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CIS-ACTING ELEMENTS IN 5'-FLANKING REGION OF RAT α-FETOPROTEIN MEDIATING RETINOIC ACID RESPONSIVENESS

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A distal RA responsive *cis*-acting element has been identified in the 5'-flanking region of the α-fetoprotein gene by transfection of different deletion mutants of AFP-CAT fusion gene. The retinoic acid receptor specifically binds to this RA responsive *cis*-acting element in mobility shift assays. Furthermore, this *cis*-acting element functions in exogenous TK promoter in transient cotransfection assays. This study suggests a role for the RA responsive *cis*-acting element in the RA induction of α-fetoprotein gene expression. © 1994 Academic Press, Inc.

Retinoic acid (RA), a vitamin A derivative, has a profound effect on cell growth and differentiation, and on embryonic development (1). RA induces differentiation of embryonal carcinoma (EC) cells. One of the most extensively studied EC cell lines is mouse F9 teratocarcinoma stem cells which are induced to differentiation by RA (2). When RA treated F9 cells are allowed to grow in suspension to form aggregates, most of the cells on the outer surface will differentiate into visceral endoderm which is able to produce α-fetoprotein (AFP) (3, 4). In addition, RA enhances AFP gene expression in both McA-RH 8994 and 7777 rat hepatoma cells (5, and unpublished data from this laboratory). The effects of RA are thought to be mediated by two families of receptors including the retinoic acid receptors (RARα, β and γ) and the retinoid X receptors (RXRs), which belong to the steroid/thyroid hormone receptor superfamily (1, 6). The retinoid receptors are transcription factors which bind to specific DNA sequences and regulate gene expression (7, 8). Research data has shown that each of the retinoid receptors has distinct distributions during

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development as well as in the adult organism, which suggest that receptors may regulate the expression of different genes (1,9).

We have undertaken studies of the rat AFP gene to identify the *cis*-acting elements that mediate a stimulatory effect of RA. In this paper we report the identification of a RA *cis*-acting element in the 5'-flanking region of the rat AFP gene.

MATERIALS AND METHODS

Plasmid construction. pAPCAT and deletion mutants p4.3AFPCAT (-4285 to +7), p3.1AFPCAT (-3127 to +7), p2.9AFPCAT (-2865 to +7), p2.6AFPCAT (-2611 to +7), and p1.9AFPCAT (-1855 to +7) were generated as described previously (10). All the mutants used here were confirmed by sequencing.

Cell culture and transfection. Cell culture for rat hepatoma McA-RH 7777, mouse F9, and CV1 cells was described previously (4, 10). Transient transfection was performed using calcium phosphate coprecipitation method (10). Unless stated otherwise, each plate was transfected with 0.6 μg of reporter plasmid, and 0.6 μg of an expression vector containing either the RAR (α, β, or γ) or RXRα (11). Each transfection mixture also contained 0.6 μg of β-galactosidase expression vector as an internal control for transfection efficiency. The total amount of transfected DNA was adjusted to 4 μg with pBluescript. Assays for β-galactosidase and CAT were described previously (12). Stable transfection of F9 cells has been described by Dong et al. (4).

Gel retardation assays. Gel retardation assays were performed as previously described (10). Briefly, partially purified bacterial expressed RARγ was used in the binding assays. For competition studies, 25-fold molar excess of RARE oligonucleotide, which is from the RARβ gene promoter, was used. For super-shifting experiments, 1 μl of anti-bacterial expressed RARγ antiserum was incubated with RARγ protein before the addition of DNA probe.

