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Retinoic Acid Induction of Major Histocompatibility Complex Class I Genes in NTera-2 Embryonal Carcinoma Cells Involves Induction of NF-κB (p50-p65) and Retinoic Acid Receptor β-Retinoid X Receptor β Heterodimers

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Retinoic acid (RA) treatment of human embryonal carcinoma (EC) NTera-2 (NT2) cells induces expression of major histocompatibility complex (MHC) class I and β-2 microglobulin surface molecules. We found that this induction was accompanied by increased levels of MHC class I mRNA, which was attributable to the activation of the two conserved upstream enhancers, region I (NF-κB like) and region II. This activation coincided with the induction of nuclear factor binding activities specific for the two enhancers. Region I binding activity was not present in undifferentiated NT2 cells, but binding of an NF-κB heterodimer, p50-p65, was induced following RA treatment. The p50-p65 heterodimer was produced as a result of de novo induction of p50 and p65 mRNAs. Region II binding activity was present in undifferentiated cells at low levels but was greatly augmented by RA treatment because of activation of a nuclear hormone receptor heterodimer composed of the retinoid X receptor (RXRβ) and the RA receptor (RARβ). The RXRβ-RARβ heterodimer also bound RA responsive elements present in other genes which are likely to be involved in RA triggering of EC cell differentiation. Furthermore, transfection of p50 and p65 into undifferentiated NT2 cells synergistically activated region I-dependent MHC class I reporter activity. A similar increase in MHC class I reporter activity was demonstrated by cotransfection of RXRβ and RARβ. These data show that following RA treatment, heterodimers of two transcription factor families are induced to bind to the MHC enhancers, which at least partly accounts for RA induction of MHC class I expression in NT2 EC cells.

Retinoic acid (RA) induction of embryonal carcinoma (EC) cell differentiation has been utilized as an in vitro model to study changes in gene regulation accompanying differentiation and early mammalian development in vivo (44, 62, 72). Similar to the more widely studied murine F9 and P19 cells, human NTera-2 (NT2) EC cells have been shown to undergo differentiation with RA treatment to produce a neuronal lineage in addition to other cell types (1). The initial event triggering RA-induced differentiation is almost certainly the activation of RA receptors (RARs) which heterodimerize with retinoid X receptors (RXRs) to bind specific DNA sequences, RA response elements (i.e., RAREs), thus leading to induction of a diverse set of transcription factors (12, 28, 41, 48, 61, 78). RA treatment of NT2 cells has been shown to induce transcription factors such as AP-2 (46) and a series of homeobox genes (70, 71). Undifferentiated NT2 cells conversely express Oct3 mRNAs at high levels, and the levels fall precipitously soon after RA treatment (57, 63). Likewise, extensive changes in regulatory gene expression have been reported for F9 and other EC cells after RA treatment (40, 74). While it seems clear that RA treatment influences expression of a number of regulatory factors that determine cellular differentiation, the mechanism responsible for induction of specific genes during EC cell differentiation in most instances remains to be deciphered.

Expression of major histocompatibility complex (MHC) class I genes is an important hallmark of EC cell differentiation, since cells concomitantly become able to produce interferons, become sensitive to cytotoxic T cells, and acquire an immunologically competent status (30).

Developmental and tissue-specific regulation of MHC class I gene expression is conferred by a conserved upstream regulatory region (7, 13, 14, 18, 32, 38), which includes the region I (κB-like [see below]) and region II enhancer elements (see Fig. 2A for scheme). The region II element has been shown to function as a moderate enhancer in fibroblasts (13) and contains a core sequence, AGGTCA, found in many hormone responsive elements (51), including RAREs (19, 75). We isolated RXRβ (formerly H-2RIIBP) on the basis of binding to region II (29), and later showed that RXRβ is capable of activating a reporter containing region II in a RA-dependent fashion when transfected into NT2 cells (56). Since then, it has been demonstrated that RXRs heterodimerize with other members of the nuclear hormone receptor superfamily, including RARs, to avidly bind region II (48) and other RAREs in vitro (12, 41, 78). While it is likely that RARs and RXRs execute important functions in RA-induced regulation of many early responsive genes in EC

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cells, the precise identification of the receptors involved and their in vivo target genes has yet to be determined. This issue is further complicated by the diversity of receptor members which may be involved (e.g., RXRα, -β, and -γ [47] and RARα, -β, and -γ [19, 79]).

Region I, a conserved κB-like sequence (67), has been shown to bind NF-κB transcription factors in addition to PRDIIBF1, H-2TF1, and KBF1 (7, 24, 37, 77). In some cells and tissues the presence of region I binding activity correlates with MHC class I expression (13, 22, 52). NF-κB binding activity consists of a heterodimeric complex of p50 and p65 proteins, the subunits of which belong to a large family of *rel*-related proteins (9, 10, 27, 37, 64, 65; reviewed in references 5 and 42). Despite the report that NF-κB homologs are involved in tissue differentiation (36), no information is available regarding expression of NF-κB factors during EC cell differentiation.

In this report we have studied induction of MHC class I gene expression by RA as an example of a developmentally regulated gene that is induced during EC cell differentiation. We show that two DNA-binding activities, NF-κB subunits (p50-p65) and RXRβ-RARβ heterodimers, are induced by RA treatment of NT2 cells and are specific for region I and region II, respectively. Moreover, induction of these complexes was found to coincide with the induction of the region I and region II enhancer activity and to at least partly account for the observed increase in MHC class I promoter activity after RA treatment. In addition, we show that region I enhancer activity can be reconstituted in untreated NT2 cells by introduction of NF-κB subunit cDNA corresponding to p50 and p65. Similarly, we show that RXRβ and RARβ are capable of enhancing MHC class I promoter activity in NT2 cells in response to RA, thus supporting a functional role for this heterodimer pair. Taken together, these data show that the absence of MHC class I gene expression in undifferentiated NT2 cells is due to the absence of these factors and the induction of MHC expression involves induction of these factors.

MATERIALS AND METHODS

Cell culture. NT2 cells (1, 2) were obtained from L. Staudt (National Institutes of Health) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), gentamicin (50 μg/ml), and glutamine (20 mM) at 37°C in 7% CO_2 at a density of $>4 \times 10^6$ cells per 75-cm^2 flask. Cells were treated with 1×10^{-5} M or 5×10^{-7} M RA (all-*trans*; Sigma) for indicated periods of time. The human B-cell line Namalwa (American Type Culture Collection [ATCC]) and murine pre-B-cells line 70Z/3 (ATCC) were cultured in RPMI 1640 supplemented with 5 to 7% FBS, gentamicin, and glutamine as described above.

Flow cytometry. Suspensions of NT2 cells (10^5 cells per tube) were incubated with hybridoma supernatants containing W6/32 (anti-human leukocyte antigens [HLA] A, B, and C [8]; ATCC), or anti-human β-2 microglobulin antibody (BBM.1 (β-2m) [11]; ATCC), both diluted 1:2, for 60 min at 4°C. Cells were washed with phosphate-buffered saline (PBS) supplemented with 1% FBS and 0.02% NaN_3 and then incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin $F(ab')_2$ diluted 1:40 and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin Fc (Cappel) diluted 1:50. Monoclonal anti-mouse H-$2L^d$/D^d (28-14-8 [59]; ATCC) was used as a negative control. To monitor expression of a neuronal marker, cells were incubated with a mixture of tetanus toxin (2 μg) and rabbit anti-tetanus toxin C antibody (Calbiochem) diluted at 1:1,500 under the same conditions as described above. Cells were then incubated with phycoerythrin-labeled goat anti-rabbit immunoglobulin G (Southern Biotechnology) diluted 1:40. Normal rabbit serum was used as a control. Cells were washed three times in PBS with the above supplements and analyzed on an EPICS II flow cytometer (Coulter Electronics).

