MOLECULAR AND CELLULAR BIOLOGY, Apr. 1995, p. 1961–1967 Vol. 15, No. 4
0270-7306/95/$04.00+0

Tissue-Specific Distribution of a Novel C-Terminal Truncation Retinoic Acid Receptor Mutant Which Acts as a Negative Repressor in a Promoter- and Cell-Type-Specific Manner

TAKASHI MATSUI* AND SHUNSUKE SASHIHARA

Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan

Received 11 August 1994/Returned for modification 15 September 1994/Accepted 4 January 1995

A cDNA clone which encodes a truncation form of the γ subtype of the retinoic acid receptor (RARγ) has been isolated. The mutant RARγ (RARγBm382) has lost its 65 C-terminal amino acids, thus truncating a part of the dimerization and activation domains. By using a reverse transcription-coupled PCR technique, it was shown that RARγBm382 is expressed at different levels in various mouse tissues and that the level of its expression does not correlate with that of normal RARγB. Cotransfection studies revealed that RARγBm382 acts as a repressor of normal RARs in a promoter- and cell-type-specific manner. Transcription of βRARE and TREinv promoters was inhibited by RARγBm382 in both HeLa and F9 cells. Unlike these two promoters, however, RARγBm382 did not inhibit transcription of the TREpal promoter in HeLa cells but did so in F9 cells. Moreover, while transcription of the lamRARE promoter was inhibited by RARγBm382 in both HeLa and F9 cells, the inhibition was not observed when F9 cells were induced to differentiate with retinoic acid and dibutyryl cyclic AMP. DNA-binding analysis revealed that RARγBm382 is able to form a heterodimer with the retinoid X receptor and bind to the different types of retinoic acid response elements with almost the same efficiency as normal RAR. By comparison with effects of other truncation mutants created in vitro, it was suggested that the C-terminal end of the ligand binding domain of RAR is crucial for determining the specificity of transactivation by RAR. Given these observations, we discuss the possibility that protein factors which mediate retinoic acid response element- and cell-type-specific transactivation by RAR are present.

Retinoic acid (RA), a derivative of retinoids, has a divergent biological effects on vertebrate development, differentiation, and cell growth (38, 48). In vitro, RA induces a variety of cell types to differentiate (52, 53), and in vivo, RA is also known to be teratogen in mammals, causing limb malformations and defects in brain development (32, 49).

The identification of the nuclear receptor for RA, RAR (3, 5, 19, 29, 34, 46, 62), provided a basis for understanding how RA exerts its biological effects. RAR, a member of steroid/thyroid hormone receptor superfamily, is a ligand-inducible transcription factor in target tissues (2, 14). RAR has three subtypes (RARα, -β, and -γ), which show different expression patterns during vertebrate embryogenesis, suggesting their involvement in transcriptional regulation of a distinct class of target genes (31, 34, 36, 40, 62, 63). In addition, a novel class of retinoid receptor, the retinoid X receptor (RXR), has been identified, leading to the discovery of a second retinoid transduction pathway (26, 35, 40). RXR is activated only by 9-*cis* RA, whereas RARs are activated by all-*trans* RA as well (27, 37, 39).

RAR binds to a specific DNA sequence termed the RA-responsive element (RARE) and activates or represses transcription of adjacent target genes in a ligand-dependent manner. It has been shown that RARE consists of repeats of a consensus half-site sequence, AGGTCA, which is shared among a subgroup of nuclear receptors including thyroid hormone and vitamin D_3 receptors (15, 44, 56). Further, the specificity of a receptor to bind its cognate responsive element appears to be determined by the orientation and the spacing of the repeats. For instance, RAR binds preferentially to a direct repeat of the half-sites with 5-bp spacing (DR5), whereas the thyroid hormone and vitamin D_3 receptors bind to DR4 and DR3, respectively (the so-called 3-4-5 rule) (44, 56). Naturally occurring RAREs, however, are known to be more diverse and consist of half-sites which are variable in number, orientation, and spacing (17, 55, 58). RARs bind to RAREs as homodimers through a dimerization domain (16, 17). More recently, much evidence has been accumulating that RAR forms heterodimers with nuclear proteins, so-called coregulators (9, 17, 18, 21, 24). Formation of such RAR heterodimers results in a higher affinity for RARE and thereby an enhanced transactivation of target genes (65). One group of such coregulators has been identified to consist of RXRs, which serve as hetrodimeric partners not only with RARs but also with other members of nuclear receptors (7, 18, 25, 33, 35, 42, 60, 65). Other nuclear proteins distinct from RXRs are also known to interact with and modify RARs, as suggested previously (4, 9, 21). It is still unclear how RAR discriminates between different types of RARE promoter to activate or repress in target tissues. It is therefore essential to determine the identity and characteristics of nuclear proteins which interact (via dimerization or other interactions) with RAR.

During attempts to isolate new members of nuclear hormone receptor superfamily, we isolated a cDNA clone which encodes a C-terminal truncation mutant of RARγ. In this report, we showed that the mutant RARγ is expressed at different levels in various mouse tissues. Moreover, despite its ability to form a heterodimer with RXR and to bind with RAREs, the mutant RAR acted as a negative repressor of normal RARs in a RARE- and cell-type-specific manner.

* Corresponding author. Phone: 93-691-7423. Fax: 93-692-2766.

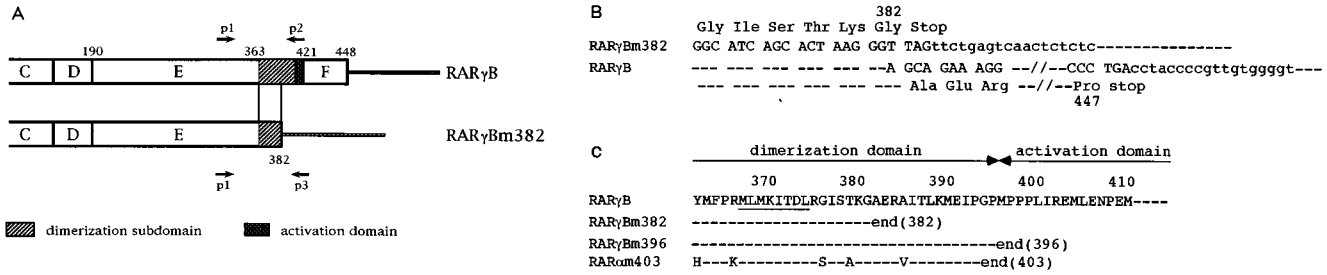

FIG. 1. (A) Schematic representation of C-terminal regions of normal and mutant RARγB. The structure of the C-terminal region of RARγBm382 is compared with that of normal RARγB. The primers for RT-PCR analysis are indicated by arrows. (B) Sequence comparison between RARγBm382 and normal RARγB. Nucleotide and amino acid sequences around the ends of the coding regions of RARγBm382 and normal RARγB are shown. (C) Amino acid sequences in the dimerization and activation domains of normal and mutant RARs. The ninth heptad repeat is underlined.