RESULTS AND DISCUSSION

Delineation of a RA cis-acting element in the 5'-flanking region of AFP gene. To determine the DNA sequences mediating the observed RA induction of the endogenous AFP gene during F9 cell differentiation, a series of AFP 5'-flanking deletion mutant constructs, p3.1AFPCAT, p2.9AFPCAT, p2.6AFPCAT, p1.9AFPCAT, and pAPCAT were stably transfected into F9 cells and tested for their ability to respond to RA induction during differentiation. As shown in Figure 1A, RA can induce CAT expression 3-4 fold in F9 cells transfected with p3.1AFPCAT, p2.9AFPCAT and p2.6AFPCAT. These data indicate that RA can still effectively induce AFP gene expression after removing most of the 5' end in 5'-flanking region up to -2611. However, a loss of induction was observed in deletions between position -2611 to -1855. Deletion of this DNA fragment caused a decrease in RA induction by 3.4-fold. Comparable data were also obtained in hepatoma 7777 cells transiently transfected with these deletion mutants and RARα expression vector (Fig. 1B). Deletion of DNA region from -2611 to -1855 caused a two-fold decrease of RA responsiveness. These results

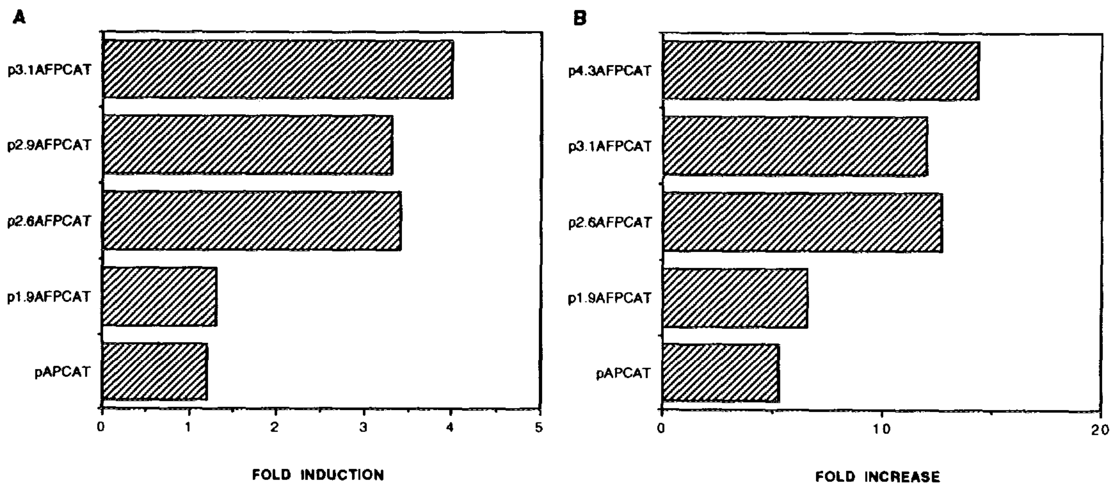

Fig. 1. Localization of the *cis*-acting element mediating the activation effect of RA on AFP 5'-flanking region. (A) F9 cells were stably transfected with various 5' deletion mutants indicated on the left, and then induced by 5 x 10^{-8} M RA for 9 days and assayed for CAT activity. Results are expressed as fold induction of CAT activity and represent the average of 4 different stable transfections. (B) McA-RH 7777 cells were transiently transfected with various 5' deletion mutants indicated on the left and with the RARα expression vector, treated with 10^{-6} M RA. Results are expressed as fold induction of CAT activity and represent the average of three different transient transfections.

indicate that a RA inducible *cis*-acting element is located between -2611 to -1855 in the rat 5'-flanking region.

DNA sequence analysis between -2611 to -1855. Sequence analysis based on the nucleotide sequence for the 5'-flanking region of rat AFP gene (13) revealed that AFP gene from -1972 to -1905 contains the sequence TGACC (which is found in most RAREs), as well as three sequences that resemble TGACC: TGATT, TGAGT and CGACC (Fig. 2A). They are located at -1912, -1948 and -1928. These four TGACC or TGACC-like sequences form two groups of direct repeats with a spacing of 3 and 11 nucleotides, respectively. Direct repeats of TGACC or TGACC-like sequence exist in reported RAREs. It is therefore highly possible that these RARE-like sequences may function as RA *cis*-acting elements in mediating the RA induction of AFP gene expression.

