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Retinoic Acid-Induced Granulocytic Differentiation of HL-60 Myeloid Leukemia Cells Is Mediated Directly through the Retinoic Acid Receptor (RAR-α)

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Retinoic acid (RA) induces terminal granulocytic differentiation of the HL-60 promyelocytic leukemia cell line as well as certain other human myeloid leukemias. Specific RA receptors that are members of the steroid-thyroid hormone superfamily of nuclear transcription factors have recently been identified. We developed an HL-60 subclone that was relatively resistant to RA-induced differentiation. Specific nuclear RA receptors in this RA-resistant subclone had a decreased affinity for RA and exhibited a lower molecular weight compared with nuclear RA receptors from the RA-sensitive parental HL-60 cells. Retroviral vector-mediated transduction of a single copy of the RA receptor (RAR-α) into this RA-resistant HL-60 subclone restored the sensitivity of these cells to RA. These observations indicate that RAR-α plays a critical and central role in mediating RA-induced terminal differentiation of HL-60 leukemia cells.

Retinoids and in particular retinoic acid (RA) exert a wide range of biologic effects. RA influences epithelial cell growth and differentiation (33), suppresses malignant transformation both in vitro and in vivo (3, 35), induces differentiation of mouse embryonal carcinoma cells in vitro (43), and influences the development of the regenerating amphibian limb (44). In addition, this compound stimulates the differentiation of certain types of human acute myelogenous leukemia (AML) cells and cell lines. For example, RA induces terminal granulocytic differentiation of the HL-60 myeloid leukemia cell line (8) as well as certain fresh human AML cells in vitro (7). Moreover, as a single agent this compound induces complete remissions in certain patients with AML without inducing marrow aplasia (25, 34) and thus is of potential therapeutic importance in certain leukemic disorders.

The mechanism by which RA induces these diverse biologic effects is unclear. Initially, it was suggested that these effects were mediated through a highly specific cellular RA-binding protein (CRABP) (9, 26). However, some cells responding to RA, including HL-60, do not appear to express this protein (19). More recently a number of specific RA receptors (RARs) that possess discrete DNA-binding and ligand-binding domains and that are structurally related to members of the steroid-thyroid hormone receptor superfamily of nuclear transcription factors have been identified. To date, these include the following: RAR-α, which exhibits widespread expression in numerous different tissues and cell lines including HL-60 (22, 41); RAR-β, which is expressed in a wide variety of epithelial cell types (1, 6, 17); and RAR-γ, whose expression appears to be confined primarily to skin (28). The identification of these receptors suggests that RA may exert its biologic effects in a manner similar to that of glucocorticoids; i.e., the interaction of RA with its specific receptor allows binding of the receptor to specific *cis*-acting DNA sequences regulating the transcription of certain target genes. Nevertheless, in human myeloid leukemia cells the relationship between RAR expression and response to RA is unclear, since many AML samples from different patients express RAR-α but do not differentiate in response to RA (21, 30, 47).

In the present study we describe the development and analysis of an HL-60 subclone (designated HL-60R) that exhibited relative resistance to RA-induced terminal granulocytic differentiation. This RA-resistant subclone harbored nuclear RARs with a decreased affinity for RA and a decreased molecular weight compared with the parental HL-60 RARs. When a single copy of retrovirus-transduced RAR-α cDNA was introduced into these RA-resistant HL-60 cells, the sensitivity of these cells to RA-mediated granulocytic differentiation was restored. Our observations provide direct evidence that RA-induced terminal differentiation of HL-60 is mediated directly through RAR-α.

MATERIALS AND METHODS

Development of an RA-resistant HL-60 subclone. HL-60 cells are cultured in liquid suspension in RPMI medium supplemented with 5% fetal bovine serum. An RA-resistant subclone of HL-60 was derived by continuously culturing HL-60 cells in growth medium containing 1 μM RA for approximately 30 passages over a 6-month period. Approximately 10^4 of these cells were then suspended in 35-mm petri dishes containing 1.1 ml of RPMI medium supplemented with 0.75% agar, 10% fetal bovine serum, and 1 μM RA. After 14 days of culture at 37°C in a humid environment, individual colonies containing at least 50 to 100 cells were picked from the agar plates and suspended in liquid medium in 0.2-ml microdilution wells. These cells were progressively expanded into larger wells, and an individual subclone (designated HL-60R) was chosen for further analysis.

Retroviral vector construction and production. The recently described LXSN retroviral vector (37) was utilized in these studies. This vector harbors a unique cloning site such

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that inserted cDNAs are driven by the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR). The vector also contains the neomycin phosphotransferase gene (*neo*^{r}) driven by the simian virus 40 promoter for use as a selectable marker. A recombinant plasmid containing a 2.8-kilobase (kb) RAR-α cDNA cloned into the *Eco*RI site of plasmid pHC624 (4) was obtained from Vincent Giguere and Ron Evans (22). A 2,022-base-pair (bp) *Bam*HI fragment from this recombinant plasmid was inserted into the *Bam*HI cloning site of the LXSN retroviral vector plasmid. This 2,022-bp *Bam*HI fragment consists of 12 bp of polylinker sequence between the *Bam*HI and *Eco*RI sites of the pHC624 plasmid, 103 bp of 5′ untranslated RAR-α cDNA, 1,386 bp of RAR-α-coding sequence, and 521 bp of 3′ untranslated RAR-α sequence. Virus was generated from the LXSN plasmid harboring this *Bam*HI insert (now designated LRARSN) as previously described (36). Briefly 10 μg of cesium chloride-banded LRARSN plasmid was transfected via $CaPO_4$ precipitation into the ψ2 ecotropic retrovirus packaging cell line (36). The supernatant was harvested after 2 days and used to infect the PA317 amphotropic packaging cell line. These infected PA317 cells were selected in 1 mg of G418 (GIBCO Laboratories) per ml, and individual G418-resistant clones were isolated and expanded. Total cellular RNA was extracted from these clones and analyzed by utilizing Northern (RNA) blot hybridization with an RAR-α probe to screen for the expected full-length 4.9-kb Mo-MuLV LTR-initiated retroviral vector RAR-α mRNA. Titers of retroviral vector production from the clonal PA317 producers were determined on NIH3T3 thymidine kinase-negative target cells with G418 selection as previously described (36). Supernatant from a LRARSN PA317 producer line harboring an unrearranged LRARSN provirus with a titer of 3×10^5 per ml was harvested and used to infect HL-60R cells.

