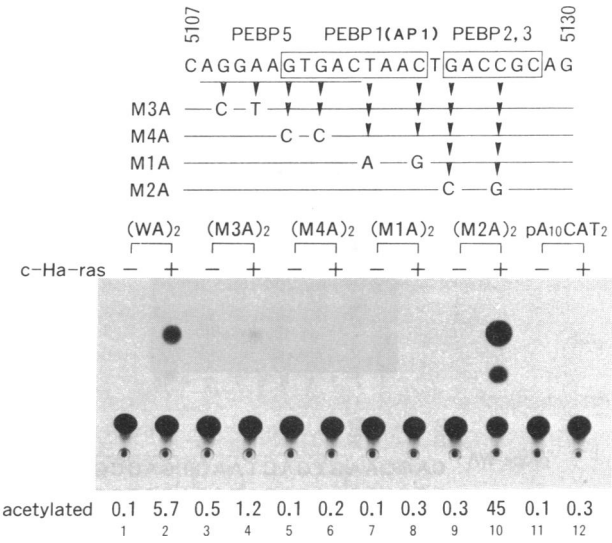

FIG. 1. Effect of transiently expressed Ha-*ras*(Val-12) on the A-element activity of polyomavirus enhancer in F9 cells. (*Upper*) Sequences of the wild-type (WA) and four kinds (M1A–M4A) of mutated A element are shown in the upper panel. Boxes indicate the binding sites of PEBP1(AP1) and PEBP2/3 (13). The binding site of PEBP5 is underlined (41). (*Lower*) CAT activity in cell extracts. Each CAT construct under the control of the A-element dimer was cotransfected into F9 cells with Ha-*ras*(Val-12) (even-numbered lanes) or with control plasmid pBR322 (odd-numbered lanes). CAT activity is expressed as the percentage of acetylated chloramphenicol.

AP1 or PEBP5 binding sites virtually abolished the response of the A element to Ha-*ras*(Val-12) (lanes 3–8). Therefore, Ha-*ras*(Val-12) was able to induce the AP1 as well as the PEBP5 DNA-binding activity in F9 cells. This induction was a relatively early response, observed as early as 24 hr after transfection (data not shown). The induction of PEBP5 binding activity suggests that the target of the activation by Ha-*ras*(Val-12) was multiple. The characteristics of PEBP5 have been reported (41). Mutations in the PEBP2/3 site increased the response to Ha-*ras*(Val-12) nearly 10-fold (compare lanes 2 and 10). This is related to the derepression response observed by Wasylyk *et al.* (24) and has been discussed fully elsewhere (25). The backbone vector containing no insert did not respond to cotransfected Ha-*ras*(Val-12) (lanes 11 and 12).

The induction of the AP1 DNA-binding activity by Ha-*ras*(Val-12) was also examined by mobility-shift assay. Whole cell extract was prepared from F9 cells that were cotransfected with pSV2neo and Ha-*ras*(Val-12) and selected with G418 for 4 days. The extract was incubated with a ^{32}P-labeled A-element probe and analyzed by mobility-shift assay (Fig. 2, lanes 3–10). The slowly migrating band represents AP1, since its formation was specifically abolished by inclusion of excess amounts of the AM oligonucleotide as a competitor in the binding reaction but was not affected by the inclusion of the AE oligonucleotide. The AM and AE oligonucleotides represent the AP1 and PEBP2/3 binding sites, respectively (Fig. 2 *Lower*). The formation of the band indicated with the asterisk was abolished by the AE oligonucleotide, suggesting that this band is related to PEBP2/3. On the other hand, the cell extract prepared from F9 cells transfected only with pSV2neo did not give rise to a discernible band (lane 1). This indicates that the expression of Ha-*ras*(Val-12) in F9 cells induced the AP1-site DNA-binding activity. Since G418 selection was used to enrich the transfected cell population, the above experiment could not be performed much earlier than 4 days after transfection.

