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Expression of retinoic acid nuclear receptors in the mouse embryonal carcinoma cell line PCC7-Mz1

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Mouse embryonal carcinoma cell line PCC7-Mz1 can serve as a model of mammalian neural development [1989, J. Cell. Biol. 109, 2481–2493]. Upon exposure to all-*trans* retinoic acid (RA), Mz1 cells differentiate into a stable pattern of neurons, astroglia and fibroblasts whereas variants of the parental cell line either are restricted in their patterns of derivatives or do not respond at all to RA. Using gene probes specific for the α_1, α_2 and β_2 isoforms of the retinoic acid nuclear receptor, we have studied by Northern blot analysis the expression of these transcription factors in uninduced and induced cells of clone Mz1 and in variants with different developmental potential. α_1-RAR is expressed constitutively in all variants independent of whether RA is present or not. Soon after addition of 10^{-7} M RA, α_2-RAR is induced in RA-responsive cells reaching within a few hours a plateau level that remains unchanged throughout the developmental process. In contrast, the β_2 isoform is expressed only transiently after RA-induction despite the continuous presence of RA. Other RAR isoforms are expressed only in trace amounts

Retinoic acid receptor; Isoforms; Expression; dbcAMP; Embryonic carcinoma cell line PCC7-Mz

1. INTRODUCTION

All-*trans* retinoic Acid (RA) has been shown to be involved in morphogenesis and teratogenesis of vertebrates. When applied experimentally, RA may alter the specification of body axes [1] and may lead to malformation of, among others, cranial [2] and limb structures [3]. Similarly, duplications of certain organ structures including digits [4], neural tube and notochord [5] have been observed under these conditions. Recently, even homoeotic transformations of whole body segments upon RA treatment have been reported [6]. These observations all suggest a close involvement of retinoic acid receptors in gene regulatory decisions in the course of cellular determination and differentiation.

Teratocarcinoma cell lines are useful model systems to study the gene regulatory effects of RA [7]. One cell line particularly advantageous in this respect, is clone 1009 of PCC7-S-AzaR$_1$ [8] (denoted here as PCC7-Mz1 [9]) which, after exposure to RA, differentiates into a stable pattern of neural phenotypes, i.e. neurons, glial cells and fibroblasts [9]. Moreover, the derivatives are generated with a time course resembling that of natural development of the embryonic mouse brain, with neurons appearing first, followed a few days later by phenotypic astroglia and fibroblast cells. By single cell subcloning, we also have isolated several variants of this cell line (MzNn, MzOn, MzPn) that differ in the patterns of derivatives produced after treatment with RA [9]. Thus, the MzN variants differentiate exclusively into neurons when cultured under standard conditions, whereas the MzP variants behave similar to the parental cell line in that they form the full pattern of neural derivatives. Another type of variants termed MzRn is resistent to RA-induced differentiation (Lang, E. et al., in preparation).

RA-dependent gene regulation is mediated by retinoic acid nuclear receptors (RAR), belonging to the superfamily of steroid/thyroid hormone receptors and functioning like these as transcription factors. Three major subtypes of RARs (α-RAR, β-RAR, γ-RAR) have been identified by gene cloning in mouse, newt and man [10–15], with their gene and protein sequences being highly conserved among these species. Each major subtype of RAR can exist in several isoforms generated by differential splicing at their 5′-ends and/or differential promoter usage [16–18]. As was shown for other members of this superfamily of nuclear receptors, the corresponding N-terminal protein domain is participating in the activation of transcription of target genes, probably by interaction with other transcription factors [19–21].

The family of clonal variants of cell line PCC7-Mz1 may help to clarify the functions of specific RAR isoforms within a developmental process, such as neural differentiation of a stem cell-like cell population [9]. We therefore have cloned full-length cDNAs coding for the α_1 and β_2 isoforms, and partial length cDNA coding for

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the α_2, β_1 and β_3 isoforms of RAR. The cloned cDNAs did not differ in sequence from those of normal mouse cells, nor did we observe any gross differences in their promotor regions [22]. We therefore have begun to investigate whether the clonal variants differ with regard to the levels or time courses of transcription of RAR isoforms.