Retroviral infection of HL-60R cells. The RA-resistant HL-60R subclone was suspended at 10^5 cells per ml in 5-ml flasks containing standard culture medium supplemented with 4 μg of Polybrene per ml. Supernatant from the PA317-LRARSN retroviral vector producer line was added at a multiplicity of infection of approximately 1:1. After 24 h of incubation at 37°C, the cells were suspended in growth medium supplemented with 1 mg of G418 per ml. Selection in this growth medium continued for at least 21 days. Individual clones of infected HL-60R cells were obtained by suspending approximately 10^3 of these infected G418 selected cells in 35-mm petri dishes containing 1.1 ml of growth medium supplemented with 0.75% agar and 500 μg of G418 per ml. After 14 days of culture in a humid environment, individual colonies containing at least 50 to 100 cells were picked, suspended in growth medium in 0.2-ml microdilution wells, and progressively expanded into larger culture wells and flasks.

Preparation of nuclear extracts. We modified the technique of Shapiro et al. (42) to obtain nuclear extracts from HL-60 cells. Cells (2×10^8 to 3×10^8) were washed once in SSC (0.15 M NaCl, 0.015 M sodium citrate [pH 7.4]) and once in phosphate-buffered saline (pH 7.4) containing 2 mM EDTA, and the cell pellet was suspended on ice for at least 15 min in 2 volumes of hypotonic buffer consisting of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9), 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid, 1 mM dithiothreitol, 10 mM KCl, and protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, 1,000 kIU of aprotinin (Calbiochem) per ml, and 10 μM leupeptin (Calbiochem). The cells were then subjected to 20 strokes with an A pestle in a Dounce homogenizer to release the nuclei followed by the addition of 0.1 volume of a 68% sucrose solution in a hypotonic buffer (42). The nuclei were pelleted with a 30-s spin at 10,000 rpm at 4°C in 15-ml Corex tubes in an SS34 Sorvall. The nuclear pellet was washed once with 1 ml of hypotonic buffer and again spun for 30 s at 10,000 rpm. The nuclear pellet was then suspended in 1 ml of extraction buffer consisting of 10 mM Tris hydrochloride (pH 8.5), 1.5 mM EDTA, 10 mM thioglycerol, 10% glycerol, 0.8 M KCl, and the same protease inhibitors as above. The extraction proceeded in a Beckman polycarbonate ultracentrifuge tube on ice for 60 min with gentle swirling every 5 to 10 min. This tube was then centrifuged at 130,000 × *g* for 30 min in a Beckman 70.1 TI rotor (45,000 rpm). The protein concentration of the supernatant extracts was quantitated by the Coomassie blue method (5), and the extracts were stored at −70°C before analysis.

RA binding and size-exclusion HPLC analysis. We modified the technique of Nervi et al. (39) to assess specific RA binding in the nuclear extracts. The nuclear extracts (from 200 to 800 μg in a total volume of 200 μl) were incubated with various amounts of [^{3}H]RA (50 Ci/mmol; Dupont, NEN Research Products) for 18 h at 4°C in the presence or absence of a 200-fold excess of unlabeled RA. To remove excess free RA, the extract was successively spun through three G-15 minicolumns prepared from Eppendorf tubes as described previously (32). The extracts were then fractionated by high-performance liquid chromatography (HPLC) over a Bio-Sil TSK-250 size exclusion column (600 by 7.5 mm; Bio-Rad Laboratories) at a flow rate of 1 ml/min; the eluent was 5 mM sodium phosphate (pH 6.8), 10 mM thioglycerol, 10% glycerol, and 0.4 M KCl. Fractions of 0.5 ml were recovered, and the radioactivity in each fraction was determined. For equilibrium binding studies the nuclear extracts were incubated with different concentrations of [^{3}H]RA, and specific RA binding in the peak HPLC fractions was determined by subtracting the disintegrations per minute obtained from extract incubations containing 200-fold excess of unlabeled RA from those obtained from incubations containing [^{3}H]RA alone.

Northern and Southern blot hybridizations. DNA extractions were performed by digesting nuclei with protease K, followed by phenol-chloroform extraction and ethanol precipitation. Southern blots of restriction endonuclease-digested genomic DNA were performed as previously detailed (12). RNA was extracted with guanidine hydrochloride and subjected to Northern blotting in formaldehyde denaturing gels as previously detailed (14).

Molecular probes. Molecular probes utilized in the Northern and Southern blots included the following: RAR-α, the 1,309-bp *Eco*RI-*Sma*I fragment from the hT1R RAR-α cDNA clone (22); actin, the pA 1β chicken actin 2-kb *Pst*I fragment (10); c-*myc*, a 1.8-kb human *myc* cDNA clone from David Bentley; CD18, a 1.8-kb *Eco*RI fragment isolated from a human cDNA library (24); and *neo*^{r}, a 0.9-kb *Pst*I fragment from the bacterial neomycin phosphotransferase gene isolated from N2 proviral DNA (27). All probes were labeled by nick translation before hybridization.

RESULTS

Characteristics of the RA-resistant subclone of HL-60. RA induces morphologic and functional granulocytic differenti-

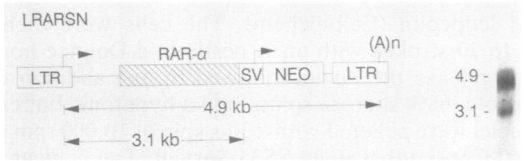

FIG. 1. Structure of the LRARSN retroviral vector. This vector was constructed, as detailed in Materials and Methods, by inserting the coding sequence of the RAR-α cDNA (22) into the *Bam*HI cloning site of the LXSN retroviral vector (37). The transcription start sites within the Mo-MuLV LTR and simian virus 40 promoter (SV) are indicated by the arrows. At right is a Northern blot of total RNA (5 μg) extracted from the PA317 packaging cell line producing this LRARSN retroviral vector and hybridized with a human RAR-α-specific probe. Under the hybridization and wash conditions utilized, this probe did not detect any endogenous mouse RAR-α transcripts.

