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Retinoic acid regulation of the expression of retinoic acid receptors in wild-type and mutant embryonal carcinoma cells

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The embryonal carcinoma cell line P19 undergoes terminal differentiation upon induction by retinoic acid (RA). The effects of RA on the retinoic acid receptor (RAR) mRNA level were investigated in P19 cells and an RA non-responsive mutant RAC65. RA induced a rapid accumulation of RARα within 2 h and this was followed by an increase in the RARβ mRNA. RAC65 cells differed from P19 cells in that the accumulation of RARα mRNA was delayed and that an aberrant pattern of RARα transcript was observed.

Retinoic acid; Retinoic acid receptor; Embryonal carcinoma

1. INTRODUCTION

It has long been postulated that the spatial organization of a developing multicellular organism depends on morphogenic signals which regulate gene expression and cell differentiation. Recent evidence indicates that one such morphogen in vertebrates is retinoic acid (RA) [1]. RA has also been known for its pleiotropic effects on cell proliferation and differentiation [2].

With the recent cloning of cDNAs for RA receptors (RAR), it has become clear that RA can mediate its action via a mechanism similar to that of steroid hormones [3–6]. Sequence analysis has revealed that RAR belongs to a superfamily of related nuclear receptor proteins which include the receptors for steroid hormones and thyroid hormones [7,8]. The recent study by Umesono et al. [9] shows that RAR can induce gene expression through a thyroid hormone responsive element, thus indicating that RAR also acts as a *cis*-acting enhancer factor. There are at least two different RARs, α and β, whose genes map to chromosomes 17 and 3, respectively [10,11].

Embryonal carcinoma (EC) cells provide a simple model for the study of cell differentiation since certain EC cell lines differentiate into one or two cell types upon RA induction. The EC cell line P19 undergoes terminal cell differentiation into neuronal and glial cells when treated with RA [12]. Since RAR is believed to play an essential role in mediating RA action, we have examined the expression of RARα and β mRNAs in P19 and an RA non-responsive mutant [13].

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

EC cell lines, P19 and RAC65 (obtained from Dr M. McBurney, University of Ottawa, Ontario), were cultured in alpha minimum essential medium containing penicillin and streptomycin (Gibco) and supplemented with 10% fetal bovine serum (Flow). Cells were maintained in their exponential growth phase by subculturing every 2 days. To induce cell differentiation, cells (8×10^4 cells/ml) were allowed to form aggregates in the presence of 5×10^{-7} M all-trans-RA (Sigma) for 48 h in bacteriological grade dishes. Aggregates were then allowed to reattach onto tissue culture dishes with fresh medium containing no RA and maintained in culture with medium changes every two days. HL-60 cells were cultured in RPMI

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1640 medium containing the same antibiotics and supplemented with 50 nM β-mercaptoethanol, 2 mM L-glutamine, and 10% fetal bovine serum.

2.2. *RNA blot analysis*

Total RNA was isolated from cells using the guanidine thiocyanate-CsCl centrifugation procedure [14]. Equal amounts of RNA were denatured in 2.2 M formaldehyde for 15 min at 60°C, separated by electrophoresis on a 1% agarose gel [15], and transferred to a nitrocellulose filter. The filter was prehybridized in a buffer containing 50% formamide, 5 × SSC, 5 × Denhardt's solution [0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl pyrrolidone], 50 mM sodium phosphate buffer, pH 6.0, 10% dextran sulfate, and 250 μg/ml denatured sperm DNA at 42°C for 8 h. The full length cDNAs of human RARα [4] and RARβ [16] were labelled with [α-^{32}P]dATP by the random priming method [17]. Hybridization was carried out with 1×10^6 cpm/ml of probes at 42°C for 18 h. The filter was washed under stringent conditions (0.1 × SSC, 0.1% SDS at 65°C) and autoradiographed at −70°C with an intensifying screen for 16 h. Autoradiograms were usually exposed for two different lengths of time and traced using a densitometer. The area of each peak was estimated by the cut and weigh method. All experiments were repeated three times.

3. RESULTS AND DISCUSSION

P19 cells remained as undifferentiated stem cells when maintained in exponential growth phase. Upon RA induction, they underwent terminal differentiation giving rise to neuronal and glial cells. Fig.1A shows the differentiated morphology of P19 cells 4 days after the RA induction period. In contrast, RAC65 cells, a non-responsive mutant derived from P19 cells, did not undergo cell differentiation upon RA induction (fig.1B).