Binding of retinoic acid receptor to RA cis-acting element. We then analyzed the ability of the retinoic acid receptor to bind to the DNA sequence between -1972 to -1820 by using the mobility shift assay. We demonstrated that this DNA fragment can bind to bacterial expressed RARγ (Fig. 2B). Anti-RARγ antibody was able to up shift the RARγ binding band which further confirmed the RARγ binding (Fig. 2C). Furthermore the RARγ binding is specifically competed out by RARE oligonucleotides from the RARβ promoter (Fig. 2D). The specific binding of the retinoic acid

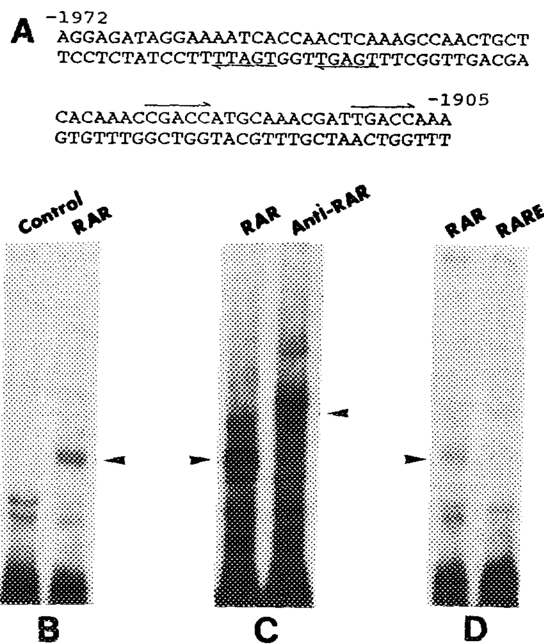

Fig. 2. (A) Organization of retinoic acid response elements in the 5'-flanking region of AFP gene. The arrows indicate the location of the TGACC and TGACC-like motifs in the nucleotide sequence from -1972 to -1905. (B) Binding of RA responsive *cis*-acting element to bacterial synthesized RARγ protein. Control is carried out by incubating probe with unprogrammed bacterial lysate. (C) Upshift assays. In analogous assays, antiserum was incubated with RAR for 45 min before performing the binding reaction. (D) Competition assays. An 25 molar excess of unlabeled βRARE, which is from the RARβ gene, was used for the competition assays.

receptor to the DNA fragment between -1972 to -1820 further confirmed the existence of a RA responsive *cis*-acting element in this region.

Cotransfection of RARs and RXR with AFP-RARE/CAT construct. In order to determine that the DNA sequence between -1972 to -1820 can functionally respond to RA induction, the 153 bp DNA fragment was inserted into vector pBLCAT$_2$ (14) upstream of the TK promoter/CAT gene. The construct, designated as p0.15tkCAT, was transiently transfected into CV1 cells (a monkey kidney cell line which contains low level of endogenous retinoid receptors) with or without cotransfection of RAR expression vectors for RARα, β and γ, as well as RXRα. As shown in Fig. 3, RA had no significant effect on CAT expression in cells cotransfected with vector pBLCAT$_2$ lacking insert gene (experiment 1). However, the addition of 10^{-6} M RA causes a significant increase in CAT expression in cells cotransfected with p0.15tk CAT and retinoid receptors (Fig. 3A, experiments 2-5). RARα exerted the strongest effect with an increase of 3-fold compared to RA untreated transfected cells (experiment 2). The cotransfection of RARs or RXR enhanced the basal expression of CAT in p0.15tkCAT transfected cells to various degrees which may be caused by the trace amount of RA in cell culture medium.