Transfection and reporter assays. MHC class I chloramphenicol acetyltransferase (CAT) reporter constructs pL^d1400.CAT, pL^d237.CAT, pL^d123.CAT, and pL^d60.CAT have been described (23). Mutant pL^d1400.CAT constructs were prepared by the oligonucleotide-directed mutagenesis procedure, using polymerase chain reaction (see Fig. 2A). Mutations in these constructs were confirmed by dideoxy sequencing. To test MHC class I promoter activity, NT2 cells (6×10^5 to 10×10^5 cells per plate) were transfected with 5 μg of reporter construct, 5 μg of PCH110 (Pharmacia) or 0.6 μg of Rous sarcoma virus luciferase (20), and 10 μg of carrier DNA (pUC18) by the calcium phosphate precipitation method with the BES buffer (56) at pH 6.96. After overnight incubation, cells were washed and cultured in complete culture media for 36 h. CAT assays were performed as previously described (56). Transfection efficiency was monitored by β-galactosidase or luciferase activity. Cotransfection assays with p50 and p65 expression plasmids were performed with PMT2T vectors containing p50 cDNA or p65 cDNA (9). NT2 cells (2×10^6) were transfected with 5 μg of pL^d1400.CAT or pL^d1400MI.CAT (see Fig. 2) and indicated amounts of expression plasmids as previously described (9).

PL^d1400.LUC was constructed with an *Xho*I-*Hin*dIII fragment excised from pL^d1400.CAT that encompassed the entire 1,400 bp of the MHC class I promoter region. The *Xho*I-*Hin*dIII fragment was then subcloned into the comparable site of pGL2 basic (Promega). Correct insertion was confirmed by dideoxy sequencing. The mammalian expression vector RSV-RXRβ was previously described (56). human RARβ in pSV-SPORT was a gift from A. DeJean (Pasteur Institute; supplied through A. Zimmer, National Institutes of Health). Reporter assays with pL^d1400.LUC were performed in 12-well flat-bottom plates seeded with 2×10^5 NT2 cells per well. After overnight incubation, 0.3 μg of reporter, 0.3 to 0.5 μg of expression vector, and 0.2 μg of PCH110 (Pharmacia) was transfected by the BES buffer method (56). The total amount of expression vector was held constant by the addition of expression vector lacking insert. Following 8 h of incubation, cells were washed with PBS, and 10 μM of all-*trans* RA (or vehicle) was added for an additional 24-h incubation. Cells were washed and then lysed by freeze-thaw three times in KH_2PO_4 (100 mM)–1 mM dithiothreitol (DTT) at pH 7. Cellular debris was separated by centrifugation, supernatants were collected, wells were standardized to β-galactosidase activity, and luciferase activity was determined as previously described (20).

Gel mobility shift assay. Nuclear and cytoplasmic extracts were prepared by the method of Dignam et al. (21) with minor modifications. All buffers contained the proteinase inhibitor phenylmethylsulfonyl fluoride (0.5 mM). Extracts were used without dialysis. Nuclear extracts containing 5 to 8 μg of protein were incubated with ^{32}P-labeled double-stranded oligonucleotides (0.1 to 0.5 ng [about 10^3 to 10^4 cpm]) (see below and Fig. 2) and 2 to 4 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) in binding buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.2

mM EDTA, and 1 mM DTT for 30 min at 4°C. The oligonucleotides depicted in Fig. 2B were used as probes as indicated. For competition experiments, unlabeled oligonucleotides were added at 50-fold molar excess 5 min prior to addition of labeled oligonucleotides. The following antibodies were tested in gel mobility shift assays. Rabbit antibodies to p50, p65, c-Rel, and Rel 50B were prepared and used as previously described (9). These antibodies (1 μl of serum) were added to 8 μg of nuclear extract proteins and incubated in 15 μl of buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) in the presence of 3 μg of poly(dI-dC) · poly(dI-dC), 20 μg of bovine serum albumin (BSA), and 0.2 ng of labeled probe for 30 min before the addition of labeled probe (0.2 ng). Monoclonal antibody specific for RXRβ (immunoglobulin G1) (MOK 13-17) and control antibody MOK 15-42 were previously described (48). Ascites (1 μl) containing this monoclonal antibody was added to 5 μg of nuclear extracts in binding buffer as described above and incubated for 30 min at 30°C prior to the addition of labeled region II probe.

To test the effect of sodium deoxycholate (DOC; Sigma) treatment, cytoplasmic extracts (8) μg) were incubated with 1% DOC in binding buffer as described above for 40 min at 4°C prior to the addition of a region I probe. Reaction mixtures were incubated and electrophoresed as described above.

UV cross-linking. UV cross-linking experiments were performed as previously described (53). Briefly, bromodeoxyuridine-substituted region I probe was mixed with 4 μg of nuclear extract proteins in 20 μl of binding buffer and electrophoresed as described above. The labeled complexes were exposed to a 300-nm UV light source (Fotodyne) for 20 min. Cross-linked complexes were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Chemical cross-linking and immunoprecipitation assay. Four microliters of in vitro-translated ^{35}S-labeled RXRβ or RARβ transcribed in rabbit reticulocyte lysate (Promega) was mixed with 20 μg of NT2 nuclear extract proteins and 10 pmol of biotinylated region II oligonucleotide (5'-biotin-GCCAGGCGGTGAGGTCAGGGGTGGGGAA-3') to a 50-μl volume in buffer A as previously described (49). After 90 min at 4°C, 1 μl of 12.5 mM disuccinimidyl-suberate (DSS; Boehringer) in dimethyl sulfoxide (Aldrich) was added and incubated for 30 min at 4°C. The mixture was quenched with 1 μl of 1 M NH_4Cl for 5 min at 4°C. Next, 15 μl of prewashed (buffer A plus 0.05% BSA) streptavidin-agarose beads in 0.05% BSA, 50 μg of poly(dI-dC) · poly(dI-dC) per ml was added, and the mixture was rocked at 4°C for 30 min. Beads were collected and washed three times with buffer A plus 0.05% BSA. A total of 50 μl of HS buffer (buffer A plus 0.5% Nonidet P-40, 0.5% DOC, and 0.5% SDS) was added, and the mixture was heated to 100°C for 10 min and then centrifuged at 13,000 rpm in a Hermle microcentrifuge to pellet the streptavidin-agarose. The supernatant was recovered, incubated for 5 min with 3 μl of specific antibody or control serum at 4°C overnight, and then combined with 30 μl of protein A-agarose beads at 4°C for 2 h with rocking. A polyclonal rabbit antibody raised against a fusion protein containing the N-terminal domain of the human RARβ was used. This antibody specifically immunoprecipitates RARβ expressed in various tissue culture cells (73). Rabbit polyclonal anti-RXRβ antibody was previously described (49). The beads were collected, washed twice with buffer A plus 0.05% BSA and once with buffer A. SDS sample buffer (50 μl) was added to the washed beads, the mixture was heated to 100°C for 10 min, beads were separated by centrifugation at 13,000 rpm for 3 min, and the supernatant was electrophoresed by SDS–8% PAGE.

Northern (RNA) blot hybridization. RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde as described previously (22). RNA was blotted onto either nitrocellulose or nylon filters and hybridized with ^{32}P-labeled cDNA probes (10^6 cpm/ml) for 16 h at 42°C. The hybridization solution was either 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution and 0.1% SDS (nitrocellulose) or 0.1% SDS and 100 μg of salmon sperm (nylon) per ml. Restriction fragments from the following plasmids were ^{32}P labeled by the random priming method: (i) a 1.5-kb HLA A33 cDNA *Eco*RI-*Eco*RI fragment from pcEXV-3-AW33, a kind gift from S.-Y. Yang (Memorial Sloan-Kettering Cancer Research Institute, New York, N.Y.); (ii) a 3.8-kb *Eco*RI fragment from clone 243 encoding p50, a DNA binding subunit of NF-κB (10); (iii) a 1.5 kb *Bgl*II-*Bam*HI fragment from p65 cDNA (64); (iv) a 1.5-kb *Eco*RI-*Acc*I fragment from the mouse RXRβ cDNA (29); (v) a 1.4-kb *Hin*dIII-*Sac*I fragment from the human RARβ cDNA (19); (vi) a 331-bp *Nco*I-*Eco*RI fragment from the chicken β-actin (60); (vii) GADPH cDNA (3). Filters were washed either three times in 0.1 × SSC at 42°C (nitrocellulose) or twice in 2× SSC–0.1% SDS for 30 min at room temperature and then twice in 1× SSC–0.1% SDS at 65°C for 30 min (nylon), and autoradiography was performed.