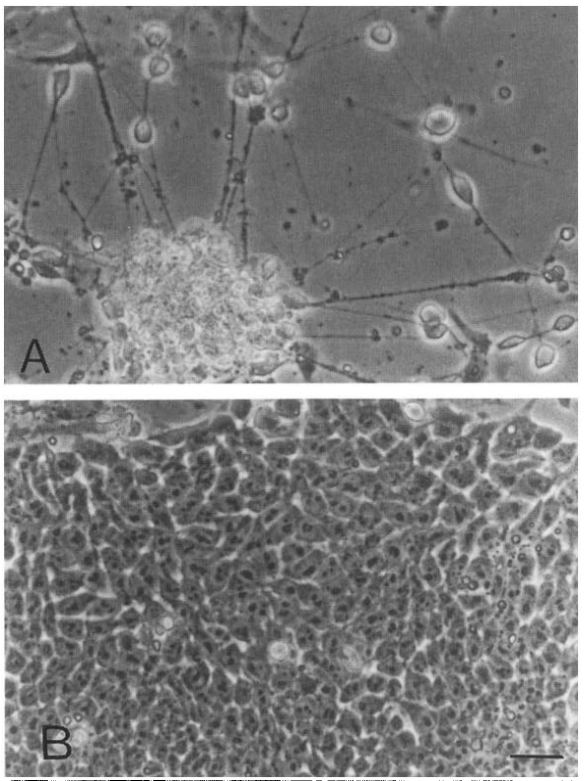

Fig.1. RA induced EC cell differentiation. P19 and RAC65 cells were allowed to form aggregates in the presence of 5×10^{-7} M RA for 48 h and the aggregates were allowed to reattach on tissue culture dishes with fresh medium containing no RA. Four days later, P19 cells (A) differentiated into neuronal cells, whereas RAC65 cells (B) remained undifferentiated. Bar, 10 μm.

As a first step to investigate the role of RAR in RA action, the expression of RARα and RARβ mRNA in P19 and RAC65 cells was examined. Uninduced P19 cells expressed only a low level of RARα mRNA (fig.2A). Upon RA treatment, a rapid increase in RARα mRNA accumulation took place in P19 cells, reaching a maximal level at 2 h of induction. Quantitative analysis based on densitometric tracings showed a greater than 8-fold increase in mRNA level at 2 h, while actin mRNA remained more or less constant during the first 12 h of induction. The cDNA probe hybridized with two distinct mRNA species of 3.2 kb and 2.3 kb. Interestingly, these two mRNA species appeared to be regulated differently. The maximal level of the 3.2 kb species was attained at 2 h while that of the 2.3 kb species was observed at 12 h. Both species showed a slight decrease by 24 h. In addition, the level of the 2.3 kb mRNA was at least 2-fold higher than that of the 3.2 kb mRNA. This was different from the pattern reported for several rat tissues [4]. Therefore, HL-60 cells were also analyzed for comparison. Interestingly, the major RARα transcript was the 3.2 kb species (fig.2C). The basal level of RARα mRNA was very high in these cells and there was only a less than 2-fold increase in the steady-state mRNA level during the first 24 h of RA induction.

RAC65 cells expressed a low basal level of RARα mRNA similar to P19 cells. However, there was a much slower rate of mRNA accumulation during the first few hours of RA induction. The mRNA level only peaked at 12 h and it was about 7-fold higher than the basal level (fig.2B). Quite unexpectedly, the predominant RARα mRNA had