2. MATERIALS AND METHODS

All enzymes were purchased from Boehringer, Biolabs or Pharmacia and were used according to the manufactures instructions. All other chemicals were from Merck, Baker, Sigma or Serva. Cetryltrimethylammonium bromide was obtained from EGA.

2.1. *Cell culture*

PCC7-Mz1 cells and variants were grown as monolayers in 225 cm^2 flasks in DMEM-medium containing 15% fetal calf serum in an humidified athmosphere of 10% CO_2. For induction kinetics, 3 flasks per time point were inoculated with $3–4 \times 10^4$ cells/cm^2. After 2 days of incubation, i.e. when the cells had reached a density of approximately $1–1.5 \times 10^5$ cells/cm^2, 10^{-7} M RA in 10% DMSO and, if desired, 1 mM dbcAMP in water was added. Control cells were treated with the corresponding amount of 10% DMSO, without RA, for 5 h. After the appropriate incubation time, the medium was discarded and the cells were washed once with PBS to remove non-adherent cells. The cells were detached from the surface by incubation with shaking for 5 min in ice cold PBS containing 1 mM EDTA, collected by centrifugation at $1,500 \times g$ for 3–4 min and washed once with ice-cold PBS without EDTA. The resulting cell pellet was frozen in liquid nitrogen and stored at −80°C. For the 48-h time point, i.e. when the cells adhere much less firmly to the plastic surface, the procedure for the collection of cells was slightly modified. The first washing step was omitted, the medium was carefully removed, and the cells were directly treated with PBS/EDTA. To remove residual medium, these cells were washed twice with PBS by centrifugation.

2.2. *Isolation of RNA for Northern blot analysis*

Poly(A$^+$) RNA was enriched by batch absorption of 1 mg of total RNA to approximately 300 μl of packed bed volume of oligo dT-cellulose. After washing three times each with 1 ml of high and low salt buffer, bound RNA was eluted twice with 700 μl of elution buffer at 65°C, precipitated with ethanol and, after determination of the concentration by UV absorption, was dissolved in formamide loading buffer. Alternatively, the CsCl cushion centrifugation was omitted and batch absorption was directly performed from the guanidinium solution [23], diluted threefold with elution buffer.

2.3. *Northern blots and hybridizations*

3–6 μg poly(A)-enriched RNA were separated on 1.1% formaldehyde/agarose gels. After staining with ethidium bromide and removal of most of the formaldehyde, the RNA was transferred by diffusion in 10×SSC to a nylon membrane (Pall Biodyne B). The RNA was fixed by baking the membrane for 2 h at 80°C.

For hybridization, fragments were labeled by the random primed labeling procedure of Feinberg and Vogelstein [24] to a specific activity of approximately $5 \times 10^8–1 \times 10^9$ cpm/μg (Cerenkov counting).

Hybridizations were carried out in 6×SSC/0.1% SDS/5× Denhardt's containing 100 μg/ml single stranded salmon sperm DNA at 65°C. After prehybridization, the radioactive probe was added at a concentration of $5 \times 10^6–1 \times 10^7$ cpm/ml. To enhance the hybridization, in some experiments 1% PEG 6000 was included. After 16–20 h, the membrane was washed twice at room temperature in 2×SSC/0.1% SDS for 5 and 20 min, respectively. These washes were followed by two washes for 20 min at 65°C under the desired stringency (usually 1×SSC or 0.5×SSC containing 0.1% SDS). The blots were exposed to pre-flashed Fuji RX films at −70°C with an intensifying screen.