RESULTS

Induction of surface MHC class I and mRNA molecules in NT2 cells following RA treatment. We studied RA induction of HLA and β-2m surface expression in NT2 cells by flow cytometric analysis. Cells were treated with RA at 10^{-5} M for up to 9 days and tested for surface expression with two monoclonal antibodies, W6/32 and BBM.1, specific for a nonpolymorphic epitope of HLA-A, -B, and -C and for β-2m, respectively. Consistent with previous reports (2), undifferentiated NT2 cells did not show binding to either of the antibodies (Fig. 1A, and C). After RA treatment at 1×10^{-5} M (or 5×10^{-7} M [not shown]) for 9 days, >50% of cells were positive for MHC class I expression and exhibited a biphasic staining pattern. MHC class I positive cells were first detectable 4 to 5 days after RA treatment (not shown), and levels of staining gradually increased up to 9 days of treatment. As shown in Fig. 1C, anti-β-2m antibody staining paralleled MHC class I staining and produced a biphasic profile (compare Fig. 1A with Fig. 1C). These data indicate that MHC class I and β-2m molecules are coordinately induced in NT2 cells following RA treatment. We further examined the RA treated cells with tetanus toxin, which binds neuronally differentiated NT2 cells (1). Results in Fig. 1B, D, and E indicate that a significant fraction of cells expressing MHC class I were of the neuronal cell type.

To determine whether the increase in surface expression correlated with an increase in RNA levels, we performed Northern analysis of RNA harvested from NT2 cells during RA treatment by using the HLA Aw33 probe. As seen in Fig. 1F, undifferentiated NT2 cells expressed very low levels of MHC class I mRNAs, but a significant increase was observed after 4 and 7 days of RA treatment. These results demonstrate that steady-state levels of MHC class I mRNA increase in NT2 cells after RA treatment.

Induction of MHC class I promoter activity by RA treat-

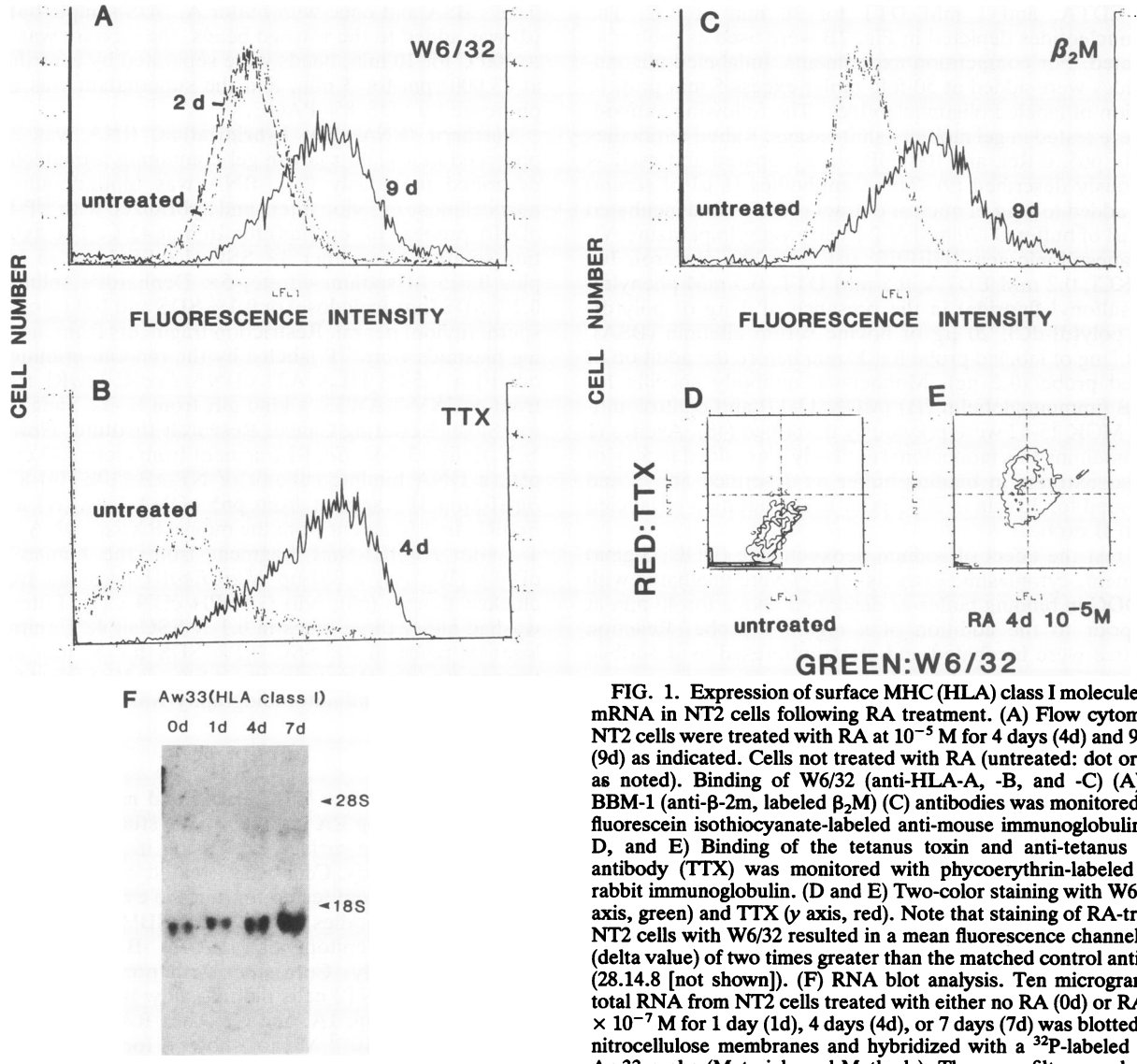


FIG. 1. Expression of surface MHC (HLA) class I molecules and mRNA in NT2 cells following RA treatment. (A) Flow cytometry. NT2 cells were treated with RA at 10^{-5} M for 4 days (4d) and 9 days (9d) as indicated. Cells not treated with RA (untreated: dot or dash as noted). Binding of W6/32 (anti-HLA-A, -B, and -C) (A) and BBM-1 (anti-β-2m, labeled β_2M) (C) antibodies was monitored with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin. (B, D, and E) Binding of the tetanus toxin and anti-tetanus toxin antibody (TTX) was monitored with phycoerythrin-labeled anti-rabbit immunoglobulin. (D and E) Two-color staining with W6/32 (x axis, green) and TTX (y axis, red). Note that staining of RA-treated NT2 cells with W6/32 resulted in a mean fluorescence channel shift (delta value) of two times greater than the matched control antibody (28.14.8 [not shown]). (F) RNA blot analysis. Ten micrograms of total RNA from NT2 cells treated with either no RA (0d) or RA at 5×10^{-7} M for 1 day (1d), 4 days (4d), or 7 days (7d) was blotted onto nitrocellulose membranes and hybridized with a ^{32}P-labeled HLA Aw33 probe (Materials and Methods). The same filter was hybridized with a β-actin probe (Fig. 4E, left panel).

ment. MHC class I gene expression is regulated by a highly conserved class I regulatory complex (−203 to −139) located in the 5′ promoter region of the gene (Fig. 2). We and others have previously described two conserved enhancer elements in the MHC class I regulatory complex, region I (also called enhancer A) and region II (Fig. 2) that are located adjacent to additional elements, the interferon response element and the negative regulatory element (7, 25, 68, 77). To study the contribution of the MHC class I regulatory complex to the induction of MHC class I gene expression by RA, promoter activity was examined in NT2 cells before and after RA treatment. CAT reporters driven by the 5′ upstream region of the H-2L^d gene (see Fig. 2 for map) were tested. pL^{d}1400.CAT and pL^{d}237.CAT included the upstream regulatory complex (located from −203 to −139) and contained 1,400 and 237 bp, respectively, of the gene. This regulatory complex is deleted in pL^{d}123.CAT and pL^{d}60.CAT, which contain only 123 and 60 bp of upstream sequence, respectively (Fig. 2). As seen in Fig. 3A, all reporter constructs, pL^{d}1400.CAT, pL^{d}237.CAT, and pL^{d}123.CAT, gave low CAT activities in untreated NT2 cells. pL^{d}123.CAT gave slightly higher CAT activity than pL^{d}237.CAT and pL^{d}1400.CAT, presumably because of modest activity of a downstream regulatory element(s) present in pL^{d}123.CAT (23) or lack of negative control by an upstream repressor element. Negative regulation of pL^{d}123.CAT was observed in F9 EC cells (25). In NT2 cells treated with RA for 7 days, however, pL^{d}1400.CAT and pL^{d}237.CAT gave much higher activity than pL^{d}123.CAT. The results indicate that the MHC class I promoter activity is weak in untreated NT2 cells, but a strong promoter activity is induced after RA treatment mediated by the sequence between −237 and −123 bp.

