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Multiple Members of the Retinoic Acid Receptor Family Are Capable of Mediating the Granulocytic Differentiation of HL-60 Cells

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The complex and diverse biological effects of retinoic acid (RA) are mediated through specific receptors that are members of the steroid hormone family of nuclear transcription factors. The RA receptor family consists of multiple structurally distinct RA receptors, which diverge primarily at the NH_2-terminal domain. The evolutionary conservation of this divergent region in individual RA receptors among different species together with their tissue-specific patterns of expression suggest that the biological function and activity of the individual RA receptors may be confined to specific tissues. To test this hypothesis in hematopoietic cells, we used retrovirus-mediated gene transduction to introduce the RA receptors RAR-α, RAR-β, and RAR-γ as well as RXR-α into a mutant subclone of the HL-60 promyelocytic leukemia cell line (designated HL-60R) that is relatively resistant to RA-induced granulocytic differentiation. We found that each of these structurally distinct RA receptors could restore sensitivity of the HL-60R cells to RA. A critical threshold number of transduced receptors per cell appears to be necessary to restore this functional activity. Thus, the capability to mediate granulocytic differentiation of HL-60 cells is shared among distinctly different RA receptors.

The retinoids and in particular retinoic acid (RA) exert a wide range of biological effects related to cell proliferation and differentiation. RA functions as a natural morphogen in regulating fetal development during vertebrate embryogenesis (2, 34). RA also influences adult epithelial cell growth and differentiation (27) and suppresses malignant transformation both in vitro and in vivo (5, 20, 29). In addition, RA induces the terminal differentiation of human promyelocytic leukemia cells (7, 8, 21, 36). RA is thought to mediate these diverse and complex biological effects through specific receptors that are members of the steroid/thyroid hormone receptor superfamily of nuclear transcription factors (15). These receptors exhibit a modular structure that includes discrete DNA-binding and hormone (ligand)-binding domains. The RA receptors presumably bind to specific *cis*-acting DNA sequences that are important for regulating transcription of specific target genes, and this target gene expression may be modified in the presence of ligand.

The RA receptors, in contrast with the glucocorticoid receptors, exhibit a relatively large number of structurally distinct isoforms. The RAR-α, RAR-β, and RAR-γ receptors share marked structural similarity in their DNA-binding and ligand-binding domains but markedly diverge in their NH_2-terminal regions. In contrast, the RA receptors designated RXR exhibit marked divergence in both the NH_2-terminal and ligand-binding domains compared with the RAR-α, RAR-β, and RAR-γ receptors and appear to define a distinct and separate family of RA receptors (28).

Why are there so many different types of RA receptors? What specific physiological role does each distinct receptor play in regulating cell growth and differentiation? Several lines of indirect evidence suggest that the activity of each of these RA receptors may be tissue specific. For example the structurally divergent NH_2-terminal domain appears to be functionally important, since for a given RA receptor there is marked interspecies conservation of this NH_2-terminal domain (25). Moreover, the homologous NH_2-terminal domain in the progesterone receptor may mediate target gene activation that is cell type specific (35). In addition, the different RA receptor isoforms exhibit a distinct tissue-specific pattern of expression (14, 23, 28). These observations suggest that each of the different RA receptors may control the expression of distinct subsets of target genes in specific cell types and thus may mediate functionally distinct biological activities. However, to date there have been few if any studies to determine whether these different RA receptors in fact exhibit such tissue-specific activity.

In this study, we directly addressed this question by comparing the activities of RAR-α, RAR-β, RAR-γ, and RXR-α in hematopoietic cells. We use as a model system the RA-induced granulocytic differentiation of the HL-60 human promyelocytic leukemia cell line (7). We previously isolated a mutant subclone of this cell line (designated HL-60R) which is relatively resistant to RA and observed that retrovirus-mediated transduction of the RAR-α gene into this subclone restored sensitivity of these cells to RA (10). We now describe the transduction and differentiative activity of the RAR-β, RAR-γ, and RXR-α receptors in these RA-resistant HL-60R cells. We note that each of these distinctly different transduced RA receptors, if expressed in sufficient numbers, has the capacity to mediate granulocytic differentiation of HL-60 cells.