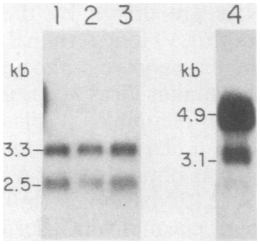

FIG. 2. Expression of RAR-α transcripts in uninfected and LRARSN-infected cells. Total RNA (5 μg per lane) was subjected to Northern blot analysis by using a human RAR-α-specific probe. The 3.3- and 2.5-kb endogenous RAR-α transcripts and the 4.9- and 3.1-kb LRARSN retroviral vector RAR-α transcripts (Fig. 1) are indicated. Lanes: 1, HL-60R cells; 2, parental HL-60 cells; 3, parental HL-60 cells incubated for 3 days with 1 μM RA; 4, HL-60R cells infected with the LRARSN retroviral vector.

ation of the HL-60 human promyelocytic leukemia cell line (8). Using methods detailed in Materials and Methods we isolated a subclone of HL-60 (designated HL-60R) exhibiting a markedly diminished response to RA. These cells had a doubling time in liquid suspension culture similar to that of the parental HL-60 cells. The approximately 20-fold genomic amplification of the c-*myc* gene noted in the parental HL-60 cells (11, 16) was also exhibited by the HL-60R cells (data not shown). Approximately 5 to 10% of the parental HL-60 cells exhibit spontaneous morphological and functional differentiation in the absence of inducing agents (15), but the HL-60R subclone exhibited virtually no such spontaneous differentiation. Whereas the parental HL-60 cells exhibit morphological and functional granulocytic differentiation associated with decreased proliferation when exposed to 0.01 to 1 μM RA (8), the HL-60R cells continued proliferating and exhibited markedly diminished morphological changes in concentrations of RA as high as 10 μM. The HL-60R cells have been continuously cultured in liquid suspension for over 1 year, and this same RA-resistant phenotype has been consistently displayed.

Retroviral vector-mediated gene transfer of RAR-α cDNA. As detailed in Materials and Methods, we inserted a cDNA fragment harboring the complete coding sequence of RAR-α (22) into the LXSN retroviral vector (37). This vector includes a convenient cloning site downstream from the Mo-MuLV LTR as well as the neomycin phosphotransferase gene (*neo*^{r}) for use as a selectable marker. The retroviral vector harboring RAR-α is designated LRARSN (Fig. 1) and was packaged in the amphotropic PA317 cell line (36). When RNA from LRARSN producer PA317 cell lines was hybridized to an RAR-α probe, the expected full-length 4.9-kb retroviral RNA was noted (Fig. 1). In addition, an unexpected 3.1-kb RAR-α transcript was also observed in these producer cells (Fig. 1). This latter transcript did not hybridize to a *neo*^{r} probe and most likely represented a Mo-MuLV LTR-initiated transcript that was truncated at a cryptic poly(A) site within the approximately 500 bp of 3′ untranslated sequences that were included in the RAR-α cDNA insert (see Materials and Methods).

The RA-resistant HL-60R cells were infected with the LRARSN retroviral vector, and individual G418 resistant subclones were isolated as described in Materials and Methods. A Southern blot analysis utilizing the enzyme *Hin*dIII, which cuts once within the LRARSN vector, and a *neo*^{r} probe indicated that each of these subclones harbored a single integrated copy of LRARSN proviral DNA (data not shown). These infected cells (now designated HL-60R+LRARSN) expressed the retroviral 4.9- and 3.1-kb RAR-α transcripts in addition to the endogenous 3.3- and 2.5-kb RAR-α mRNA (Fig. 2, lane 4).

RA-induced growth inhibition of infected HL-60R cells. Comparisons were made among the phenotypic response of the uninfected RA-resistant HL-60R subclone, the infected HL-60R+LRARSN cells, and the parental HL-60 cells after exposure to various concentrations of RA (Fig. 3 through 6). RA induced terminal differentiation of parental HL-60 cells as evidenced by the markedly diminished proliferation of these cells after exposure to RA (Fig. 3A). In contrast, little inhibition in the proliferation of the RA-resistant HL-60R subclone was observed in concentrations of RA as high as 10 μM (Fig. 3B). However, these same RA-resistant cells infected with the LRARSN vector exhibited markedly diminished proliferation when incubated with concentrations of RA as low as 0.1 μM (Fig. 3C). The RA-induced growth inhibition of the HL-60R+LRARSN cells was as great as if not greater than the growth inhibition observed in parental HL-60 cells after RA exposure (compare Fig. 3A and 3C).

RA-induced morphologic and functional differentiation of infected HL-60R cells. RA-induced granulocytic differentiation of parental HL-60 cells is characterized by marked morphological changes, including the indentation, convolution, and segmentation of nuclei that characterize maturing myelocytes, metamyelocytes, and banded and segmented neutrophils (8). Over 80% of parental HL-60 cells exhibited these morphological changes when cultured with RA (Fig. 4A). In contrast, the RA-resistant HL-60R cells exhibited only very slight morphologic changes at relatively high concentrations of RA (Fig. 4B and 5B). Nevertheless, the same RA-resistant HL-60R cells infected with the LRARSN vector and exposed to RA exhibited prominent morphologic changes characteristic of granulocytic differentiation (Fig. 4C and 5C).

The functional differentiation induced by exposure to RA was also compared among the different HL-60 cell types. Upon membrane stimulation with TPA, mature, differentiating HL-60 cells but not immature uninduced HL-60 cells will generate superoxide, which can be detected with the Nitro Blue Tetrazolium (NBT) dye reduction test (40). Cells capable of reducing NBT predominated in RA-induced cultures of parental HL-60 cells (Fig. 6A) but were exceedingly rare in the RA-treated resistant HL-60R cells (Fig. 6B). In contrast, NBT-positive cells were significantly increased in RA-treated HL-60R cells infected with the LRARSN retro-