To determine the contribution of region I and region II to the RA-induced promoter activity, CAT reporters containing a 4-bp mutation of region I (pL^{d}1400MI.CAT), region II (pL^{d}1400MII.CAT), both region I and region II (pL^{d}1400 MI+MII.CAT), or of a control region (pL^{d}1400MX.CAT) were examined (Fig. 2A). These mutations were placed in nucleotide sequences critical for factor binding to region I or

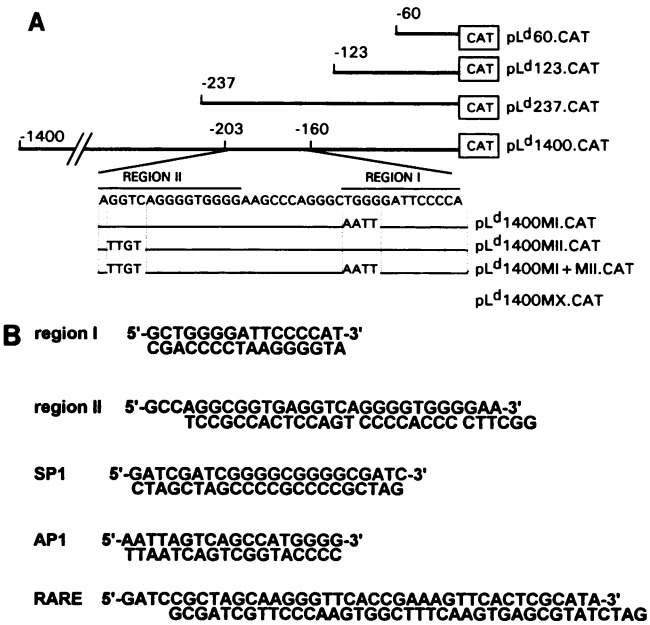


FIG. 2. Schematic representation of MHC class I enhancer elements and CAT constructs. (A) Reporter constructs. Region I (κB-like) and region II are part of the conserved MHC class I regulatory complex. pL^{d}1400.CAT and pL^{d}237.CAT (derived from the H-2L^d gene) contain region I and region II (−160 to −203), while truncated pL^{d}123.CAT and pL^{d}60.CAT do not. Mutant reporter constructs are similar to pL^{d}1400.CAT but contain 4-bp substitutions in region I (pL^{d}1400MI+MII.CAT), region II (pL^{d}1400MII.CAT), or both region I and region II (pL^{d}1400MI.CAT) at sites shown to be critical for factor binding (68). The control mutant construct pL^{d}1400MX.CAT is similar to pL^{d}1400.CAT but contains a 4-bp mutation (−137 to −141) in a region (X) not involved in known factor binding. (B) Oligonucleotide probes studied. Nucleotide sequences of oligonucleotides used for mobility shift analysis are depicted. Region I and region II sequences encompass the discrete enhancer elements previously identified (20). Region I, position −175 to −160; region II, −210 to −184 (20). Sequences of AP1 (13), SP1 (Stratagene), and βRARE (RARE) (19) are also shown.

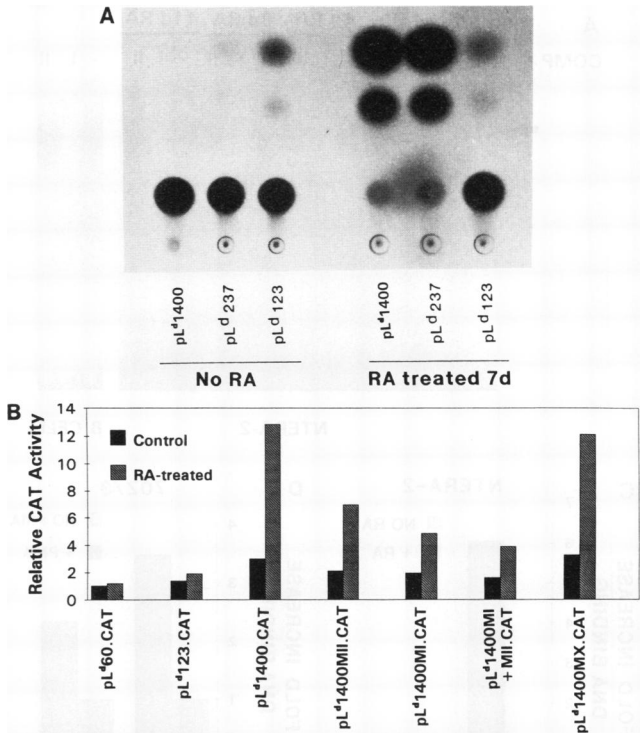
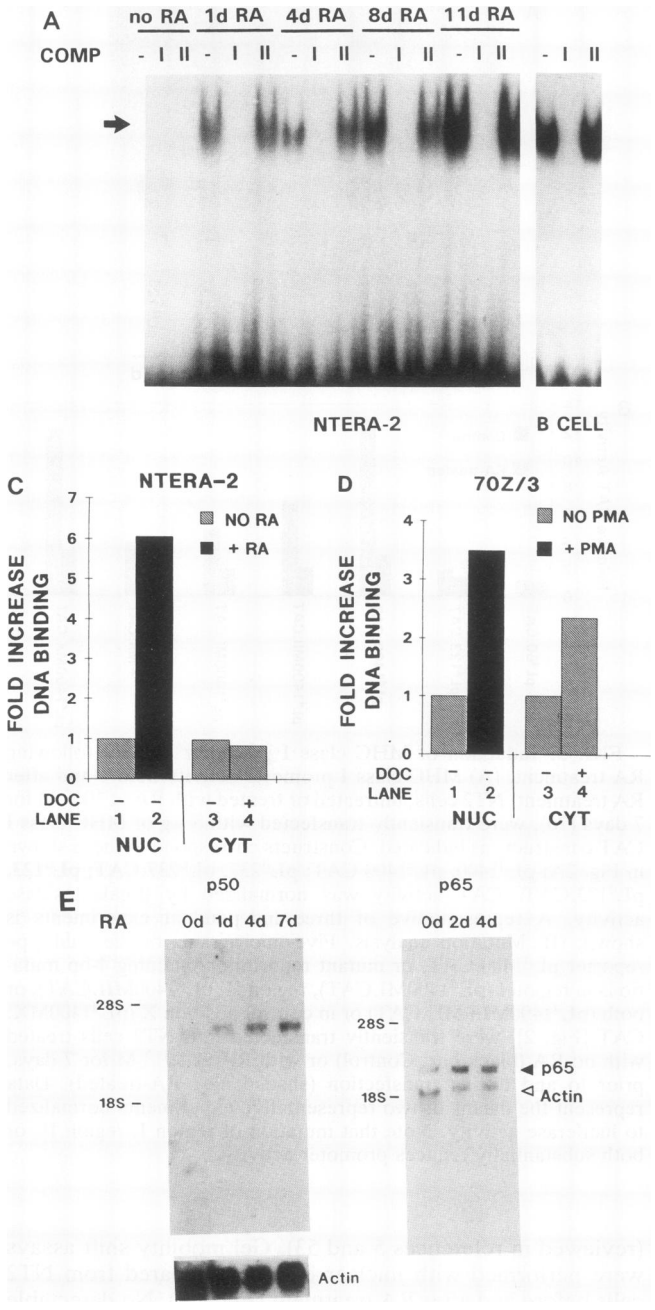


FIG. 3. Induction of MHC class I promoter activity following RA treatment. (A) MHC class I promoter activity before and after RA treatment. NT2 cells, untreated or treated with RA at 10^{-5} M for 7 days (7d), were transiently transfected with 5 μg of MHC class I CAT constructs as indicated. Constructs correspond to those shown in Fig. 2A: pL^{d}1400, pL^{d}1400.CAT; pL^{d}237, pL^{d}237.CAT; pL^{d}123, pL^{d}123.CAT. CAT activity was normalized by β-galactosidase activity. A representative of three independent experiments is shown. (B) Mutation analysis. Five micrograms of the wild-type reporter pL^{d}1400.CAT, or mutant reporters containing 4-bp mutations in region I (pL^{d}1400MI.CAT), region II (pL^{d}1400MII.CAT), or both (pL^{d}1400MI+MII.CAT) or in control position X (pL^{d}1400MX.CAT [Fig. 2]) were transiently transfected into NT2 cells treated with no RA (black bar; Control) or with RA at 10^{-5} M for 7 days, prior to and during transfection (shaded bar; RA-treated). Data represent the means of two representative experiments normalized to luciferase activity. Note that mutation of region I, region II, or both substantially reduces promoter activity.

region II (68). As shown in Fig. 3B, mutation of region II resulted in a 50% reduction in RA-induced MHC class I promoter activity. Mutation of region I resulted in a >50% reduction in promoter activity. Conversely, no reduction in promoter activity was seen with mutation of the control region X. The region I and region II double mutant substantially reduced the induction of MHC class I promoter activity by RA to levels slightly less than mutation of region I alone. The fact that the region I and region II double mutant still responded to RA (albeit at a reduced level) suggests that an additional (previously unknown) element(s) upstream from −123 is weakly activated by RA. These results show that the region I and region II enhancers are involved in induction of MHC class I transcription. These data also indicate that both enhancers contribute to MHC class I promoter activity following RA treatment.