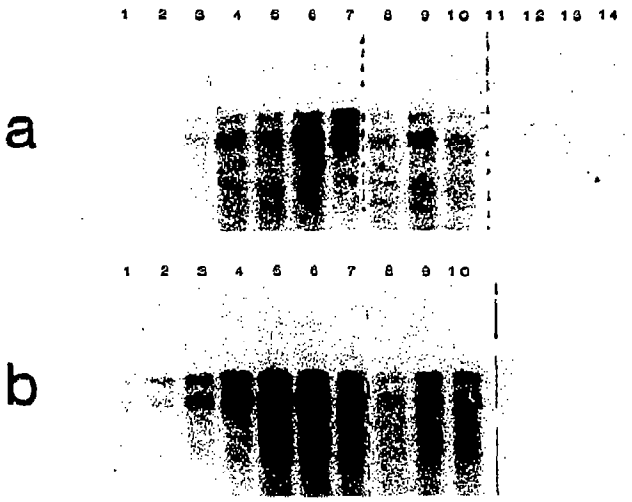

Fig. 1. Expression of α-RAR mRNA in MzN1, MzR3 and MzP1 cells. The cells were treated with RA either in the presence (lanes 1–7, 11–14) or in the absence of dbcAMP (lanes 8–10). Poly(A)$^+$ RNA was isolated and 5 μg were applied to each lane of a 1.2% agarose–formaldehyde gel. After transfer to nylon membrane, the blot was hybridized with a probe recognizing all α-RAR isoforms. (a) RNA from MzN1 cells treated for 0, 0.5, 1, 3, 5, 10 and 22 h with RA in the presence of dbcAMP (lanes 1–7), or for 5, 10 and 22 h in the absence of dbcAMP (lanes 8–10). RNA from MzR3 cells treated for 0, 5, 10 and 22 h in the presence of dbcAMP (lanes 11–14). (b) RNA from MzP1 cells treated for 0, 0.5, 1, 3, 5, 10 and 22 h with RA in the presence of dbcAMP (lanes 1–7), or for 5, 10 and 22 h in the absence of dbcAMP (lanes 8–10).

3. RESULTS

Previous studies by others [25,26] have suggested that structural alterations in RAR mRNA may be responsible for alterations in RA-responsiveness, including RA-resistance. We did not detect any differences in the gene structures of RAR isoforms cloned from Mz cell lines as compared to those from normal mouse cells [22]. This has prompted us to begin analysing the expression of RAR isoforms in the course of RA-induced differentiation.

3.1. *Expression of αRAR-isoforms*

Initially, we used in our studies of αRAR expression as hybridization probe a *Sma*I-fragment of the 3'-untranslated region of the α-RAR-cDNA that discriminated neither between αRAR isoforms nor between the type of alternatively spliced/polyadenylated transcripts that also exists in Mz cell lines [11,22]. With this probe we observed an induction of RARα-mRNA within 3–5 hours in MzP1 cells (Fig. 1b), and within 10–20 hours in MzN1 (Fig. 1a) and Mz1 cells. The plateau reached after this time period remained unchanged for at least the next 12–24 h, within the margin of experimental error (e.g. variations in the amount of RNA loaded per gel slot).

