Evidence for Impaired Retinoic Acid Receptor-Thyroid Hormone Receptor AF-2 Cofactor Activity in Human Lung Cancer

NADEEM MOGHAL* AND BENJAMIN G. NEEL

Molecular Medicine Unit, Beth Israel Hospital, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02215

Received 31 January 1995/Returned for modification 27 February 1995/Accepted 24 April 1995

Retinoic acid (RA) is required for normal airway epithelial cell growth and differentiation both in vivo and in vitro. One of the earliest events following the exposure of bronchial epithelial cells to RA is the strong induction of RA receptor beta (RAR β) mRNA. Previous work established that many lung cancer cell lines and primary tumors display abnormal RAR β mRNA expression, most often absence or weak expression of the RAR β 2 isoform, even after RA treatment. Restoration of RAR β 2 into RAR β -negative lung cancer cell lines has been reported to inhibit tumorigenicity. Since RAR β 2 inactivation may contribute to lung cancer, we have investigated the molecular mechanism of defective RAR β 2 expression. Nuclear run-on assays and transient transfections with RAR β 2 promoter constructs indicate the presence of *trans*-acting transcriptional defects in most lung cancer cell lines, which map to the RA response element (RARE). These defects cannot be complemented by RAR-retinoid X receptor cotransfection and can be separated into two types: (i) one affecting transcription from direct repeat RAREs, but not palindromic RAREs, and (ii) another affecting transcription from both types of RARE. Studies using chimeras between RAR α , TR α , and other transcription factors suggest the existence of novel RAR-thyroid hormone receptor AF-2-specific cofactors, which are necessary for high levels of transcription. Furthermore, these factors may be frequently inactivated in human lung cancer.

Retinoic acid (RA) is a retinol (vitamin A) metabolite that exerts profound effects on cell growth and differentiation (106). In the trachea and bronchi, an RA-sensitive stem cell gives rise to mucosecretory and ciliated cells (35, 45, 105). In vivo, vitamin A deficiency results in replacement of mucociliary epithelium with keratinized squamous epithelium (79, 126). Squamous metaplasia is reversed if vitamin A is restored to the diet (80). Squamous metaplasia also occurs during the early stages of lung carcinogenesis, raising the possibility that perturbations in retinoid signaling contribute to pathogenesis (88). This requirement of RA for proper mucociliary differentiation can be reproduced in vitro. If grown in the absence of retinoids, cultures of airway epithelial cells undergo squamous differentiation (47, 103). Physiological concentrations (nanomolar) of RA prevent the squamous phenotype (46, 47, 103) and, in the presence of the appropriate extracellular matrix, trigger mucosecretory differentiation (104, 128). Since lung cancers display defective control of differentiation, understanding the molecular details of retinoid action in these pathways may yield insights into physiological and pathophysiological processes.

Much progress has been made in understanding RA signaling. There are at least two biologically active RA stereoisomers, all-*trans*- and 9-*cis*-RA (37, 63), and two classes of retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (60). RARs bind all-*trans*- and 9-*cis*-RA (1, 37), whereas RXRs bind only 9-*cis*-RA (1, 37, 63). Both receptors are members of the nuclear hormone receptor superfamily of sequence-specific, ligand-activated transcription factors (8, 23). These receptors have modular structures, consisting of domains A to F. The C domain, also known as the DNA binding domain (DBD), directs DNA binding to specific sequences, termed hormone response elements. The E, or

hormone binding domain (HBD), confers ligand binding, as well as a ligand-dependent transactivation function (AF-2). The A-B domain contains a ligand-independent transactivation function (AF-1), which at least for some members of this family has been shown to modulate the activity of AF-2 in a cell-type- and promoter-specific manner (24, 93, 94, 118, 119). The D domain probably functions as a flexible hinge between the DBD and HBD, although it also has a direct role in DNA binding in some family members (55, 59, 100, 125, 131). The function of the F domain is unknown. In mammalian cells, there are three RAR genes (termed α , β , and γ) (9, 12, 17, 27, 53, 99) and three RXR genes (33, 61, 74, 75, 129). Further complexity is generated by alternative splicing, resulting in isoforms differing only in their A-B domains (48, 61, 62, 66, 95, 132). Such isoforms have identical DNA binding specificity but may display distinct promoter and cell type transcriptional capabilities.

Retinoids stimulate transcription from target genes containing specific hormone response elements, termed RA response elements (RAREs). Three types of RARE have been identified. Most consist of direct repeat (DR) arrangements of the core half-site 5'-AGGTCA-3' or a highly related half-site (19, 39, 111). Other RAREs consist of inverted (palindromic [PAL]) (120) or everted (102, 117) arrangements of the same or related half-sites. High-affinity binding of an RAR to any of these RAREs in vitro requires heterodimerization with an RXR (51, 61, 77, 117, 129, 133). Likewise, strong transactivation of RARE-containing reporter constructs in vivo requires coexpression of RARs and RXRs (32, 36, 51, 117, 129, 133).

The precise biological functions of the RARs are not well defined. Gene disruption experiments suggest that they perform both unique and redundant functions (11, 64, 67, 68, 70, 83, 84). Several lines of evidence suggest that RAR β plays an important role in normal lung development. In vivo, it is highly expressed in adult lung tissue (26) and in the upper airway epithelium of developing mice (20). In addition, its expression in airway epithelia begins at the onset of mucociliary differen-

* Corresponding author. Phone: (617) 667-2901. Fax: (617) 667-2913.

tiation (20). In vitro, one of the earliest events to occur during airway epithelial cell exposure to RA is the induction of RAR β mRNA (26, 96). Furthermore, the level of induction is tightly correlated with the ability of the cells to undergo mucosecretory differentiation (87).

We (26) and others (40, 96) reported that RAR β is abnormally expressed in approximately 50% of human lung cancer cell lines and in a comparable number of primary tumors (26, 69). Most RAR β expression abnormalities involve reduced or absent expression of the RAR β 2 isoform, the most abundant isoform in normal human lung tissue (41, 87). One-third of lung cancer cell lines do not express RAR β 2, even after exposure to RA (26). Recent in situ hybridization studies confirm that up to 50% of primary tumors lack RAR β expression and that loss of expression is an early event in lung carcinogenesis (69). Restoration of RAR β 2 into RAR β -negative lung cancer cell lines has been reported to inhibit tumorigenicity in nude mice (42). In addition, introduction of RAR β 2 into RAR β -negative HeLa cells inhibits cell proliferation in vitro in an RA-dependent manner (25). Although RAR β 2 does not appear to be necessary for normal lung development in the mouse (84), it is a relatively minor RAR β isoform in murine lung tissue (132) in contrast to human lung tissue (41, 87). Since absence of the predominant RAR β isoform could contribute to the pathogenesis of lung cancer, we sought to uncover the molecular mechanism through which RAR β 2 expression is lost.

Using nuclear run-on studies and transient transfections, we have identified at least two types of *trans*-acting transcriptional defects that prevent lung cancer cell lines from expressing high levels of RAR β after exposure to RA. One type of defect results in a selective impairment of RA-dependent transcription from DR RAREs, but not PAL RAREs, whereas the other type of defect impairs RA-dependent transcription from both types of RARE. Using receptor chimeras, we have determined that at least one of the defects clearly blocks the activity of the RAR and thyroid hormone receptor α (TR α) ligand-dependent transactivation function, AF-2. Our data define different mechanisms for ligand-dependent transcription by RARs on DR versus PAL RAREs and strongly suggest the existence of novel factors, distinct from RXRs and the general transcription machinery, which are necessary for high levels of hormone-receptor-mediated transcription. Furthermore, these factors may be frequently inactivated in human lung cancer.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized on a MilliGen Cyclone Plus DNA synthesizer. Nytran filters were purchased from Schleicher and Schuell. Tissue culture media and serum were purchased from Gibco. All-*trans*-RA and activated charcoal were purchased from Sigma. 9-*cis*-RA was obtained from Hoffmann-La Roche, Nutley, N.J. Triiodothyronine (thyroid hormone [T3]) was purchased from Aldrich. Luciferin was purchased from Analytical Luminescence Laboratory. [*acetyl*-³H]coenzyme A and Econofluor-2 were purchased from Dupont/NEN.

Plasmids. RAR β promoter constructs were derived from human RAR β 2 promoter sequences contained within plasmid pD1 (19). pD1 was digested with *Bgl*II-*Bam*HI, and the fragment spanning -747 to +156 of the RAR β 2 promoter was ligated into the *Bam*HI site of pBluescript II SK⁺ (pBSSK) (Stratagene) to generate pB747. pX60 was constructed by *Sma*I-*Sac*I digestion of pB747 and ligation of the fragment spanning -60 to +156 of the RAR β 2 promoter into the *Sma*I-*Sac*I sites of the luciferase vector pXP2 (97). pB5 was generated by removal of the 903-bp insert in pB747 by *Eco*RI digestion and replacement with the 5-kb *Eco*RI promoter fragment from pD1. pX5 was made by ligating the 5-kb *Hind*III-*Sac*I fragment from pB5 into the *Hind*III-*Sac*I sites of pXP2. pBRAREtk was made by annealing the oligonucleotides 5'-AGCTGGGTAGG GTTACCGAAAGTTCACTCG-3' and 5'-TCGACGAGTGAACITTCGGT GAACCTACCC-3' and ligating the resulting duplex into the *Hind*III-*Xho*I sites of pTK109luc (97). A region of pTK147 (123), containing the alcohol dehydrogenase promoter (ADH3) RARE and the herpes simplex virus thymidine kinase (TK) promoter, was amplified by PCR with the primer pair 5'-CGGGG

TACCATGATTACGAATTCGGAG-3' and 5'-GTGTAAGCTTGGTGTTCG AGGCCACACGCG-3', digested with *Kpn*I-*Hind*III, and ligated into the *Kpn*I-*Hind*III sites of the luciferase vector pA₃LUC (127), generating pADH3. pX60 DR4 was constructed by amplification of the region spanning -60 to +156 from the RAR β 2 promoter by two sequential rounds of PCR (5'-AGGTCACAG GAGGTCACTCGCATATATTAGGC-3' and 5'-GAATGCGTTCCGGATC CTACCCC-3', followed by 5'-CGGGGTACCTTCAGGTACAGGAGGT CA-3' and 5'-GAATGCGTTCCGGATCCTACCCC-3') and ligation of the *Kpn*I-*Bam*HI-digested final product into the *Kpn*I-*Bgl*II sites of pXP2. pGAL4tkCAT (110) was digested with *Hind*III and *Bgl*II, and the fragment containing the five GAL4 binding sites along with the TK promoter was ligated into the *Hind*III-*Bgl*II sites of pXP2, generating pGAL4₅tk. Plasmids pBRARE₃tk (19) and pPAL₂tk (92) contain hormone response elements cloned upstream of a minimal TK promoter (-109 to +52) in luciferase expression vectors. pBRARE₃tk contains three copies of the RARE from the RAR β 2 promoter, and pPAL₂tk contains two copies of the PAL element 5'-AGGTCAT GACCT-3'.

The TR DBD-RAR HBD chimera, encoded in the expression vector pJTR α RAR α , was constructed as follows. An *Eco*RI fragment from pHK1 (27), containing the human RAR α coding region, was ligated into the *Eco*RI site of pBSSK to yield pBRAR α . The primer set 5'-CGGGATCCTTGTGGGGGAGC TCATT-3' and 5'-CAGCGGCTCTGCAGCATGTCC-3' was used to amplify a region of RAR α from pBRAR α , beginning with the nucleotides encoding amino acid 184 and ending at the most proximal internal 3' *Pst*I site. This fragment was digested with *Bam*HI and *Pst*I and ligated with the *Pst*I-*Hind*III fragment from pBRAR α , containing the complementary portion of the 3' end of the coding region, into the *Bam*HI-*Hind*III sites of pBSSK, yielding pBRAR-DEF. The murine TR α DBD (amino acids 1 to 150) was amplified from pBS13 (13) with the primer set 5'-CGGAATTCACCATGGAAAGCAAGCAAG-3' and 5'-CGCGGATCCCCATCTCCTCTTCG-3', digested with *Eco*RI and *Bam*HI, and ligated into the *Eco*RI-*Bam*HI sites of pBSSK to yield pBTRABC. The RAR α HBD, obtained as a *Bam*HI fragment from pBRARDEF, was ligated into the *Bam*HI-*Bgl*II sites of the expression vector pJ3 (89) to yield pJRAR-DEF. Finally, the TR α DBD was excised from pBTRABC by *Bam*HI-*Hind*III digestion and was ligated into the *Bam*HI-*Hind*III sites of pJRARDEF. The resulting plasmid, pJTR α RAR α , encodes the first 150 amino acids of murine TR α fused to the carboxy terminus of human RAR α (amino acids 184 to 462) in a pJ3 backbone. As a result of the subcloning, a stretch of three amino acids (glycine, isoleucine, and leucine) separates the two receptor domains.