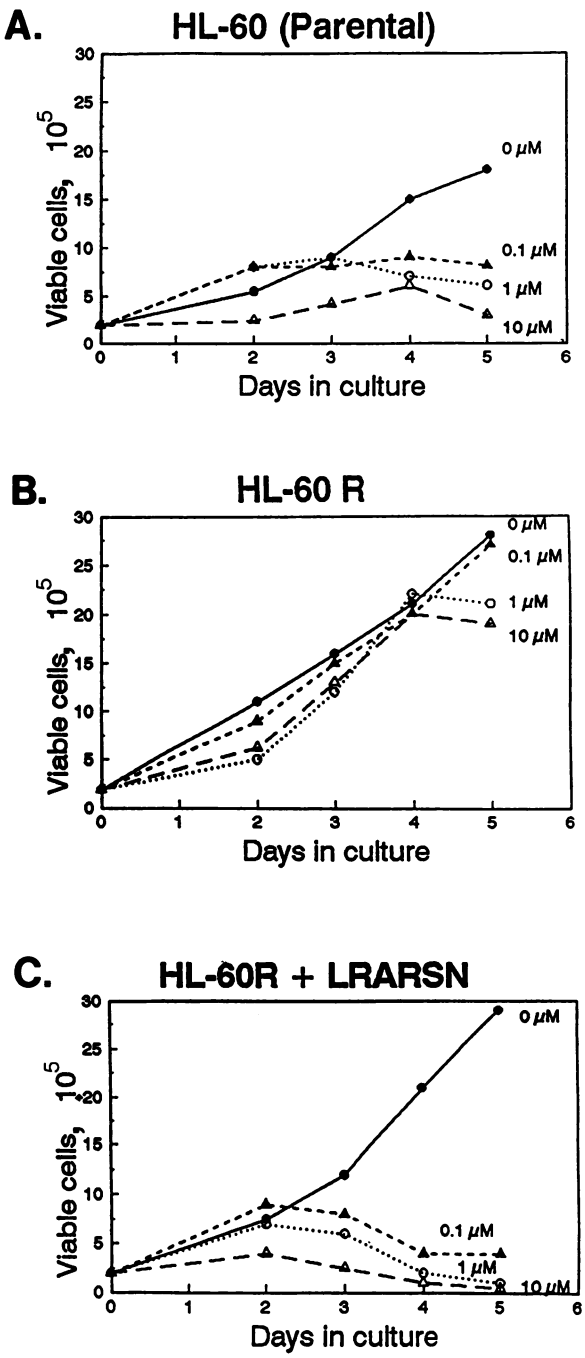

FIG. 3. Growth of various HL-60 lines in different concentrations of RA. Different HL-60 lines were incubated in liquid suspension in the indicated concentrations of RA, and the total viable cell number was determined at daily intervals by counting in a hemacytometer chamber. The indicated points represent the mean of two independent experiments.

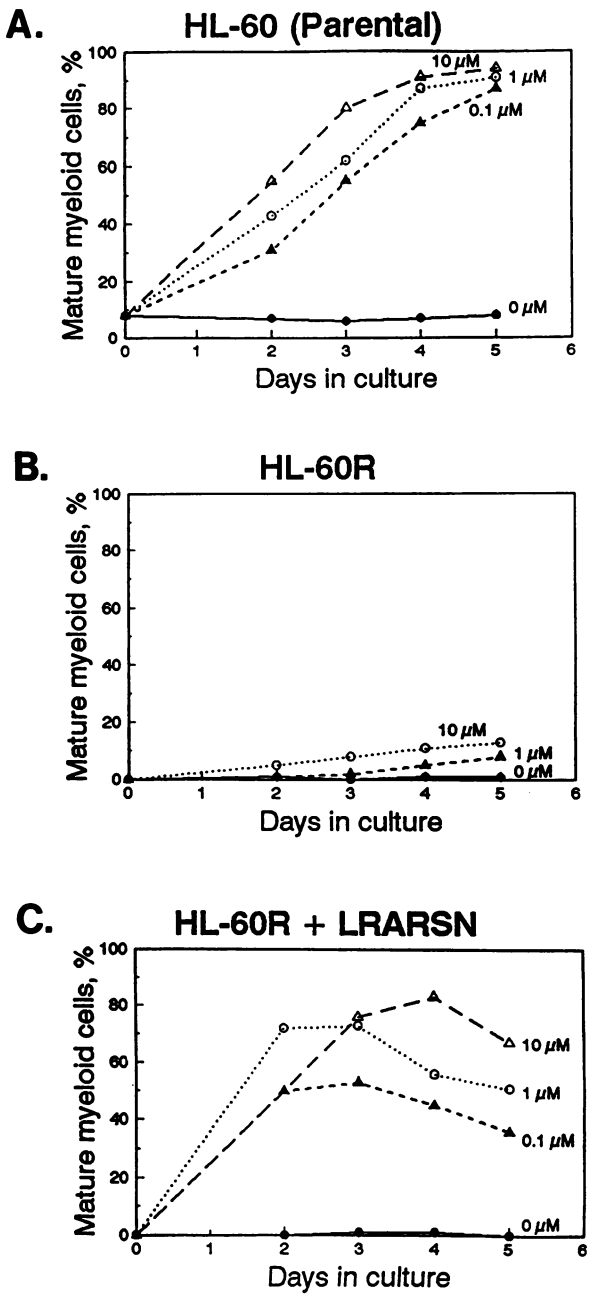

FIG. 4. RA-induced morphological differentiation of various HL-60 lines. Different HL-60 lines were incubated in liquid suspension in the indicated concentrations of RA. At the intervals noted, differential counts were performed on Wright-Giemsa-stained Cytospin preparations of 0.2-ml samples of the cell suspensions. "Mature myeloid cells" refers to myelocytes, metamyelocytes, and banded and segmented neutrophils. The indicated points represent the mean of two independent experiments.

viral vector (Fig. 6C), although the number of such positive cells induced in these cultures was consistently less than the number of NBT-positive cells induced in the parental HL-60 cells.

We also infected the RA resistant HL-60R cells with a retroviral vector (N2) containing the *neo*^{r} gene but no RAR-α cDNA insert (13). These cells exhibited an RA-resistant phenotype virtually identical to that of the uninfected HL-60R cells (data not shown). Thus the enhanced response to RA of the HL-60R cells infected with the LRARSN vector can be directly attributed to the RAR-α cDNA insert.

Differential gene expression in infected HL-60 cells. RA-induced granulocytic differentiation of HL-60 is associated with the modulation of expression of specific genes. For example, the c-*myc* gene is transcriptionally down-regulated