MATERIALS AND METHODS

Isolation of cDNA. cDNA library from F9 cells was screened with a DNA fragment encompassing DNA binding domain of RARγ. Among several cDNA clones encoding RARγ, we isolated a clone (RARγBm382) which shows a pattern of restriction enzyme digestion different from that of normal RARγ cDNA.

Reverse transcription-coupled PCR. Total RNA was prepared from F9 cells and adult mouse tissues. cDNA was synthesized in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 75 mM KCl, 0.5 mM each of four deoxynucleoside triphosphates (dNTPs), 10 mM dithiothreitol (DTT), 25 μg of oligo$(dT)_{12–18}$ per ml, 10 μg of total RNA, and 100 U of avian myeloblastosis virus reverse transcriptase. After 2 h of incubation at 42°C, the reaction was terminated by heating at 94°C for 5 min. One microliter of the reaction mixture was used for amplification by PCR. PCR was carried out in 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mg of gelatin per ml, 1 mM DTT, 200 ng of each primer, 0.2 mM each of four dNTPs, and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer). Thirty cycles of reaction were performed at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. Three primers were used; primers P1 and P2 encompassed amino acids 311 to 318 and 410 to 417, respectively of RARγB, and P3 encoded the 3′ untranslated region specific for RARγBm382. The amplified DNA was run on a 3% NuSieve gel (Takara Shuzo Co.) and blotted onto a Hybond-N filter (Amersham). The filter was hybridized with an oligonucleotide encompassing amino acids 340 to 347 of RARγB.

Poly(A) RNA was selected by using oligo(dT) latex beads (Takara Shuzo). About 5 μg of poly(A) RNA from adult mouse tissues and F9 cells was run on a 1% agarose gel. After transfer of RNA onto a Hybond-N filter, the filter was hybridized with ^{32}P-labeled RARγB cDNA. The same filter was rehybridized with ^{32}P-labeled β-actin cDNA to normalize the amount of RNA used.

Plasmid DNA. A synthetic oligonucleotide encoding TREpal (23), TREinv (44), βRARE (13, 54), or lamRARE (45, 58, 59) was inserted upstream of thymidine kinase gene promoter of ptkCAT DNA and used as a reporter DNA in transient expression experiments. As a receptor expression plasmid, pSGRARα, pSGRARγB, or pSGRARγBm382 was constructed by inserting each cDNA into the *Eco*RI site of the expression vector pSG5. pSGRARαm403 was constructed by deleting the *Sma*I fragment from pSGRARα. For construction of pSGRARγBm396, pSGRARγB was digested from its C-terminal end with exonuclease III and then with S1 nuclease. The coding regions of these two in vitro truncation mutants were terminated by inserting an oligonucleotide encoding stop codons, 5′-TTAAGTTAACTTAA-3′.

Transfection of cells. Transfection of HeLa or F9 cells was performed by the DEAE-dextran (43) or calcium phosphate (8) method, respectively. For transfection of differentiated F9 cells, F9 cells were treated with 1 μM RA and 0.5 mM dibutyryl cyclic AMP for 2 days before transfection. In a typical transfection, 1 μg of the reporter plasmid, 1 to 3 μg of the respective expression plasmid, and 1 μg of an internal control plasmid (pRSVβgal) were used. The amount of simian virus 40 promoter was held constant in all transfections by addition of pSG5. For transfection of cells in a 6-cm-diameter dish, the total amount of DNA was adjusted to 5 μg for HeLa cells and 10 μg for F9 cells with pUC19 DNA. All transfections were done in duplicate and were repeated at least three times. Cell extracts were prepared by use of Reporter Lysis buffer (Promega). The amounts of cell extracts for the chloramphenicol acetyltransferase (CAT) assay were normalized by the level of β-galactosidase activity.

In vitro synthesis of RARγBm382 protein and its binding with RAREs. pSGRARγB, pSGRARγBm382, and pSGRXRα, which was provided by P. Chambon, were linearized with *Bgl*II. Capped mRNAs were synthesized in vitro, using T7 polymerase in 50 μl of reaction mixture containing 40 mM Tris-HCl (pH 7.5), 80 mM NaCl, 8 mM $MgCl_2$, 10 mM DTT, 1 mM each ATP, CTP, and UTP, 0.2 mM GTP, 1 mM capped GTP, 40 U of RNase inhibitor, and 2 μg of template DNA. After incubation for 60 min at 37°C, RNA was purified and dissolved in 20 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. One microliter of each RNA was used to synthesize RARγB, RARγBm382, or RXRα protein in vitro, using rabbit reticulocyte lysates (Promega). A total of 3 μl of programmed reticulocyte lysate was added to each reaction mixture containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM KCl, 5% glycerol, 1 mM DTT, 0.05% Nonidet P-40, 1 μg of poly(dI-dC) · poly(dI-dC), and approximately 10^4 cpm of each probe in a final volume of 10 μl. As a control, probes were also incubated with the same amount of unprogrammed lysate. A synthetic oligonucleotide encoding βRARE, TREpal, or lamRARE (see Fig. 3) was inserted at the *Sma*I site of pUC19 DNA. The isolated *Eco*RI-*Hin*dIII fragments were labeled at the 5′ ends with T4 polynucleotide kinase and were used as probes.

