ROLE OF THE VARIOUS ISOFORMS OF RETINOIC ACID RECEPTORS DURING THE FINAL COMMITMENT STEP OF THE ERYTHROCYTIC DIFFERENTIATION SEQUENCE IN THE CHICKEN.

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The v-erbA oncogene, carried by the Avian Erythroblastosis Virus (AEV), is one of the most clear-cut case of an oncogene that acts by blocking a differentiation program. When this oncogene is expressed in an erythrocytic progenitor cell at the BFU-E stage of differentiation, this cell progresses along the differentiation pathway to the downstream early CFU-E stage where it stops differentiating (Samarut and Gazzolo, 1982). Although this effect was first recognised when acting in conjunction with the v-erbB oncogene (Sealy et al., 1983a, 1983b), the use of a selectable retrovirus carrying the v-erbA oncogene as the sole oncogene has directly demonstrated that the v-ErbA gene product induces by itself a blockade of the erythrocytic differentiation program at the CFU-E stage both *in vitro* and *in vivo* (Gandrillon et al.,1989; Casini and Graf, 1995).

The current view of this phenomenon is that during the BFU-E to CFU-E transition, the expression levels of a number of genes would have to be modulated by ligand-activated endogenous nuclear hormones receptors of the c-erbA family (belonging to the large superfamily of structurally and functionally related receptors that includes the receptors for thyroid hormone (T3R) and retinoids (RARs and RXRs)) thereby committing the cell from a proliferation to a differentiation program. These genes could either be a small number of master controllers of the erythrocytic differentiation program or an entire bank of genes each of which playing its own peculiar and discrete role in the combined phenomenon of erythrocytic differentiation. In any case, the v-ErbA protein would constitutively repress the actions of those endogenous nuclear hormone receptors and thus block the differentiation sequence (see Gandrillon et al., 1995 for a review).

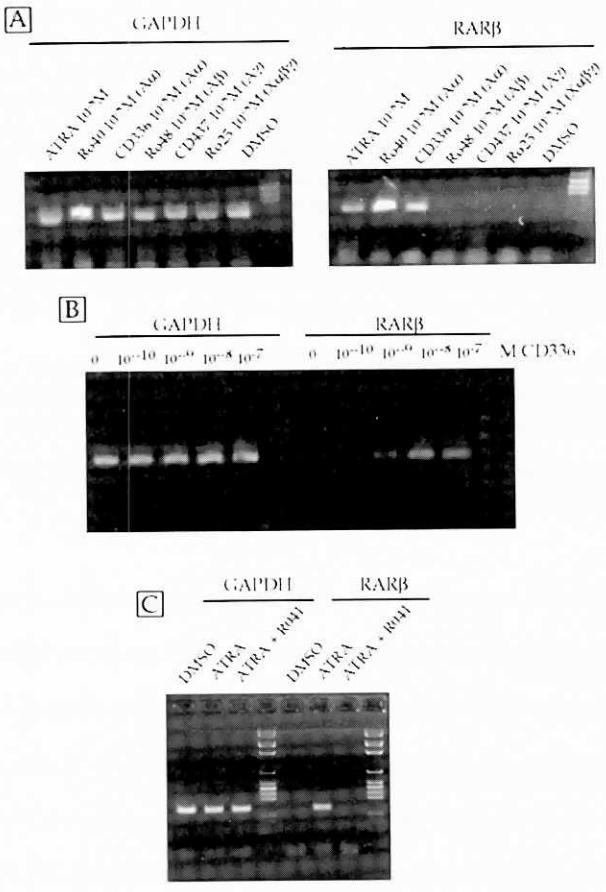

Figure 1: A: activation of RARß gene expression by RARα-specific ligands, but not by ligands specific for the other isoforms of RARs or for all RXRs isoforms; B: dose reponse of RARß gene expression in the presence of various concentrations of CD336 (= Am580), a RARα-specific ligand (Reichert et al., 1993); C: blockade of RARß gene expression induced by 10 nM *all-trans* retinoic acid by the addition of 1 µM Ro41, a RARα-specific antagonist (Apfel et al., 1992).

In order to substantiate this view, the isolation and characterization of v-erbA target genes, in its natural target cell, is an absolute necessity. We therefore have screened the expression of a number of genes by RT-PCR in normal early erythrocytic precursor cells treated with various hormones. Most of the tested genes were found to be expressed in those cells and no variation under hormonal influence could be detected in their expression pattern. The expression of some of the tested genes could not be detected in any condition in those progenitor cells (not shown).

The RARß gene was found to be strongly induced by retinoids addition (either *all-trans* or 9-cis retinoic acid) starting from undetectable levels in untreated cells. On the other side, no RARß gene expression was detected after *all-trans*-retinoic acid treatment in AEV-transformed cell lines (Sande et al., 1993). This gene therefore represents a promising putative target gene for the v-erbA oncogene.