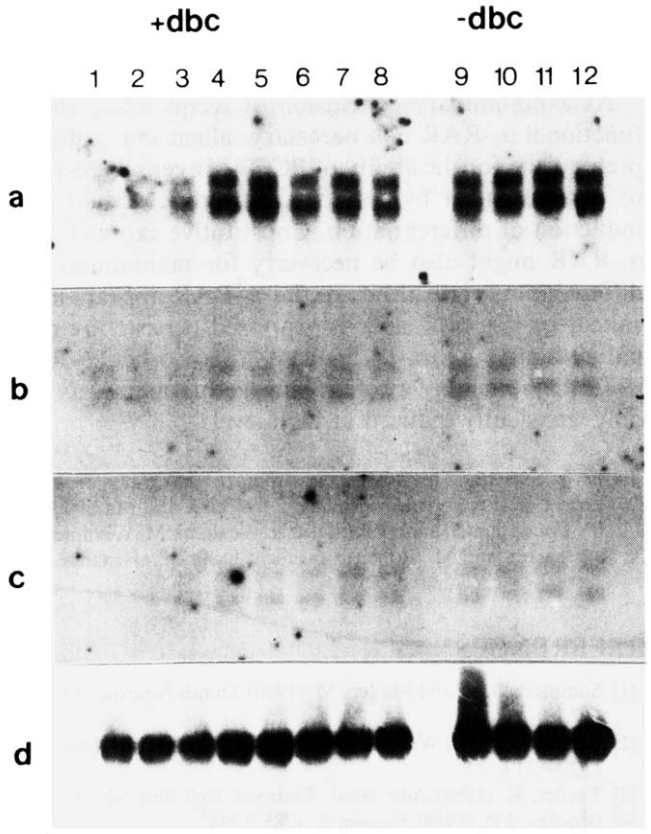

Fig. 2. Expression of α_1-RAR and α_2-RAR mRNA in MzN1 cells. The cells were treated with RA either in the presence of dbcAMP for 0, 0.5, 1, 3, 5, 12, 24 and 48 h (lanes 1–8) or in the absence of dbcAMP for 3, 5, 12 and 24 h (lanes 9–12). Poly(A)$^+$ RNA was isolated and 5 μg were applied to each lane of a 1.2% agarose–formaldehyde gel. After transfer to nylon membrane, the blot was hybridized with a probe recognizing (a) all α-RAR isoforms, (b) α_1-RAR only, (c) α_2-RAR only, (d) GAPDH, as control.

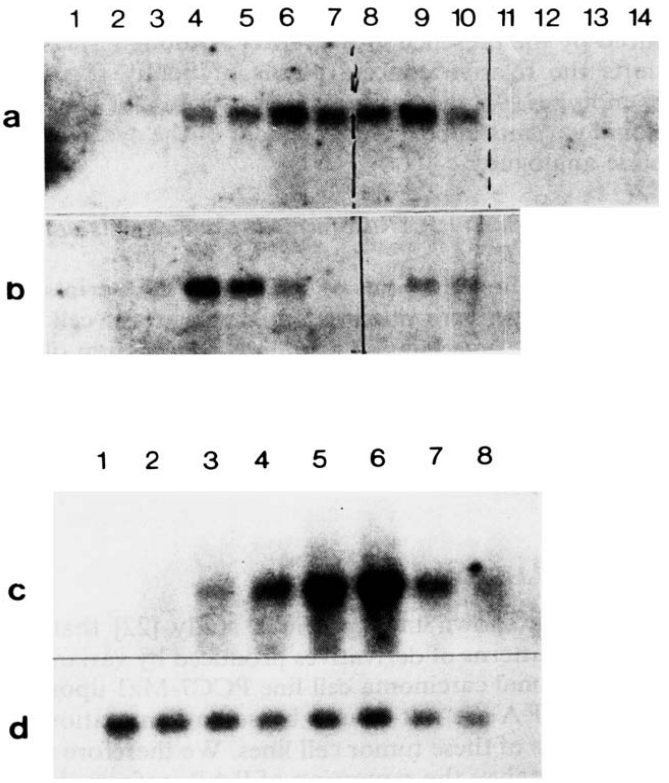

Fig. 3. Expression of β-RAR mRNA in MzN1 and MzR3, MzP1 and Mz1-cells. The cells were treated with RA either in the presence or in the absence of dbcAMP. Poly(A)$^+$ RNA was isolated and 5 μg were applied to each lane of a 1.2% agarose–formaldehyde gel. After transfer to nylon membrane, the blot was hybridized with a probe recognizing all β-RAR isoforms. (a) RNA from MzN1 cells treated for 0, 0.5, 1, 3, 5, 10 and 22 h with RA in the presence of dbcAMP (lanes 1–7), or for 5, 10 and 22 h in the absence of dbcAMP (lanes 8–10). RNA from MzR3 cells treated for 0, 5, 10 and 22 h with RA in the presence of dbcAMP (lanes 11–14). (b) RNA from MzP1 cells treated for 0, 0.5, 1, 3, 5, 10 and 22 h with RA in the presence of dbcAMP (lanes 1–7), or for 5, 10 and 22 h in the absence of dbcAMP (lanes 8–10). (c) RNA from Mz1 cells treated for 0, 0.5, 1, 3, 5, 10, 22 and 48 h with RA in the presence of dbcAMP (lanes 1–8). (d) Same experimental conditions as in (c), except that the blot was hybridized with a GAPDH probe as control.