MATERIALS AND METHODS

Leukemic cell culture and assessment of differentiation. Parental HL-60 cells and the RA-resistant HL-60R subclone

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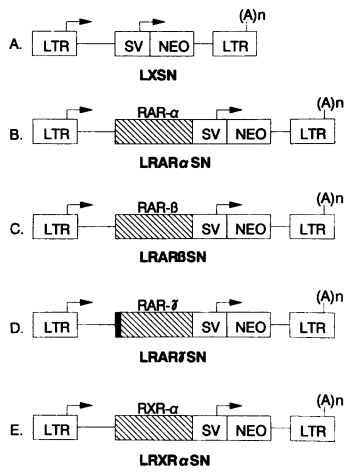

FIG. 1. Structures of retroviral vectors harboring RA receptor-coding sequences. These vectors were constructed by inserting the appropriate cDNAs for RAR-α, RAR-β, RAR-γ, and RXR-α into the cloning sites of LXSN as detailed in Materials and Methods. The shaded region in the cDNA insert of the LRARγSN vector corresponds to 94 bp of 5′ untranslated adenosine deaminase sequences as described in Materials and Methods. SV, SV40.

were cultured in liquid suspension in RPMI medium supplemented with 5% fetal bovine serum. To assess morphological differentiation induced by RA, cells were seeded at 10^5/ml in 2-ml (22-mm-diameter) wells in 12-well plates (Corning Glass Works, Corning, N.Y.). RA, diluted from a stock 2 mM solution made up fresh in ethanol, was added. After various times of incubation, differential counts were performed on Wright-Giemsa-stained Cytospin preparations of 0.2-ml samples of the cell suspensions. Cell number was quantitated on trypan blue-stained cell suspensions, using a hemocytometer.

Retroviral vector construction and production. We subcloned different human RA receptor cDNAs into the LXSN retroviral vector in which the cDNA inserts are driven by the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR) and which harbors the neomycin phosphotransferase (Neo^r) gene, driven by the simian virus 40 (SV40) promoter, for use as a selectable marker (31) (Fig. 1A). We previously described the construction of an LXSN-derived retroviral vector (designated LRARαSN) harboring an RAR-α insert (10) (Fig. 1B). Retroviral vectors containing the coding sequences of the RAR-β, RAR-γ, and RXR-α genes were constructed as follows.

(i) **RAR-β.** We obtained a plasmid (designated pGEM-RARβ Δ5′) harboring the coding sequences of the RAR-β gene (3) from Richard Heyman (Salk Institute). A *Pst-Sst* digest of this plasmid released a 1.6-kb fragment which includes 44 bp of 5′ untranslated sequence, the complete coding sequence of RAR-β, and 60 bp of 3′ untranslated sequence. This fragment was blunted with T4 polymerase, ligated to *Bam* linkers (New England Biolabs), digested with *Bam*, and then ligated into the *Bam* cloning site of the LXSN retroviral vector to give the recombinant vector designated LRARβSN (Fig. 1C).

(ii) **RAR-γ.** To construct the RAR-γ retroviral vector, we obtained a plasmid (designated pGEM3Z-hRARγ) containing RAR-γ cDNA (22) from Richard Heyman. This sequence corresponds to the RAR-γA (RAR-γ1) isoform of RAR-γ (17). An *Nco*I-*Pst* digest of this plasmid released a 1.6-kb fragment harboring the complete coding sequence of RAR-γA (without 5′ untranslated sequences) and approximately 100 bp of 3′ untranslated sequences. To provide for a presumably better translational context for this cDNA, we used an LXSN-based retroviral vector designated pLASN containing adenosine deaminase cDNA (19). An *Nco-Xba*I digest of this plasmid released a 4.3-kb fragment, and an *Xba*I-*Bam*HI digest released a 1.7-kb fragment. A three-way ligation involving these two fragments together with the 1.6-kb *Nco*I-*Pst* RAR-γ fragment was performed. The resulting recombinant retroviral vector construct includes 94 bp of 5′ untranslated sequences from the human adenosine deaminase gene immediately preceding the RAR-γ initiator ATG and is designated LRARγSN (Fig. 1D).