The RAR DBD-TR HBD chimera encoded in the expression vector, pLC7 Δ SRAR α TR α , was constructed as follows. The primer set 5'-CGCGGA TCCATTCGCTCACTGCAG-3' and 5'-GTGTCTCTGACGCCATCTTTG AA-3' was used to amplify a region of murine TR α from pBS13, beginning with the nucleotides encoding amino acid 151 and ending with the most proximal internal 3' *Bsa*HI site. This fragment was digested with *Bam*HI and *Bsa*HI and ligated along with the *Bsa*HI-*Sall* fragment from pBS13, encoding the remainder of the 3' end of the coding region, into the *Bam*HI-*Sall* sites of pUC19 (pUC TRDEF). A region of human RAR α encoding the first 183 amino acids was amplified from pBRAR α with primers 5'-TGCTCTAGACCCTGCCAGACT GTC-3' and 5'-ACGCTGACGCCGAGGATCCGCG-3' and was digested with *Xba*I and *Bam*HI. The TR α HBD was removed from pUCTRDEF by *Bam*HI-*Hind*III digestion and was ligated with the *Xba*I-*Bam*HI-digested RAR α PCR product into the *Avr*II-*Hind*III sites of pLC7 Δ SR α . The resulting clone was digested with *Bam*HI, filled in with Klenow fragment, and self-ligated to generate the final clone, pLC7 Δ SRAR α TR α , which encodes the first 183 amino acids of human RAR α fused to the carboxy terminus of murine TR α (amino acids 151 to 410). As a result of the subcloning, a stretch of three amino acids (leucine, glycine, and serine) separates the two receptor domains. The parental vector pLC7 Δ SR α will be described in more detail elsewhere. Briefly, it is derived from the retroviral vector pLNSX (85) and has a portion of the human CD7 gene substituted for the neomycin resistance gene.

To construct pJVP16RAR α , encoding a VP16-RAR α fusion protein, a region of DNA encoding amino acids 410 to 490 of VP16 was amplified from plasmid pRB3441 (81) with primers 5'-CGGGGTACCTGTGCGACGGCCCCCGGA CC-3' and 5'-CGCGGATCCCCACCGTACTCGTCAATTCC-3', and a region of the RAR α cDNA spanning nucleotides 351 to 772 was similarly amplified from pHK1 with primers 5'-CGCGGATCCATCTACAAAGCCTTGCTTTG TC-3' and 5'-GGTCAATGTCCAGAGACACGTTGTTC-3'. These fragments were digested with *Kpn*I-*Bam*HI and *Bam*HI-*Sst*I, respectively. A *Bam*HI fragment containing the entire RAR α open reading frame was released from pBRAR α and ligated into the *Bam*HI-*Bgl*II sites of pJ3 to generate pJRAR α . Finally, an internal portion of the RAR α coding region was removed from pJRAR α by *Kpn*I-*Sst*I digestion and replaced with a fusion between the VP16 and RAR α PCR products. This expression vector encodes a fusion protein that begins with the first 19 amino acids of RAR α , followed by amino acids 410 to 490 of VP16 fused to amino acids 84 to 462 of RAR α . As a consequence of the subcloning, a glycine and a serine residue are inserted between the last VP16 amino acid and amino acid 84 of RAR α .

To construct pGAL4RAR α , DNA encoding amino acids 184 to 351 of RAR α was amplified from pBRAR α , with primers 5'-CCGGAATTCGTGGGGGA GCTCATTTAG-3' and 5'-CCGGAATTCACGGGATCTCAGTCTTCAG-3'. This PCR product was digested with *Eco*RI and *Pst*I and ligated along with a

PstI-HindIII fragment from pBRAR α (encoding the receptor carboxy terminus) into the *EcoRI-HindIII* sites of pBSSK. A fragment encoding the RAR α HBD, including the RAR α stop codon, was released by *EcoRI-BamHI* digestion of this clone and subcloned into the *EcoRI-BamHI* sites of pGAL4VP2 (49), which encodes a GAL4 DBD (amino acids 1 to 147) fusion to two copies of the VP16 transactivation domain (amino acids 413 to 454). pGAL4RAR α encodes a fusion of the GAL4 DBD (amino acids 1 to 147) to the RAR α HBD (amino acids 184 to 462) and is separated from downstream VP16 sequences by the RAR α translation stop codon. To obtain a fragment encoding the TR α HBD for the expression clone pGAL4TR α , pPUCTRDEF was digested with *HindIII*, filled in with Klenow fragment, and then digested with *BamHI*. The fragment encoding amino acids 151 to 410 of the TR α HBD was then ligated into pGAL4VP2, which had been previously digested with *XbaI*, filled in with Klenow fragment, and digested with *BamHI*.

pCDMRAR α (102) and pCDMRXR β (88a) contain cDNAs for human RARs and RXRs, respectively, cloned into pCDM8 (108). pLTrE2BP is a retroviral expression vector containing the human TATA-binding protein (TBP) cDNA (136), and pEIA contains the 13S viral EIA clone (35a) inserted into pCMV-Neo-Bam (4). pEFCAT (86) contains the human elongation factor 1- α promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene.

Cell culture. Cell lines were obtained from the American Type Culture Collection (Calu6, Calu1, Calu3, H441, SK-MES-1, ChaGo K-1, H596, and H146); A. Gazdar, University of Texas Southwestern Medical Center (H661 and H322); and S. Bernal, Boston University School of Medicine (U1752). They were maintained in either Dulbecco modified Eagle medium (DMEM) or RPMI supplemented with 10% fetal calf serum (FCS).

Nuclear run-on assays. Nuclear run-on assays were performed essentially as described previously (3). Cells grown in 150-mm dishes were exposed to 1 μ M RA for 4 h prior to isolation of nuclei by Nonidet P-40 lysis. Each transcription reaction mixture typically contained 5 mM Tris-HCl (pH 8.0); 150 mM KCl; 2.5 mM MgCl₂; 2.5 mM dithiothreitol; 250 μ M (each) ATP, CTP, and GTP; 2×10^7 nuclei; and 30 μ l of 800-Ci/mmol [α -³²P]UTP, in a final volume of 300 μ l. Hybridizations to Nytran filters containing slot blotted DNA (10 μ g per plasmid) were performed at 65°C for 2 days. Filters were washed twice at room temperature in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min and once at 60°C in $0.1 \times$ SSC–0.1% SDS for 30 min before being exposed in a PhosphorImager cassette (Molecular Dynamics). DNAs used for hybridization included pBR327, containing the full-length human RAR β 2 cDNA cloned into pBR327 (17); p β actin, containing the human β actin cDNA cloned into pBR322 (30); and pBR327.

Transient transfections and reporter gene assays. Calu6 (1.5×10^6), H157 (5×10^6), Calu1 (1.2×10^6), SK-MES-1 (3×10^6), ChaGo K-1 (3.5×10^6), H441 (2×10^6), and H322 (2×10^6) cells were transfected on 100-mm dishes at the indicated optimal densities, with 15 μ g of reporter gene construct, 2 μ g of pEFCAT to control for transfection efficiency, and pBSSK to a total of 25 μ g of plasmid DNA. H322 (1.5×10^6), H661 (5×10^5), and CV1 (4×10^5) cells were transfected on 60-mm dishes at the indicated optimal densities with 6 μ g of reporter gene construct, 800 ng of pEFCAT, and pBSSK to a total of 10 μ g of plasmid DNA. Cells were seeded in medium containing 10% charcoal-stripped FCS at 18 to 24 h prior to transfection. All cells were refed with fresh DMEM–10% charcoal-stripped FCS at 1 to 4 h prior to transfection and were exposed to calcium phosphate-DNA coprecipitates (3) for 12 to 18 h. After transfection, cells were washed and refed with fresh medium containing charcoal-stripped serum and hormones. Where indicated, all-*trans*- or 9-*cis*-RA were added to final concentrations of 1 μ M, and T3 (thyroid hormone) was added to a final concentration of 10^{-8} M. Cell lysates were prepared for reporter gene assays 24 h after refeeding with hormones.

Charcoal stripping was required to observe strong inductions in luciferase activity in response to added hormones. Serum was charcoal stripped by the addition of 6 g of activated charcoal per 100 ml of serum, followed by constant stirring for 7 to 12 h at room temperature. Charcoal was removed by centrifugation at 7,500 rpm in a Sorvall GS-3 rotor for 30 min and filtration through a 0.2- μ m-pore-size Nalgene filter.

To analyze RA inducibility of pX5 in H146, both H146 and Calu6 were transfected with liposomes. H146 (24×10^6) suspension cells were transfected in 6.4 ml of RPMI in 100-mm dishes by the addition of a mixture of 15 μ g of pX5, 80 μ l (80 μ g) of lipofectin (Gibco/BRL), and 1.6 ml of RPMI. Calu6 (7×10^5) cells were transfected in 4 ml of DMEM in 60-mm dishes by the addition of 5 μ g of pX5–670 ng of pEFCAT–6 μ l (6 μ g) of lipofectin contained in 600 μ l of DMEM. After 5 h, H146 cells were diluted by the addition of 8 ml of RPMI containing 10% charcoal-stripped FCS and split into two parallel cultures, one of which was exposed to 1 μ M all-*trans*-RA, whereas Calu6 cells were refed with 4 ml of DMEM containing 10% charcoal-stripped FCS and either 1 μ M all-*trans*-RA or an equivalent volume of the retinoid solvent, dimethyl sulfoxide. Cell lysates were prepared for reporter gene assays 24 h later.

Transfected cells were washed once and collected in phosphate-buffered saline, transferred to Eppendorf tubes, and pelleted by centrifugation in a microcentrifuge (Baxter) at 2,000 rpm for 10 min at 4°C. Pellets were subjected to three rounds of freeze-thaw lysis in 100 to 150 μ l of 100 mM Tris-HCl (pH 7.5). Debris was removed by centrifugation in a microcentrifuge (Baxter) at 13,000 rpm for 5 minutes at 4°C, and the supernatants were used in reporter gene assays.

FIG. 1. Transcriptional defects in RAR β expression in lung cancer cell lines. Nuclear run-on transcription reactions were performed with nuclei from RAR β -negative (H322, H596, Calu1, and H661) and RAR β -positive (Calu6, Calu3, H460, and U1752) cell lines. Labeled probes (11×10^6 cpm, H322; 11×10^6 cpm, H596; 5×10^6 cpm, Calu1; 30×10^6 cpm, H661; 7.5×10^6 cpm, Calu6; 28×10^6 cpm, H460; 30×10^6 cpm, Calu3; 5×10^6 cpm, U1752) were hybridized to filters containing cDNAs for RAR β 2 and β actin and the control vector pBR327.