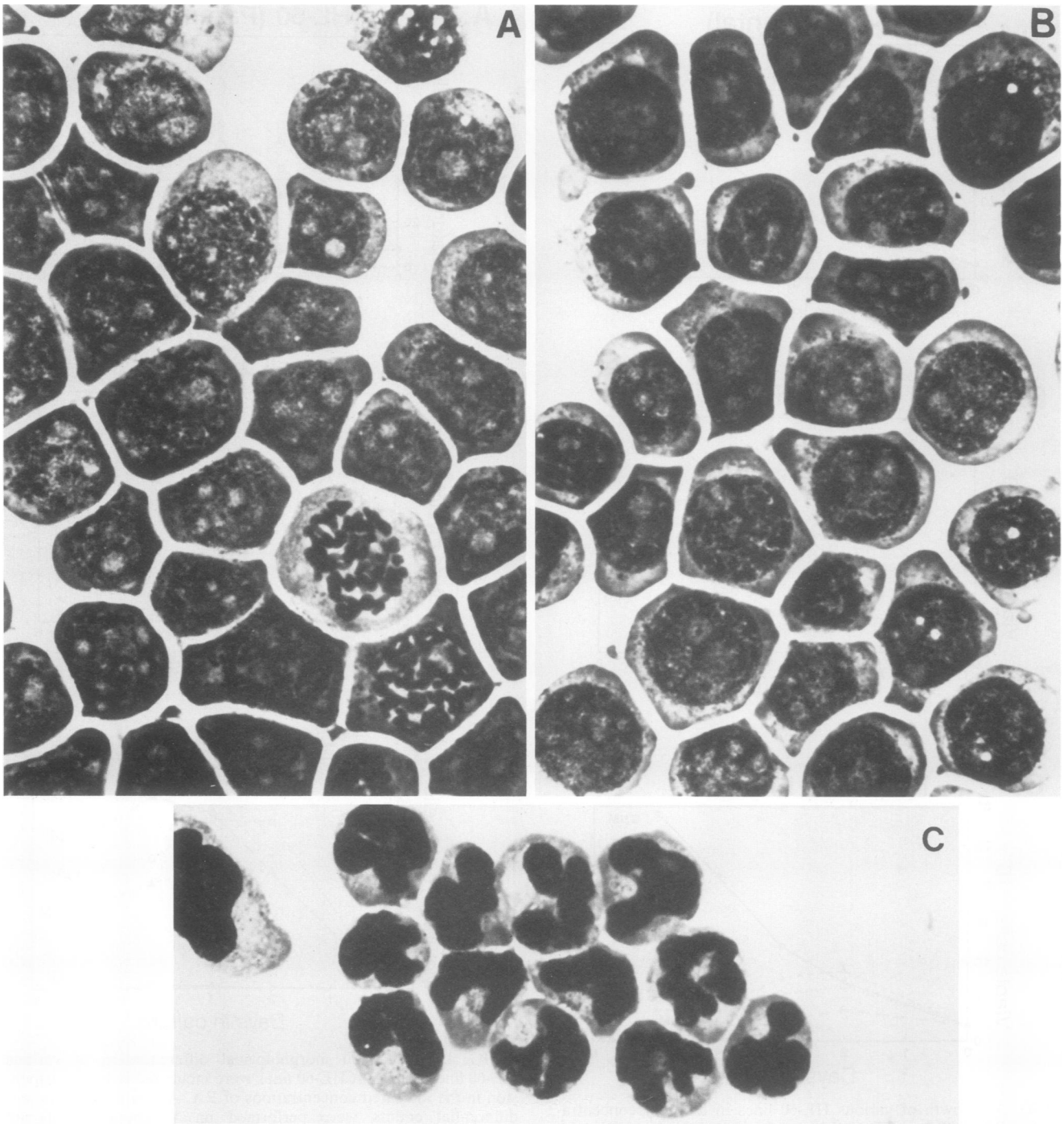

FIG. 5. Morphological appearance of uninduced and induced HL-60R cells. Cytospin preparations of HL-60R suspension cell cultures were stained with Wright-Giemsa before and after treatment with RA. (A) RA-resistant HL-60R subclone cultured without RA; (B) HL-60R cultured for 3 days in 1 μM RA; (C) HL-60R infected with the LRARSN retroviral vector (HL-60R+LRARSN) cultured for 3 days in 1 μM RA.

during RA-induced HL-60 differentiation (2), whereas the gene coding for the β subunit of the leukocyte adherence receptor CD18 is transcriptionally upregulated by RA (24). We compared the level of c-*myc* and CD18 transcripts in uninfected and LRARSN-infected HL-60R cells as well as in the parental HL-60 cells after exposure to various concentrations of RA (Fig. 7). Whereas the parental HL-60 cells exhibited a marked decrease in steady-state levels of c-*myc* mRNA after exposure to RA (Fig. 7A), only a slight decrease in c-*myc* expression was noted in the RA-resistant HL-60R cells exposed to RA concentrations as high as 10 μM (Fig. 7B). In contrast, c-*myc* mRNA expression was significantly

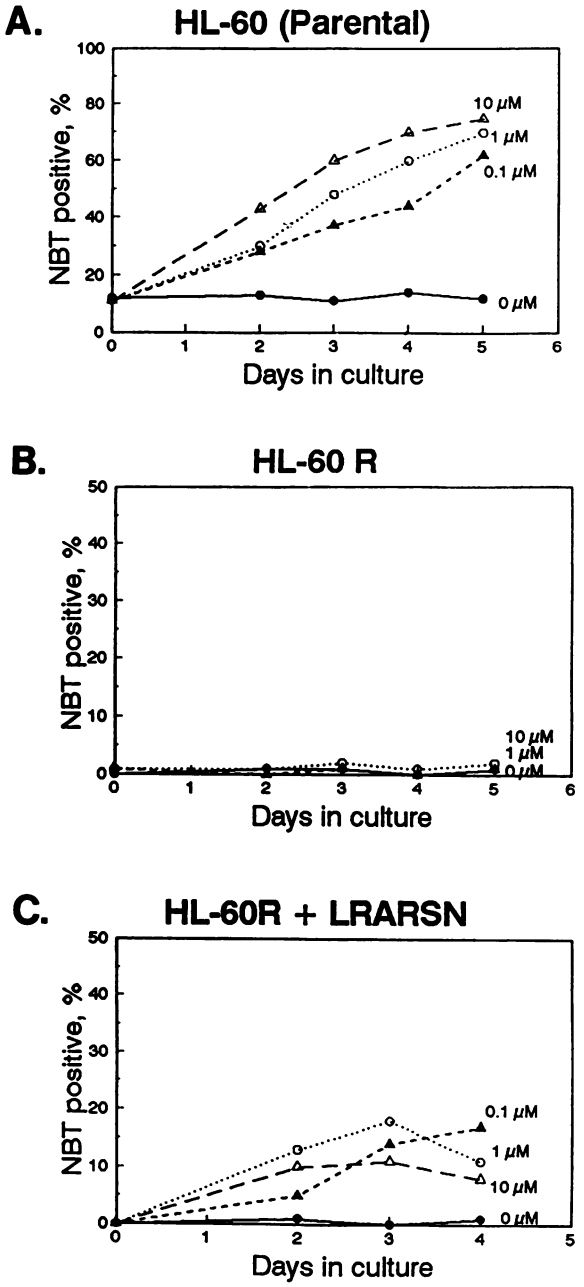

FIG. 6. NBT reduction of RA-induced HL-60 cells. Different HL-60 lines were incubated in liquid suspension in the indicated concentrations of RA. At the intervals noted, the percentage of cells capable of reducing NBT (40) was determined. The indicated points represent the mean of two independent experiments.

reduced in RA-treated HL-60R cells infected with the LRARSN retroviral vector (Fig. 7C). Similar studies on the transcriptionally upregulated CD18 gene revealed markedly enhanced steady-state levels of CD18 mRNA in RA-induced parental HL-60 cells (Fig. 7A). A slight increase in steady-state CD18 transcripts was also noted in the uninfected RA-resistant HL-60R cells but only at a relatively high (10 μM) concentration of RA (Fig. 7B). In contrast, the same cells infected with the LRARSN vector exhibited a marked increase in CD18 transcripts after exposure to concentrations of RA as low as 0.1 μM (Fig. 7C). In these cells the levels of steady-state actin mRNA remained approximately the same after RA exposure, indicating that the above differences in gene expression did not result from inadvertently loading different amounts of RNA on the gel.