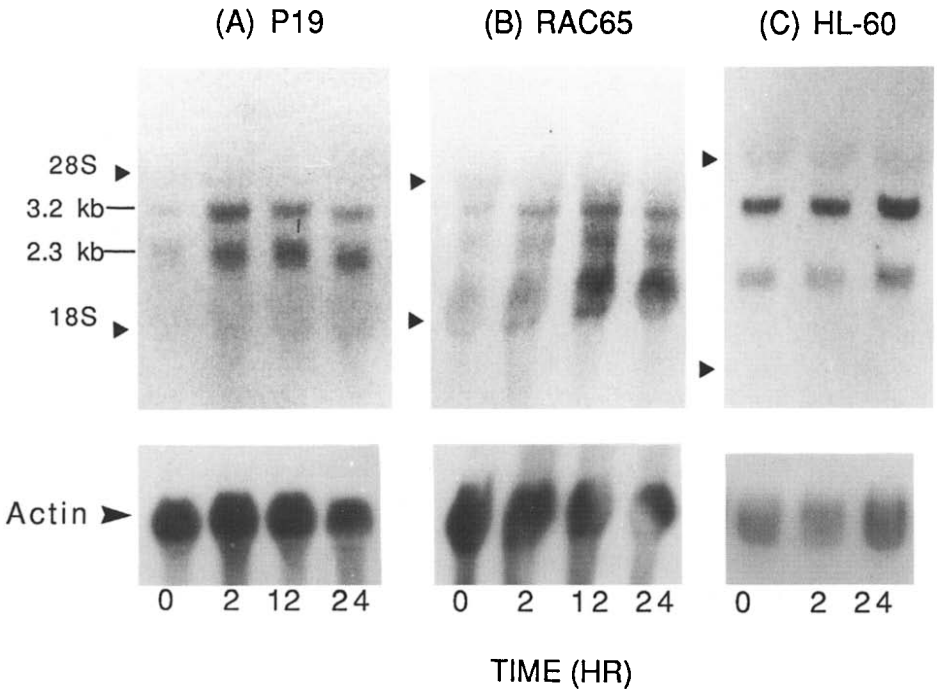

Fig.2. Expression of RARα mRNA in P19, RAC65 and HL-60 cells. Cells were induced with 5×10^{-7} M RA. Samples were collected at indicated time points and total RNA was prepared. For P19 and RAC65 cells, 40 μg of RNA was analyzed and, for HL-60 cells, 10 μg of RNA was analyzed. The blots were hybridized with the full length RARα cDNA probe. The solid circle designates the novel 2.1 kb mRNA in RAC65 cells. Positions of the 28 S and 18 S rRNA are marked by an arrowhead. These filters were rehybridized with a β-actin probe [20] and the actin probes are shown below the autoradiograms of each corresponding filter.

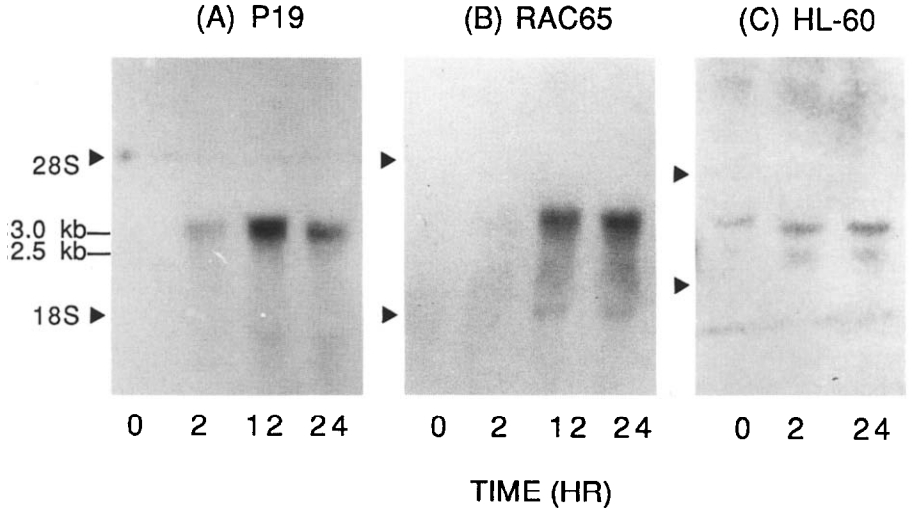

Fig.3. Expression of RARβ mRNA in P19, RAC65 and HL-60 cells. For P19 and RAC65 cells, the same blots from fig.2 were removed of the RARα probe and rehybridized with the full length RARβ cDNA probe. For HL-60 cells, 80 μg of RNA was subjected to agarose gel electrophoresis and the blot was hybridized with the same RARβ probe.

a molecular size of 2.1 kb. The 3.2 kb and 2.3 kb species constituted less than one-third of the total RARα transcripts. All three mRNA species decreased slightly by 24 h.

There was no detectable amount of RARβ mRNA in both P19 and RAC65 cells in the uninduced state (fig.3). Upon RA induction, there was a delayed but substantial increase in the RARβ mRNA level in both cell lines. The probe hybridized with a single mRNA species of 3.0 kb. The mRNA level was maximal at 12 h in P19 cells, but it continued to rise slightly for another 12 h in RAC65 cells. For comparison, HL-60 cells expressed transcripts of two sizes, 3.0 kb and 2.5 kb. Both mRNA species were expressed at a very low level, which remained relatively unchanged during the first 24 h of induction.

These results indicate that the expression of both RARα and RARβ is regulated by their ligand and the regulation is probably at the transcriptional level. This further suggests that these genes may contain RA-responsive elements in the promoter region. Hormone receptors, such as glucocorticoid receptors and vitamin D_3 receptors, have also been shown to be autoregulated by their respective ligands [18,19]. The initial rapid increase in RARα expression may be crucial to enable cells to respond maximally to RA stimulation and to embark a specific differentiation pathway. The subsequent induction of RARβ expression may be required to augment this response but not sufficient to trigger pathway choice. The subsequent gradual decrease in RAR expression may be the result of aggregate formation, when many cells will be shielded from direct exposure to RA.

The most striking differences between P19 cells and RAC65 cells are (i) the lag in RARα mRNA accumulation and (ii) the appearance of the novel 2.1 kb RARα mRNA. It has been suggested that transcripts of different sizes might have been the result of alternative RAR splicing [16]. The 2.1 kb transcript might have arisen via an aberrant RNA processing mechanism. It is conceivable that this 2.1 kb mRNA might code for a defective RARα protein, resulting in the delay of the induction of RARα mRNA expression and the eventual RA non-responsive phenotype of the RAC65 cells. Further studies involving the cloning of these different cDNA species from wild-type and mutant cells should provide new insight on the function of these receptors.

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