For luciferase assays, the volume of lysate was adjusted to 100 μ l by the addition of 100 mM Tris-HCl (pH 7.5), and then 300 μ l of assay buffer was added per sample, yielding final concentrations of 25 mM Gly-Gly (pH 7.8), 11 mM potassium phosphate (pH 7.5), 15 mM MgSO₄, 4 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 1 mM dithiothreitol, 1.5 mM ATP, and 0.25% Triton X-100. Following injection of 100 μ l of 1 mM luciferin, luciferase activity was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Fluor-diffusion CAT assays were performed by adjusting the volume of lysate to 50 μ l by the addition of 100 mM Tris-HCl (pH 7.5) and then 200 μ l of assay buffer, containing 0.5 μ Ci of [*acetyl*-³H]coenzyme A, yielding final concentrations of 80 mM Tris-HCl (pH 7.5) and 1 mM chloramphenicol. Reaction mixtures were overlaid with 3 ml of Econofluor-2, incubated at 37°C for 15 to 30 min, and then subjected to scintillation counting to quantitate the amount of acetylated chloramphenicol.

Luciferase activity is expressed as light units normalized to CAT activity (relative light units [RLU]), and data are plotted as the mean from duplicate plates $\pm$ the standard deviation from a representative experiment.

RESULTS

Defective RAR β 2 expression is due to a transcriptional mechanism(s). We previously identified many lung cancer cell lines that fail to express high levels of RAR β mRNA after exposure to RA (26). In other cells, such as HepG2 hepatocellular carcinoma cells (18) and F9 embryonal carcinoma cells (78), it has been shown that RA-dependent increases in RAR β mRNA are due to increased transcription. To determine whether transcriptional defects in RA-regulated RAR β gene expression occur in RAR β -negative lung cancer cell lines, we performed nuclear run-on studies. Full-length human RAR β 2 cDNA was slot blotted onto filters along with human β actin cDNA as a positive control for transcription and pBR327 as a negative control. Nuclei from RA-treated RAR β -positive cell lines (Calu6, Calu3, H460, and U1752) demonstrated strong RAR β - and β actin-specific transcription (Fig. 1). In contrast, nuclei from RA-treated RAR β -negative cell lines (H322, H596, Calu1, and H661) demonstrated no or weak RAR β -specific transcription relative to the signal from the pBR327 control (Fig. 1). β Actin-specific transcription, however, was clearly evident in these nuclei. These data demonstrate that transcriptional defects in RA-regulated RAR β expression occur in lung cancer cell lines.

Defective RAR β 2 transcription involves *cis*- and *trans*-acting mechanisms. Since RAR β 2 is the most abundant RAR β isoform in normal human lung tissue (41, 87), we investigated the molecular mechanism for its defective transcription in more detail. Defective RAR β 2 transcription could result from either *cis*- or *trans*-acting mechanisms. *cis*-acting mechanisms could result from mutations in positive control elements in the RAR β 2 promoter itself or from alterations in chromatin structure which prevent transcription factors from positively regulating this gene. *trans*-acting mechanisms could involve either

FIG. 2. Transcriptional defects are due to *trans*-acting mechanisms. A 5-kb fragment of the RAR β promoter fused to luciferase was transiently cotransfected with pEFCAT into the indicated cell lines. The cells were subsequently cultured in the absence or presence of all-*trans*-RA, before luciferase activity was measured and normalized to CAT activity. Fold inductions in normalized luciferase activity after RA treatment were calculated and are expressed as a percentage of the fold induction in Calu6. Data are expressed as the mean from duplicate plates $\pm$ the standard deviation. Also indicated are the histologic subtypes of the lung cancer cell lines. AP, anaplastic; SQ, squamous; SC, small cell; BA, bronchioalveolar; AC, adenocarcinoma; LC, large cell.

an absence of functional positive regulatory factors or an excess of negative regulatory factors.

To distinguish between *cis*- and *trans*-acting mechanisms, 5 kb of sequence upstream of the RAR β transcription start site was fused to the luciferase reporter gene to generate pX5. pX5 was transiently cotransfected with the control plasmid pEFCAT, in which CAT expression is driven by the translation elongation factor EF1- α promoter, into lung cancer cell lines displaying defective RAR β transcription. pEFCAT activity was used to normalize luciferase activity for variations in transfection efficiency in these and all subsequent transfection experiments. Initial experiments established that pEFCAT activity was not affected by RA treatment in any of the cell lines tested (data not shown). Failure of RA to induce reporter gene activity in RAR β -negative cell lines would indicate *trans*-acting defects, whereas induction of reporter gene activity by RA would suggest *cis*-acting defects. Although we previously described a large number of lung cancer cell lines that do not express RAR β mRNA (26), only a subset of these cells could be transfected with sufficient efficiency to permit analysis.

In Calu6, RA normally induces high levels of endogenous RAR β mRNA (26). As expected, luciferase activity from pX5 was also strongly induced (40-fold) by RA in Calu6. This level of induction is comparable to that observed with similar constructs in other cell lines displaying high levels of endogenous RAR β mRNA (19, 111). The 40-fold induction in luciferase activity in Calu6 was set as the standard with which inductions in several RAR β -negative cell lines were compared. There was a strong correlation between RA inducibility of reporter gene activity and RA inducibility of endogenous RAR β mRNA (Fig. 2). RA induces trace amounts of RAR β mRNA in H157, Calu1, SK-MES-1, and ChaGo K-1, whereas in H322 and H441, RA fails to induce any detectable message (26). Although H146 does not express detectable RAR β mRNA basally (26), the RA inducibility of the endogenous promoter in this cell line has not been examined. Nevertheless, our data suggest that impaired RA-dependent transcription accounts for the lack of RAR β expression in this cell line, as well as in

most of the other cell lines examined, and are consistent with the results obtained from the nuclear run-on studies (Fig. 1). Lack of RA responsiveness of pX5 in these cell lines is not likely to reflect cell-specific differences in the kinetics of the transcriptional response, since H322 fails to induce significant levels of luciferase activity when assayed at 12, 24, 48, and 72 h after RA treatment (data not shown). Rather, these lung cancer cell lines (but not all, e.g., H661; see Discussion) appear to contain genuine *trans*-acting transcriptional defects in retinoid responsiveness of the RAR β promoter.

The *trans*-acting defect(s) localizes to the RARE and cannot be rescued by cotransfection with RARs and RXRs. To better understand the nature of these *trans*-acting defects, we characterized the defect in the RAR β -negative cell line H322 in more detail. Within the RAR β promoter, a 17-bp RARE, consisting of an imperfect DR of the core sequence 5'-AGG TCA-3', confers high levels of RA-dependent transcription on heterologous promoters (19, 39, 111). The inability of RA to induce transcription from the transfected RAR β promoter in H322 could be mediated through the RARE, other upstream promoter elements, or both. To address this question, the RAR β promoter was truncated at -60 relative to the transcription start site and fused to the luciferase reporter gene, generating pX60. Since the RARE begins at -54 and ends only 6 bp away from the TATA box, this construct is effectively free of other upstream promoter elements and preserves the RARE in the context of its natural environment (see Fig. 5A). In addition to containing the RARE, pX60 also contains 184 bp of RAR β sequence downstream from the TATA box.

pX60 was transiently transfected into Calu6 and H322, and the cells were cultured in the absence or presence of RA (Fig. 3). In Calu6, RA treatment resulted in a 19-fold increase in luciferase activity, confirming the ability of RA to induce high levels of transcription from this isolated element. In H322, however, RA inducibility was severely diminished. Thus, the difference in RA-induced activity of the -5-kb construct between Calu6 and H322 is largely reproduced with a minimal RAR β promoter and does not involve elements upstream of the RARE.

RARs and RXRs are the best-characterized factors known to mediate activity from the RARE found in the RAR β promoter (β RARE). In vitro, the β RARE is bound with highest affinity by RAR-RXR heterodimers (51, 61, 129, 133). In both mammalian cells and in *Saccharomyces cerevisiae*, coexpression of RARs and RXRs enhances RA-dependent transcription from the β RARE and related RAREs (32, 36, 51, 129). We expected that the weak β RARE activity in H322 resulted from defective RAR and/or RXR activity. To investigate whether functional RARs or RXRs were limiting in H322, we cotransfected various combinations of RAR and RXR expression vectors with pX60 into these cells. Surprisingly, however, cotransfection of either RAR α or RAR β expression vectors over a wide range of concentrations with pX60 had only minimal effects on RA-induced transcription in H322 (Fig. 3 and data not shown). Furthermore, RA inducibility was not improved by cotransfection of either RXR α or RXR β expression vectors (Fig. 3 and data not shown). These data suggest that RARs and RXRs, alone or in combination, are not sufficient to mediate high levels of RA-dependent transcription from the β RARE in this cell line. In contrast to the results obtained in H322, cotransfection of these expression vectors with pX60 into CV1 cells significantly increased reporter gene expression both in the absence and in the presence of RA (Fig. 3A). Thus, our RAR-RXR expression vectors are capable of stimulating transcription from the β RARE in cells containing limiting amounts of RARs and RXRs. These data

FIG. 3. The *trans*-acting transcriptional defect(s) localizes to the RARE and cannot be complemented by RAR or RXR cotransfection. (A) pX60 was cotransfected with RAR and RXR expression vectors into the indicated cell lines. The expression vectors pCDMRARα, pCDMRARβ, and pCDMRXRβ were each used at a dose of 100 ng per 60-mm dish (H322, CV1) or 250 ng per 100-mm dish (Calu6), whereas pKCRRXRα was used at a dose of 1 μg per 60-mm dish (H322, CV1) or 2.5 μg per 100-mm dish (Calu6). Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of all-*trans*-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations. (B) pX60 was cotransfected with the indicated doses of expression vectors into H322 cells on 60-mm dishes. Further analysis was performed as described for panel A.

indicate that in H322 defective RA-induced transcription from the βRARE cannot be explained solely by limiting RARs, RXRs, or both.

Defective RAR activity is not due to limiting TBP and/or E1A-like activities and affects transcription mediated by both all-*trans*- and 9-*cis*-RA. Recently, it was reported that TBP and the 13S viral E1A protein synergize with RARs in transcriptional activation (10). The proposed mechanism for this synergy is that E1A serves as a bridge between RARs and TBP. E1A is believed to mimic a naturally occurring molecule(s), since endogenous E1A-like activity has been described in some mammalian cells (44, 56). To determine whether defective RAR activity on DR RAREs is due to limiting TBP and/or E1A-like activities, we cotransfected increasing amounts of RARα, human TBP, and 13S viral E1A expression vectors with pX60 (Fig. 4A). In this particular experiment, H322 was transfected with 2.5-fold less total DNA than Calu6 because H322 cells were transfected on 60-mm dishes and Calu6 cells were transfected on 100-mm dishes. Cotransfection of all three vectors had little effect on pX60 expression in Calu6 or H322. Thus, the defective activity in H322 appears to involve novel proteins, distinct from RARs, RXRs, E1A-like activity, and the general transcription factors.

It was recently reported that multiple RARα agonists, but

not an antagonist, induce a similar conformational change in at least one region of the receptor (50). This suggests that naturally occurring ligands, such as all-*trans*- and 9-*cis*-RA, may stimulate transcription through a similar mechanism. However, all-*trans*- and 9-*cis*-RA bind to two overlapping but distinct sites in the RAR HBD (116), suggesting that there may be subtle differences between the conformational changes induced by these two ligands. To better characterize the mechanism of transcriptional activation by these ligands, we examined the response of pX60 to 9-*cis*-RA in H322 and Calu6. In H322, 9-*cis*-RA (as was the case with all-*trans* RA [Fig. 3A]) was unable to induce significant transcription from pX60 (Fig. 4B), although in Calu6, 9-*cis*- and all-*trans*-RA induced comparable amounts of luciferase activity (Fig. 3A and 4B). These data indicate that the *trans*-acting defect(s) in lung cancer cells affects a common feature of transcription mediated by all-*trans*- and 9-*cis*-RA.

RAR activity is impaired on DR but not PAL RAREs. Recent studies focusing on the mechanism of RAR and TR heterodimerization with RXRs suggest that the heterodimers are configured differently, depending on the spacing between, and the arrangement of, the core half-sites (54, 73, 98, 131). Thus, an RAR-RXR heterodimer bound to a DR RARE is not likely to have the same overall conformation as an RAR-RXR heterodimer bound to a PAL RARE. If heterodimers are configured differently on different elements, they could use different mechanisms for ligand-dependent transactivation. Since RAR-RXR activity is impaired on the βRARE in H322, we asked whether this was a general defect or one that showed promoter specificity.

