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Increased Expression of c-*jun*, But Not Retinoic Acid Receptor β, Is Associated With F9 Teratocarcinoma Stem Cell Differentiation Induced by Polyamine Depletion

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Abstract α-Difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase, and all-*trans*-retinoic acid (RA) are known to induce F9 teratocarcinoma stem cell differentiation. Both compounds induce the formation of the same cell type, i.e., parietal endoderm-like cells expressing tissue plasminogen activator and collagen type IV α-1. The present study shows that DFMO and RA induce terminal differentiation of F9 cells through different pathways. Thus, retinoic acid receptor (RAR) α mRNA is weakly expressed during DFMO treatment, but strongly induced during an early phase of RA treatment. RAR β mRNA is not detectable in DFMO-treated cells, but very strongly induced by RA and maintained at a high level throughout the differentiative process. RAR γ mRNA is relatively strongly expressed in untreated control cells and remains at approximately the same level during DFMO-induced differentiation. In RA-treated cells, however, RAR γ mRNA is rapidly down-regulated and becomes nondetectable during the final course of differentiation. These experiments show that the differentiation of F9 cells into parietal endoderm-like cells does not necessarily involve changes in any of the RAR mRNA subtypes. Even though the steady-state levels of the RAR α and RAR γ transcripts may be sufficient to support the differentiative process, our data clearly show that induction of RAR β mRNA transcription is neither a prerequisite for F9 cell differentiation, nor an absolute consequence of the elevated c-*jun* mRNA expression that is consistently observed during the course of parietal endoderm differentiation. J. Cell. Biochem. 67:378–385, 1997. © 1997 Wiley-Liss, Inc.

Key words: α-difluoromethylornithine; ornithine decarboxylase; parietal endoderm; retinoic acid receptor α; retinoic acid receptor γ; tissue plasminogen activator

F9 teratocarcinoma stem cells resemble the cells of the inner cell mass of the mouse preimplantation embryo [Hogan et al., 1983]. They exhibit a very low rate of spontaneous differentiation, but can be induced to differentiate by treatment with all-*trans*-retinoic acid (RA) [Strickland and Mahdavi, 1978], one of the most potent biologically active forms of vitamin A, or by α-difluoromethylornithine (DFMO) [Heby et al., 1983; Oredsson et al., 1985], an enzyme-activated irreversible inhibitor of ornithine decarboxylase [Metcalf et al., 1978]. Both inducers cause similar changes in the expression of several genes, and the F9 cells gradually differentiate into parietal endoderm-like cells, which exhibit a morphology that differs entirely from that of undifferentiated teratocarcinoma stem cells [Oredsson et al., 1985; Rickles et al., 1988]. The terminally differentiated parietal endoderm cells correspond to the extraembryonic endoderm of the parietal yolk sac surrounding the early mouse embryo [Hogan et al., 1983]. It should also be emphasized that the differentiation of F9 cells in response to RA or DFMO is accompanied by loss of tumorigenicity and by loss of the transformed phenotype.

RA-induced differentiation of F9 cells is believed to be primarily mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are members of the nuclear receptor superfamily [Chambon, 1994; Gudas et

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al., 1994]. There is evidence to suggest that each RAR and RXR species may regulate the expression of particular subsets of target genes in specific cell types, thereby mediating different biological activities [Leid et al., 1992; Boylan et al., 1993, 1995; Chambon, 1994; Taneja et al., 1995]. Nevertheless, there seems to be at least a partial functional redundancy between the three receptor subtypes [Taneja et al., 1995].

The mechanism by which DFMO induces teratocarcinoma stem cell differentiation is not fully understood. One possibility is that the depletion of the two growth-promoting polyamines, putrescine and spermidine, acts as a differentiation signal [Heby et al., 1983; Schindler et al., 1983, 1985; Jetten and Shirley, 1985; Oredsson et al., 1985]. Another possibility is that the dramatic accumulation of decarboxylated AdoMet, which is a consequence of the depletion of putrescine and spermidine, induces differentiation by interfering with DNA methylation [Heby et al., 1988; Heby, 1995; Frostesjö et al., 1996].

In view of the large number of studies demonstrating consistent patterns of changes in RAR mRNA expression during the course of RA-induced F9 cell differentiation, it may be tempting to conclude that these changes are obligatory events in the differentiative process. The purpose of the present study was therefore to determine whether F9 cells go through the same changes in RAR mRNA expression if the differentiation to parietal endoderm is induced by DFMO, instead of RA.