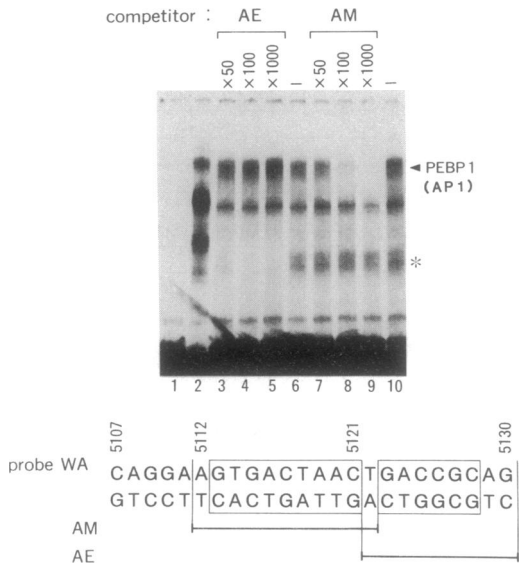

FIG. 2. Mobility-shift assay of factors expressed in Ha-*ras*(Val-12)-transfected F9 cells. Whole cell extracts from pSV2neo- (lane 1) or Ha-*ras*(Val-12)- (lanes 3–10) transfected F9 cells were incubated with ^{32}P-labeled A-element (WA) probe before electrophoresis. AM or AE oligonucleotide (sequences indicated below the autoradiogram) were included in the reaction mixture at 50-, 100-, or 1000-fold molar excess. Two boxes within the AM and AE fragments indicate the minimum binding sites of PEBP1 (AP1) and PEBP2/3, respectively. The nuclear extract from murine NIH 3T3 fibroblasts was used in lane 2 and the position of PEBP1 (AP1) is indicated at right. The band marked by the asterisk may represent PEBP2/3 (see text).

Induction of c-*jun* Gene Expression by Ha-*ras*(Val-12). To analyze the mechanism of induction of AP1 activity by Ha-*ras*(Val-12), poly(A)$^+$ RNA was prepared from F9 cells 20 hr after transfection with Ha-*ras*(Val-12). Northern analysis with c-*jun*-specific ^{32}P-labeled DNA revealed that the amount of the 2.7-kilobase (kb) c-*jun*-specific transcript was significantly higher in Ha-*ras*(Val-12) transfectants than in control cells (Fig. 3). By densitometric scanning of the autoradiogram, the increase in the c-*jun* transcript was calculated to be 2.4-fold after normalization using an internal control of β-actin transcript. The efficiency of transfection was found to be 1% by transfecting a parallel culture with the *lacZ* gene driven by the long terminal repeat (LTR) promoter of the Rous sarcoma virus and subsequently counting the number of cells stained blue after the addition of 5-bromo-4-chloro-3-indolyl β-D-galactoside. By taking this transfection efficiency into consideration, the overall increase of c-*jun* expression by Ha-*ras*(Val-12) was estimated to be 140-fold. The c-*fos* transcript could not be detected under the conditions used. This result suggests that the induction of the AP1 binding activity in F9 cells by Ha-*ras*(Val-12) was due, at least in part, to the increase of c-*jun* expression.

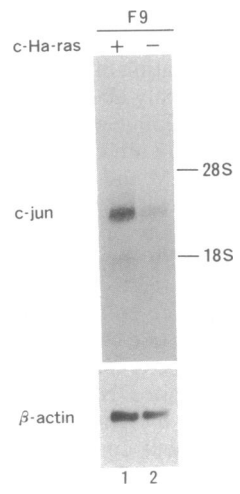

FIG. 3. Induction of endogenous c-*jun* expression by Ha-*ras*(Val-12) in F9 cells. RNA was extracted from nontransfected F9 cells (lane 2) or from cells transfected with Ha-*ras*(Val-12) (lane 1). Five micrograms of poly(A)$^+$ RNA, recovered by passing 250 μg of total RNA through an oligo(dT)-cellulose column twice, was electrophoresed through an agarose gel. The blotted filter was hybridized with a ^{32}P-labeled c-*jun* probe (*Upper*) and rehybridized with a mouse β-actin probe (*Lower*). Positions of 18S and 28S rRNA are shown as size markers.