RA treatment of NT2 cells induces region I binding by the p50-p65 NF-κB complex. We next examined whether RA treatment influenced factor binding to region I and region II. Region I binds a factor designated either KBF1 (77) or H-2TF1 (7) as well as the NF-κB components, p50 and p65 (27, 37), and other proteins belonging to the Rel family (reviewed in references 5 and 53). Gel mobility shift assays were performed with nuclear extracts prepared from NT2 cells before and after RA treatment (Fig. 4A). No detectable band was observed when the region I probe was added to extracts from untreated NT2 cells (Fig. 4A). Region I binding activity was negligible in nuclear extracts prepared from untreated NT2 cells despite the use of increased amounts of extract proteins or prolonged autoradiography of gels (not shown). This result is supported by similar observations using other κB-like elements (9, 26). However, extracts from cells treated with RA for 1 day produced a detectable retarded band (black arrow) at the position identical to that produced by B-cell extracts used as a positive control (Fig. 4A, right panel). The intensity of region I binding activity in NT2 cell extracts gradually increased after exposure to RA for up to 11 days. This band was specific for region I, since it was eliminated by excess unlabeled region I competitor but not by region II competitor (Fig. 4A). These results were highly reproducible and were



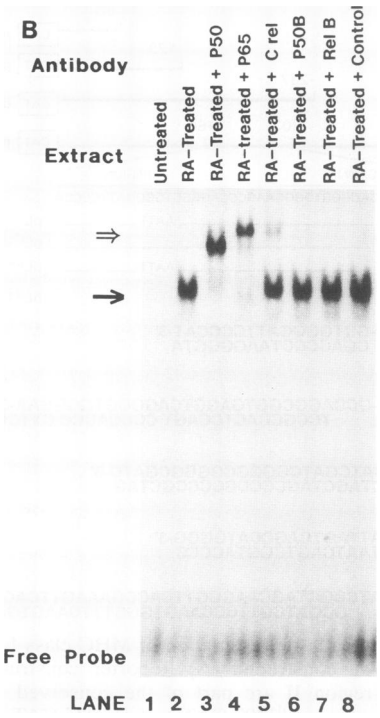


FIG. 4. RA-induced binding of NF-κB (p50-p65) to region I. (A) Analysis of region I binding activity in NT2 cells following RA treatment. Gel mobility shift assays were performed with a ^{32}P-labeled region I probe (Fig. 2) by using 5 μg of nuclear extracts from NT2 cells before and after RA treatment (5×10^{-7} M) for the indicated number of days (d). Competitor oligonucleotides were added at a 50-fold molar excess (I, region I competitor; II, region II competitor). Nuclear extracts from the human B-cell line Namalwa were used as a positive control (right panel). (B) RA-induced region I binding activity in NT2 cells consists of NF-κB factors. Gel mobility shift assays were performed with a region I probe as described above with 8 μg of NT2 nuclear extracts before (Untreated) and after RA treatment (at 10^{-5} M for 8 days). Extracts were preincubated with 1 μl of antibody for 30 min. Lane 1, extracts from untreated NT2 cells; lane 2, extracts from RA-treated cells; lanes 3 to 9, extracts from RA-treated cells preincubated with antibodies against p50 (lane 3), p65 (lane 4), c-Rel (lane 5), p50B (lane 6), RelB (lane 7), or preimmune serum (lane 8). (C and D) The absence of cytoplasmic NF-κB factors in untreated NT2 cells. Cytoplasmic (CYT) extracts (8 μg) from untreated NT2 cells (C) or pre-B cell 70Z/3 (D) were incubated with 1% DOC in binding buffer for 40 min at 4°C and were tested in gel mobility shift assays with a region I probe as described above. Nuclear (NUC) extracts (8 μg) prepared from NT2 cells treated with RA (10^{-5} M for 8 days) were used as a positive control. Extracts from 70Z/3 cells treated with phorbol ester phorbol myristate acetate at 50 ng/ml for 8 h were also tested as a positive control. The intensity of the specific region I band was quantitated by phosphorimager scanning and is expressed relative to region I band intensity produced by extracts of untreated cells. (E) RNA blot analysis. Three micrograms of poly(A)$^+$ RNA prepared from NT2 cells treated with RA for the indicated days (d) were blotted onto nitrocellulose membranes (left panel) or nylon membranes (right panel) and hybridized with ^{32}P-labeled cDNAs corresponding to p50 (left panel) and p65 (right panel). The same blots were probed with a β-actin cDNA.

verified with several separately prepared sets of nuclear extracts. These findings show that region I binding activity is absent or very low in untreated NT2 cells and is induced after RA treatment.

To identify the factors constituting binding to region I in NT2 cells, supershift analysis was performed with antibodies directed against various members of the Rel family. As shown in Fig. 4B, addition of antibody directed against NF-κB subunits, p50 (lane 3) or p65 (lane 4), supershifted (open arrow) the RA-induced region I band in NT2 nuclear extracts. The complex was almost entirely supershifted by either antibody, leaving little residual region I band (black arrow). No effect upon region I binding was seen with preimmune serum (lane 8) or with antibodies directed against RelB (lane 7) or p50B (lane 6). This effect was specific, since the antibodies had no effect upon gel shift complexes generated with the region II probe (not shown). Because of the presence of some c-Rel–p50 complex (9), antibody directed against c-Rel did show a weak partial supershift, but little

ablation (Fig. 4B, lane 5). Removal of the majority of the RA-induced region I complex by anti-p50 and anti-p65 antibodies indicates that the RA-induced region I complex is largely composed of a p50 and p65 heterodimer (37, 45). Consistent with these results, UV cross-linking experiments showed that nuclear extracts from RA-treated NT2 cells contained two protein species of about 50 and 65 kDa that interacted with a bromodeoxyuridine-substituted region I probe (not shown).

It has been extensively documented that in many cells NF-κB is present in the cytoplasm bound to an inhibitory protein belonging to the IκB family of proteins (5, 67). Treatment of cytoplasmic extracts with DOC has been shown to release the inhibitor and permit DNA binding (6). Since RA treatment led to induction of NF-κB factor binding to region I, it was possible that cytoplasmically partitioned NF-κB proteins translocated to the nucleus following RA treatment. To test this possibility, cytoplasmic extracts from untreated NT2 cells were treated with DOC and tested for region I binding activity in gel shift assay. As a control, cytoplasmic extracts from 70Z/3 pre-B cells known to release NF-κB activity in response to DOC treatment (67) were tested in gel shift assay. DOC treatment of cytoplasmic extracts from untreated NT2 cells did not result in region I binding activity (Fig. 4C, lane 4). In contrast, identical treatment of 70Z/3 cytoplasmic extracts did result in increased region I binding activity (Fig. 4D, lane 4). As expected, nuclear extracts from RA-treated NT2 cells and phorbol myristate acetate-treated 70Z/3 cells had much greater region I binding activity than extracts from untreated cells. These results demonstrate that RA induction of region I binding activity was not due to the release of preexisting NF-κB factors (26) but was largely due to increased NF-κB protein levels following RA treatment. Consistent with this interpretation, Northern analysis (Fig. 4E) of poly(A)$^+$ mRNA prepared from NT2 cells during RA treatment showed that message levels for both p50 (left panel) and p65 (right panel, black arrowhead) were very low in untreated cells and were substantially upregulated following RA treatment. These results indicate that RA induction of p50-p65 heterodimer binding to region I occurs as a result of their de novo synthesis, initiated by RA induction of both p50 and p65 mRNAs.