Contrary to the major changes in RAR mRNA expression induced by RA (which are largely in agreement with those reported by others [Zelent et al., 1989; Hu and Gudas, 1990]), the RAR α and RAR γ mRNA levels proved not to change significantly during DFMO treatment, and the RAR β mRNA level remained nondetectable. The latter finding clearly shows that RAR β induction is not an obligatory step in teratocarcinoma cell differentiation, which is contradictory to the conclusion drawn in another study [de Groot et al., 1990]. In the same study [de Groot et al., 1990] it was also suggested that the up-regulation of RAR β in P19 mouse teratocarcinoma cells is mediated by c-Jun, a proto-oncogene product, which, together with c-Fos, forms a heterodimer that binds to AP-1 sites, thus stimulating transcription of the associated gene [Rauscher et al., 1988]. At variance, we find that even though c-*jun* mRNA expression is more strongly up-regulated by DFMO than by RA, there is no increase in RAR β mRNA expression during the course of DFMO-mediated F9 cell differentiation.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The F9 teratocarcinoma stem cell line was initially isolated from embryoid bodies of the transplantable tumor OTT 6050-970 [Bernstine et al., 1973], a pluripotent teratocarcinoma, which originated from the grafting of a 6-day male mouse embryo to the testis of a strain 129 mouse [Stevens, 1970]. The F9 cells were grown in the absence of antibiotics in Dulbecco's Modified Eagle Medium (containing 4.0 mM L-glutamine, 5.6 mM D-glucose, 1.0 mM sodium pyruvate, and 25 mM HEPES) supplemented with 20% fetal calf serum. The cells were plated in gelatinized Costar flasks at a density of 4,000 cells/cm^2 (2.0×10^4 cells/ml of growth medium) and incubated at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 75–80%. In order to retain the undifferentiated phenotype characteristic of teratocarcinoma stem cells, the F9 cells were thoroughly dissociated (in a solution of 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.2), and subcultured every second day, i.e., while in exponential growth. Under these conditions there was no detectable spontaneous differentiation, i.e., no change in cell morphology and no induction of tissue plasminogen activator (tPA) gene transcripts.

Induction of Terminal Differentiation

F9 cells were induced to differentiate by supplementing the growth medium with 5 mM DFMO or 1 μM RA. RA was always freshly prepared as a 1 mM stock solution in absolute ethanol. The final concentration of ethanol in the medium was $<0.1\%$ and did not affect growth or differentiation of the cells. In all procedures involving RA, exposure to light was avoided. The growth medium was changed every second day to a fresh medium containing the same concentration of DFMO or RA.

RNA Isolation

Total RNA was isolated from F9 cells according to the method of Chomczynski and Sacchi [1987] as modified by Vauti and Siess [1993]. After each experiment, the adherent F9 cells

were briefly rinsed with a 37°C solution containing 0.02% EDTA in phosphate-buffered saline (pH 7.2). The cells were then removed and dispersed by trypsinization. The trypsin was inactivated by adding growth medium to the cell suspension. After centrifugation at room temperature, the cell pellet was resuspended in ice-cold phosphate-buffered saline (pH 7.2) and an aliquot was removed for cell counting. The remaining cells were diluted and then sedimented by centrifugation and lysed in 500 μl of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.72% (0.1 M) freshly added β-mercaptoethanol, and 0.1% anti-foam A, in order to denature the proteins (ribonuclease especially). Thus, the cell suspension was vortexed and forced 10 times through a 0.6 × 25 mm hypodermic needle. The resulting cell lysate was supplemented with 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of phenol (water-saturated), and 0.2 volumes of chloroform:isoamylalcohol (24:1), vortexed and kept on ice for 15 min. The aqueous phase was then subjected to chloroform extraction. After centrifugation (10,000*g* for 20 min at 4°C), the RNA was precipitated from the aqueous phase by adding 1 volume of ice-cold isopropanol. The mixture was kept at −20°C for at least 1 h. After centrifugation (10,000*g* for 20 min), the precipitates were dissolved in RNAse-free water, and precipitated by addition of 0.1 volume of 8 M LiCl and 1 volume of isopropanol (−20°C for 1 h) and subsequent centrifugation (10,000*g* for 10 min). The RNA was washed in 70% ethanol and finally dissolved in RNAse-free water and stored at −70°C.