Morphological Changes of F9 Cells Induced by Ha-*ras*(Val-12) and c-*jun*. Since AP1 was detectable in F9 cells after differentiation by retinoic acid (9), it was of interest to determine the long-term effect of Ha-*ras*(Val-12) on F9 cells. When F9 cells were cotransfected with Ha-*ras*(Val-12) and pSV2neo and selected for G418 resistance for 4–6 days, practically all the surviving cells were morphologically different from the parental F9 cells. The majority of the cells formed colonies and were multiplying, although they seemed to stop growing after several cycles of division. These growing cells were flattened polygonal cells, similar in morphology to retinoic acid-treated F9 cells (Fig. 4*A*, *c*).

The induction of AP1 activity by Ha-*ras*(Val-12) appeared to precede the morphological differentiation of F9 cells (24–36 hr vs. 4–6 days). Hence, a causative role of AP1 in this differentiation was envisaged. As mentioned earlier, the introduction of c-*fos* into F9 cells induced differentiation but only partially (12). Therefore, we tested the effect of c-*jun*. The morphological differentiation of c-*jun*-transfected F9 cells was very similar to that of Ha-*ras*(Val-12)-transfected F9 cells (Fig. 4*A*, *a*). As a control, we also transfected a plasmid that has the potential to express a truncated form of the Jun protein that lacks the DNA-binding domain. The morphology of such F9 cells (Fig. 4*A*, *b*) was indistinguishable from that of parental F9 cells. We also observed no morphological change in F9 cells when the nonactivated c-Ha-*ras* protooncogene was introduced (data not shown).

Northern analysis of the RNAs extracted from the c-*jun*- or mutant c-*jun*-transfected cells 20 hr after transfection revealed the expected 2.0-kb c-*jun*-specific RNA (Fig. 5, lanes 2 and 3). The amounts of each transcript detected were comparable, indicating that the wild-type and the mutated c-*jun* genes were transfected and expressed at about the same efficiency. Since relatively small amounts of poly(A)$^+$ RNA were loaded on the gel compared to that used in Fig. 3, the endogenous mouse c-*jun* transcript was not detected under these conditions (lane 1). Therefore, the morphological differentiation of F9 cells was likely to be due to the function of Jun. In the case of Ha-*ras*(Val-12)-transfected cells, a 1.2-kb c-Ha-*ras*-specific RNA was detected, which was the expected size for the transcript from the transfected plasmid (lane 5). The endogenous c-Ha-*ras* transcript was not detected under the conditions used (lane 4).

Characterization of F9 Cells Induced to Differentiate by Ha-*ras*(Val-12) and c-*jun*. The stage-specific embryonic antigen SSEA-1 (26) is a known stem-cell marker present in F9 cells, but it disappears when cells are induced to differentiate by retinoic acid. The presence of SSEA-1 was examined by the indirect immunofluorescence method. SSEA-1 was detected on the surface of F9 cells or on those expressing mutated c-*jun* (Fig. 4*B*, *1b*) but was completely absent from Ha-*ras*(Val-12)-(1*c*) or c-*jun*-(*1a*) transfected cells.