RESULTS

Expression of a truncation RAR mutant in vivo. Multiple isoforms of RARγ which differ from one another in their 5′ untranslated regions and N-terminal A domains have been identified (20, 31, 34). A cDNA clone isolated here was identical to the RARγB (or RARγ2) isoform from its 5′ untranslated region to the E domain (Fig. 1 and data not shown). However, the coding region of the cDNA (RARγBm382) was terminated at amino acid 382, thus truncating its 65 C-terminal amino acids. RARγBm382 lacked a part of its dimerization domain as well as the activation domain. The 3′ untranslated region differed totally from that of normal RARγ (Fig. 1B). To rule out the possibility that the cDNA is an artificial product of the cloning process, we first examined whether mRNA corresponding to RARγBm382 is present in F9 cells from which a cDNA library was created. To distinguish RARγBm382 from normal RARγ, we carried out RT-PCR of total RNA from F9 cells. In this experiment, three oligonucleotide primers, P1, P2, and P3, were used for RT-PCR analysis (Fig. 1A). As shown in Fig. 2A, with these three primers, 323 or 373 bp of DNA fragment was specifically amplified from normal RARγB cDNA (lane 1) or from RARγBm382 cDNA (lane 2), respectively. Each of these two bands was amplified specifically with P1 and P2 or with P1 and P3, respectively (data not shown). Next, when total RNA from F9 cells was amplified with the three primers by RT-PCR, two major bands identical in size to the bands amplified from the cloned DNAs were amplified (lane 3). Under the conditions used here, almost the same efficiency of amplification was observed from each of the two cDNAs in the same reaction (compare lanes 1 and 2). This result clearly indicated that mRNA for RARγBm382 is present in F9 cells. We further investigated its expression in various adult mouse tissues and determined its amount relative to normal RARγ. As shown in Fig. 2B, RARγBm382 was expressed in all tissues examined. Interestingly, the ratio of its amount to normal RARγ was variable among various tissues. The relative level of its expression in the spleen was very high, about 70% of total RARγ. Brain tissue also expressed RARγBm382 at a high level, about 45% of total. In contrast, only low-level expression, less than 5% of total, was observed

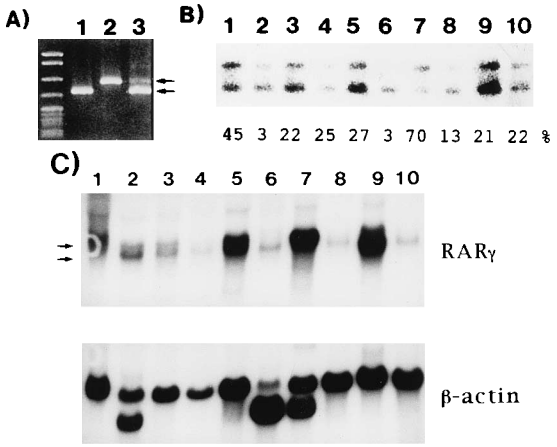

FIG. 2. Expression of mutant RARγ. (A). Oligo(dT)$_{12-18}$-primed cDNA synthesis was carried out with total RNA from F9 cells. An aliquot of the reaction mixture was amplified with primers P1, P2, and P3 (lane 3). For a control, pSGRARγB DNA (lane 1) and pSGRARγBm382 DNA (lane 2) were also amplified with the three primers. The left-most lane represents a digest of pBR322 DNA with *Hpa*II as a size marker. The amplified DNAs were analyzed on a 3% NuSieve-agarose gel. The gel stained with ethidium bromide was photographed. (B) RT-PCR was carried out with total RNA from mouse tissues. The amplified DNA was electrophoresed as for panel A. The DNA was transferred onto a Hybond-N filter (Amersham) and hybridized with an oligonucleotide encompassing amino acids 340 to 347. The amount of each band was determined by using a Fujix BAS2000 image analyzer. The numbers at the bottom represent the amounts of RARγBm382 as percentages of total RARγ. Lanes: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung; 6, muscle; 7, spleen; 8, skin; 9, undifferentiated F9 cells; 10, differentiated F9 cells. (C) Poly(A) RNA was run on a 1% agarose gel and then subjected to Northern blot hybridization with RARγB cDNA as a probe (top). The same blot was rehybridized with β-actin cDNA to normalize the amount of RNA applied on the gel (bottom). Lanes: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung; 6, muscle; 7, skin; 8, spleen; 9, undifferentiated F9 cells; 10, differentiated F9 cells. The upper and lower arrows indicate RARγA and RARγB isoforms, respectively.

in heart and muscle tissue. In other tissues such as kidney, liver, lung, and skin, the mutant RARγB accounted for about 10 to 30% of total expressed RARγ. In F9 cells, the amount of RARγBm382 relative to normal RARγ (about 20% of total) did not change during the differentiation, although total RARγ expression was negatively regulated after induction of differentiation by RA and cAMP (Fig. 2C). Next, to determine whether expression of RARγBm382 correlates with that of the normal RARγB isoform, we performed Northern (RNA) blot analysis of RARγ mRNA from mouse tissues. As shown in Fig. 2C, two major mRNAs, about 3.3 and 3.1 kb in size, were detected on the blots. From their sizes, the larger and smaller bands represented mRNAs for the RARγA and RARγB isoforms, respectively (31). This was confirmed by hybridization using the N-terminal region specific for each isoform (data not shown). Consistent with previous reports (20, 31, 40, 62), high-level expression of RARγ was observed in skin, lung, and F9 cells, whereas brain, heart, kidney, liver, and muscle cells expressed RARγ at very low levels. In addition, each of the two isoforms showed a tissue-specific pattern of expression. RARγA was predominant in skin and brain cells, whereas RARγB was predominant in liver, muscle, spleen, and F9 cells. In heart, kidney, and lung cells, the two isoforms were expressed at roughly similar levels. Comparison with the results shown in Fig. 2B and C demonstrated that the abundance of RARγBm382 in each tissue does not correlate with the level of normal RARγB or total RARγ.