To compare the activities of receptors on different types of RARE, RAREs (Fig. 5A) were removed from potentially confounding natural promoter elements and placed upstream of the heterologous TK promoter in a luciferase reporter vector. pβRAREtk and pβRARE₃tk contain one or three copies of the βRARE, respectively, and pADH3tk contains a single copy of the ADH3 RARE (21). The ADH3 RARE consists of a DR arrangement of core half-sites, but the half-sites are slightly different in nucleotide sequence than those found in the βRARE and are oriented with opposite polarity (Fig. 5A). pPAL₂tk consists of two copies of an idealized thyroid hormone response element, with core half-sites in a PAL arrangement (29, 52). This element is positively regulated by both RAR-RXR and TR-RXR heterodimers (32, 133). These reporter constructs were transfected into Calu6 and H322, either

FIG. 4. Inability of TBP, E1A, or 9-*cis*-RA to restore activity to pX60. (A) pX60 was cotransfected with the indicated doses of pCDMRARα, pLTR-TBP, and pE1A expression vectors into Calu6 (100-mm dishes) and H322 cells (60-mm dishes). Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of all-*trans*-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations. (B) pX60 was cotransfected with RAR and RXR expression vectors into Calu6 and H322 cells as described for Fig. 3. Luciferase activity normalized to pEFCAT activity (RLU) from cells cultured in the absence or presence of 9-*cis*-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations.

FIG. 5. Structures of RAREs and fusion proteins. (A) Structures of RAREs used in transfection experiments. Core half-sites and TATA boxes are capitalized and in boldface. The polarity of core half-sites is indicated with arrows. "N" denotes the presence of additional nucleotides. (B) Structures of fusion proteins used in transfection experiments. Numbers in parentheses refer to amino acid sequence information.

alone or in combination with the indicated hormone receptor expression vector (Fig. 6).

The contribution of non- β RARE sequences in pX60 to its defective activity in H322 was assessed by transfecting p β RAREtk into this cell line. Even in the presence of a co-transfected RAR α expression vector, H322 was unable to support the same level of RA-dependent transcription as Calu6. However, the difference between the two cell lines in ligand-dependent transcription from this promoter was not as great as with the β RARE in the context of the natural RAR β 2 promoter. This suggested that sequences within the TK promoter alleviated some of the impairment in RAR activity in H322. To further characterize the intrinsic RA responsiveness of the isolated β RARE and to validate the existence of a defect which specifically affects activity from this element, the β RARE was trimerized and placed upstream of the TK promoter. Transfection of this construct into H322 and Calu6 clearly demonstrated severe impairment in retinoid responsiveness of isolated RAREs in H322, even in a heterologous promoter context. To determine whether the defect in H322 was specific for the β RARE or affected other types of RARE, pADH3tk and pPAL₂tk were also transfected into H322 and Calu6. Whereas H322 displayed substantially less RA-depen-

FIG. 6. RAR activity is impaired on DR but not PAL RAREs. The indicated RARE-containing luciferase constructs were cotransfected with 250 ng (per 100-mm dish) (Calu6) or 100 ng (per 60-mm dish) (H322) of pCDMRAR α expression vector. Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of all-trans-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations.

dent transcription from the DR RARE-based promoter (pADH3tk) than Calu6, it was completely unimpaired in its ability to support strong ligand-dependent transcription from the PAL RARE. Thus, H322 contains a very specific transcriptional defect that blocks the activity of RARs only on DR (pX60, p β RAREtk, p β RARE₃tk, and pADH3tk) but not PAL (pPAL₂tk) RARE-containing promoters.

RAR activity is impaired only in the context of the full-length receptor. Defective RAR activity on DR RAREs could reflect impairment in element-specific DNA binding or impairment in a downstream step in the transcriptional response. To map the RAR domain that displays defective function in H322, we took advantage of the modular structure of nuclear hormone receptors. By constructing chimeric receptors between RAR α and TR α , we examined the activity of the RAR α DBD and HBD in isolation. RARs and TRs bind as heterodimers with RXRs to the same core half-sites (5'-AGGTCA-3') (90, 121), with hormone receptor specificity on DR elements arising through receptor-specific preferences for spacing between half-sites (90, 121). RARs prefer spacings of 1, 2, or 5 bp (DR1, DR2, or DR5), and TRs prefer a spacing of 4 bp (DR4). Dimerization surfaces confined to the DBDs of these receptors dictate the DR spacing preference of the particular heterodimer (73, 98, 100, 130, 131), whereas dimerization surfaces within the HBDs appear to be universally used by RAR-RXR and TR-RXR heterodimers for solution dimerization (51) and for high-affinity binding to DR (61, 129) and PAL (55, 73, 133) hormone response elements. We constructed a chimera consisting of the RAR α amino terminus and DBD (amino acids 1 to 183) fused to the TR α HBD and carboxy terminus (amino acids 151 to 410). This chimeric receptor, RAR α TR α (Fig. 5B), should interact with RXRs and bind to DR5 hormone response elements, such as the DR RAREs described in Fig. 3, 5, and 6.

Three of the DR RAREs previously shown to have impaired activity in H322 (Fig. 3 and 6) were cotransfected with the RAR α TR α expression vector, and increases in luciferase activity were measured after exposure to thyroid hormone (T3). In the presence of wild-type RAR α , the difference in hormone-activated transcription from pX60 between Calu6 and H322 was 19-fold versus 5-fold (Fig. 3A). However, in the presence of the chimera, there was virtually no difference (6.5-fold versus 4.5-fold) (Fig. 7A). This suggests that the difference in transcriptional activity of full-length RAR α does not result from a difference in its DNA binding activity. Indeed, when tethered to the RAR α DBD, the TR α HBD also demonstrated comparable hormone-dependent transcription from the β RARE- and ADH3 RARE-containing TK promoters in H322 and Calu6 (Fig. 7A). Thus, the function of the RAR HBD, but not the DBD, appears to be compromised specifically on DR RAREs in H322.

FIG. 7. Analysis of RAR DBD activity in H322. (A) The indicated reporter constructs were cotransfected with either 2.5 μ g (per 100-mm dish) (Calu6) or 1 μ g (per 60-mm dish) (H322) of pLC7 Δ SRAR α TR α expression vector. Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of T3 is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations. (B) p β RARE3tk was cotransfected with 2.5 μ g of pJVP16RAR α expression vector per 100-mm dish of Calu6 and H322 cells. The experiment was performed as described for panel A, but with all-*trans*-RA. The left subpanel depicts an expanded scale for data obtained in the absence of RA.

Ligand-dependent transcriptional activity could be impaired at the level of hormone binding or at a downstream step. Defective ligand binding could be due to alterations in the activities of RA-metabolizing enzymes, RA storage proteins, or proteins that influence the hormone binding properties of the HBD through enzymatic or physical mechanisms. Alternatively, defects in post-ligand-binding events could involve an alteration in the activity of an accessory protein(s) necessary for transcriptional stimulation by the ligand-dependent transactivation function, AF-2. Since full-length RAR α is capable of RA-dependent transcription from pPAL₂tk (Fig. 6), it does not appear that H322 contains a defect affecting the activities of RA-metabolizing enzymes or RA storage proteins. Instead, our data suggest that the defect in H322 affects either the ligand-binding properties of RARs or the transcriptional activation properties of liganded RARs in an element-specific manner.

To confirm that RAR AF-2 activity is impaired in H322, we constructed the converse chimeric receptor. The amino terminus and DBD of TR α (amino acids 1 to 150) were fused to the HBD and carboxy terminus of RAR α (amino acids 184 to 462) (Fig. 5B). This chimeric receptor, TR α RAR α , should recognize DR4 hormone response elements while strongly interacting with RXRs through its DBD and HBD. The TR α RAR α expression vector was cotransfected with pPAL₂tk or p60DR4 into Calu6 and H322. p60DR4 is identical to pX60 but contains a single copy of a DR of the consensus 5'-AGGTCA-3' half-site separated by 4 bp instead of the DR5 β RARE (Fig. 5A). PAL and DR4 elements are both efficiently bound by TR-RXR heterodimers (61, 77, 133) and therefore should be recognized by TR α RAR α . As expected, both the full-length TR α and the TR α RAR α chimera demonstrated equivalent hormone-dependent transcriptional activity on the PAL RARE in Calu6 and H322 (Fig. 8A). When these receptors were cotransfected with p60DR4, full-length TR α again induced comparable levels of activity in response to hormone binding but, surprisingly, so did the TR α RAR α chimera (Fig. 8A). Interestingly, in the absence of hormone, the TR α RAR α chimera

FIG. 8. Analysis of RAR HBD activity in H322. (A) The indicated reporter constructs were cotransfected with 2.5 μ g (per 100-mm dish) (Calu6) or 1 μ g (per 60-mm dish) (H322) of pLC7 Δ STR α or pTR α RAR α expression vector. Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of T3 or all-*trans*-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations. (B) pGAL4₅tk was cotransfected with 2.5 μ g of pGAL4RAR α (left and middle subpanels) or pGAL4VP2 (right subpanel) expression vector per 100-mm dish of Calu6 and H322 cells. The experiment was performed as described for panel A, but with all-*trans*-RA. The left subpanel depicts an expanded scale for data obtained in the absence of RA. (C) pGAL4₅tk was cotransfected with 2.5 μ g of pGAL4TR α expression vector per 100-mm dish of Calu6 and H322 cells. The experiment was performed as described for panel A, but with T3 only. The left subpanel depicts an expanded scale for data obtained in the absence of T3.

repressed transcription threefold from pPAL₂tk in Calu6, whereas it stimulated transcription threefold from the same construct in H322. This differential repressing/activating activity of the TR α RAR α chimera in Calu6 and H322 may, therefore, still reflect a difference in RAR HBD configuration and/or activity in these two cell lines.

Since both the RAR α DBD and HBD have apparently normal activity when separated from each other and fused to the complementary portions of TR α , we suspected that the TR α sequences were altering the activities of one or both of these RAR domains from that normally observed in the native full-length RAR. We therefore fused the RAR α HBD to the completely heterologous GAL4 DBD (Fig. 5B). When cotransfected with a reporter construct containing five GAL4 binding sites upstream of a minimal TK promoter (Fig. 5A), GAL4 RAR α actually induced higher levels of activity in H322 than in Calu6, strongly demonstrating the transcriptional activating capabilities of this isolated region of RAR α in H322 (Fig. 8B, middle panel). Notably, in the absence of RA, GAL4RAR α strongly repressed basal transcription in Calu6 (24-fold), whereas it stimulated transcription in H322 (5-fold) (Fig. 8B, left panel). These data are in accordance with another report

TABLE 1. Repressing and activating properties of RAR and TR HBDs in lung cancer cell lines

Cell line	Repression ^a		Activation ^b		
	GAL4RAR α	GAL4TR α	GAL4RAR α	GAL4TR α	GAL4VP16
Calu6	100	100	100	100	100
H441	14	7.5	14	18	114
Calu1	10	8.5	24	14	107
ChaGo K-1	29	10	14	18	59
H322	0.8	6.8	184	185	540

^a Cells were transfected with the indicated constructs, and repression of reporter gene activity in the absence of ligand was calculated. The fold repression for each construct in Calu6 was assigned a value of 100, and the fold repression in the other cell lines is expressed relative to that observed in Calu6.

^b Cells were transfected with the indicated constructs, and activation of reporter gene activity in the presence of the appropriate ligand, or no ligand (GAL4VP16), was calculated. The fold activation for each construct in Calu6 was assigned a value of 100, and the fold activation in the other cell lines is expressed relative to that observed in Calu6.

which indicates that, when fused to the GAL4 DBD, the RAR α HBD exhibits cell-type-specific differences in ligand-independent repression (5). Thus, although the GAL4RAR α chimera exhibits strong RA-dependent transcriptional activity in both Calu6 and H322, its widely different activity on basal transcription suggests that the RAR α HBD still may be differentially configured or contacted in these cell lines and is consistent with the TR α RAR α chimera data described above.