The expression of several differentiation marker proteins including laminin (27), plasminogen activator (8), and a heat shock protein, Hsp47, which is associated with collagen type IV (28), were also examined. Cytoplasmic laminin (Fig. 4*B*, *2a* and *2c*) and Hsp47 (*3a* and *3c*) were detectable by indirect immunofluorescence in cells transfected with c-*jun* or Ha-*ras*(Val-12) but were undetectable in cells transfected with mutated c-*jun* (*2b* and *3b*). Plasminogen activator was similarly induced in c-*jun* or Ha-*ras*(Val-12)-transfected cells (Fig. 4*C*, *a* and *c*) but not in cells transfected with the mutated c-*jun* (*b*), as judged by the transparent area generated by proteolysis of casein in overlaid milk. Expression of Ha-*ras*(Val-12) or c-*jun* in F9 cells did not preferentially select a differentiated subpopulation of cells that existed in the orig-

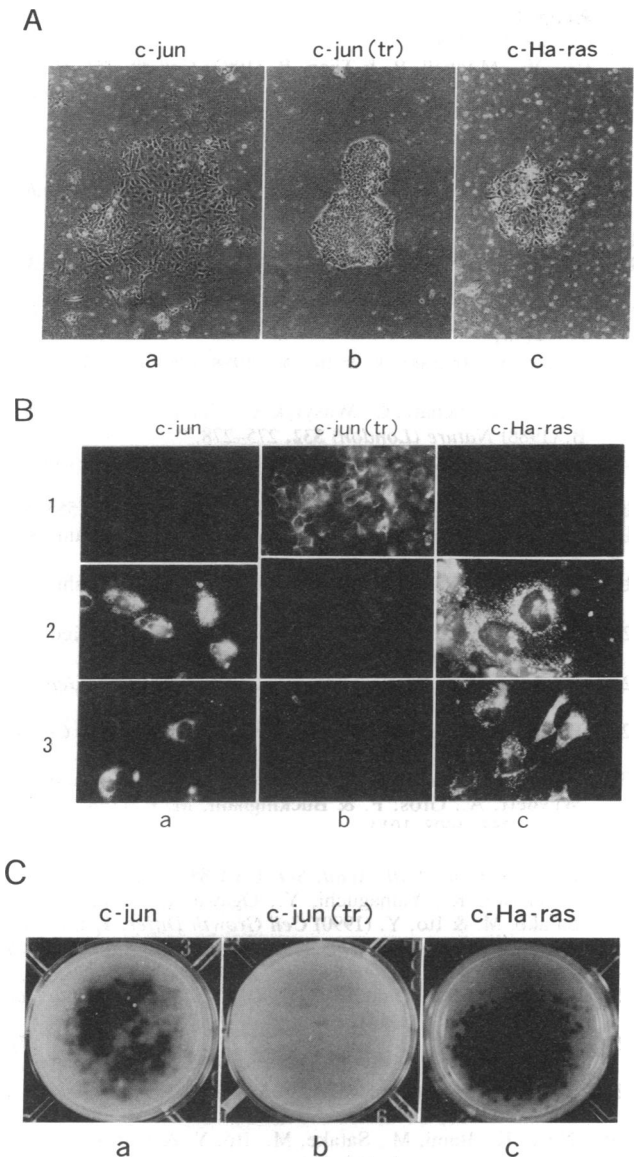

FIG. 4. Induction of F9 differentiation by c-*jun* or Ha-*ras*(Val-12). (*A*) Phase-contrast photomicrographs of G418-resistant F9 cells after transfection with c-*jun* and pSV2neo (*a*), with mutated c-*jun* and pSV2neo (*b*), or with Ha-*ras*(Val-12) and pSV2neo (*c*). (*B*) Immunofluorescent staining of stem-cell and differentiation-specific markers in F9 cells after transfection with c-*jun* (*a*), mutated c-*jun* (*b*), or Ha-*ras*(Val-12) (*c*). Antigens stained were SSEA-1 (*1*), laminin (*2*), or Hsp47 (*3*), respectively. (*C*) Plasminogen activator in F9 cells transfected with c-*jun* (*a*), mutated c-*jun* (*b*), or Ha-*ras*(Val-12) (*c*).

inal F9 cell population, since the increase in the number of the cells secreting plasminogen activator after transfection of these genes (Fig. 4*c*, compare the clear areas in *a* and *c* with that in *b*) could not have resulted from cell division within 48 hr. As judged from the known properties of the stem-cell and differentiation markers tested above, F9 cells induced to differentiate by both Ha-*ras*(Val-12) and c-*jun* resembled the cells between the primitive and parietal endoderm stages of early mouse development.