In order to determine the α-RAR isoform that is induced in response to RA, the hybridization was repeated with isoform-specific probes from the 5′-ends of α_1-RAR and α_2-RAR. As shown in Fig. 2 for clone MzN1, in all clonal variants tested only the α_2-RAR gene is induced by exposure to RA while the α_1-RAR gene is constitutively expressed throughout the time period investigated (48 h).

3.2. *Expression of β_2-RAR*

Previous studies have reported for other embryonal carcinoma cell lines, embryonic stem cells and the chicken limb bud that their exposure to RA leads to a rapid transient increase of the amount of β_2-RAR-mRNA [27–29]. As shown in Fig. 3, the same applied to our family of cell lines. As early as 60 min after addition of 10^{-7} M RA to the cell culture medium, an increase in the level of β_2-RAR-mRNA was detected in the three cell lines tested. In MzP1 cells, the maximal level of induction was observed already after 3 h while in MzN1 and Mz1 cells the maximal level of β_2-RAR-mRNA was reached only after 10 h of exposure to RA (Fig. 3). The expression of the β_2-RAR gene then declined over the next 24 hours, approaching nearly basal levels after 48 h. At this stage in the developmental process, the neuronal phenotype has developed, as is demonstrated by the typical cell morphology and the expression of neurofilaments [9].

3.3. *Effect of dbcAMP on the induction of β_2-RAR mRNA*

The presence or absence of 1 mM dbcAMP in the cell culture medium has no effect on the induction of β_2-RAR mRNA (Figs. 2 and 3). Neither the kinetics nor

the level of induction in MzP1 and MzN1 cells is influenced by the presence of dbcAMP. Although dbcAMP shifts the relative concentrations of finally produced phenotypes [2], the early response to RA in all three clonal variants appears independent of the cyclic nucleotide analogue.

3.4. *Expression of RAR isoforms in the RA-resistant cell line MzR3*

None of the inductions of RAR gene transcripts described above were observed in the resistant cell line MzR3 in the time interval examined. Expression of the α_1-RAR (Fig. 1) remained constant at its basic level even after 22 h of exposure to RA, whereas the β_2-RAR was barely detectable (Fig. 3). Furthermore, the basal levels of α_1-RAR-mRNA in these three cell lines were similar.

4. CONCLUSIONS

We have shown in a previous study [22] that the different patterns of derivatives produced by variants of the embryonal carcinoma cell line PCC7-Mz1 upon exposure to RA are not caused by somatic mutations in RAR genes of these tumor cell lines. We therefore have begun to analyse the expression of RAR isoforms in the clonal variants including the RA-resistant clone MzR3.

The transcript coding for α_1-RAR was present in all cells, independent of whether RA was present or not, suggesting that this isoform presumably represents the primary target of RA action. The α_1-RAR is likely to regulate transcription of those RAR genes that are detected in substantial amounts only after RA induction, i.e. the α_2 and β_2 genes. Transcription of both is greatly enhanced within a short time after RA administration in all RA-responsive Mz clones, albeit at different levels and with different time courses. As the most prominent characteristics, α_2-mRNA reaches a maximal level within a few hours after exposure of the cells to RA, and maintains this level throughout the lifespan of the cells, while β_2-mRNA is only transiently produced. In contrast to previous reports on F9 embryonal carcinoma cells [27–29], dbcAMP does not affect the expression of RAR isoforms in PCC7-Mz cell lines.

It appears unlikely that the limited quantitative differences in RAR isoform expression in the clonal variants tested should account for the remarkable qualitative differences in their patterns of RA-induced derivatives. Similarly, it remains an open question how the unresponsiveness to RA is produced in the respective variants. In P19 cells, α and β RAR isoforms can transactivate the β_2-RAR gene, with the RA-resistance of variant RAC65 caused by an altered α-RAR transcript present in these cells [25,26]. Further insights may be gained by in situ hybridization on the single cell level and by PCR analysis of the expression of RAR genes the levels of which are too low to be monitored by Northern blot analysis. Preliminary results suggest that also β_1-RAR and β_3-RAR mRNA is present in Mz variants, albeit at very low levels.