Negative function of the mutant RAR. Because of deletion of its activation domain, RARγBm382 is expected to lose the

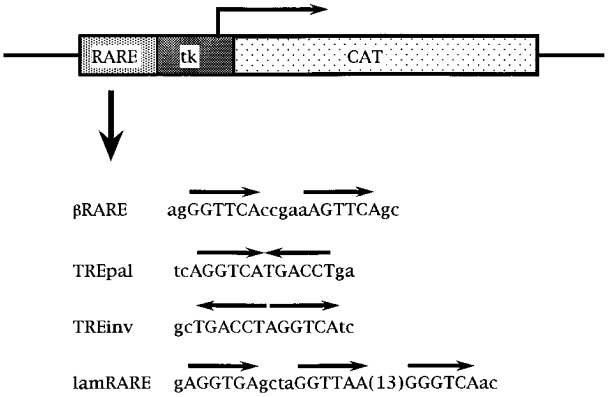

FIG. 3. Structures of reporter CAT DNAs. Each reporter construct contains one copy of each synthetic oligonucleotide as shown. The consensus half-sites are indicated by arrows.

ability to activate transcription of an RA-responsive promoter. To confirm this, we carried out a transient expression experiment using pTREpaltkCAT DNA as a reporter (Fig. 3). As shown in Fig. 4, no RA-dependent stimulation of CAT activity was observed in cells cotransfected with pSGRARγBm382, whereas cotransfection of a normal RARα or RARγB expression plasmid increased CAT activities by more than 25-fold in response to RA. The other three RARE promoters (Fig. 3) were also not activated by RARγBm382 (data not shown).

Recently, Damm et al. (10) have demonstrated that C-terminal truncation mutants of RARs, which were created in vitro, acted as dominant negative repressors of normal RARs. In contrast to such mutants, RARγBm382 lost a part of its dimerization domain essential for protein-protein interaction. We therefore examined whether RARγBm382 is able to act as a negative repressor of normal RARs. In the following experiments, we analyzed the effects of RARγBm382 on transcrip-

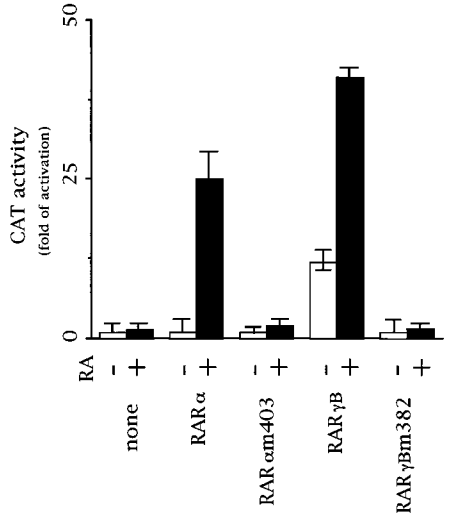

FIG. 4. Loss of transactivation function in mutant RARs. One microgram of normal or mutant RAR-expressing plasmid DNA was cotransfected with 1 μg of pTREpaltkCAT DNA in HeLa cells, and the cells were incubated for 2 days in the presence (+) or absence (−) of 1 μM RA. The amount of the acetylated form of [^{14}C]chloramphenicol was determined by using a Fujix BAS2000 image analyzer and normalized by estimation of the level of β-galactosidase activity used as an internal standard. The CAT activity in cells transfected with the reporter DNA alone and cultured in the absence of RA is represented as 1.

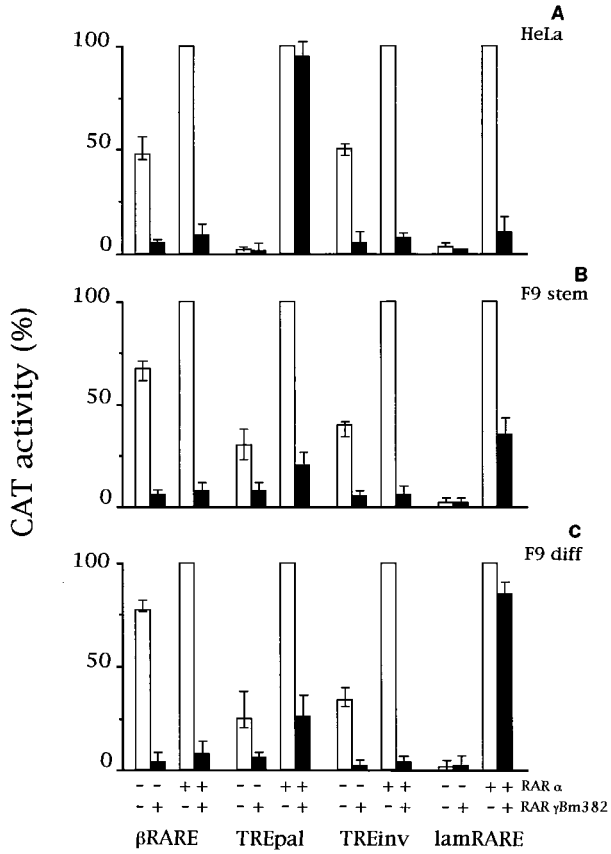

FIG. 5. Promoter- and cell-type-specific negative effects of RARγBm382. One microgram of the indicated reporter CAT DNA was cotransfected with 2 μg of pSGRARγBm382 and 0.5 μg of pSGRARα into HeLa, F9 stem, or differentiated (diff) F9 cells. After transfection, the cells were cultured in the presence of 1 μM RA. For each RARE promoter, relative CAT expression is presented in comparison with that measured in cells cotransfected with the reporter and pSGRARα, arbitrarily defined as 100. In the presence of exogenous RARα, the CAT level of TREpal, TREinv, or lamRARE reporter relative to βRARE as 100 was 60, 95, or 14, respectively, in HeLa cells, 93, 120, or 21, respectively, in F9 cells, and 88, 105, or 26, respectively, in F9 differentiated cells.

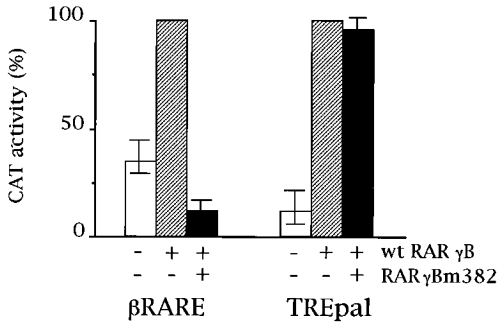

FIG. 6. Negative effects of RARγBm382 on normal RARγB. One microgram of pβRAREtkCAT (left) or pTREpaltkCAT (right) was cotransfected with 0.5 μg of pSGRARγB and 2 μg of pSGRARγBm382 into HeLa cells. After transfection, the cells were cultured in the presence of 1 μM RA. For each RARE promoter, relative CAT expression is presented in comparison with that measured in cells cotransfected with each reporter and pSGRARγB, arbitrarily defined as 100. wt, wild type.