DISCUSSION

Two major conclusions can be drawn from this study. (*i*) The activated c-Ha-*ras* [Ha-*ras*(Val-12)] gene can markedly increase the steady-state level of c-*jun* transcripts and induce the AP1-site DNA-binding activity in F9 cells. (*ii*) Ha-

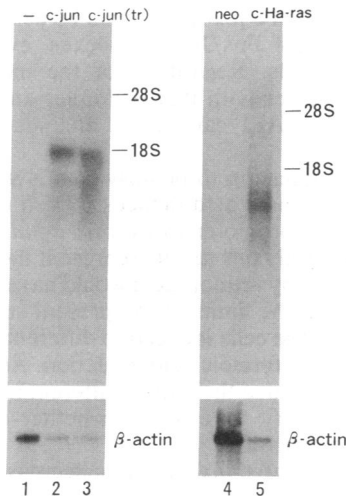

FIG. 5. Northern blot analysis of RNA obtained from F9 cells transfected by c-*jun* or Ha-*ras*(Val-12). RNA was extracted from nontransfected F9 cells (lane 1) or F9 cells transfected with c-*jun* (lane 2), mutated c-*jun* (lane 3), pSV2neo (lane 4), or Ha-*ras*(Val-12) (lane 5). Poly(A)$^+$ RNA (10 μg, lanes 1 and 4; 5 μg, lanes 2, 3, and 5), recovered by passing 100 μg of total RNA through an oligo(dT)-cellulose column, was electrophoresed in an agarose gel. The blotted filter was hybridized with a ^{32}P-labeled c-*jun* probe (lanes 1–3) or with an Ha-*ras*(Val-12) probe (lanes 4 and 5) (*Upper*) and rehybridized with a mouse β-actin probe (*Lower*). Positions of 18S and 28S rRNA are shown as size markers.

ras(Val-12) as well as c-*jun* can induce the differentiation of F9 cells.

Transcriptional activation of the endogenous c-*fos* gene by transiently expressed Ha-*ras* has been reported (29). This induction was transient and persisted for only several hours. In our case, c-*fos* transcripts were undetectable in F9 cells 20 hr after transfection with Ha-*ras*(Val-12). However, we observed a significant increase in c-*jun* transcripts by introducing Ha-*ras*(Val-12). Similar results have been reported for mouse osteocytes stably transformed by Ki- or Ha-*ras*: high levels of c-*jun* RNA were detected, whereas very low levels of c-*fos* transcripts were present in these cells (30). The induction of the AP1-site DNA-binding activity by Ha-*ras*(Val-12) observed in this study may, therefore, have been largely due to the increase in c-*jun* transcript and hence its product, Jun. However, an alternative explanation, that a small amount of Fos protein induced might be sufficient to form Jun/Fos complexes for the activation of the AP1 site, cannot be excluded.

When F9 cells were transfected with c-*fos*, only a small fraction of the cells expressing c-*fos* underwent morphological differentiation (12). In contrast, almost all of the F9 cells that received c-*jun* [or Ha-*ras*(Val-12)] manifested a differentiated phenotype. One explanation of the contrast between the effects of c-*jun* and c-*fos* is that a homodimer of Jun, which is expected to be formed in c-*jun*-transfected cells, is sufficient to exceed the threshold level of the AP1 DNA-binding activity required to trigger differentiation, although the Jun/Jun homodimer has much weaker DNA-binding activity than the Jun/Fos heterodimer (31). It was confirmed, by using the plasmid containing the CAT reporter gene under the control of the AP1 site, that cotransfection of this plasmid into F9 cells with c-*fos*, c-*jun*, or the combination of the two genes increased the CAT activity in this order (data not shown). Fos does not form a stable homodimer (31). In F9 cells transfected with c-*fos*, the availability of limited amounts of endogenous Jun to form Jun/Fos complexes might have been different in individual cells, thus inducing the differentiation in only a limited number of cells. Lack of

laminin expression in c-*fos*-transfected F9 cells (11) might be due to low levels of Fos/Jun complexes, even if they are formed, in the cells. Regardless of the mechanism, our observation, together with that of Müller and Wagner (11) demonstrates that AP1 can act as an inducer of F9 cell differentiation.