Expression of RAR-α in HL-60 parental versus HL-60R cells. The above observations indicate that the introduction of a single copy of the RAR-α gene into HL-60R cells, which are relatively resistant to RA, will render these cells sensitive to RA-induced terminal granulocytic differentiation. This indicates that RAR-α plays a critical, direct role in mediating RA-induced granulocytic differentiation of HL-60. One implication of this finding is that there may be an abnormality in the structure and/or expression of RAR-α in the HL-60R subclone to account for its resistance to RA. To address this question, we first examined the mRNA expression of the endogenous RAR-α gene in both the RA-sensitive parental HL-60 cells and the RA-resistant HL-60R subclone. RAR-α transcripts of approximately 3.3 and 2.5 kb, similar in size to those previously reported in human hematopoietic cells (18, 21, 30, 47), were noted in both the resistant HL-60R cells and the sensitive HL-60 parental cells (Fig. 2, lanes 1 and 2). Moreover there was essentially no quantitative difference in the amount of these RAR-α transcripts in the RA-sensitive versus -resistant HL-60 cells (Fig. 2).

In contrast to the Northern blot analysis, we noted significant differences in specific RA-binding activity in nuclear extracts of parental HL-60 cells compared with that in extracts from the RA-resistant HL-60R cells (Fig. 8). Specific RA-binding activity that most likely represents RAR-α-mediated RA binding has been recently demonstrated in HL-60 nuclei utilizing size-exclusion HPLC analysis (39). Using a similar approach on [^{3}H]RA-labeled nuclear extracts, we noted RA-binding activity in parental HL-60 cells that eluted with a symmetrical peak on a size exclusion column at an approximate molecular weight of 100,000 (Fig. 8A). This peak represented specific RA-binding activity, since it was abolished when the same nuclear extracts were incubated with a 200-fold excess of unlabeled RA (Fig. 8A). In contrast, nuclear extracts from HL-60R cells exhibited specific RA-binding activity that peaked at a significantly lower molecular weight of approximately 60,000 (Fig. 8B). A similar analysis on the HL-60R cells infected with the LRARSN retroviral vector revealed specific nuclear binding activity that peaked at an approximate molecular weight of 100,000 (Fig. 8C), which is virtually identical to the peak of specific binding in the parental HL-60 cells (Fig. 8A).

Equilibrium binding curves of nuclear extracts incubated with various concentrations of [^{3}H]RA indicated that the specific binding activity of the peak HPLC fractions in both the parental HL-60 cells and the RA-resistant HL-60R cells was saturable at higher [^{3}H]RA concentrations (Fig. 9). A Scatchard plot analysis of these data (insets, Fig. 9) indicated that the dissociation constant (K_d) of the specific RA receptor was significantly higher in the RA-resistant HL-60R cells (K_d, 12.5 ± 2.5 nM; n = 3) than in parental HL-60 cells (K_d, 0.88 ± 0.10 nM; n = 2), indicating that the specific nuclear RA receptors in the RA-resistant HL-60R cells had a significantly lower affinity for RA compared with the parental HL-60 nuclear RA receptors. Moreover, these Scatchard plots indicate that the average number of measurable nuclear RA receptors appears to be lower in HL-60R cells (160 ± 90; n = 3) than in the parental HL-60 cells (537 ± 12; n = 2).

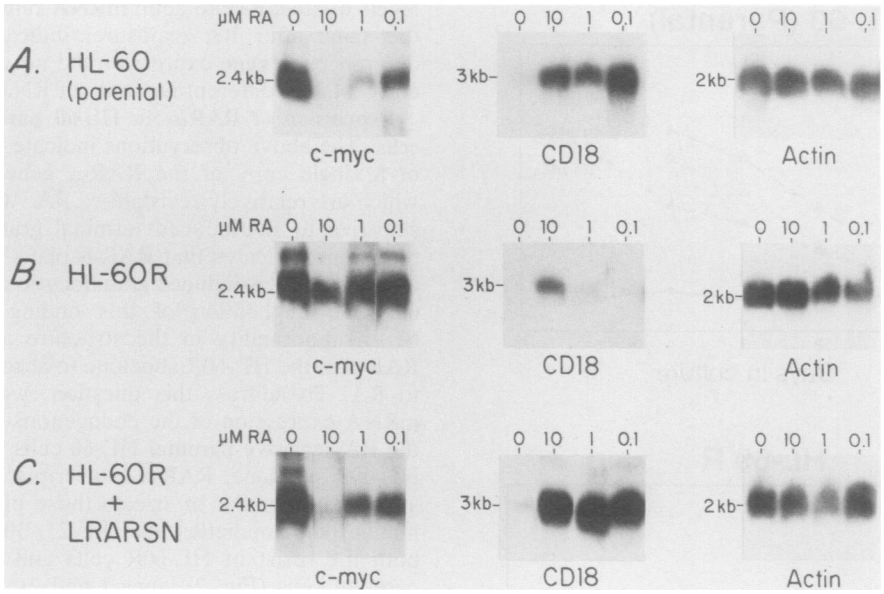

FIG. 7. Differential gene expression in RA-treated HL-60 cell lines. RNA was extracted from the indicated HL-60 cell lines that had been incubated in the indicated concentrations of RA for 3 days. Total RNA (5 μg per lane) was subjected to Northern blot hybridization with the indicated probes. These probes are described in detail in Materials and Methods.

DISCUSSION

Retinoids and in particular RA exert a wide range of effects in a number of different biologic systems. RA induces terminal differentiation in a small subset of human AML cells, and RA-induced terminal granulocytic differentiation of the HL-60 myeloid leukemia cell line serves as the prototype model system for studying this antileukemic effect of retinoids (8). At least three distinct RARs (RAR-α, -β, and -γ) have been molecularly cloned to date. Transcripts for RAR-α but not RAR-β or -γ are expressed in HL-60 cells (18), and thus RAR-α is a candidate receptor for mediating RA-induced granulocytic differentiation of HL-60. In this study we utilized a retroviral vector to introduce RAR-α cDNA into a subclone of HL-60 (HL-60R) that is relatively resistant to RA. Retroviral vectors provide a precise means of transferring a stable integrated single copy of a particular gene into the target cell genome. This technology is particularly appropriate for HL-60 cells, which are generally resistant to other techniques of gene transfer, including electroporation and DEAE-mediated transfection. We found that transfer of a single copy of RAR-α cDNA into the RA-resistant HL-60R subclone restored sensitivity of these cells to RA. This observation provides direct evidence that RA-induced granulocytic differentiation of HL-60 is mediated directly through RAR-α. RAR-α is structurally and functionally related to the steroid-thyroid hormone receptor superfamily of nuclear transcription factors, which are thought to mediate biologic effects by interacting with specific *cis*-acting DNA sequences that regulate the expression of distinct target genes (20). Thus, the implication of our observation is that the DNA-binding domain of the activated RAR-α interacts with a specific regulatory gene(s) or a specific network of target genes, resulting in terminal granulocytic differentiation of HL-60.