We therefore have characterized the RARß gene response for both kinetics and dose-response parameters. We first have established that this gene was still activated by *all-trans* retinoic acid in the presence of anisomycine, a potent translation inhibitor thereby establishing this response as being a direct phenomenon that does not require ongoing protein synthesis (not shown). Then we established the kinetics of the response, in the absence or presence of cycloheximide. The RARß gene response was found to be a late event requiring at least 4 hours of *all-trans* retinoic acid addition to start being detected (not shown). We then established the dose-response profile: a sharp increase was noted at 10 nM *all-trans* retinoic acid, thereby establishing a molecular basis for differential effects between low and high doses of retinoic acid (not shown).

We then investigated the RARß gene response under the influence of isoform-specific synthetic retinoids (Figure 1A). Only the RARα-activating ligands were able to elicit a RARß gene

response at discriminating concentrations (10 nM). We performed the dose-response to CD336, a RARα-specific ligand (Figure 1B). As expected, this ligand was shown to be slightly more potent than *all-trans* retinoic acid as a faint induction signal could be detected at 1 nm CD336. We further confirmed that the RARα isoform was responsible for the RARß gene response by using Ro41, a RARα-specific antagonist. At 1 µM, this molecule completely abolished the RARß gene response induced by the addition of 10 nM *all-trans* retinoic acid (Figure 1C). Taken together all these results indicate that the RARß gene is activated directly in chicken erythrocytic cells via the RARα isoform.

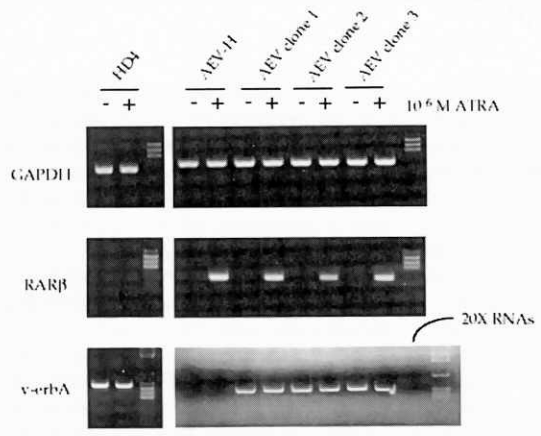

Figure 2: RT-PCR analysis of RARß gene expression in AEV-transformed cell line (HD4) and in three different clones from bone marrow cells freshly transformed by AEV infection. 20X RNAs: non-reverse transcribed RNAs control.

We then explored whether or not the expression of the v-erbA oncogene could alter the RARß gene response. We first reproduced the previously published results by using HD4 cells, an erythrocytic chicken cell line expressing v-erbA (Beug et al., 1982): those cells were unable to respond to *all-trans* retinoic acid by a detectable increase in RARß gene expression (Figure 2, left panel). Nevertheless, the situation was radically different when we investigated the response of freshly transformed cells obtained by transformation of chicken bone marrow cells with AEV. Those cells are not immortalized and, as shown in figure 2 (right panel), they do respond to *all-trans* retinoic acid by a detectable increase in RARß gene expression. It thus appears that a cell can at the same time express the v-erbA oncogene and keep an normal *all-trans* retinoic acid-mediated molecular response. An analogous situation could be demonstrated for cells transformed with the E26 virus carrying the $p135^{gag\text{-}myb\text{-}ets}$ oncogene: although an established cell line (HD57-E cells) was unable to respond to *all-trans* retinoic acid (Rascle et al., 1996), bone marrow cells freshly transformed by E26 virus did respond to *all-trans* retinoic acid by an increase in RARß gene expression (not shown). We therefore investigated the possibility that the loss of RARß gene response was correlated with the immortalization process. For this we investigated ABTCE cells, a chicken erythrocytic cell line transformed by AEV and the SV40 largeT antigen that was generated in our laboratory. This cell line did respond to *all-trans* retinoic acid by an increase in RARß gene expression (not shown). Thus, although is is quite clear that the loss of RARß gene response is not under the direct control of the v-erbA oncogene, the rationale for its loss in certain cell lines remains to be solved.

We are presently investigating whether the events that are specifically mediated by the RARα isoform are or not relevant toward the retinoid ability to induce the commitment of erythrocytic progenitor cells toward either differenciation or apoptosis. This should help us define which isoform-specific pathway, if any, should be a v-erbA target.

Acknowledgements: We wish to thank Dr. M. Klaus, from Hoffmann-La Roche Ltd. (Basel), and Dr. U. Reichert, from CIRD (Sophia Antipolis) for their very generous gift of isoform-specific ligands and for helpful discussions.

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