An important question to be answered is whether AP1 is involved when retinoic acid induces F9 cell differentiation. We used a strong promoter to express c-*jun* in the present study. How faithfully our results represent the physiological processes induced by retinoic acid would have to be carefully evaluated. Within the limits of the present study, however, the phenotype of the cells induced to differentiate by c-*jun* is similar to that after retinoic acid induction. Adenovirus E1A protein can also induce the partial differentiation of F9 cells (32). It is necessary to examine whether the E1A gene induces c-*jun* expression.

The expression of c-*myc* rapidly decreases as F9 cells differentiate. Since antisense c-*myc* RNA induces F9 differentiation, c-*myc* appears to have an active role in maintaining F9 cells in an undifferentiated state (33). It is interesting that the silencer found in the regulatory region of human c-*myc* harbors the AP1 binding site to which a factor containing Fos binds (34). The role of AP1 might be to inhibit the expression of c-*myc* in this case.

Neuronal differentiation of a pheochromocytoma cell line, PC12, can be induced by nerve growth factor (35) or by introducing activated Ha-*ras* into the cells (36, 37). There are some apparent similarities in gene regulation between F9 and PC12 cells during differentiation. Treatment of PC12 cells with nerve growth factor transiently induces the expression of both c-*fos* and c-*jun* (38). The induction of PC12 differentiation by Ha-*ras*(Val-12) also appears to be mediated by AP1, since the transcriptional activating function of AP1 can be induced by Ha-*ras* in the cells (39). In this case, the transcription of c-*fos* is augmented by Ha-*ras*(Val-12) and the activation occurs through the serum-responsive element in the c-*fos* regulatory region (39). When both F9 and PC12 differentiation are taken into consideration, it appears that AP1 is involved in triggering cell differentiation at multiple levels of the developmental hierarchy.

It must be noted, however, that AP1 may antagonize the process of cell differentiation in a different situation. Introduction of c-*fos* or activated *ras* has been reported to suppress the MyoD1-induced differentiation of C2 fibroblasts into myoblasts (40).

AP1 has been shown to play an essential role in growth regulation and oncogenesis (2, 3). The present study and those from other laboratories suggest that it has an important role in developmental regulation as well.

We thank Drs. K. Nagata and R. Kannagi (Kyoto University) for gifts of anti-Hsp47 and anti-SSEA-1 antibodies, respectively. We thank Dr. Y. Ogiso (Medical College of Kyoto Prefecture) for advice on plasminogen activator assay. This work was supported in part by a Grant-in-Aid for Cancer Research (subject no. 63010036) and by a Grant-in-Aid for Scientific Research (subject no. 63440027) from the Ministry of Education, Science, and Culture, Japan.