tion of four different types of RARE promoters by normal RAR (Fig. 3). TREpal is a synthetic palindrome of the AG GTCA motif, a modified version of the rat growth hormone T3 response element (22). TREinv is a synthetic inverted form of TREpal (44). βRARE is a direct repeat of the element with 5-bp spacing which is found within the RARβ gene promoter (13, 54). lamRARE is a complex element consisting of three motifs which is found in the upstream region of the mouse laminin B1 gene (45, 58, 59). These RAREs were inserted just upstream of the *tk*-CAT DNA. As shown in Fig. 5A, βRARE and TREinv promoters were activated by endogenous RAR in response to RA and activated further by ectopically expressed RARα in HeLa cells, whereas RA-dependent activation of TREpal and lamRARE promoters required exogenous RARα. When RARγBm382 was coexpressed in HeLa cells, the transcription level of the βRARE or TREinv promoter activated by endogenous and exogenous RAR decreased to about 5 to 10% of control levels. Transcription of the lamRARE promoter activated by exogenous RARα was similarly inhibited by RARγBm382. In the contrast, transcription of the TREpal promoter activated by exogenous RARα was not inhibited by RARγBm382. In contrast to HeLa cells, however, in F9 cells, RARγBm382 inhibited transcription of the TREpal promoter as well as the other three RARE promoters, although the degrees of inhibition of TREpal and lamRARE promoters were lower than those of βRARE and TREinv promoters (Fig. 5B). On the other hand, almost no inhibition of transcription of the lamRARE promoter by RARγBm382 was observed when F9 cells were induced to differentiate with RA and cAMP (Fig. 5C). The selective negative effects of RARγBm 382 on different RARE promoters were also observed with transcription by its normal counterpart, RARγB (Fig. 6). RARγBm382 again failed to inhibit transcription of TREpal promoter activated by normal RARγB in HeLa cells, whereas it inhibited activation of transcription of the βRARE promoter. Activation of the lamRARE promoter by RARγB was also not inhibited by RARγBm382 in differentiated F9 cells (data not shown). These data together with the results in Fig. 5 indicated that RARγBm382 acts as a negative repressor of both RARα and RARγB in a promoter- and cell-type-specific manner.

To determine the mechanism of the negative effects of RARγBm382 on normal RARs, we investigated whether the mutant RARγB is able to form a heterodimer with RXR and to bind with RAREs. For this purpose, RARγB, RARγBm 382, and RXRα proteins were synthesized in vitro by using rabbit reticulocyte lysates and analyzed for their abilities to bind with the three RAREs, βRARE, TREpal, and lamRARE, by a gel retardation assay. As shown in Fig. 7, none of the receptors alone bound efficiently with all three RAREs. Efficient binding of normal RARγB occurred with the three RAREs in the presence of RXRα protein (and also RXRβ and -γ [data not shown]), although βRARE was the most efficiently bound of the three RAREs. RARγBm382 also bound to the three RAREs with a little lower efficiency than normal RARγB, probably reflecting a fluctuation of efficiency of in vitro transcription and translation. Importantly, its efficiency of binding to each RARE was almost the same as that of normal RARγB. After long exposure of the gels, a gel-retarded band was visible in the reaction supplemented only RARγBm382 or normal RARγB (data not shown). These results clearly indicate that RARγBm382 does not lose the ability to dimerize with itself and with RXR and to bind with the three different types of RARE. RARγBm382 inhibited transcription by normal RARs in a RARE- and cell-type-dependent manner, which suggested that its negative effect on transcription does not result simply from its binding with RAREs in competition with normal RAR heterodimer.

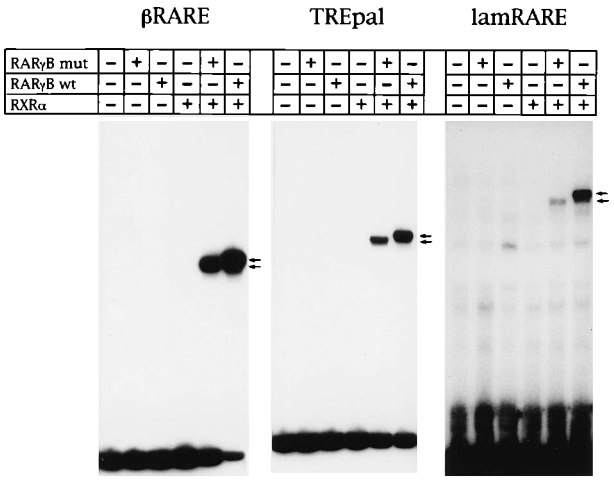

FIG. 7. Binding of RARγBm382 with RAREs. Radiolabeled βRARE, TREpal, and lamRARE were incubated with reticulocyte lysate programmed with RARγB, RARγBm382, or RXRα alone or in combination. Probes were also incubated with unprogrammed lysate as a control (the left-most lane in each panel). Specific complexes are indicated by arrows. mut, mutant; wt, wild type.

Finally, to examine whether the RARE promoter- and cell-type-specific negative effect on normal RARs is characteristic of RARγBm382, we constructed truncation forms of RARγB and RARα which were deleted of their activation domains but retained their dimerization domains (Fig. 1C) and compared their negative effects on transcription with that of RARγBm 382. As shown in Fig. 8, unlike RARγBm382, RARγBm396 inhibited transcription of the TREpal promoter in HeLa cells and the lamRARE promoter in differentiated F9 cells. Similarly, RARαm403 efficiently inhibited transcription of βRARE, TREpal, and lamRARE promoters irrespective of cell type. Thus, it appears that a region from amino acids 382 to 396 of RARγB determines the specificity of RAR function in transactivation of RA-responsive promoters.

DISCUSSION

The experiments described here demonstrated the presence of a novel isoform of RAR which is deleted of its C-terminal region and part of the dimerization and activation domains. All isoforms of RAR identified so far differ from one another in their 5′ untranslated and N-terminal regions and are produced from a common gene by alternative splicing (20, 31, 34, 36, 63). Thus, this is the first report to demonstrate the presence of a C-terminal truncation mutant of RAR present in normal tissues and cells. It has been previously demonstrated that a truncation mutant of RARα is present in a mutant cell line of P19 cells nonresponsive to RA (47). In that case, the mutant RARα was shown to be produced from a RARα gene mutated at the C-terminal region. Southern hybridization analysis of mouse genomic DNA showed that any recombination within the RARγ gene does not occur in normal cells and tissues (our unpublished observation). RARγBm382 was exactly the same as normal RARγB except for its C-terminal truncation. These observations suggest that RARγBm382 is transcribed from the normal gene and is produced by alternative splicing at its C-terminal region.