Increased region II binding activity by the RARβ-RXRβ heterodimer following RA treatment. We next tested whether region II binding activity was altered following RA treatment of NT2 cells. As seen in Fig. 5A, mobility shift analysis of extracts from untreated NT2 cells produced a weak, but detectable, retarded band which was inhibited by excess region II but not by the previously implicated region II factor, AP-1 (13). RA treatment (for both 1 and 8 days) induced a new region II band (Fig. 5, open arrow). In addition, RA treatment increased the intensity of the upper constitutive band, and both bands were inhibited by oligomers corresponding to region II. The region II sequence is similar to RARE identified in RA responsive genes (51, 75). As seen in Fig. 5B (lane 8), region II binding activity was efficiently inhibited by oligomers corresponding to the RARE from the RARβ gene (19), supporting a close relationship between region II and the βRARE. To examine whether the increase in region II binding activity involved RXRβ, we tested the effect of an antibody specific for RXRβ (MOK 13.17 [49]) upon the region II binding activity. As shown in Fig. 5C, addition of MOK 13.17 ablated both constitutive and RA-induced region II-specific bands. Conversely, an isotype-matched control antibody (MOK 15.42; C) did not affect either the constitutive or the induced bands. Since MOK 13.17 only detected a 44-kDa RXRβ band in NT2 nuclear extracts in Western blot (immunoblot) analysis, and not other related nuclear hormone receptors (48), it is clear that RXRβ contributes to region II binding activity before and after RA treatment. To confirm that the increase in both region I and region II binding activities was not attributable to a nonspecific change in the properties of the nuclear extracts, an unrelated oligonucleotide probe corresponding to the SP1 sequence was tested in mobility shift assays (Fig. 5D). This probe produced retarded bands of comparable intensity in both untreated and RA-treated NT2 cells. These results indicate that RA treatment of NT2 cells results in a specific increase in both region I and region II binding activities.

We have previously shown that RXRβ alone binds poorly to region II in vitro, but binding is augmented when the receptor is heterodimerized with RAR and other receptors (48). In addition, heterodimerization is a critical requirement for RAR binding to target DNA sequences (12, 41, 78). Since region II binding was inhibited by a RARE, a RAR was a likely heterodimerization partner for RXRβ. Because RARβ (but not RARα or RARγ) has been shown to be induced after RA treatment of NT2 cells (70) and F9 EC cells (79), we tested an antibody specific for RARβ (73) in mobility shift assays. Since this antibody did not yield a clean supershift effect, we performed immunoprecipitation assays of chemically cross-linked NT2 nuclear extracts. ^{35}S-labeled in vitro-translated RXRβ was mixed with nuclear extracts from NT2 cells, bound to biotinylated region II oligonucleotides, and cross-linked with DSS. Bound materials were eluted and precipitated with anti-RARβ antibody, and the antibody-bound materials were eluted and resolved by SDS-PAGE. As shown in Fig. 5E, much of ^{35}S-labeled RXRβ bound to region II was precipitated by anti-RARβ antibody (lane 2). The cross-linked complex migrated at about 100 kDa, indicating that the band represents a RARβ-RXRβ heterodimer. No labeled proteins were precipitated when extracts from untreated cells were used (lane 4). Likewise, experiments performed with control serum (lanes C) gave no specific precipitated band (lanes 1, 3, 5, and 7). Use of an antibody that recognizes all RARs revealed a similar precipitation pattern to that observed with the anti-RARβ antibody (66). To further confirm that the induced region II binding activity consisted of an RXRβ-RARβ heterodimer, the converse experiment was performed: ^{35}S-labeled RARβ was chemically cross-linked with NT2 nuclear extracts, precipitated with biotinylated region II oligonucleotide, reprecipitated by anti-RXRβ antibody, and resolved in an SDS gel (Fig. 5E, lanes 5 to 8). Labeled RARβ was likewise precipitated by anti-RXRβ antibody and formed two closely migrating bands of approximately 100 kDa. Extracts from both untreated and RA-treated NT2 cells produced RARβ cross-linked bands, consistent with the presence of RXRβ both before and after RA treatment (see below). These data indicate that the increased region II binding activity in NT2 cells following RA treatment represents binding of a RXRβ-RARβ heterodimer. These results, however, do not exclude the possibility that a minor component of the region II band is RXRβ heterodimerized with other receptors.

We next examined whether RA-induced RARβ-RXRβ binding to region II was due to modification of an existing receptor or de novo synthesis of the receptors. Northern analysis of mRNA prepared from NT2 cells following RA treatment did not reveal any change in steady-state levels of RXRβ message (Fig. 5F, right panel, open arrowhead).