1. Maki, Y., Bos, T. J., Davis, C., Starbuck, M. & Vogt, P. K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2848–2852.
2. Vogt, P. K. & Tjian, R. (1988) *Oncogene* **3**, 3–7.
3. Curran, T. & Franza, B. R., Jr. (1988) *Cell* **55**, 395–397.
4. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. & Karin, M. (1987) *Cell* **49**, 729–739.
5. Lee, W., Mitchell, P. & Tjian, R. (1987) *Cell* **49**, 741–752.
6. Murakami, Y., Asano, M., Satake, M. & Ito, Y. (1990) *Oncogene* **5**, 5–13.
7. Martin, G. R. (1980) *Science* **209**, 768–776.
8. Strickland, S. & Mahdavi, V. (1978) *Cell* **15**, 393–403.
9. Kryszke, M.-H., Piette, J. & Yaniv, M. (1987) *Nature (London)* **328**, 254–256.
10. Chiu, R., Angel, P. & Karin, M. (1989) *Cell* **59**, 979–986.
11. Müller, R. & Wagner, E. F. (1984) *Nature (London)* **311**, 438–442.
12. Rüther, U., Wagner, E. F. & Müller, R. (1985) *EMBO J.* **4**, 1775–1781.
13. Satake, M., Ibaraki, T. & Ito, Y. (1988) *Oncogene* **3**, 69–78.
14. Piette, J. & Yaniv, M. (1987) *EMBO J.* **6**, 1331–1337.
15. Imler, J. L., Schatz, C., Wasylyk, C., Chatton, B. & Wasylyk, B. (1988) *Nature (London)* **332**, 275–278.
16. Yamaguchi, Y., Satake, M. & Ito, Y. (1989) *J. Virol.* **63**, 1040–1048.
17. Imbra, R. J. & Karin, M. (1986) *Nature (London)* **323**, 555–558.
18. Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. (1982) *Nature (London)* **298**, 343–347.
19. Sakai, M., Okuda, A., Hatayama, I., Sato, K., Nishi, S. & Muramatsu, M. (1989) *Cancer Res.* **49**, 5633–5637.
20. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y. & Kedes, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4831–4835.
21. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327–341.
22. Reichel, R., Kovesdi, I. & Nevins, J. R. (1987) *Cell* **48**, 501–506.
23. Minty, A. J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. & Buckingham, M. E. (1981) *J. Biol. Chem.* **256**, 1008–1014.
24. Wasylyk, B., Imler, J. L., Chatton, B., Schatz, C. & Wasylyk, C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7952–7956.
25. Furukawa, K., Yamaguchi, Y., Ogawa, E., Shigesada, K., Satake, M. & Ito, Y. (1990) *Cell Growth Differ.* **1**, 135–147.
26. Solter, D. & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5565–5569.
27. Kahan, B. & Adamson, E. D. (1983) *Cold Spring Harbor Conf. Cell Prolif.* **10**, 131–141.
28. Saga, S., Nagata, K., Chen, W.-T. & Yamada, K. M. (1987) *J. Cell Biol.* **105**, 517–527.
29. Schönthal, A., Herrlich, P., Rahmsdorf, H. J. & Ponta, H. (1988) *Cell* **54**, 325–334.
30. Nose, K., Itami, M., Satake, M., Ito, Y. & Kuroki, T. (1989) *Mol. Carcinogen.* **2**, 208–216.
31. Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E. & Leder, P. (1988) *Cell* **55**, 917–924.
32. Montano, X. & Lane, D. P. (1987) *Mol. Cell. Biol.* **7**, 1782–1790.
33. Griep, A. E. & Westphal, H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6806–6810.
34. Hay, N., Takimoto, M. & Bishop, J. M. (1989) *Genes Dev.* **3**, 293–303.
35. Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.
36. Noda, M., Ko, M., Ogura, A., Liu, D.-G., Amano, T., Takano, T. & Ikawa, Y. (1985) *Nature (London)* **318**, 73–75.
37. Bar-Sagi, D. & Feramisco, J. R. (1985) *Cell* **42**, 841–848.
38. Wu, B.-Y., Fodor, E. J. B., Edwards, R. H. & Rutter, W. J. (1989) *J. Biol. Chem.* **264**, 9000–9003.
39. Sassone-Corsi, P., Der, C. J. & Verma, I. M. (1989) *Mol. Cell. Biol.* **9**, 3174–3183.
40. Lassar, A. B., Thayer, M. J., Overell, R. W. & Weintraub, H. (1989) *Cell* **58**, 659–667.
41. Asano, M., Murakami, Y., Furukawa, K., Yamaguchi-Iwai, Y., Satake, M. & Ito, Y. (1990) *J. Virol.*, in press.