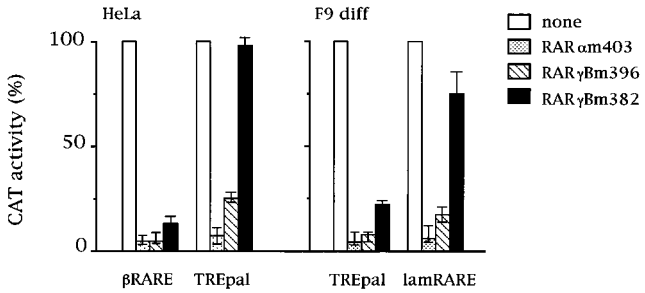

FIG. 8. Promoter-specific negative effect of RARγBm382 by deletion of its dimerization domain. One microgram of the indicated reporter DNA was cotransfected with 2 μg of the mutant RAR-expressing plasmid in the presence of 0.5 μg of pSGRARα into HeLa or differentiated (diff) F9 cells. After culture in the presence of 1 μM RA for 2 days, CAT activity was measured. For each RARE promoter, relative CAT expression is presented in comparison with that measured in cells cotransfected without the mutant RAR expressing plasmid, arbitrarily defined as 100.

The amounts of mRNA for RARγBm382 relative to normal RARγB were different in various adult mouse tissues. For instance, the mutant RARγB accounted for about 70% of total RARγ in the spleen, which expresses predominantly (about 70% of total RARγ) the RARγB isoform. About 10% of total RARγ was the mutant RARγB in the skin, which expresses predominantly (more than 80% of total RARγ) the RARγA isoform. Thus, almost all RARγB in these two tissues appears to be the mutant form. In contrast, heart and muscle tissues, in which RARγB is predominant, expressed RARγBm382 at only low levels, about 3% of total RARγ. Thus, almost all RARγB appears to be the normal form in these tissues. In the other tissues and F9 cells, the mutant form was estimated to account for about 25 to 40% of total RARγB. As in the case of normal RARγ, under the conditions used in RT-PCR, the same size of DNA would be amplified from any similar truncation mutants of other RARγ isoforms. Moreover, it is not clear at present whether RARγB382 is expressed stably at the translational level. More studies are required to understand its physiological significance, although the differential distribution among various tissues raises the possibility that it plays some role in the RA-signaling pathway.

On the basis of their structural similarity, RARγBm382 resembles the v-*erbA* oncogene product, a mutated form of c-ErbA (thyroid hormone receptor) (50). Like v-ErbA (11, 51, 64), RARγBm382 acted as a negative repressor of normal RARs. Interestingly, however, the negative effects on normal RARs were dependent not only on the type of RARE promoter but also on the cell type. For instance, the TREpal promoter in HeLa cells and the lamRARE promoter in differentiated F9 cells escaped the negative effects of RARγBm382. These observations suggest that transcriptional repression by RARγBm382 results neither from its direct interaction with normal RARs nor from competitive binding of its homodimer to the respective RAREs. Despite its deletion of the dimerization domain, RARγBm382 was able to dimerize with RXRs and to bind in vitro with at least three different types of RARE (βRARE, TREpal, and lamRARE) as efficiently as normal RARγB. If binding of the RAR-RXR heterodimer with RARE simply confers activation of transcription, RARγBm 382 should act as a negative repressor irrespective of RARE promoter and cell type. It has been previously demonstrated that cooperativity in transactivation between RAR and TFIID requires an activity analogous to E1a (4). Such an activity (mediator) might mediate an interaction between the RAR-RXR heterodimer with the basal transcription machinery. Further, since specificity of conformation of the RAR-RXR heterodimer appears to depend on the spacing and orientation of the AGGTCA repeats (61), mediators might be distinct from every RARE type and might be cell type specific. Therefore,

RARE-type-dependent negative effects of RARγBm382 may be based on its selective interaction with mediators. However, this model requires the assumption that RAR-RXR heterodimers bind stably to RARE in vivo only when they interact with mediators to form an active transcription complex.

Alternatively, since RAR is known to form a heterodimer with cell-type-specific nuclear proteins as well as RXRs (21), it is also possible that RARE- and cell-type-dependent negative effects of RARγBm382 reflect the heterogeneity of such heterodimeric partners. Unlike RARγBm382, the two truncation mutants RARα403 and RARγBm396, which retain their dimerization domains, acted as negative repressors of normal RARs irrespective of RARE promoter and cell type. This finding indicates that the region from amino acids 382 to 396 may be essential for mediating the interaction of RAR with mediators and/or its heterodimeric partners, while the nine heptad repeats within the dimerization domain conserved among nuclear hormone receptors have been shown to mediate diverse responses by affecting homodimer and heterodimer formation (1, 17, 40, 42, 66). In either case, RARγBm382 may provide an important tool with which to identify proteins interacting with RAR which are involved in discrimination and activation of a variety of RARE promoters.

It should be noted that unlike βRARE, TREpal promoter was not activated by endogenous RAR in HeLa cells. Since in vitro binding of the RAR-RXR heterodimer to βRARE was at most three- to fourfold higher than that to TREpal, the differential activation in response to endogenous RAR appears not to result simply from the difference in their binding efficiencies. Rather, because of their low level of expression in HeLa cells, RARs might interact only with a distinct class of heterodimeric partners and/or mediators and thereby activate βRARE but not the TREpal promoter. Upon its ectopic expression, however, RAR could interact with a partner or mediator which is involved in activation of the TREpal promoter. In contrast to HeLa cells, F9 cells express RAR at a relatively high level, and so the TREpal promoter is activated by endogenous RAR in response to RA. Therefore, the relative concentrations of RAR and its heterodimeric partners and mediators within different cells or tissues are likely to determine which target genes are activated.