One measure of functional differentiation of HL-60 cells that we utilized in these studies was the NBT dye reduction test, which detects superoxide production by membrane-stimulated mature HL-60 cells (8, 40). We consistently noted that the number of cells capable of producing superoxide was significantly less in the RA-treated RAR-α transduced HL-60R cells than in the RA-treated parental HL-60 cells (compare Fig. 6C and 6A). Superoxide production in mature neutrophils is dependent on activation of a complex NADPH oxidase system consisting of at least one membrane and two or more cytosolic components (46). It is possible that one or more of the components of this complex are disrupted or regulated aberrantly in the HL-60R+LRARSN cells, resulting in the relatively poor superoxide production by these RA-treated cells compared with that of RA-treated parental HL-60 cells.

Possible mechanisms of HL-60R resistance to RA. Although our observations indicate that RAR-α directly mediates RA-induced differentiation of HL-60, we observed no significant difference in the amount and size of RAR-α specific mRNA transcripts when comparing the RA-sensitive parental HL-60 cells with the RA-resistant HL-60R subclone (Fig. 2). Nevertheless, our analysis of specific RA nuclear binding activity by size-exclusion HPLC of nuclear extracts indicated that there are at least two major differences in specific nuclear RA-binding activity between parental HL-60 cells and the RA-resistant HL-60R cells. First, there appeared to be a significant difference in the elution profile of the parental HL-60 nuclear RA-binding activity versus the HL-60R activity, with the parental HL60 cells exhibiting peak RA nuclear binding activity at an approximate molecular weight of 100,000 and the RA-resistant HL-60R cells exhibiting such peak activity at an apparent molecular weight of 60,000 (Fig. 8). Second, the Scatchard plot analysis of equilibrium binding curves of this peak RA nuclear binding activity indicated that the affinity of the nuclear RARs for RA was significantly less in the RA-resistant HL-60R cells (K_d, 12.5 nM) than in the parental HL-60 cells (K_d, 0.88 nM) (Fig. 9). Point mutations or deletions in

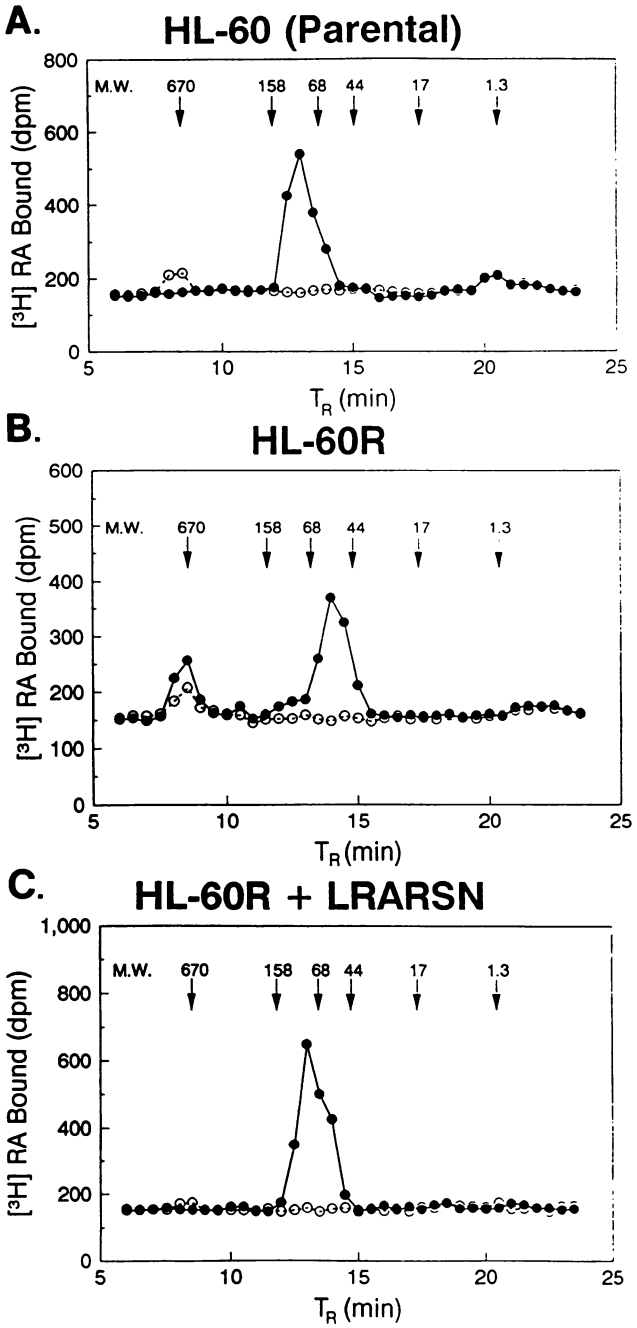

FIG. 8. Specific nuclear RA-binding activity in various HL-60 cell lines. Nuclear extracts from the indicated cell lines were incubated with [^{3}H]RA in the presence or absence of a 200-fold excess of unlabeled RA and subjected to size-exclusion HPLC analysis as detailed in Materials and Methods. The radioactivity in each fraction (0.5 ml) was then determined. Symbols: ●, [^{3}H]RA only; ○, [^{3}H]RA and 200-fold excess of unlabeled RA. The elution times of specific molecular weight markers (in thousands) as determined by A_{280} are indicated by the arrows. These protein markers include: thyroglobulin, 670,000; gamma globulin, 158,000; human albumin, 68,000; ovalbumin, 44,000; myoglobin, 17,000; and vitamin B_{12}, 1,300.

critical domains of the RAR-α-coding region might account for these observed differences in the nuclear RARs. For example, mutations or deletions within certain regions of RAR-α in the HL-60R cells might interfere with dimerization of the RAR. The glucocorticoid and estrogen receptors appear to bind as dimers to their respective response elements (29, 45), and there is indirect evidence that the RAR may also form dimers (23). Although our HPLC-determined molecular weights are only approximate, the predicted molecular weight of RAR-α from its cDNA sequence is approximately 50,000 (22, 41), and therefore the estimated molecular weight for peak nuclear RA-binding activity in parental HL-60 cells (100,000) versus that for RA-resistant HL-60R cells (60,000) is consistent with lack of dimerization of the RAR in the HL-60R cells. Since dimerization of the RAR may be critical for its optimal binding to target DNA sequences, mutations that interfere with dimerization may inhibit the transcriptional activity of the activated RARs.