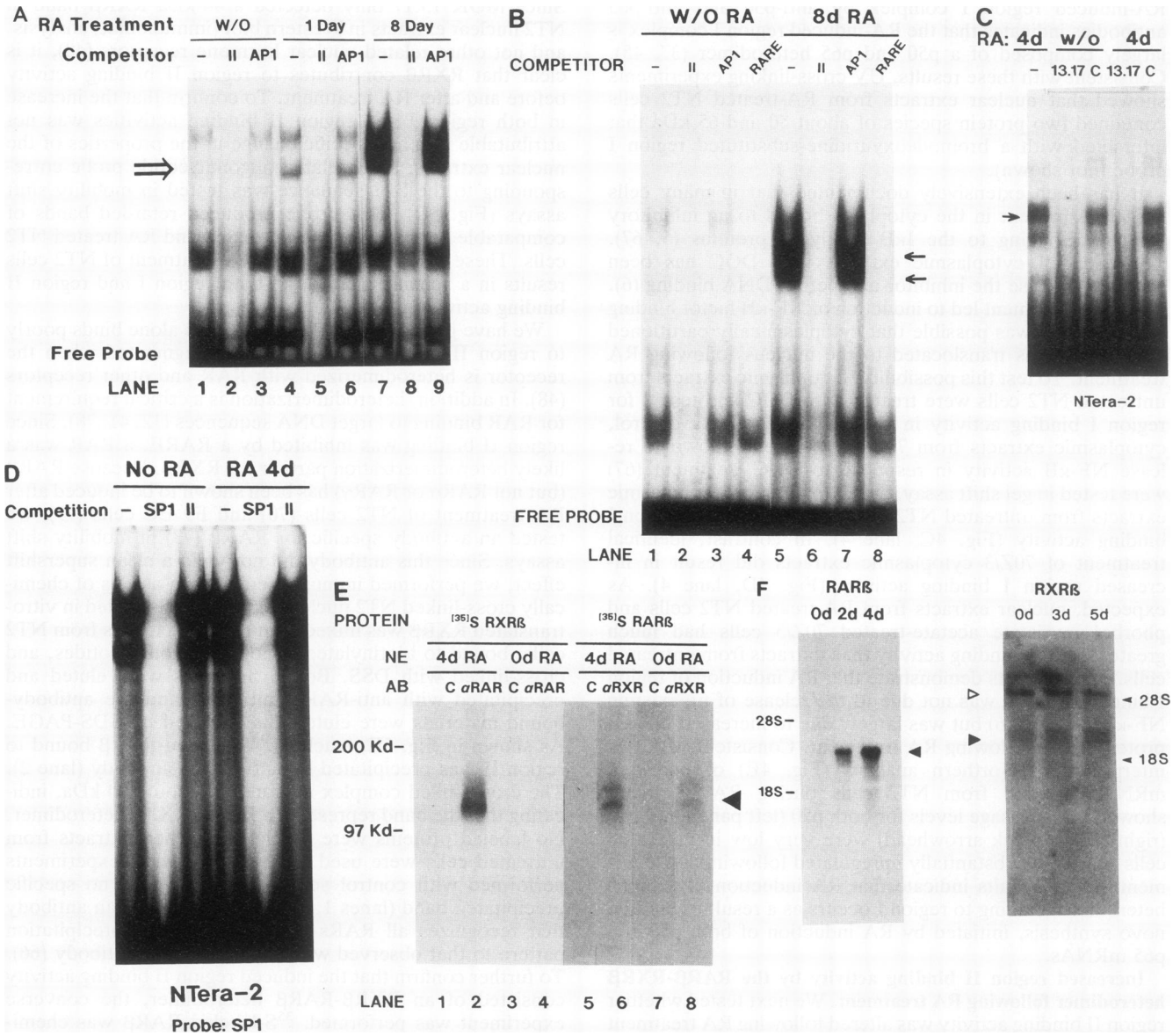


FIG. 5. Increased region II binding activity after RA treatment is due to RXRβ and RARβ heterodimers. (A and B) Analysis of region II binding activity in RA-treated NT2 cells. Gel mobility shift assays were performed with a ^{32}P-labeled region II probe (Fig. 2) by using nuclear extracts from NT2 cells following RA treatment at 10^{-5} M for 1 or 8 days. RA-induced band is marked with an open arrow (A) or with two black arrows (B). Competitor oligonucleotides were added at a 50-fold molar excess (II, region II competitor; AP-1 competitor; and a RARE from the RARβ gene). (C) Ablation of region II binding activity by anti-RXRβ antibody. Gel shift assays were performed as described above with nuclear extracts from untreated NT2 cells (w/o) or NT2 cells treated with RA for 4 days (4d) preincubated with ascites (1 μl) of a monoclonal antibody specific for RXRβ (lanes 13.17) or control (lanes C) isotype-matched ascites of MOK 15.42 (1 μl). (D) Lack of an RA-induced change in SP1 binding activity in NT2 cells. Gel mobility shift experiments were performed with a SP1 probe by using nuclear extracts (8 μg) from NT2 cells as described above. Unlabeled DNA was added at a 50-fold molar excess. (E) Chemical cross-linking and immunoprecipitation. In vitro translated, ^{35}S-labeled RXRβ protein (lanes 1 to 4) was mixed with nuclear extracts (NE) from untreated NT2 cells (0d RA) or cells treated with RA at 10^{-5} M for 4 days (4d RA), bound to biotinylated region II oligomer, cross-linked with DSS, washed, eluted, and reprecipitated with rabbit anti-human RARβ antibody (αRAR) or preimmune serum (lanes C). In vitro-translated ^{35}S-labeled RARβ (lanes 5 to 8) was similarly cross-linked, precipitated by region II oligomer, and reprecipitated with anti-RXRβ antibody (αRXR) or control antibody (lanes C). Black arrowhead identifies in vitro-translated labeled protein which has been cross-linked to a binding protein in nuclear extracts and specifically precipitated by antibody directed against the opposite (putative) heterodimer partner. (F) RNA blot analysis. Five (right panel) and three (left panel) micrograms of poly(A)$^+$ RNA were blotted onto a nylon membrane (right panel) or a nitrocellulose membrane (left panel) hybridized with a cDNA probe for RARβ (left panel) or RXRβ (right panel). The blot in the left panel was probed with β-actin cDNA as a control (shown in Fig. 4E, right panel). RNA in the right panel was probed with a control GAPDH cDNA (open arrowhead). Black arrowhead (right panel) identifies RXRβ-specific transcript. d, days of RA treatment.

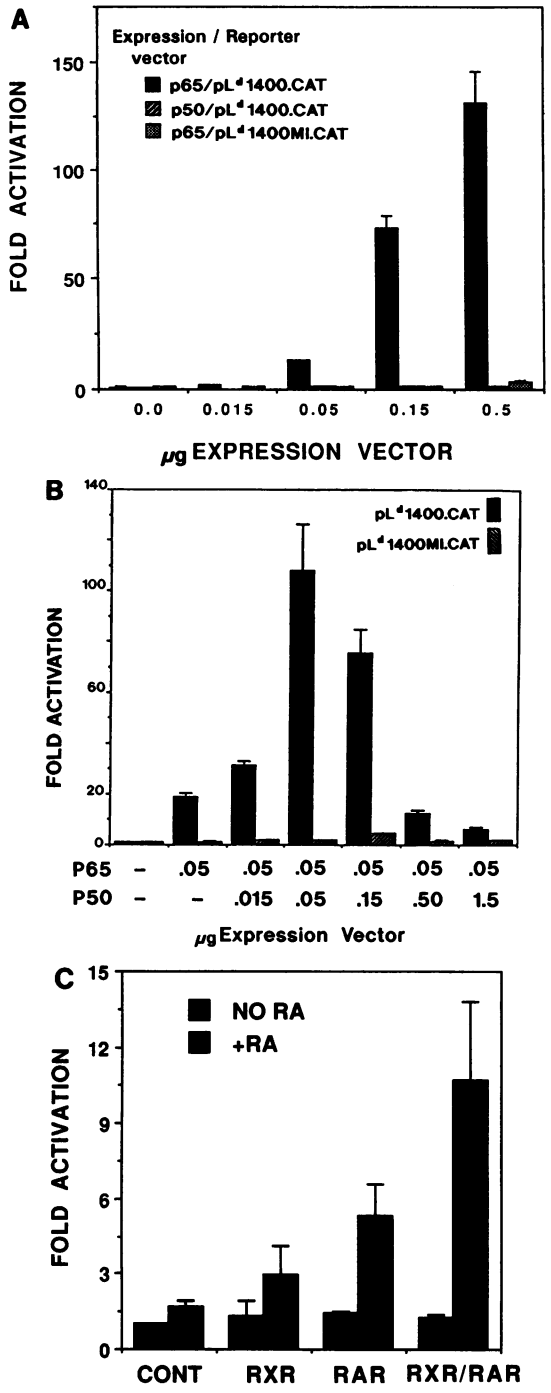


FIG. 6. Addition of NFκB p50-p65 or RARβ-RXRβ to untreated NT2 cells activates the MHC class I reporter. (A) Activation of region I-dependent reporter activity by p65 alone, but not p50. Untreated NT2 cells were transfected with 5 μg of pL^{d}1400.CAT or a mutant reporter, pL^{d}1400MI.CAT (Fig. 2), and increasing amounts of an expression vector for p50 or p65 as shown. Lane 1, no expression vector; lanes 2 through 6, all had expression vector (lane 2, 0.015 μg; lane 3, 0.05 μg; lane 4, 0.15 μg; lane 5, 0.5 μg). Values represent the means of three experiments +/− standard deviation. (B) Synergistic, dose-dependent activation of region I by p50-p65 cotransfection. pL^{d}1400.CAT or mutant pL^{d}1400MI. CAT was transfected into untreated NT2 cells with 0.05 μg of the p65 expression vector and increasing amounts of p50 as indicated. Data represent mean of three experiments +/− standard deviation. Note that maximal activation of region I-dependent reporter activity in the presence of p50 and p65 occurs at amounts of expression vector less than that observed with p65 alone (A). (C) Transfection of RXRβ and RARβ activate the MHC promoter in NT2 cells not pretreated with RA. pL^{d}1400.LUC (500 μg) was transfected into untreated NT2 cells with 300 μg of expression construct containing the cDNA for RXRβ (RXR), RARβ (RAR), or both (RXR/RAR) or the control expression plasmid lacking insert (CONT). A total of 10 μM of RA (+RA) or vehicle (NO RA) was added for 24 h. Data represent the fold activation (mean + standard deviation) of three experiments normalized for β-galactosidase activity.

Likewise, western analysis of NT2 nuclear extracts showed that RXRβ protein levels did not change after RA treatment (not shown). Conversely, RARβ mRNA was strongly induced after 2 and 4 days of RA treatment (Fig. 5F, left panel). Taken together, the data in Fig. 5 indicate that the observed increase in region II enhancer activity is attributable to heterodimerization of the existent RXRβ with RARβ which is induced following RA treatment.

Transfection of p50-p65 and RARβ-RXRβ into untreated NT2 cells activates MHC class I promoter activity. We reasoned that if MHC class I gene expression in undifferentiated cells was limited solely by the lack of positively acting factors, expression of NF-κB factors in untreated NT2 cells should result in an increase in MHC promoter activity. To test this possibility, untreated NT2 cells were transfected with expression vectors for p50, p65, or both (9) together with the pL^{d}1400.CAT reporter or the mutant reporter which carries a mutation in region I, pL^{d}1400MI.CAT (Fig. 2). Transfection of various concentrations of p50 alone failed to activate pL^{d}1400.CAT (Fig. 6A). Conversely, transfection of relatively large amounts of p65 (>0.15 μg) resulted in a dose-dependent increase in pL^{d}1400.CAT promoter activity. This increase was dependent on region I, since mutation of region I abolished the promoter activity (Fig. 6A). Cotransfection experiments were also performed with 0.05 μg of p65 (an amount which gave little activation alone) and increasing amounts of p50. As seen in Fig. 6B, strong synergistic activation was observed when the amount of transfected p50 was less than 0.15 μg. Transfection of greater amounts of p50 resulted in a dose-dependent inhibition of promoter activity (9, 26 [and references therein]). The effect was again dependent upon the region I element, since the pL^{d}1400MI. CAT reporter was unresponsive. These results indicate that an increase in region I enhancer activity does not depend on RA treatment; rather, it requires expression of p50 and p65.