Finally, aberrant forms of nuclear receptors, as dominant negative repressors for their normal receptor counterparts, have been found in patients with a variety of endocrine and neoplastic diseases and in cell lines nonresponsive to ligands (6, 12, 28, 30, 47, 57). Expression of RARγ and its isoforms is strictly regulated at specific stages of early embryogenesis and at development of specific tissues (20, 31, 40, 62). It was previously demonstrated that a dominant negative RAR mutant, RARα403, caused malformations of the palate in transgenic mice, which died within 24 h after birth (10). Since, unlike the case for RARα403, the function of RARγBm382 as a negative repressor was dependent on RARE promoter and cell type, it might cause more moderate and restricted effects on development of such transgenic mice. Analysis of their developmental fates would give clues to the mechanism by which RAR regulates early embryogenesis, development, and cellular differentiation in response to RA.

ACKNOWLEDGMENTS

We thank S. Kato and P. Chambon for providing RXR expression DNAs, and we thank M. Banno and M. Ohotsuka for technical assistance.

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas and by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. **Au-Fliegner, M., E. Helmer, J. Casanova, B. M. Raaka, and H. H. Samuels.** 1993. The conserved ninth C-terminal heptad in thyroid hormone and retinoic acid receptors mediates diverse response by affecting heterodimer but not homodimer formation. Mol. Cell. Biol. **13:**5725–5737.
2. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56:**335–344.
3. **Benbrook, D., E. Lernhardt, and M. Pfahl.** 1988. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature (London) **333:**669–672.
4. **Berkenstam, A., M. M. V. Ruiz, D. Barettino, M. Horikoshi, and H. G. Stunnenberg.** 1992. Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. Cell **69:**401–412.
5. **Brand, N., M. Petkovich, A. Krust, P. Chambon, H. de The, A. Marchio, P. Tiollais, and A. Dejean.** 1988. Identification of a second retinoic acid receptor. Nature (London) **332:**850–853.
6. **Brinkman, A. O., and J. Trapman.** 1992. Androgen receptor mutants that affect normal growth and development. Cancer Surv. **14:**95–111.
7. **Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg.** 1992. RXRa, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11:**1409–1418.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
9. **Chen, H.-W., Z., and M. L. Privalsky.** 1993. The *ervA* oncogene represses the action of both retinoid X and retinoid A receptors but does so by distinct mechanisms. Mol. Cell. Biol. **13:**5970–5980.
10. **Damm, K., R. Heyman, K. Umezono, and R. M. Evans.** 1993. Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants. Proc. Natl. Acad. Sci. USA **90:**2989–2993.
11. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature (London) **339:**593–597.
12. **de The, H., C. Lavau, A. Marchio, C. Chomienne, L. Degos, and A. Dejean.** 1991. The PML-RARα fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell **66:**675–684.
13. **de The, H., M. M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, and A. Dejean.** 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. Nature (London) **343:**177–180.
14. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240:**889–895.
15. **Forman, B. M., J. Casanova, B. M. Raaka, J. Ghysdael, and H. H. Samuels.** 1992. Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers. Mol. Endocrinol. **6:**429–442.
16. **Forman, B. M., and H. H. Samuels.** 1990. Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol. Endocrinol. **4:**1293–1301.
17. **Forman, B. M., C. R. Yang, M. Au, J. Casanova, J. Ghysdael, and H. H. Samuels.** 1989. A domain containing leucine zipper like motifs mediate novel in vivo interactions between the thyroid hormone and retinoic acid receptors. Mol. Endocrinol. **3:**1610–1626.
18. **Gearing, K. L., M. Gottlicher, M. Teboul, E. Widmark, and J.-A. Gustafsson.** 1993. Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor. Proc. Natl. Acad. Sci. USA **90:**1440–1444.
19. **Giguere, V., E. S. Ong, P. Segui, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) **330:**624–629.
20. **Giguere, V., M. Shago, R. Zirngibl, P. Tate, J. Rossant, and S. Varmuza.** 1990. Identification of a novel isoform of the retinoic acid receptor γ expressed in the mouse embryo. Mol. Cell. Biol. **10:**2335–2340.
21. **Glass, C. K., O. V. Devary, and M. G. Rosenfeld.** 1990. Multiple cell-type specific proteins differentially regulate target sequence recognition by the a retinoic acid receptor. Cell **63:**729–738.
22. **Glass, C. K., R. Franco, C. Weinberger, V. R. Albert, R. M. Evans, and M. G. Rosenfeld.** 1987. A c-erb-A binding site in rat growth hormone gene mediates transactivation by thyroid hormone. Nature (London) **329:**738–741.
23. **Glass, C. K., J. M. Holloway, O. V. Devary, and M. G. Evans.** 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. Cell **54:**313–323.
24. **Glass, C. K., S. M. Lipkin, O. V. Devary, and R. M. Evans.** 1989. Positive and negative regulation of transcription by a retinoic acid-thyroid hormone receptor heterodimer. Cell **59:**697–708.
25. **Hall, B. L., Z. Smit-McBride, and M. L. Privalsky.** 1993. Reconstitution of retinoid X receptor function and combinatorial regulation of other nuclear hormone receptors in the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **90:**6929–6933.
26. **Hamada, K., S. L. Gleason, B.-Z. Levi, S. Hirschfeld, E. Appella, and K. Ozato.** 1989. H-2RIIBP, a member of the nuclear hormone receptor super-