Since the RAR α chimeras indicated that the defect in H322 affects only full-length RARs, we used a second approach to study the activities of the DBD and the ligand-independent (AF-1) and the ligand-dependent (AF-2) transactivation functions. We removed most of the A-B domain of RAR α , which contains AF-1 (93), and substituted the transactivation domain of VP16 (Fig. 5B). This region of VP16 confers transcriptional activation properties on heterologous DBDs, especially when assayed on multimerized response elements (49). Since our VP16RAR α fusion retains the RAR DBD and HBD, it should still bind RAREs through heterodimerization with RXRs, whereas the VP16 transactivation domain should stimulate higher levels of ligand-independent transcription than AF-1. Moreover, the VP16RAR α fusion should still be able to support ligand-dependent transactivation through AF-2 in the HBD. We, therefore, cotransfected an expression vector encoding this fusion protein with p β RARE₃tk into Calu6 and H322 and asked whether, in the absence of RA, this chimeric receptor could enhance transcription from this promoter. In the absence of RA, this receptor produced a threefold transcriptional stimulation in Calu6 and an eightfold stimulation in H322 (Fig. 7B, left panel). These data strongly support the RAR α TR α chimera data that the RAR α DBD can still bind to DR RAREs in H322 (Fig. 7A). The greater transcriptional stimulation by the VP16 chimera (in the absence of RA) in H322 probably reflects an intrinsically higher activity (approximately fivefold) (Fig. 8B, right panel, and Table 1) of the VP16 transactivation domain in H322. When the activity of the VP16RAR α fusion is normalized to this difference in VP16 activity, the fusion protein's activity is roughly equivalent in the two cell lines. Strikingly, RA dramatically enhances the transcriptional activity of VP16RAR α in Calu6 but only slightly stimulates its activity in H322 (Fig. 7B, right panel). Together, although we cannot completely rule out impaired DBD activity (see Discussion), our studies suggest a very specific defect in H322 that affects RAR AF-2 activity, only in the context of the full-length receptor.

Defective RAR activity on DR RAREs can occur through

FIG. 9. H441 displays a general defect in RAR and TR AF-2 activity. (A) The indicated reporter constructs were cotransfected with 250 ng of pCDM-RAR α expression vector per 100-mm dish of Calu6 and H441 cells. Luciferase activity normalized to CAT activity (RLU) from cells cultured in the absence or presence of all-*trans*-RA is shown. Data are expressed as the means from duplicate plates $\pm$ the standard deviations. (B) p β RARE₃tk was cotransfected with 250 ng of pCDMRAR α or 2.5 μ g of pJVP16RAR α expression vector per 100-mm dish of Calu6 or H441 cells. Further analysis was performed as described for panel A. The right subpanel depicts an expanded scale for data obtained in the absence of RA. (C) pGAL4₅tk was cotransfected with 2.5 μ g of pGAL4RAR α (left and middle subpanels) or pGAL4VP2 (right subpanel) expression vector per 100-mm dish of Calu6 and H441 cells. Further analysis was performed as described for panel A. The left subpanel depicts an expanded scale for data obtained in the absence of RA. (D) pGAL4₅tk was cotransfected with 2.5 μ g of pGAL4TR α expression vector per 100-mm dish of Calu6 and H441 cells, and analysis was performed as described for Fig. 8C. The left subpanel depicts an expanded scale for data obtained in the absence of T3.

different mechanisms. The RAR β -negative lung cancer cell line, H441, is also unable to support significant levels of RA-induced transcription from the transfected -5-kb RAR β 2 promoter (Fig. 2). To determine if the mechanism of defective RAR β transcription in H441 is similar to that in H322, we characterized H441's inability to support RA-dependent transcription in more detail. As with H322 (Fig. 3), H441 was also

unable to support high levels of RA-dependent transcription from the DR5-containing pX60, even in the presence of co-transfected RAR α (Fig. 9A). However, in contrast to H322, RAR activity was also somewhat impaired on the PAL RARE (Fig. 9A).

To determine whether RARs are capable of binding DR RAREs in H441, we asked whether RAR activity was also impaired on the multimerized β RARE-containing construct, p β RARE₃tk. As in H322 (Fig. 6), the responsiveness of this construct to RA was severely impaired in H441 (Fig. 9B, left panel). Also as in H322, when the VP16RAR α fusion was cotransfected with this reporter construct into Calu6 and H441, the fusion protein again demonstrated higher activity in the absence of RA in the RAR β -negative cell line than in the positive cell line (Fig. 9B, right panel). Furthermore, despite the higher basal transcriptional activity of the VP16RAR α fusion protein in H441, in the presence of RA, this fusion protein still failed to stimulate transcription to the same degree as in Calu6 (Fig. 9B, left panel). Thus, in H441, as in H322, RARs appear to bind DR5 RAREs and yet because of impairment of AF-2 activity are unable to produce high levels of transcriptional stimulation.

Since H441 seemed partially impaired in its ability to respond to RA on PAL as well as DR elements, we suspected that it contained a more general defect affecting AF-2 activity. To examine this issue, we cotransfected pGAL4RAR α with the GAL4-responsive reporter construct, pGAL4₅tk, and measured luciferase activity in the absence and presence of RA. In contrast to the results obtained in H322 (Fig. 8B, middle panel), GAL4RAR α did not support high levels of RA-dependent transcription in H441 (Fig. 9C, middle panel), clearly demonstrating a general block in AF-2 activity. Notably, like H322, and in contrast to Calu6, H441 was also unable to support high levels of basal repression by the GAL4RAR α protein (3.4-fold in H441 and 24-fold in Calu6) (Fig. 8B and 9C, left panels) (see Discussion).

Nuclear hormone receptors share a conserved region of amino acid identity and predicted secondary structure in their AF-2 domains (15). Transcriptional interference studies suggest that this conservation of AF-2 structure is functionally relevant and that some downstream signaling components of receptor-mediated transcription are shared (6). Moreover, it has recently been demonstrated that at least two HBD-associating proteins can physically interact with this domain from more than one type of receptor (31, 58). We therefore asked whether the AF-2-specific activity of other nuclear hormone receptors was also impaired in H441. pGAL4₅tk was cotransfected with an expression vector encoding a fusion of the TR α HBD to the GAL4 DBD (Fig. 5B) into Calu6, H322, and H441 cells. Whereas the GAL4TR α chimera was able to confer strong ligand-dependent transcription on the reporter construct in Calu6 and H322 (Fig. 8C, right panel), TR α AF-2 activity was clearly impaired in H441 (Fig. 9D, right panel). Also, as for the RAR α HBD, in the absence of ligand the TR α HBD was a much better repressor of transcription in Calu6 than in H322 (Fig. 8C, left panel) or H441 (Fig. 9D, left panel). Taken together, our data indicate that there are at least two ways to impair RAR ligand-dependent transcriptional responses from DR RAREs. In H441, AF-2 function is blocked by a general mechanism that affects both RAR and TR AF-2. In H322, RAR activity (most likely via AF-2), but not TR activity, is blocked, and only in the context of the full-length receptor.

Multiple lung cancer cell lines contain a general block in RAR AF-2 activity. Since we had identified two different types of transcriptional defects affecting RAR activity in H322 and

FIG. 10. Calu1 and ChaGo K-1 also display a general defect in RA-dependent transactivation from RARE-containing promoters. p β RARE₃tk was cotransfected with 250 ng of pCDMRAR α expression vector into the indicated cell lines. The experiment was performed as described for Fig. 6.

H441, we asked whether similar defects occurred in other lung cancer cell lines. Calu1, SK-MES-1, and ChaGo K-1 also demonstrated significant impairment in activity from the -5-kb RAR β 2 promoter (four- to fivefold [Fig. 2]). We cotransfected p β RARE₃tk with an RAR α expression vector into Calu1, SK-MES-1, and ChaGo K-1 (Fig. 10). Whereas Calu1 and ChaGo K-1 were unable to support high levels of RA-dependent activity from this reporter construct, SK-MES-1 did permit significant levels of RAR-mediated transcription from this promoter.

Since Calu1 and ChaGo K-1 possess general defects in DR RARE activity, independent of promoter context and the level of functional RARs, we asked whether AF-2 is the target for the block in RAR activity in these cell lines, as in H441. pGAL4₅tk was cotransfected with either pGAL4RAR α or pGAL4TR α , and the transcriptional activity of the respective HBDs was measured (Table 1). As was the case in H322 and H441, in the absence of hormone, both the RAR α and TR α HBD were unable to repress transcription as strongly as in Calu6. Moreover, in the presence of hormone, Calu1 and ChaGo K-1 also displayed impaired RAR and TR AF-2 activity as was seen in H441. Thus, of the two types of defects affecting RAR AF-2 activity in lung cancer cells, at least the one resulting in a general impairment of RAR and TR AF-2 activity occurs in multiple cell lines.

DISCUSSION

We have characterized the molecular mechanism underlying defective RAR β 2 expression in several lung cancer cell lines. Nuclear run-on studies indicate that RAR β -negative lung cancer cell lines cannot transcribe significant levels of RAR β , even in the presence of RA (Fig. 1). In most of these cell lines, RA cannot induce high levels of activity from an exogenously supplied -5-kb fragment of the RAR β 2 promoter (Fig. 2), indicating the presence of *trans*-acting transcriptional defects in retinoid responsiveness. Two of the lung cancer cell lines used in our studies do not express any detectable RAR β mRNA (26). These cell lines, H322 and H441, did not support significant levels of RA-dependent transcription from either the -5-kb RAR β 2 promoter (Fig. 2) or a minimal version of this promoter truncated immediately upstream of the β RARE (Fig. 3B and 9A). At present, in addition to the general transcription machinery, RARs and RXRs are the only clearly defined components of RA-dependent transcriptional responses. However, limiting amounts of RARs and/or RXRs cannot account for the observed transcriptional defects in lung cancer cells, since cotransfection of various combinations of RAR and RXR expression vectors could not restore high lev-

els of RA-dependent transcription to a minimal RAR β 2 promoter containing the RARE (Fig. 3).

Although multiple genes and isoforms have been identified for both RARs and RXRs, we tested only two types of RAR (α and β) and two types of RXR (α and β) in these studies. Previous studies demonstrated that RAR α and RAR β are the best mediators of RA-dependent transcription from the RAR β 2 promoter and other DR RAREs (94). Furthermore, *in vitro*, all three RXRs are equally efficient in heterodimerizing with RARs on the β RARE (51, 61, 129, 133), and in transactivation assays, each RXR enhances RAR-mediated transcription from DR RAREs (32, 36, 51, 129). Therefore, we think it is unlikely that absence of a specific RAR and/or RXR accounts for our results. Despite the apparent functional redundancy between RAR and RXR subtypes at the biochemical level in these assays, we cannot rule out the possibility that, in airway epithelial cells, and/or in lung carcinoma cells derived from them, specific and distinct receptor combinations are required for RA-mediated transcriptional responses on RAREs.

However, it is more plausible that lung cancer cells either contain an excess of negative regulatory factors or lack critical positive cofactors that affect the activity of all RARs. We favor the latter hypothesis since cotransfection of RARs and RXRs over a 10-fold dose range could not restore transcription from DR RAREs (Fig. 3B and data not shown) but could clearly stimulate transcription from a PAL RARE (Fig. 6 and data not shown). We might have expected an inhibitor to be titrated under such conditions. *In vitro* transcription experiments suggest that most, if not all, eukaryotic transcription factors require coactivator proteins for optimal activity (22, 38, 72, 82, 101, 114). Although cellular E1A-like activity has been implicated as a natural coactivator for transcription factors such as CREB/ATFs (14, 65), Oct-4 (107), and RARs (10), our data indicate that the defects involving the RAR β 2 promoter cannot be complemented by viral 13S E1A in H322 (Fig. 4A) or H441 and ChaGo K-1 (data not shown). We therefore believe that lung cancer cells lack novel factors, necessary for strong RAR-RXR-mediated transcription from RAREs. These factors are most likely protein coactivators, although we cannot exclude enzymes that may modify RARs-RXRs or such activators.