In preliminary conclusion, it seems likely that a functional α_1-RAR is a necessary, although not sufficient, prerequisite for the ability of PCC7-Mz cells to respond to RA treatment by cessation of mitotic activity and induction of differentiation. Constitutive expression of α_1-RAR might also be necessary for maintaining the differentiated state although the α_2-RAR appears more suited for this task, as it is expressed persistently once induced. In contrast, β_2-RAR (and other β-RAR isoforms] may play a role in lineage branching, as it is only transiently induced at high levels.

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REFERENCES

[1] Summerbell, D. and Maden, M. (1990) Trends Neurosci.13, 142–147.
[2] Tassin, M.T. and Weill, R. (1981) J. Craniofacial Gen. Dev. Biol. 1, 229–313.
[3] Theiler, K. (1988) Adv. Anat. Embryol. Cell Biol. 90 , 1–20.
[4] Brockes, J.P. (1989) Neuron 2, 1285–1294.
[5] Tibbles, L. and Wiley, M.J. (1988) Teratology 37, 113–125.
[6] Kessel, M. and Gruss, P. (1991) Cell 67, 89–104.
[7] Strickland, S. and Mahdavi, V. (1978) Cell 15, 393–403.
[8] Paulin, D., Jakob, H., Jacob, F., Weber, K. and Osborn, M. (1982) Differentiation 22, 90–99.
[9] Lang, E., Mazauric-Stüker, M.-L. and Maelicke, A. (1989) J. Cell Biol. 109, 2481–2493.
[10] Giguere, V., Ong, E.S., Evans, R.M. and Tabin, C.J. (1987) Nature 337, 566–569.
[11] Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989) Nature 339, 714–717.
[12] Brand, N., Petkovich, M., Krust, A. Chambon, P., de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1988) Nature 332, 850–853.
[13] Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. (1987) Nature 330, 444–450.
[14] Benbrook, D., Lernhardt, E. and Pfahl, M. (1988) Nature 333, 669–672.
[15] Ragsdale, C.W., Petkovich, M., Gates, P.B., Chambon, P. and Brockes, J.P. (1989) Nature 341, 654–657.
[16] Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.-M., Kastner,P., Dierich, A. and Chambon, P. (1991) EMBO J. 10, 59–69.
[17] Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J.-M., Ruffenach, M., Leroy, P. and Chambon, P. (1991) EMBO J. 10, 71–81.
[18] Kastner, P., Krust, A., Mendelsohn, C., Zelent, A., Leroy, P., Staub A. and Chambon, P. (1990) Proc. Natl. Acad. Sci. USA 87, 2700–2704.
[19] Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R. and Chambon, P. (1987) Cell 51, 941–951.
[20] Giguere, V., Hollenberg, S.M., Rosenfeld, M.G. and Evans, R.M. (1986) Cell 46, 645–652.
[21] Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E. and Chambon, P. (1989) Cell 59, 477–487.
[22] Heiermann, R., Rentrop, M., Lang, E. and Maelicke, A. (1992) J. Receptor Res., in press.

[23] Jacobson, A. (1987) Methods Enzymol. 152, 254–261.
[24] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[25] Kruyt, A.F., van den Brink, C.E., Beems, L.H.S., Dorath, M.J., Kastner, P., Kruijer, W., Chambon, P. and van der Saag, P.T. (1991) Mech. Develop. 33, 171–178.
[26] Kruyt F.A.E., van der Veer L., Mader S., van den Brink C.E., Feijen, A., Jonk, L.J., Kruijer, W. and van der Saag P.T. (1992) Differentiation 49, 27–37.
[27] Martin, C.A., Ziegler, L.M. and Napoli, J.L. (1990) Proc. Natl. Acad. Sci. USA, 87, 4804–4808.
[28] de The, H., Marchio, A., Tiollais, P. and Dejean, A. (1989) EMBO J. 8, 429–433.
[29] Hu, L. and Gudas, L.J. (1990) Molec. Cell. Biol. 10, 391–396.