Northern Blot Analysis

From each sample an aliquot containing 25 μg of RNA was mixed with 2 volumes of sample buffer (75% formamide, 7.8% formaldehyde, 15 mM sodium phosphate, pH 6.5) and 0.4 volumes of loading buffer (45% glycerol, 0.9 mM EDTA, 1 mg/ml ethidium bromide, 0.25% xylene cyanole FF [Sigma, St. Louis, MO] and 0.25% bromophenol blue [Sigma]), and then incubated at 55°C for 15 min. After denaturation, the RNA was fractionated on a 1% agarose gel (containing 1.9% formaldehyde and 25 mM sodium phosphate buffer, pH 6.5) using 10 mM sodium phosphate as running buffer, and transferred by vacuum blotting to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL), using 0.1 M sodium phosphate buffer (pH 6.5). A UV Stratalinker 1800 (Stratagene, La Jolla, CA) was used to cross-link the RNA to the membrane.

The membrane was prehybridized for 1–3 h at 50°C in prehybridization solution (6 × SSC, 0.5% SDS, 5 × Denhardt's solution, 100 μg/ml of salmon sperm DNA [sheared by sonication and denatured by boiling]). Hybridization was performed at 50°C for 12–18 h in hybridization solution (6 × SSC, 0.5% SDS, 100 μg/ml of salmon sperm DNA). The membrane was washed at a final stringency of 0.2 × SSC (0.1% SDS) at 55°C. Autoradiography was performed at −70°C with the use of intensifying screens (Kodak, Rochester, NY).

DNA Probes

DNA probes were made from the following cDNAs: a 2.1-kb mouse RAR α *Eco*RI fragment, a 1.95-kb mouse RAR β *Bam*HI-*Bst*ZI fragment, a 2.0-kb mouse RAR γ *Eco*RI fragment [Zelent et al., 1989], a 0.94-kb mouse c-*jun* *Eco*RI-*Bgl*I fragment [Ryder and Nathans, 1988] (American Type Culture Collection, Rockville, MD), a 0.4-kb rat tPA (cDNA clone 15) *Eco*RI fragment [Ny et al., 1988], and a 0.55-kb human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *Hind*III-*Xba*I fragment (American Type Culture Collection) [Tso et al., 1985]. The probes were labelled with [α-^{32}P]dCTP according to Feinberg and Vogelstein [1983], using the Megaprime DNA labelling kit (Amersham) with nonamer oligonucleotide primers.

Chemicals

DL-α-Difluoromethylornithine monohydrochloride monohydrate (DFMO) was generously donated by Marion Merrell Dow Research Institute, Cincinnati, OH. All-*trans*-retinoic acid (RA) was purchased from Sigma Chemical Co.

RESULTS

When F9 teratocarcinoma stem cells were treated with either DFMO or RA, their proliferation rate declined (Fig. 1). At the concentrations used (5 mM for DFMO and 1 μM for RA), DFMO exerted a stronger antiproliferative effect, which was apparent from the second day on. The cell morphology changed progressively toward a more differentiated phenotype. For both DFMO and RA, the terminal differentiation product was a parietal endoderm-like cell.

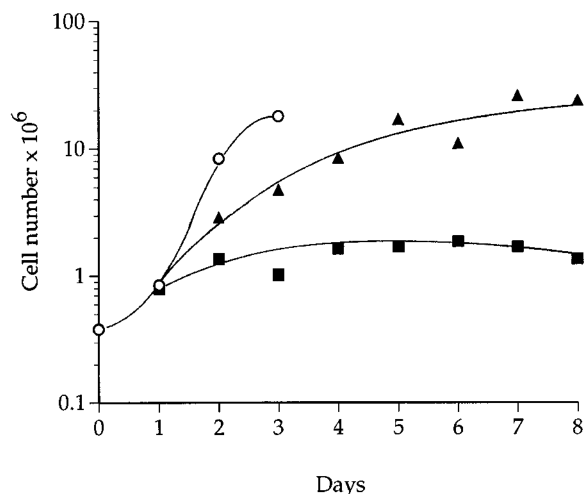

Fig. 1. Effects of DFMO and RA on F9 teratocarcinoma stem cell growth. ○, untreated control cells; ■, cells treated with 5 mM DFMO from day 0 on; ▲, cells treated with 1 μM RA from day 0 on.