Transfection studies with DR and PAL RARE-driven reporter constructs and chimeric receptors indicate that at least two types of cofactor activities may be required for strong RA-dependent transcription by RARs on these elements. H322 displays a general loss of DR RARE responsiveness without any impairment in PAL RARE activity (Fig. 6). The exact mechanism for this loss in activity is unclear since both the RAR DBD and HBD have apparently normal activity when examined in isolation in these cells. However, when the activities of these domains are reexamined in the context of an RAR containing only a substitution of the VP16 transactivation domain for AF-1, an apparent defect is revealed in AF-2. Since this receptor more closely resembles the full-length receptor than the other chimeric receptors used in our study, we think it very likely that AF-2 is indeed the target for the block in RAR activity in H322. Likewise, RAR DBD activity is not impaired in H441 (Fig. 9B, right panel), but instead, there is a general block in RAR AF-2 function, which is revealed most dramatically by the weak RA-dependent transcriptional response conferred by the GAL4RAR α fusion protein (Fig. 9C, middle panel). The latter type of defect accounts for impaired RAR-mediated transcription from DR RARE-containing promoters in three of four lung cancer cell lines tested (Table 1).

These data imply that the mechanism of retinoid receptor-mediated transcription may be more complex than previously

appreciated. It has recently been proposed that a flexible hinge between a receptor's DBD and HBD prevents the DBD from being rotationally constricted, so that an RAR-RXR heterodimer can bind to both DR and PAL RAREs (55). Since the transcriptional response from DR RAREs can be functionally dissociated from that on PAL RAREs, we propose that RAR-RXR heterodimers acquire different conformations on these structurally distinct RAREs and hence use different mechanisms for transactivation. This model is supported by other data indicating that DBD-HBD contacts between heterodimer partners are different on DR and PAL RAREs. For example, DBDs have been found to contain all of the information necessary for correct and cooperative DR element recognition (55, 73, 98, 100, 130, 131), but modeling studies predict that these contacts cannot be maintained on PAL elements (98, 131). Furthermore, some mutations in the HBD of the TR result in a selective loss of heterodimerization with RXRs on DR but not PAL hormone response elements (91). Although it was previously reported that RARs display promoter-specific activity (94), these activities were not defined in a system that normally responds biologically to retinoids, and it was not reported that RAR α could use different mechanisms for transactivation on DR5 versus PAL elements. Whereas a dissociation between DR5 and PAL RARE transcriptional responses was demonstrated for RAR β and RAR γ , it was implied that, on the same type of RARE, RAR β and RAR γ did not necessarily use the same signaling pathway for transactivation.

Our studies go beyond this previous work by defining two types of retinoid signaling defects in a biologically relevant system, one of which clearly affects both types of tested RAR (Fig. 3). We propose a model for RAR-RXR-mediated transactivation that places more steps in the transduction of a transcriptional response from a DR5 RARE than from a PAL RARE. Because of the specific conformation of a DR5-bound heterodimer, RAR AF-2 may be less accessible to downstream coactivators of transcription than it is in the context of a PAL-bound dimer involving either the full-length receptor or a GAL4 fusion. Furthermore, since the RAR DBD uses different amino acids than the TR DBD to dimerize with RXRs on DR elements (130), it is possible that a receptor's DBD also affects the overall configuration of the heterodimer and hence the accessibility of the AF-2 domain. This is supported by our finding that the ligand-dependent transcriptional activity of RAR α is impaired in H322 in the context of the full-length receptor, which recognizes DR5 elements, but not when the RAR HBD is fused to the TR α DBD, which binds to DR4 elements. Interestingly, the polarity and transcriptional activation properties of RAR-RXR heterodimers are greatly influenced by the spacing between DR half-sites (54). In the case of DR1 versus DR5 elements, the functional differences in the RAR-RXR transactivating capabilities are clearly attributable to spacing-dependent differences in the orientations of the DNA-bound heterodimers. We propose that, on a DR5 RARE, either two proteins or a single protein with two different surfaces is required to elicit a complete transcriptional response, whereas on a PAL RARE, only one protein or one surface of the multisurfaced protein is required for a comparable response (Fig. 11B). In this model, these accessory factors could be modifying enzymes or actual binding proteins that are physically recruited into the transcriptional activation complex. They would be predicted either to regulate the binding of ligand to the RAR or to control the accessibility of AF-2 to the general transcription machinery once ligand was bound to the receptor.

Interestingly, although the four cell lines (H322, H441,

A **B**

FIG. 11. Model for element-specific transactivation by RARs. (A) RAR-RXR heterodimers acquire different conformations on DR and PAL response elements. Consequently, ligand binding or exposure of AF-2 by the RAR is blocked on DR elements relative to PAL (including GAL4 binding site) elements. (B) In the presence of RA, a coactivator (black shape) is required to transduce the transcription-stimulating signal emanating from activated AF-2. On DR elements, specifically, an additional factor (striped shape) is required for ligand binding or exposure of activated AF-2 by the RAR. In H322, a cofactor represented by the striped pattern may have defective activity. In H441, Calu1, and ChaGo K-1, a cofactor represented by the black squares may have defective activity.

Calu1, and ChaGo K-1) proposed to have blocks in AF-2 signaling fall into two categories, all of them share one feature concerning the activity of the isolated RAR HBD. None of these cell lines support the high levels of ligand-independent repression normally conferred by GAL4RAR α in cells (e.g., Calu6) that do support strong ligand-dependent transcriptional responses (Table 1). These data thus provide additional support for the existence of aberrant interactions between protein cofactors and the RAR HBD in lung cancer cells. In addition, since a block in TR α AF-2 activity always accompanies the block in RAR α AF-2 activity in H441, Calu1, and ChaGo K-1, it is likely that some of the accessory factors necessary for AF-2 activity are functionally shared between hormone receptors. Our data thus support a complementary set of experiments which (i) indicate that overexpression of the ligand-activated AF-2 domain of one receptor can interfere with the transcriptional activity of another receptor's AF-2 domain (6) and (ii) demonstrate that at least two candidate coactivator proteins physically associate with multiple HBDs (31, 58). In further agreement with shared signaling between RARs and TRs, GAL4TR α , like GAL4RAR α , cannot repress transcription well in H322, H441, Calu1, and ChaGo K-1 (Table 1). However, it is likely that some cofactor activities are receptor specific, since full-length TR α seems to function equivalently on both DR and PAL elements in H322 and Calu6 (Fig. 8A). This may reflect receptor-specific differences in amino acid composition or in heterodimer configuration due to half-site spacing preferences.

The existence of element (DR)-specific RAR cofactors would provide an additional level of control for the regulation of gene expression by RA. Through regulation of coactivator

activity, cells could selectively alter their response to RA, without gross changes in levels of RA or RARs. Thus, a cell need not compromise all aspects of retinoid signaling in order to eliminate a subset of responses from its repertoire. This level of control could be used by normal cells to regulate the expression of genes whose inappropriate expression might inhibit growth and promote differentiation. Interestingly, exposure of several pituitary cell lines to RA results in an upregulation of growth hormone mRNA but not of RAR β mRNA (16). Whereas the RAR β RARE is a DR RARE, the growth hormone RARE is a PAL RARE (120). Thus, pituitary cells may restrict RAR β expression through negative regulation of a DR RARE-specific cofactor. This mechanism could also extend to other nuclear hormone receptors. Moreover, the ability of some nuclear receptors to also stimulate transcription as monomers (28, 34, 124) or homodimers (57, 76, 134) could allow still more possibilities for the orientations and conformations of DNA-bound receptors and hence could contribute to even greater complexity in the mechanism of transcriptional activation.

In lung cancer cells, the activity of RAR cofactors could be deregulated to restrict the expression of genes such as RAR β that may inhibit tumorigenicity. However, loss of element-specific cofactor activity is not the only cause of decreased RAR β expression in these cells. In one cell line displaying absent RAR β transcription (H661), RA induced very high levels of activity from the transiently transfected -5 -kb RAR β 2 promoter (Fig. 1). Amplification by PCR of the endogenous -5 -kb RAR β 2 promoter from H661 and fusion to the luciferase reporter gene generated a construct that was similarly induced by RA in Calu6 and H661 (data not shown).

Thus, at least this RAR β -negative lung cancer cell line contains a *cis*-acting transcriptional defect which maps outside of the regulatory sequences within the -5 -kb RAR β 2 promoter. While the manuscript was in preparation, another group reported findings concerning the absence of RAR β expression in human lung cancer (135). They also find evidence for *cis*- and *trans*-acting transcriptional defects but in addition report that one cell line, SK-MES-1, displays a more severe defect in β RARE-directed transcription in the context of the natural RAR β 2 promoter, relative to the heterologous TK promoter. Our data support this observation (Fig. 2 and 10), and we propose that this type of defect may also reflect a common mechanism for loss of RAR β expression in some epithelium-derived carcinomas, since this same defect has also been described in HeLa cells (7). Loss of RAR β expression has also been reported in head and neck squamous carcinoma (43), hepatocellular carcinoma (109), and breast carcinoma (112). The molecular details underlying defective RAR β expression in these types of cancer, as well as in other lung cancer cells, remain to be further characterized.

Since most lung cancer cell lines displaying defective RAR β expression also show impaired RAR activity, it appears somewhat paradoxical that restoration of RAR β into RAR β -negative cells could have biological consequences. We previously demonstrated that restoration of RAR β into HeLa cells results in RA-dependent suppression of proliferation (25). Since the defect in HeLa cells impairing β RARE activity displays promoter specificity (7), it is likely that the RAREs in the target genes necessary for the suppression of proliferation are activated by RAR β because of their favorable promoter context. It has also been reported that overexpression of RAR β in H157 and Calu1 lung cancer cells inhibits the tumorigenicity of these cells (42). Our data indicate that H157 is only partially inhibited in its ability to use RARs to transactivate from the β RARE contained within the natural RAR β 2 promoter (Fig. 2) or the heterologous TK promoter (data not shown). Thus, it is likely that overexpression of RAR β in these particular cells may still activate genes to a high-enough level to have biological consequences. Although the activities of the GAL4RAR α and GAL4TR α fusion proteins indicate that Calu1 and H441 contain similar defects, which comparably affect AF-2 function in isolation, Calu1 is still able to induce higher levels of activity from the natural -5 -kb RAR β 2 promoter than H441 (Fig. 2). Thus, there may be subtle differences between the defects in these two cell lines such that the activity of RARs on some promoters (such as the RAR β 2 promoter) in Calu1, as in H157, is of a sufficient level to result in the inhibition of tumorigenicity. However, regardless of RAR β 's role in the pathogenesis of lung cancer, its frequent loss of expression is still likely to serve as a marker and tool for uncovering a common underlying defect in lung cancer.

Perturbed cell growth due to inactivation of a promoter-specific coactivator has been described in at least one system and suggested to occur in at least two others. A temperature-sensitive baby hamster kidney (BHK) cell line mutant that is blocked in G₁ at the nonpermissive temperature has been identified (113). Complementation experiments demonstrated that, at the nonpermissive temperature, activity of the coactivator TAF_{II}250 is compromised (122). The result is not a global decrease in transcription but rather a specific downregulation of gene products necessary for cell cycle progression, such as cyclin A. Similarly, it has been demonstrated that, in human rhabdomyosarcomas, failure to differentiate into myotubes correlates with the loss of activity of a *trans*-acting factor(s) necessary for the transcriptional, but not DNA binding, activity of MyoD (115). Finally, after submission of the manu-

script, it was reported that, in some cells, the 12S viral E1A oncoprotein directly interacts with a family of coactivators of the CREB transcription factor to repress, instead of stimulate, cyclic AMP-dependent transcription (2, 71). It is therefore conceivable that, in lung cancer, analogous mutations can occur in cofactors necessary for the expression of tumor-suppressing genes, ultimately resulting in cellular transformation. Biochemical experiments are now required to further characterize the AF-2-specific cofactors and to determine the mechanism by which they are inactivated in lung cancer.