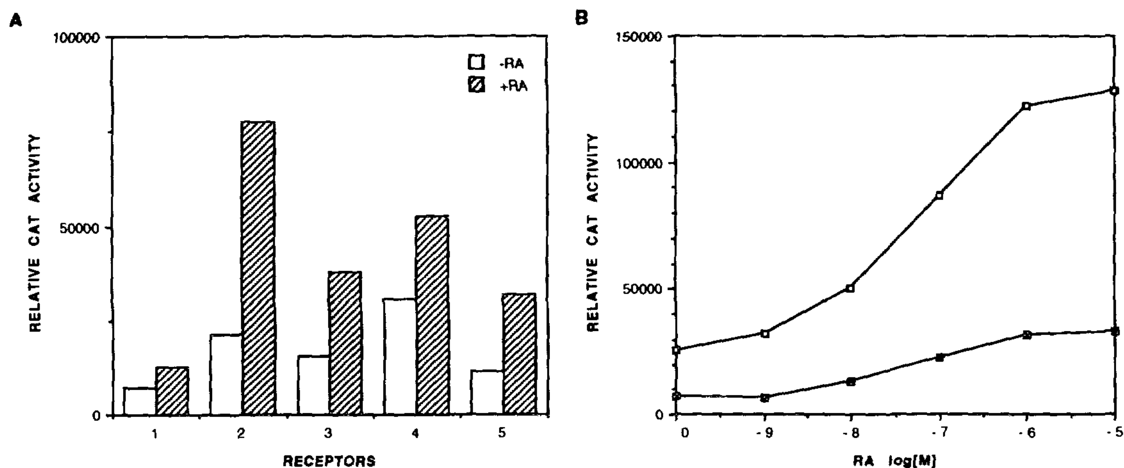

Fig. 3. (A) Activation of CAT activity by retinoid receptors. CV1 cells were cotransfected with p0.15tkCAT and with either vector (1), RARα (2), RARβ (3), RARγ (4), or RXRα (5). After transfection, 1 X 10^{-6} M of RA (hatch bar) or vehicle (ethanol, 0.01% final concentration) (open bar) were used to treat the cells. (B) Plot of CAT activity versus concentration of different RA. p0.15tkCAT (open square) or pBLCAT2 (closed square) were cotransfected with RARα and then treated with different concentrations of RA. Results represent the average of three different transfections.

Furthermore, the induction of CAT expression by RA in CV1 cells was dose-dependent. RARα was used in these experiments since it is most effective among the receptors tested. As shown in Figure 3B, a concentration of RA as low as 10^{-9} M was capable of inducing CAT activity when the RARα expression vector and p0.15tkCAT were cotransfected into CV1 cells. The activation of CAT expression reached maximal levels at 10^{-6} M RA. Cotransfection of $pBLCAT_2$ with RARα affected CAT activity only slightly, indicating that the response to RARα was sequence-dependent. Activation of p0.15tkCAT is also receptor dose-dependent (data not shown). Thus, induction of p0.15tkCAT is both RA and receptor dose dependent.

The functional studies demonstrate a preferential responsiveness of RA *cis*-acting element to RARα over other retinoid receptors. The existence of RA *cis*-acting elements or RA response elements with different specificity to various RARs or RXRs is considered to be an important mechanism for the mediation of diverse biological effects of retinoic acid (1).

In conclusion, Distal RA *cis*-acting element, located between -1972 and -1820 from the transcriptional initiation site, is able to bind specifically to the bacterial synthesized RAR protein in mobility shift assays. This RA *cis*-acting element was inserted into a reporter construct with chloramphenicol acetyltransferase (CAT) gene and a heterologous promoter. In cotransfection with RAR expression vector, the RA *cis*-acting element reporter plasmid showed a marked increase in RA induced CAT activity. Sequence analysis of this RA *cis*-acting element revealed four direct repeats of TGACC and TGACC-like motifs which have been reported to form retinoic acid response

elements. Thus, it is possible that the four TGACC and TGACC-like motif may contribute to the RA induction of AFP gene expression.

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