The reduced affinity for RA of the RAR in the RA-resistant HL-60R cells may result from mutations or deletions in the ligand-binding domain of the RAR, leading to altered receptor-ligand interaction. This would lead to fewer receptors being activated at a given concentration of RA, presumably resulting in a diminished biologic response of the HL-60R cells to RA. Molecular cloning and sequencing of RAR-α cDNA from the RA-resistant HL-60R cells should more clearly determine the molecular basis of the relative resistance of HL-60R cells to RA.

It should be emphasized that the majority of human AML cells do not exhibit a differentiative response to RA and yet, like the HL-60R subclone, commonly express RAR-α transcripts in approximately equal amounts (21, 30, 47). Thus, determining the molecular basis for the relative RA resistance of the HL-60R subclone may provide insight into why most human AML cells are also insensitive to RA.

What are the target genes for the activated RAR? Regardless of the mechanism of HL-60R resistance to RA, our observations indicate that the RAR-α plays a direct, critical role in mediating RA-induced HL-60 differentiation. Presumably this involves interaction of the activated RAR with specific *cis*-acting DNA sequences, resulting in enhanced or diminished target gene expression. RA-induced HL-60 differentiation is associated with the modulation of expression of a number of different genes including c-*myc*, which is transcriptionally downregulated (2), and CD18, which is transcriptionally upregulated (24), after RA treatment of HL-60 cells (Fig. 7). The activated RAR might directly interact with the regulatory regions of genes such as c-*myc* and CD18 or, alternatively, might alter the expression of certain other genes that in turn act as transcriptional regulatory agents. With regard to this latter possibility, it might be pertinent to note that RA-induced differentiation of the mouse F9 teratocarcinoma stem cell line is associated with the relatively early expression of genes with distinct homeobox domains (31, 38). Such early RA-induced genes may subsequently alter the expression of other target genes, resulting in the more mature F9 phenotype. Whether RA might also induce the early expression of potential regulatory genes involved in mediating the granulocytic differentiation of HL-60 cells is presently unknown.

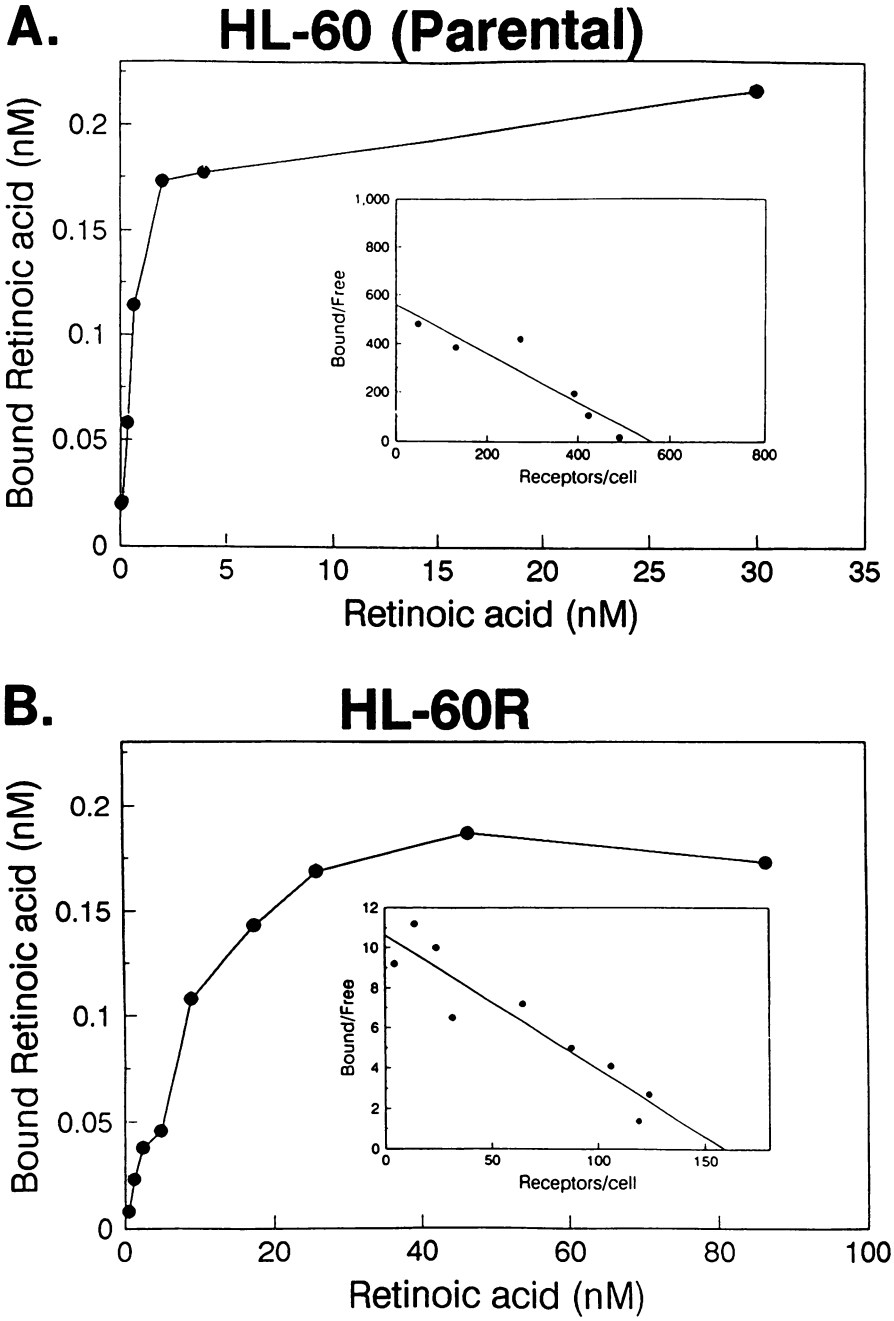

FIG. 9. Equilibrium binding of [^{3}H]RA to parental HL-60 cells (A) and HL-60R cells (B). Increasing concentrations of [^{3}H]RA were incubated with nuclear extracts from the indicated cell lines and analyzed by size-exclusion HPLC (Fig. 8). Specific binding is represented on the ordinate and represents total bound [^{3}H]RA within the peak HPLC fractions minus [^{3}H]RA bound in the same relative fractions when the extracts were incubated with a 200-fold excess of unlabeled RA. Scatchard plots of the equilibrium binding data are represented in the insets.

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