ACKNOWLEDGMENTS

We thank H. de Thé (Hôpital St. Louis, Paris, France), A. Dejean (Institut Pasteur), R. M. Evans (Salk Institute), M. E. Greenberg (Harvard Medical School), S. Izumo (University of Michigan), D. D. Moore (Massachusetts General Hospital), A. Hollenberg (Beth Israel Hospital), G. A. Brent (Brigham and Women's Hospital), Q. Zhou (Massachusetts Institute of Technology), E. Harlow (Massachusetts General Hospital), and D. M. Knipe (Harvard Medical School) for gifts of plasmids; A. Gazdar (University of Texas Southwestern Medical Center) and S. Bernal (Boston University School of Medicine) for cell lines; A. A. Levin (Hoffmann-La Roche) for 9-*cis*-RA; and M. Brown (Dana-Farber Cancer Institute), D. D. Moore, M. E. Greenberg, and A. A. Levin for helpful comments.

This work was supported by NIH grant CA49152 to B.G.N. B.G.N. is supported in part by a JFRA from the ACS. N.M. was supported by a studentship from the Medical Research Council of Canada.

REFERENCES

- Allenby, G., M. T. Bocquel, M. Saunders, S. Kazmer, J. Speck, M. Rosenberger, A. Lovey, P. Kastner, J. F. Grippo, P. Chambon, and A. A. Levin. 1993. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc. Natl. Acad. Sci. USA* **90**:30-34.
- Arany, Z., D. Newsome, E. Oldread, D. M. Livingston, and R. Eckner. 1995. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. *Nature (London)* **374**:81-84.
- Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. *Current protocols in molecular biology*. Greene Publishing Associates, New York.
- Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249**:912-915.
- Baniahmad, A., A. C. Kohne, and R. Renkawitz. 1992. A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. *EMBO J.* **11**:1015-1023.
- Barettino, D., M. D. M. Vivanco-Ruiz, and H. G. Stunnenberg. 1994. Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J.* **13**:3039-3049.
- Bartsch, D., B. Boye, C. Baust, H. zur Hausen, and E. Schwarz. 1992. Retinoic acid-mediated repression of human papillomavirus 18 transcription and different regulation of the retinoic acid receptor β gene in non-tumorigenic HeLa hybrid cells. *EMBO J.* **11**:2283-2291.
- Beato, M. 1989. Gene regulation by steroid hormones. *Cell* **56**:335-344.
- Benbrook, D., E. Lernhardt, and M. Pfahl. 1988. A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature (London)* **333**:669-672.
- Berkenstam, A., M. D. M. Vivanco-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.
- Boylan, J. F., D. Lohnes, R. Taneja, P. Chambon, and L. J. Gudas. 1993. Loss of retinoic acid receptor γ function in F9 cells by gene disruption results in aberrant *Hoxa*-1 expression and differentiation upon retinoic acid treatment. *Proc. Natl. Acad. Sci. USA* **90**:9601-9605.
- Brand, N., M. Petkovich, A. Krust, P. Chambon, H. de Thé, A. Marchio, P. Tiollais, and A. Dejean. 1988. Identification of a second human retinoic acid receptor. *Nature (London)* **332**:850-853.
- Brent, G. A. Personal communication.
- Chatton, B., J. L. Bocco, M. Gaire, C. Hauss, B. Reimund, J. Goetz, and C. Keding. 1993. Transcriptional activation by the adenovirus larger E1A product is mediated by members of the cellular transcription factor ATF family which can directly associate with E1A. *Mol. Cell. Biol.* **13**:561-570.
- Danielian, P. S., R. White, J. A. Lees, and M. G. Parker. 1992. Identification of a conserved region for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **11**:1025-1033.

16. Davis, K. D., and M. A. Lazar. 1993. Induction of retinoic acid receptor- β by retinoic acid is cell specific. *Endocrinology* **132**:1469–1474.
17. de The, H., A. Marchio, P. Tiollais, and A. Dejean. 1987. A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. *Nature (London)* **330**:667–670.
18. de The, H., A. Marchio, P. Tiollais, and A. Dejean. 1989. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. *EMBO J.* **8**:429–433.
19. de The, H., M. D. 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.
20. Dolle, P., E. Ruberte, P. Leroy, G. Morriss-Kay, and P. Chambon. 1990. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**:1133–1151.
21. Duester, G., M. L. Shean, M. S. McBride, and M. J. Stewart. 1991. Retinoic acid response element in the human alcohol dehydrogenase gene ADH3: implications for regulation of retinoic acid synthesis. *Mol. Cell. Biol.* **11**:1638–1646.
22. Dynlacht, B. D., T. Hoey, and R. Tjian. 1991. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66**:563–576.
23. Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**:889–895.
24. Folkers, G. E., B. J. M. van der Leede, and P. T. van der Saag. 1993. The retinoic acid receptor- β 2 contains two separate cell-specific transactivation domains, at the N-terminus and in the ligand-binding domain. *Mol. Endocrinol.* **7**:616–627.
25. Frangioni, J. V., N. Moghal, A. Stuart-Tilley, B. G. Neel, and S. L. Alper. 1994. The DNA binding domain of retinoic acid receptor β is required for ligand-dependent suppression of proliferation. *J. Cell Sci.* **107**:827–838.
26. Gebert, J. F., N. Moghal, J. V. Frangioni, D. J. Sugarbaker, and B. G. Neel. 1991. High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* **6**:1859–1868.
27. 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.
28. Giguere, V., M. Tini, G. Flock, E. Ong, R. M. Evans, and G. Otulakowski. 1994. Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR α , a novel family of orphan hormone nuclear receptors. *Genes Dev.* **8**:538–553.
29. Glass, C. K., R. Franco, C. Weinberger, V. Albert, and R. M. Evans. 1987. A *c-erbA* binding site in the rat growth hormone gene mediates transactivation by thyroid hormone. *Nature (London)* **329**:738–741.
30. Greenberg, M. E. Personal communication.
31. Halachmi, S., E. Marden, G. Martin, H. MacKay, C. Abbondanza, and M. Brown. 1994. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264**:1455–1458.
32. 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.
33. 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 superfamily 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.
34. Harding, H., and M. A. Lazar. 1993. The orphan receptor rev-erbA α activates transcription via a novel response element. *Mol. Cell. Biol.* **13**:3113–3121.
35. Harkema, J., A. Mariassy, J. St. George, D. M. Hyde, and C. G. Plopper. 1991. Epithelial cells of the conducting airways, p. 3–39. *In* S. G. Farmer and D. W. P. Hay (ed.), *The airway epithelium*. Marcel Dekker, Inc., New York.
- 35a. Harlow, E. Personal communication.
36. Heery, D. M., T. Zacharewski, B. Pierrat, H. Gronemeyer, P. Chambon, and R. Losson. 1993. Efficient transactivation by retinoic acid receptors in yeast requires retinoid X receptors. *Proc. Natl. Acad. Sci. USA* **90**:4281–4285.
37. Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller. 1992. 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**:397–406.
38. Hoey, T., R. O. J. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian. 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* **72**:247–260.
39. Hoffmann, B., J. M. Lehmann, X. K. Zhang, T. Hermann, M. Husmann, G. Graupner, and M. Pfahl. 1990. A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol. Endocrinol.* **4**:1727–1736.
40. Houle, B., F. Leduc, and W. E. C. Bradley. 1991. Implication of RAR β in epidermoid (squamous) lung cancer. *Genes Chromosomes Cancer* **3**:358–366.
41. Houle, B., M. Pelletier, J. Wu, C. Goodyer, and W. E. C. Bradley. 1994. Fetal isoform of human retinoic acid receptor β expressed in small cell lung cancer lines. *Cancer Res.* **54**:365–369.
42. Houle, B., C. Rochette-Egly, and W. E. C. Bradley. 1993. Tumor-suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* **90**:985–989.
43. Hu, L., D. L. Crowe, J. G. Rheinwald, P. Chambon, and L. Gudas. 1991. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* **51**:3972–3981.
44. Imperiale, M. J., H. T. Kao, L. Feldman, J. Nevins, and S. Strickland. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol. Cell. Biol.* **4**:867–874.
45. Jetten, A. M. 1989. Multistep process of squamous differentiation in tracheobronchial epithelial cells *in vitro*: analogy with epidermal differentiation. *Environ. Health Perspect.* **80**:149–160.
46. Jetten, A. M., M. A. George, G. R. Petit, and J. I. Rearick. 1989. Effects of bryostatins and retinoic acid on phorbol ester- and diacylglycerol-induced squamous differentiation in human tracheobronchial epithelial cells. *Cancer Res.* **49**:3990–3995.
47. Jetten, A. M., and J. E. Shirley. 1986. Characterization of transglutaminase activity in rabbit tracheal epithelial cells. *J. Biol. Chem.* **261**:15097–15101.
48. Kastner, P., A. Krust, J. M. Garnier, A. Zelent, P. Leroy, and A. Staub. 1990. Murine isoforms of retinoic acid receptor γ with specific patterns of expression. *Proc. Natl. Acad. Sci. USA* **87**:2700–2704.
49. Katayoon, H. E., and M. Carey. 1992. A synergistic increase in potency of a multimerized VP16 transcriptional activation domain. *EMBO J.* **11**:5005–5012.
50. Keidel, S., P. LeMotte, and C. Apfel. 1994. Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. *Mol. Cell. Biol.* **14**:287–298.
51. Kliewer, S. A., K. Umeson, D. J. Mangelsdorf, and R. M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D $_3$ signaling. *Nature (London)* **355**:446–449.
52. Koenig, R. J., G. A. Brent, R. L. Warner, P. R. Larsen, and D. D. Moore. 1987. Thyroid hormone receptor binds to a site in the rat growth hormone promoter required for induction by thyroid hormone. *Proc. Natl. Acad. Sci. USA* **84**:5670–5674.
53. 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.
54. Kurokawa, R., J. DiRenzo, M. Boehm, J. Sugarman, B. Gloss, M. G. Rosenfeld, R. A. Heyman, and C. K. Glass. 1994. Regulation of retinoid signalling and allosteric control of ligand binding. *Nature (London)* **371**:528–531.
55. Kurokawa, R., V. C. Yu, A. Naar, S. Kyakumoto, Z. Han, S. Silverman, M. G. Rosenfeld, and C. K. Glass. 1993. Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. *Genes Dev.* **7**:1423–1435.
56. La Thangue, N. B., and P. W. J. Rigby. 1987. An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* **49**:507–513.
57. Lazar, M. A., T. J. Berrodin, and H. P. Harding. 1991. Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. *Mol. Cell. Biol.* **11**:5005–5015.
58. Lee, J. W., F. Ryan, J. C. Swaffield, S. A. Johnston, and D. D. Moore. 1995. Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature (London)* **374**:91–94.
59. Lee, M. S., S. A. Kliewer, J. Provencal, P. E. Wright, and R. M. Evans. 1993. Structure of the retinoid X receptor α DNA binding domain: a helix required for homodimeric DNA binding. *Science* **260**:1117–1121.
60. Leid, M., P. Kastner, and P. Chambon. 1992. Multiplicity generates diversity in the retinoic acid signaling pathways. *Trends Biochem. Sci.* **17**:427–433.
61. Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, 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.
62. Leroy, P., A. Krust, A. Zelent, C. Mendesohn, J. M. Garnier, P. Kastner, A. Dierich, and P. Chambon. 1991. Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and differential induction by retinoic acid. *EMBO J.* **10**:59–69.
63. Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzel, M. Rosenberger, A. Lovey, and J. F. Grippo. 1992. 9-*cis* Retinoic acid stereoisomer binds and activates the nuclear receptor RXR α . *Nature (London)* **355**:359–361.
64. Li, E., H. M. Suvov, K. F. Lee, R. M. Evans, and R. Jaenisch. 1993. Normal development and growth of mice carrying a targeted disruption of the α 1 retinoic acid receptor gene. *Proc. Natl. Acad. Sci. USA* **90**:1590–1594.
65. Liu, F., and M. R. Green. 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. *Cell* **61**:1217–1224.