(iii) **RXR-α.** We obtained plasmid pSKXR3-1 harboring the complete coding sequence of the RXR-α gene (28) from Ron Evans (Salk Institute). An *Eco*RI digest of this plasmid released a 1.9-kb fragment containing the coding sequences of RXR-α together with approximately 75 bp of 5′ untranslated and 400 bp of 3′ untranslated sequence. This 1.9-kb fragment was then ligated into the *Eco*RI cloning site of the LXSN retroviral vector to give the recombinant construct designated LRXRαSN (Fig. 1E).

Retroviral vectors were generated from each of these recombinant plasmids as previously described (30). Briefly, 10 μg of cesium chloride-banded retroviral construct plasmid was transfected via $CaPO_4$ precipitation into the Ψ2 ecotropic retrovirus packaging cell line (30). The supernatant was harvested after 2 days and used to infect the PA317 amphotropic packaging cell line. These cells were cultured in G418 (1 mg/ml), and individual foci of resistant cells were isolated by using cloning rings and expanded. RNA was extracted from these individual clones and analyzed by Northern (RNA) blots, using the appropriate RA receptor cDNA fragments as probes. In this manner, retroviral vector producer clones that expressed the appropriate-size MuLV LTR-initiated transcript harboring the RA receptor insert were identified. Supernatants from these producers were then titered on NIH 3T3 thymidine kinase-negative target cells as previously described (30). Those supernatants exhibiting the highest titer were used to infect HL-60R cells. Titers of these supernatants from these retroviral producers were as follows: LRARαSN, 3×10^5/ml (10); LRARβSN, 4.5×10^4/ml; LRARγSN, 7×10^4/ml; and LRXRαSN, 10^5/ml.

Retroviral infection of HL-60R cells. HL-60R cells were cultured for 2 days in medium supplemented with 5% fetal calf serum and 4 μg of Polybrene per ml together with supernatant from the appropriate PA317 retroviral vector producer cell line at a multiplicity of infection of approximately 1:1. The cells were then cultured in growth medium supplemented with G418 (1 mg/ml) for 21 to 28 days. Individual clones of infected HL-60R cells were obtained by culturing approximately 10^3 of these G418-resistant cells in 35-mm-diameter petri dishes containing 1.1 ml of growth medium supplemented with 0.75% agar and 500 μg of G418 per ml. After 10 to 14 days, individual colonies containing 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.

Determination of K_d and number of transduced RA receptors. Nuclear extracts from uninfected and retroviral vector-infected HL-60 cells were obtained exactly as previously described (10). These extracts were incubated with various concentrations of [^{3}H]RA (all-*trans* RA; 50 Ci/mmol; Dupont, NEN Research Products) and subjected to size exclu-

TABLE 1. RA receptors in uninfected and retrovirus-infected HL-60 cells

Cells	No. of RA receptors	K_d (nM)	% Differentiation[a]
HL-60 (parental)	1,000	0.6	>90
HL-60R[b]	125	0.6	<10
	100	12	
HL-60R + LRARαSN	2,300	3.2	70–80
HL-60R + LRARβSN	310	0.8	30–40
HL-60R + LRARγSN	2,080	3.3	>90
HL-60R + LRXRαSN clone 1	685	1.3	>90
HL-60R + LRXRαSN clone 5	250	0.6	<20

[a] Percentage of morphologically mature myelocytes, metamyelocytes, and banded and segmented neutrophils in cultures treated for 5 days with 10^{-5} M RA.