family that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. Proc. Natl. Acad. Sci. USA **86:**8289–8293.
27. **Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller.** 9-cis retinoic acid is a high affinity ligand for the retinoic X receptor. Cell **68:**397–406.
28. **Horowitz, K. B.** 1992. Cellular heterogeneity and mutant estrogen receptors in hormone resistant breast cancer. Cancer Surv. **14:**41–54.
29. **Ishikawa, T., K. Umezono, D. J. Mangelsdorf, H. Aburatani, B. Z. Stanger, Y. Shibatani, M. Imawari, R. M. Evans, and F. Takaku.** 1990. A functional retinoic acid receptor encoded by the gene on human chromosome 12. Mol. Endocrinol. **4:**837–844.
30. **Kakizuka, A., W. H. Miller, Jr., K. Umezono, R. P. Warell, Jr., S. R. Frankel, V. V. Murty, E. Dmitrovsky, and R. M. Evans.** 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor. Cell **66:**663–674.
31. **Kastner, P., A. Krust, C. Mendelsohn, J. M. Garnie, A. Zelent, P. Leroy, A. Staub, and P. Chambon.** 1990. Murine isoforms of retinoic acid receptor γ with specific patterns of expression. Proc. Natl. Acad. Sci. USA **87:**2700–2704.
32. **Kessel, M., and P. Gruss.** 1991. Homeotic transformations of murine vertebrae and concomitant alternation of Hox codes induced by retinoic acid. Cell **67:**89–104.
33. **Kliewer, S. A., K. Umezono, D. J. Mangelsdorf, and R. M. Evans.** 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signaling. Nature (London) **355:**446–449.
34. **Krust, A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon.** 1989. A third human retinoic acid receptor, hRAR-γ. Proc. Natl. Acad. Sci. USA **86:**5310–5314.
35. **Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Sauders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68:**377–395.
36. **Leroy, P., A. Krust, A. Zelent, C. Mendelsohn, J.-M. Garnier, P. Kastner, A. Dierich, and P. Chambon.** 1991. Multiple isoforms of the mouse retinoic acid receptor a are generated by alternative splicing and differential induction by retinoic acid. EMBO J. **10:**59–69.
37. **Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosacowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J. F. Grippo.** 1992. 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature (London) **355:**359–361.
38. **Maden, M.** 1882. Vitamin A and pattern formation in the regenerating limb. Nature (London) **295:**672–675.
39. **Mangelsdorf, D. J., U. Borgmeyer, R. A. Heyman, J. Y. Zhou, E. S. Ong, A. E. Oro, A. Kakizuka, and R. M. Evans.** 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. **6:**329–344.
40. **Mangelsdorf, D. J., and R. M. Evans.** 1992. Retinoid receptors as transcription factors, p. 1137–1167. *In* S. McKnight and K. Yamamoto (ed.), Transcriptional regulation, vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans.** 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) **345:**224–229.
42. **Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato.** 1992. H-2RIIBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. **11:**1419–1435.
43. **Matsui, T.** 1989. Novel regulation of transcription initiation of the peptide IX gene of adenovirus 2. Mol. Cell. Biol. **10:**4265–4271.
44. **Naar, A. M., J. M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Hollowey, C. K. Glass, and M. G. Rosenfeld.** 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell **65:**1267–1279.
45. **Okano, R., T. Mita, and T. Matsui.** 1992. Characterization of a novel promoter structure and its transcriptional regulation of the laminin B1 gene. Biochim. Biophys. Acta **1132:**49–57.
46. **Petkovich, M., N. J. Brand, A. Krust, and P. Chambon.** 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (London) **330:**444–450.
47. **Pratt, M. A. C., J. Kralova, and M. W. Mcburney.** 1990. A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. Mol. Cell. Biol. **10:**6445–6453.
48. **Roberts, A. B., and M. B. Sporn.** 1984. Cellular biology and biochemistry of the retinoids, p. 209–286. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), The retinoids, vol. 2. Academic Press, New York.
49. **Rosa, F. W., A. L. Wilk, and F. O. Kelsey.** 1986. Teratogen update: vitamin A congeners. Teratology **33:**355–364.
50. **Sap, J., A. Munoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennstrom.** 1986. The c-erb A protein is a high affinity receptor for thyroid hormone. Nature (London) **324:**635–640.
51. **Sap, J., A. Munoz, J. Schmitt, H. G. Stunnenberg, and B. Vennstrom.** 1989. Repression of transcription mediated by a thyroid hormone response element by the v-erb A oncogene product. Nature (London) **340:**242–244.
52. **Sporn, M. B., and A. B. Robert.** 1983. Role of retinoids in differentiation and carcinogenesis. Cancer Res. **43:**3034–3040.
53. **Strickland, S., K. K. Smith, and K. R. Marotti.** 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell **21:**347–355.
54. **Sucov, H. M., K. K. Murakami, and R. M. Evans.** 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. Proc. Natl. Acad. Sci. USA **87:**5392–5396.
55. **Tini, M., G. Otulakowski, M. L. Breitman, L.-C. Tsui, and V. Giguere.** 1993. An everted repeat mediates retinoic acid induction of the γF-crystallin gene: evidence of a direct role for retinoids in lens development. Genes Dev. **7:**295–307.
56. **Umesono, K., K. K. Murakami, C. C. Tompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65:**1255–1266.
57. **Usala, S. J., G. E. Tennyson, A. E. Bale, R. W. Lash, N. Gesundheit, F. E. Wondisford, D. Accili, P. Hause, and B. D. Weintraub.** 1990. A base mutation of the c-erb A thyroid hormone receptor in a kindred with generalized thyroid hormone resistance. J. Clin. Invest. **85:**93–100.
58. **Vasios, G. W., J. D. Gold, M. Petkovich, P. Chambon, and L. J. Gudas.** 1989. A retinoic acid-responsive element is present in the 5′ flanking region of the laminin B1 gene. Proc. Natl. Acad. Sci. USA **86:**9099–9103.
59. **Vasios, G. W., S. Mader, J. D. Gold, M. Lutz, M. P. Gaub, P. Chambon, and L. J. Gudas.** 1989. The late retinoic acid induction of laminin B1 gene transcription involves RAR binding to the responsive element. EMBO J. **10:**1149–1158.
60. **Yu, V. C., C. Delsert, B. Anderson, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kimm, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXRβ: a co-regulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67:**1251–1266.
61. **Zechel, C., X.-Q. Shen, J.-Y. Chen, Z.-P. Chen, P. Chambon, and H. Gronemeyer.** 1994. The dimerization interfaces formed between the DNA binding domains of RXR, RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. EMBO J. **13:**1425–1433.
62. **Zelent, A., A. Krust, M. Petkovich, P. Kastner, and P. Chambon.** 1989. Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. Nature (London) **339:**714–717.
63. **Zelent, A., C. Mendelsohn, P. Kastner, A. Krust, J.-M. Garnier, F. Ruffenach, P. Leroy, and P. Chambon.** 1991. Differentially expressed isoforms of the mouse retinoic acid receptor β are generated by usage of two promoters and alternative splicing. EMBO J. **10:**71–81.
64. **Zenke, M., A. Munoz, J. Sap, B. Vennstrom, and H. Boug.** 1990. v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. Cell **61:**1035–1049.
65. **Zhang, X-K., B. Hoffmann, P. B.-V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **355:**441–446.
66. **Zhang, X-K., G. Salbert, M.-O. Lee, and M. Pfahl.** 1994. Mutations that alter ligand-induced switches and dimerization activities in the retinoid X receptor. Mol. Cell. Biol. **14:**4311–4323.