66. Liu, Q., and E. Linney. 1994. The mouse retinoid-X receptor- γ gene: genomic organization and evidence for functional isoforms. *Mol. Endocrinol.* 7:651–658.
67. Lohnes, D., P. Kastner, A. Dierich, M. Mark, M. LeMeur, and P. Chambon. 1993. Function of retinoic acid receptor γ in the mouse. *Cell* 73:643–658.
68. Lohnes, D., M. Mark, C. Mendelsohn, P. Dolle, A. Dierich, P. Gorry, A. Gansmuller, and P. Chambon. 1994. Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial abnormalities in RAR double mutants. *Development* 120:2723–2748.
69. Lotan, R. Personal communication.
70. Lufkin, T., D. Lohnes, M. Mark, A. Dierich, P. Gorry, M. P. Gaub, M. LeMeur, and P. Chambon. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor α mutant mice. *Proc. Natl. Acad. Sci. USA* 90:7225–7229.
71. Lundblad, J. R., R. P. S. Kwok, M. E. Lurance, M. L. Harter, and R. H. Goodman. 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature (London)* 374:85–88.
72. Luo, Y., H. Fujii, T. Gerster, and R. G. Roeder. 1992. A novel B cell-derived coactivator potentiates the activation of immunoglobulin promoters by octamer-binding transcription factors. *Cell* 71:231–241.
73. Mader, S., J. Y. Chen, Z. Chen, J. White, P. Chambon, and H. Gronemeyer. 1993. The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificities of the DNA binding domains. *EMBO J.* 12:5029–5041.
74. 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 the three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev.* 6:329–344.
75. 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.
76. Mangelsdorf, D. J., K. Umehono, S. A. Kliewer, U. Borgmeyer, E. S. Ong, and R. M. Evans. 1991. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66:555–561.
77. Marks, M. S., P. L. Hallenback, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato. 1992. H-2RIIBP (RXR β) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.* 11:1419–1435.
78. Martin, C. A., L. M. Ziegler, and J. L. Napoli. 1990. Retinoic acid, dibutyryl-cAMP, and differentiation affect the expression of retinoic acid receptors in F9 cells. *Proc. Natl. Acad. Sci. USA* 87:4804–4808.
79. McDowell, E. M., K. P. Keenan, and M. Huang. 1984. Effects of vitamin A-deprivation on hamster tracheal epithelium: a quantitative morphologic study. *Virchows Arch. B Cell Pathol.* 45:197–219.
80. McDowell, E. M., K. P. Keenan, and M. Huang. 1984. Restoration of mucociliary tracheal epithelium following deprivation of vitamin A. *Virchows Arch. B Cell Pathol.* 45:221–240.
81. McKnight, J. L., P. E. Pellett, F. J. Jenkins, and B. Roizman. 1987. Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate α -trans-inducing factor-dependent activation of α genes. *J. Virol.* 61:992–1001.
82. Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder. 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. *Cell* 66:981–993.
83. Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, P. Chambon, and M. Mark. 1994. Function of the retinoic acid receptors (RARs) during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120:2749–2771.
84. Mendelsohn, C., M. Mark, P. Dolle, A. Dierich, M. P. Gaub, A. Krust, C. Lampron, and P. Chambon. 1994. Retinoic acid receptor β 2 (RAR β 2) null mutant mice appear normal. *Dev. Biol.* 166:246–258.
85. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 7:980–986.
86. Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18:5322.
87. Moghal, N., and B. G. Neel. Unpublished data.
88. Moon, R. C., and L. M. Itri. 1984. Retinoids and cancer, p. 327–336. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*. Academic Press, Orlando, Fla.
- 88a. Moore, D. D. Personal communication.
89. Morgenstern, J. P., and H. Land. 1990. A series of mammalian expression vectors and characterization of their expression of a reporter gene in stably and transiently transfected cells. *Nucleic Acids Res.* 18:1068.
90. Naar, A. M., J. M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Holloway, C. K. Glass, and M. G. Rosenfeld. 1991. The orientation and spacing of core DNA-binding motifs dictates selective transcriptional responses to three nuclear receptors. *Cell* 65:1267–1279.
91. Nagaya, T., and J. L. Jameson. 1993. Distinct dimerization domains provide antagonist pathways for thyroid hormone receptor action. *J. Biol. Chem.* 268:24278–24282.
92. Nagaya, T., and J. L. Jameson. 1993. Thyroid hormone receptor dimerization is required for dominant negative inhibition by mutations that cause thyroid hormone resistance. *J. Biol. Chem.* 268:15766–15771.
93. Nagpal, S., S. Friant, H. Nakshatri, and P. Chambon. 1993. RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization *in vivo*. *EMBO J.* 12:2349–2360.
94. Nagpal, S., M. Saunders, P. Kastner, B. Durand, H. Nakshatri, and P. Chambon. 1992. Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell* 70:1007–1019.
95. Nagpal, S., A. Zelent, and P. Chambon. 1992. RAR- β 4, a retinoic acid receptor isoform, is generated from RAR- β 2 by alternative splicing and usage of a CUG initiator codon. *Proc. Natl. Acad. Sci. USA* 89:2718–2722.
96. Nervi, C., T. M. Vollberg, M. D. George, A. Zelent, P. Chambon, and A. M. Jetten. 1991. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp. Cell Res.* 195:163–170.
97. Nordeen, S. K. 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* 6:454–458.
98. Perlmann, T., P. N. Rangarajan, K. Umehono, and R. M. Evans. 1993. Determinants for selective RAR and TR recognition of direct repeat HREs. *Genes Dev.* 7:1411–1422.
99. 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.
100. Predki, P. F., D. Zamble, B. Sarkar, and V. Giguere. 1994. Ordered binding of retinoic acid and retinoid-X receptors to asymmetric response elements involves determinants adjacent to the DNA-binding domain. *Mol. Endocrinol.* 8:31–39.
101. Pugh, B. F., and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* 61:1187–1197.
102. Raisher, B. D., T. Gulick, Z. Zhang, A. W. Strauss, D. D. Moore, and D. P. Kelly. 1992. Identification of a novel retinoid-responsive element in the promoter region of the medium chain acyl-coenzyme A dehydrogenase gene. *J. Biol. Chem.* 267:20264–20269.
103. Rearick, J. I., P. W. Albro, and A. M. Jetten. 1987. Increase in cholesterol sulfotransferase activity during *in vitro* squamous differentiation of rabbit tracheal epithelial cells and its inhibition by retinoic acid. *J. Biol. Chem.* 262:13069–13074.
104. Rearick, J. I., M. Deas, and A. M. Jetten. 1987. Synthesis of mucous glycoproteins by rabbit tracheal cells *in vitro*. Modulation by substratum, retinoids and cyclic AMP. *Biochem. J.* 242:19–25.
105. Rearick, J. I., and A. M. Jetten. 1989. Effect of substratum and retinoids upon the mucosecretory differentiation of airway epithelial cells *in vitro*. *Environ. Health Perspect.* 80:229–237.
106. 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*. Academic Press, Orlando, Fla.
107. Scholer, H. R., T. Ciesiolka, and P. Gruss. 1991. A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells. *Cell* 66:291–304.
108. Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid linked membrane protein homologous to its receptor, CD2. *Nature (London)* 329:840–842.
109. Sever, C. E., and J. Locker. 1991. Expression of retinoic acid α and β receptor genes in liver and hepatocellular carcinoma. *Mol. Carcinog.* 4:138–144.
110. Shi, Y., E. Seto, L.-S. Chang, and T. Shenk. 1991. Transcriptional repression by YY1, a human GLI-kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* 67:377–388.
111. Sucov, H. M., K. K. Murakami, and R. M. Evans. 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc. Natl. Acad. Sci. USA* 87:5392–5396.
112. Swisshelm, K., K. Ryan, X. Lee, H. C. Tsou, M. Peacocke, and R. Sager. 1994. Down-regulation of retinoic acid receptor β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.* 5:133–141.
113. Talavera, A., and C. Basilico. 1977. Temperature sensitive mutants of BHK cells affected in cell cycle progression. *J. Cell. Physiol.* 92:425–436.
114. Tanese, N., B. F. Pugh, and R. Tjian. 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* 5:2212–2224.
115. Tapscott, S. J., M. J. Thayer, and H. Weintraub. 1993. Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. *Science* 259:1450–1453.
116. Tate, B. F., G. Allenby, R. Janocha, S. Kazmer, J. Speck, L. J. Sturzenbecker, P. Abarzua, A. A. Levin, and J. F. Grippo. 1994. Distinct binding determinants for 9-*cis* retinoic acid are located within AF-2 of retinoic acid receptor α . *Mol. Cell. Biol.* 14:2323–2330.
117. Tini, M., G. Otulakowski, M. L. Breitman, L. C. Tsui, and V. Giguere. 1993. An everted repeat mediates retinoic acid induction of the γ F-crystallin gene: evidence of a direct role for retinoids in lens development. *Genes Dev.* 7:295–307.
118. Tora, L., H. Gronemeyer, B. Turcotte, M. P. Gaub, and P. Chambon. 1988.

- The N-terminal region of the chicken progesterone receptor specifies target gene activation. *Nature (London)* **333**:185–189.
119. Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon. 1989. The human estrogen receptor has two independent non-acidic transcriptional activation functions. *Cell* **59**:477–487.
 120. Umesono, K., V. Giguere, C. K. Glass, M. G. Rosenfeld, and R. M. Evans. 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature (London)* **336**:262–265.
 121. Umesono, K., K. K. Murakami, C. C. Thompson, 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.
 122. Wang, E. H., and R. Tjian. 1994. Promoter-selective transcriptional defect in cell cycle mutant *ts13* rescued by hTAF_{II}250. *Science* **263**:811–814.
 123. Williams, G. R., J. W. Harney, D. D. Moore, P. R. Larsen, and G. A. Brent. 1992. Differential capacity of wild type promoter elements for binding and *trans*-activation by retinoic acid and thyroid hormone receptors. *Mol. Endocrinol.* **6**:1527–1537.
 124. Wilson, T. E., T. J. Fahrner, and J. Milbrandt. 1993. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol. Cell. Biol.* **13**:5794–5804.
 125. Wilson, T. E., R. E. Paulsen, K. A. Padgett, and J. Milbrandt. 1992. Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. *Science* **256**:107–110.
 126. Wong, Y. C., and R. C. Buck. 1971. An electronic microscopic study of metaplasia of the rat tracheal epithelium in vitamin A deficiency. *Lab. Invest.* **24**:55–66.
 127. Wood, W. M., M. Y. Kao, D. F. Gordon, and E. C. Ridgway. 1989. Thyroid hormone regulates the mouse thyrotropin β -subunit gene promoter in transfected primary thyrotropes. *J. Biol. Chem.* **264**:14840–14847.
 128. Wu, R., W. R. Martin, C. B. Robinson, J. A. St. George, C. G. Plopper, G. Kurland, J. A. Last, C. E. Cross, R. J. McDonald, and R. Boucher. 1990. Expression of mucin synthesis and secretion in human tracheobronchial epithelial cells grown in culture. *Am. J. Respir. Cell Mol. Biol.* **3**:467–478.
 129. Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kim, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld. 1991. RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**:1251–1266.
 130. Zechel, C., X. Q. Shen, P. Chambon, and H. Gronemeyer. 1994. Dimerization interfaces formed between the DNA binding domains determine the cooperative binding of RXR/RAR and RXR/TR heterodimers to DR5 and DR4 elements. *EMBO J.* **13**:1414–1424.
 131. 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.
 132. 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.
 133. 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.
 134. Zhang, X.-K., J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl. 1992. Homodimer formation of retinoid X receptor induced by 9-*cis* retinoic acid. *Nature (London)* **358**:587–591.
 135. Zhang, X.-K., Y. Liu, M.-O. Lee, and M. Pfahl. 1994. A specific defect in the retinoic acid response associated with human lung cancer cell lines. *Cancer Res.* **54**:5663–5669.
 136. Zhou, Q., P. M. Lieberman, T. G. Boyer, and A. J. Berk. 1992. Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. *Genes Dev.* **6**:1964–1974.