The differentiated phenotype was characterized by morphologic criteria [Oredsson et al., 1985] and by expression of tPA mRNA (Fig. 2) and collagen type IV α-1 mRNA [Frostesjö et al., 1997], as determined by Northern blot analysis. The induction of tPA mRNA expression occurred approximately 1 day earlier and was initially somewhat stronger in RA-treated as compared to DFMO-treated cells (Fig. 2). This should not be taken to denote that RA is a more efficient inducer of terminal cell differentiation than is DFMO. In fact, RA treatment always seems to permit a small fraction of cells to continue or resume their proliferation, whereas DFMO treatment causes a more consistent effect with all cells eventually being terminally differentiated, and showing no proliferative capacity.

RA treatment caused a rapid induction of RAR α mRNA expression (Fig. 2). The 3.7- and 2.8-kb species were both dramatically elevated from day 1 through 3. With the appearance of the morphologically differentiated phenotype, however, the amounts of these species returned to the levels characteristic of undifferentiated F9 cells. RAR β mRNA expression was also rapidly induced by RA treatment (Fig. 2). In fact, this 3.4-kb message was undetectable in untreated control cells, but appeared on the first day of RA treatment. The amount of RAR β mRNA increased even further on days 2 and 3, but then remained at a high and rather constant level throughout the differentiative process. At variance with its induction of RAR α and RAR β mRNA expression, RA treatment caused down-regulation of RAR γ mRNA expression (Fig. 2). Thus, the 3.1-kb RAR γ mRNA exhibited a relatively high level in untreated control cells, decreased during the first day of RA treatment, and disappeared below the level of detectability within 2 days.

When F9 cells were induced to differentiate by treatment with the ornithine decarboxylase inhibitor DFMO, the RAR mRNA expression patterns were remarkably different from those induced by RA treatment (Fig. 2). Firstly, there were no major changes in the levels of the RAR mRNAs during the entire course of DFMO treatment. Thus, the two RAR α mRNA transcripts remained at the levels characteristic of untreated control F9 cells (with the 3.7-kb transcript dominating), RAR β mRNA was nondetectable, and RAR γ mRNA remained at roughly the same level from the undifferentiated state until the terminally differentiated state, with the possible exception of a slight increase on days 2 to 3.

In undifferentiated F9 cells the c-*jun* mRNA level was below the level of detection, both in case of the 3.5- and the 2.9-kb species. The temporal pattern for the induction of c-*jun* mRNA expression was similar in RA-treated and in DFMO-treated cells, but the message levels induced by DFMO treatment by far exceeded those induced by RA treatment (Fig. 2). This was true for both mRNA species. Both c-*jun* mRNA species remained nondetectable during the first day of treatment with RA as well as DFMO. By day 2, the 2.9-kb c-*jun* mRNA species appeared in both RA-treated and DFMO-treated cells. With increasing amount of c-*jun* message on day 3, the larger (3.5 kb) species also became detectable. The amounts of the two c-*jun* mRNAs remained roughly constant at these levels during the continued course of RA treatment, but increased even further during DFMO treatment, exhibiting the highest levels in terminally differentiated cells. In RA-treated F9 cells, the accumulation of c-*jun* mRNA is caused by increased c-*jun* transcription [Yang-Yen et al., 1990]. This increase seems to be an indirect response to RA, and requires a functional AP-1 binding site within the c-*jun* promoter.

Equal loading of the gels was ascertained by ethidium bromide staining and visual inspection of the 18S and 28S rRNA band intensities