Since RXRβ was found to heterodimerize with RARβ in NT2 cells following RA treatment, it was important to determine whether the combination of the two receptors could activate transcription of a MHC class I reporter. A luciferase reporter (pL^{d}1400.LUC) was used for these experiments. With this reporter, MHC class I promoter activity could be measured at earlier times following transfection than with CAT reporters; the increased sensitivity permitted determination of reporter activity with minimal exposure of the untreated cells to RA (note in Fig. 5F that 48 h after RA treatment, RARβ mRNA is expressed in NT2 cells). Results are shown in Fig. 6C. As expected, addition of RA in the presence of control expression constructs did not result in a substantial increase in luciferase activity. Addition of either RARβ or RXRβ alone caused a modest increase in luciferase activity, which was dependent on RA treatment. However, addition of both RARβ and RXRβ resulted in a 10-fold

increase in reporter activity in the presence of RA. These data show that a combination of RARβ and RXRβ can activate MHC class I promoter activity in NT2 cells, supporting a functional role of RARβ-RXRβ heterodimers.

DISCUSSION

Nearly 20 years ago Artzt and Jacob (4) noted that MHC class I molecules (and β-2m) were absent in undifferentiated EC cells. Since then, RA induction of MHC class I gene expression in EC cells has drawn considerable attention (2, 15, 16, 54) and is now regarded as relevant to developmental regulation of the genes in vivo (31, 35, 59). Here we have analyzed the mechanism of RA induction of MHC class I genes in NT2 cells. Our analysis led us to conclude that the respective binding of the NF-κB heterodimer, p50-p65, and the RARβ-RXRβ heterodimer to the conserved enhancer elements, region I and region II, at least partly accounts for MHC class I gene induction. It should be noted that although RA-induced activation of MHC class I enhancer activity is prominent in NT2 cells, this is not readily detected in F9 EC cells. MHC class I promoter activity is strongly repressed in undifferentiated F9 cells by a negative regulatory element that is located downstream of region I (25), which presumably obscures RA-induced enhancer activity in F9 cells. It is also noteworthy that transcriptional activation of MHC class I genes by RA (measurable 24 to 48 h after RA treatment) does not lead to immediate surface expression in NT2 cells (Fig. 1). A similar delay in surface expression after RA or interferon stimulation has been observed for undifferentiated F9 cells and other embryonic cell lines (69). This may be due to delays in peptide loading and in various steps of the transport processes affecting surface MHC class I expression.

The event that initiates the RA-induced cascade of gene regulation in EC cells is almost certainly binding of RA to the RARs and RXRs (33, 43) and subsequent receptor binding to RARE target sequences present in RA responsive genes. RXRs likely play a pivotal role in this process, since RXR (α, β, or γ) heterodimerization with RARs (α, β, or γ) leads to augmented RARE binding, as well as a cooperative activation of target gene transcription (12, 39, 41, 78). In agreement, we previously observed that either the RAR (α or β) or RXRβ receptors alone bind weakly to region II; but upon forming a heterodimer, in vitro binding to the element is significantly increased (48). Despite the apparent role of RXRs and RARs in RA-induced gene regulation, the exact heterodimeric combinations of receptors responsible for regulating specific target genes in NT2 cells were previously unknown. By using antibodies specific for RARβ and RXRβ, we show that region II binding activity (both before and after RA treatment) contains RXRβ (Fig. 5C). Furthermore, by a combination of chemical cross-linking and immunoprecipitation, we show that much of the RA-induced factor that complexes with RXRβ and binds to region II is indeed RARβ.

RARβ mRNA levels rapidly increased following RA treatment of NT2 cells (Fig. 5F). On the other hand, RXRβ was shown to be expressed regardless of RA treatment of NT2 cells: RXRβ mRNA and protein levels were not changed by RA treatment. These observations indicate that the increased region II binding activity following RA treatment is largely due to induction of a heterodimer composed of the preexisting RXRβ and the RA-induced RARβ. The increased region II enhancer activity is most likely to be due to binding of the RARβ-RXRβ heterodimer, since MHC class I reporter activity was activated by cotransfection of RXRβ and RARβ (Fig. 6). Other members of the RAR and RXR family, although expressed in EC cells (70, 79), appear not to contribute significantly to region II binding in NT2 cells (Fig. 5). Since the induced region II binding activity also bound the βRARE, we speculate that the RXRβ-RARβ heterodimer could be playing a significant role in an early phase of RA-mediated gene regulation in general (Fig. 5B). Because RARβ is not expressed at an appreciable level in untreated NT2 cells, the original triggering of RARβ expression by RA may be due to formation of a RAR (α or γ)-RXRβ heterodimer or a homodimer of RXRβ.

We observed that region I binding activity is absent in untreated NT2 cells but is induced after RA treatment. Although in vitro binding of the Rel family proteins to various κB motifs has been well documented (5, 9, 27, 37, 64), the proteins actually responsible for in vivo region I binding have rarely been identified. Our data in Fig. 4B show that the p50-p65 heterodimer constitutes much of region I binding activity in RA-treated NT2 cells. This conclusion is supported by the findings that (i) both anti-p50 and anti-p65 antibodies independently supershifted the region I band, (ii) UV cross-linking with a region I probe identified two proteins of approximately 50 and 65 kDa (data not shown), and (iii) both p50 and p65 mRNAs were induced by RA treatment (Fig. 4E; see below). The participation of p50 in region I binding activity is expected, on the basis of the previous report that KBF1, a protein isolated for its binding to region I, is in fact p50 (37). In vitro binding to region I has been demonstrated for the p50 homodimer as well as the p50-p65 heterodimer (37, 45). However, it appears that most of p50 is associated with p65 in the cell (reviewed in reference 5). In agreement, our results indicate that the p50-p65 heterodimer is likely to be responsible for RA-induced region I enhancer activation, since transfection of low amounts of both p50 and p65 led to cooperative enhancement of region I-dependent MHC class I reporter activity (Fig. 6).

The RA-induced activation of the subunits, p50 and p65, presented in this work is quite distinct from the previously documented activation of NF-κB subunits by a series of posttranslational changes (reviewed in reference 5). In many cells the p50-p65 heterodimer is sequestered in the cytoplasm by associating with an inhibitory subunit, IκB. A wide range of external stimuli initiates the dissociation of this inhibitory subunit, leading to induction of nuclear translocation and increase in DNA-binding activity (5, 6, 26). We demonstrate that in untreated NT2 cells there is no detectable cytoplasmically partitioned region I binding activity poised to be released to the nucleus and that there is very little detectable mRNA for p50 or p65 (Fig. 4). However, both p50 mRNA and p65 mRNA levels increased gradually over several days following RA treatment. Coincidentally, region I binding activity gradually increased. Thus, de novo induction of p50 and p65 most likely accounts for RA-induced region I binding activity in NT2 cells. To our knowledge, this is the first report demonstrating that de novo activation of NF-κB protein subunits leads to functional activation of a target gene. These results raise the interesting possibility that both the p50 and the p65 genes are developmentally controlled (in addition to being controlled by cellular activation [9, 10, 64]) and that their transcription is coordinately induced following RA treatment of EC cells. It will be of importance to determine whether the p50 and p65 genes have an RARE which explains their induction by RA or whether their induction is mediated by a secondary transcription factor(s) induced after RA treatment.

Over the years, extensive studies have been performed on genes that may be involved in morphological differentiation of EC cells (17, 34, 50, 58). In addition, many regulatory genes that may play a role in controlling growth and differentiation of EC cells have been investigated (40, 46, 55, 57, 63, 70, 76). Given the capacity of the two species of heterodimers studied here to bind many additional *cis* elements, the mechanistic basis for changes in gene expression outlined in this work may be involved in the developmental regulation of a number of other genes.

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The first two authors made equal contributions to this work.

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