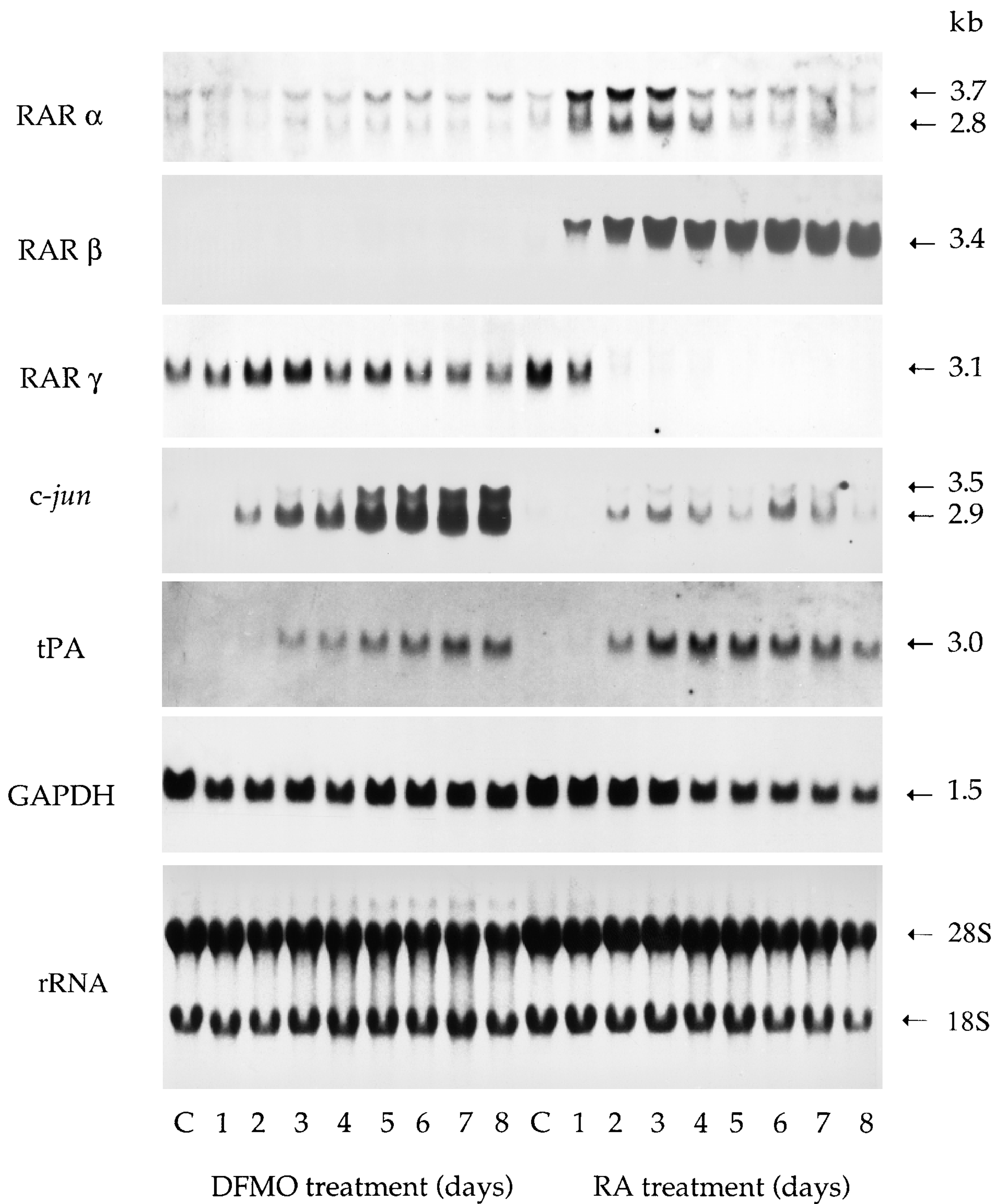

Fig. 2. Effects of DFMO- and RA-induced differentiation on the mRNA levels of RAR α, RAR β, RAR γ, c-*jun*, tPA, and GAPDH. F9 cells were cultured for 0 to 8 days in the presence of either 5 mM DFMO or 1 μM RA. Thirty micrograms total RNA of each sample was analyzed. The entire set of experiments was repeated 3 times with consistent results. All hybridizations shown have been made with the same RNA membrane, which makes all data directly comparable. Equal loading of the gel was ascertained by ethidium bromide staining of the 18S and 28S rRNA bands.

(Fig. 2). Hybridization with a GAPDH cDNA probe was also used with the intention to confirm an even loading of RNA (Fig. 2). However, the cellular GAPDH mRNA content was found to decrease during differentiation, particularly in RA-treated F9 cells. A similar observation has been made for PCC4.aza1R cells, another mouse teratocarcinoma cell line, during RA-induced differentiation [Nervi et al., 1990]. Thus, despite its frequent use as a marker of even RNA loading, the GAPDH mRNA content is clearly not suitable for this purpose in studies of teratocarcinoma cell differentiation.

DISCUSSION

When F9 cells were induced to differentiate by treatment with RA, characteristic RAR mRNA expression patterns evolved. These patterns are largely in agreement with those observed by others [Zelent et al., 1989; Hu and Gudas, 1990] and with those of other mouse teratocarcinoma stem cell lines [Nervi et al., 1990]. Thus, the RAR α mRNA levels increased temporarily, the RAR β mRNA level increased gradually, and the RAR γ mRNA level decreased gradually. The down-regulation of RAR γ mRNA expression was more extensive than that observed in other studies. In fact, the depletion may be comparable to that seen after disruption of RAR γ in F9 cells [Boylan et al., 1993, 1995; Taneja et al., 1995]. An interesting parallel is that RAR γ-null F9 cells exhibit aberrant differentiation when treated with RA, and that the strain of F9 cells used in our laboratory appears to have a lower differentiation potential in response to RA than many other F9 strains.

Treatment of F9 cells with RA has been found to strongly upregulate RAR β mRNA expression [Hu and Gudas, 1990]. This up-regulation has been proposed to be a necessary step in the differentiation of P19 teratocarcinoma cells [de Groot et al., 1990]. It is interesting to note that tumor cells usually exhibit an abnormally low level or even a loss of RAR β expression [Hu et al., 1991; Swisshelm et al., 1994; Xu et al., 1994]. Moreover, it has been demonstrated that transfection of a human epidermoid lung carcinoma cell line with an RAR β expression vector decreases the tumorigenicity of these cells in nude mice [Houle et al., 1993].

In view of the fact that the phenotype resulting from RA treatment closely resembled that induced by DFMO treatment, it was surprising to find that the RAR mRNA patterns deviated markedly. In fact, polyamine depletion induced a terminal differentiation process that seems not to depend on changes in RAR mRNA levels. Thus, the levels of RAR α mRNA and RAR γ mRNA remained constant throughout the course of DFMO-mediated F9 cell differentiation. RAR β mRNA was not detectable in undifferentiated F9 stem cells or in the progeny (primitive endoderm and parietal endoderm) resulting from DFMO treatment.

This finding is clearly inconsistent with the proposal that RAR β mRNA up-regulation is an obligatory step in teratocarcinoma cell differentiation as suggested in studies of P19 cells [de Groot et al., 1990]. However, the possibility remains that RAR γ and RAR β can replace each other, because RAR γ mRNA is present in DFMO-induced F9 cells (but not in RA-induced F9 cells) and RAR β is present in RA-induced F9 cells (but not in DFMO-induced F9 cells). However, overexpression of RAR β was found to only poorly restore differentiation, even though it could replace RAR γ for the activation of target genes [Taneja et al., 1995].

In this context it is interesting to note that mice lacking all isoforms of RAR β develop normally, are viable and fertile, and have no externally apparent abnormalities [Luo et al., 1995]. Null mutant mice for all RAR γ isoforms, however, exhibit growth deficiency, early lethality, and male sterility [Lohnes et al., 1993]. Likewise, targeted disruption of the whole RAR α gene resulted in early postnatal lethality and testis degeneration [Lufkin et al., 1993]. RAR double mutants develop multiple abnormalities at various stages of organogenesis [Lohnes et al., 1994; Mendelsohn et al., 1994].

Experiments with P19 teratocarcinoma cells have shown that ectopic expression of exogenous c-*jun* sequences leads to differentiation and loss of the transformed phenotype [de Groot et al., 1990]. Evidence was also presented in support of a direct stimulatory effect of the c-*jun* gene product on transcription of the RAR β gene. Obviously, the present data are not consistent with this view because even though c-*jun* is more strongly induced by DFMO than by RA, there was no rise in RAR β gene expression during DFMO-mediated differentiation. The RAR β mRNA level remained below the level of detection at all times analyzed.

The conclusion drawn by de Groot et al. [1990] and by Iwai et al. [1993], that c-*jun* gene expres-

sion is involved in the induction of P19 and F9 teratocarcinoma stem cell differentiation, is consistent with the present finding that c-*jun* is more strongly induced in DFMO-treated than in RA-treated F9 cells, because the DFMO-treated cells exhibit a higher degree of differentiation than do their RA-treated counterparts. On the other hand, the data of Auer et al. [1994] suggest that UV-induced c-*jun* gene activation does not suffice to induce the full program of F9 cell differentiation. It should be noted, however, that the UV-induced c-*jun* mRNA accumulation is a very early event and apparently of short duration (with a maximum already 1 h after treatment) [Auer et al., 1994], as compared to that induced by DFMO or RA (Fig. 2).

It may be concluded from the present experiments that the differentiation induced by polyamine depletion must follow a pathway that does not require any changes in the steady-state levels of the RAR α and RAR γ mRNA subtypes. However, we cannot exclude the possibility that the actual RAR α and RAR γ transcript levels are sufficient to support F9 cell differentiation. Our data also show that increased c-*jun* transcription does not necessarily lead to increased RAR β expression. Moreover, RAR β mRNA induction is clearly not a prerequisite for the differentiation of F9 teratocarcinoma stem cells into parietal endoderm cells, but c-Jun remains a prime candidate for essential steps in the differentiation process.

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