[b] Two RA receptor populations are noted in HL-60R cells.

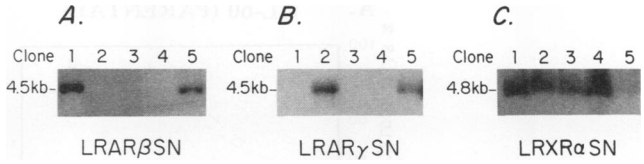

FIG. 2. Expression of transduced RA receptor transcripts in retroviral vector-infected HL-60R clones. Northern blot analysis was performed on total cellular RNA (5 μg per lane) extracted from various G418-resistant clones of HL-60R cells infected with the indicated RA receptor retroviral vectors. The probes used for hybridization correspond to the appropriate RA receptor cDNAs and are described in Materials and Methods. Endogenous RAR-β, RAR-γ, or RXR-α transcripts are not detectable in any of these blots.

sion high-pressure liquid chromatography (HPLC) analysis as described previously (10). One modification of this previously described protocol was that after incubation with [^{3}H]RA, nuclear extracts were spun through a single Sephadex G-15 minicolumn constructed from a duplicate 1-ml syringe (1,100 × g for 3 min), using Whatman glass GF/c microfiber filters to retain the Sephadex. This more vigorous centrifugation resulted in recovery of a greater number of specifically bound [^{3}H]RA counts, and the estimated number of RA receptors for a given sample is now approximately twice what we previously reported (10). (This technical modification also permitted the detection of previously unreported high-affinity RA receptors present in low numbers in the RA-resistant HL-60R cells [Table 1].) The specific binding activity exhibited by the nuclear extracts was calculated for each concentration of [^{3}H]RA, and the resultant equilibrium binding data were subjected to Scatchard plot analysis to estimate the receptor affinity for RA as well as the number of receptors per cell.

Northern blot hybridization. RNA was extracted with guanidine hydrochloride and subjected to Northern blot hybridization in formaldehyde denaturing gels exactly as previously described (9).

Molecular probes. Molecular probes used for hybridization included the following: RAR-α, the 1,309-bp *Eco*RI-*Sma*I fragment from the hT1R RAR-α cDNA clone (16); 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 (18); and Neo^r, a 0.9-kb *Pst*I fragment from the bacterial Neo^r gene isolated from N2 proviral DNA (24). Probes for RAR-β, RAR-γ, and RXR-α were the cDNA fragments described above that were used to construct the respective retroviral vectors. All probes were labeled by nick translation before hybridization.

RESULTS

Construction of retroviral vectors harboring different RA receptors. We have previously used retroviral vectors to introduce specific genes, including the RAR-α gene, into HL-60 cells (10, 12). These retroviral vectors are particularly useful in HL-60 cells since these cells are relatively resistant to gene transduction by more conventional transfection techniques. Moreover, to quantitate the transduced RA receptor number as described below, there is considerable advantage in having a stable integrated copy of the transduced gene provided by the retroviral vectors. As detailed in Materials and Methods, we used the retroviral vector LXSN (31) (Fig. 1A) to construct vectors harboring the coding sequences of the RAR-β, RAR-γ, and RXR-α genes and containing the Neo^r gene for use as a selectable marker. Diagrams and designations of each of these retroviral constructs are shown in Fig. 1.

We infected the RA-resistant HL-60R cells with each of these retroviral vectors and isolated individual G418-resistant clones as described in Materials and Methods.

Variable expression of the retrovirus-transduced genes in infected HL-60R clones. The RA receptor retroviral constructs (Fig. 1) harbor two transcriptional units: the Mo-MuLV LTR-initiated transcripts harboring the RA receptor cDNA inserts and the SV40 promoter-driven transcripts harboring the Neo^r gene. Variable, frequently reduced expression of particular transcripts from retroviral vectors harboring two such transcriptional units has been previously observed in hematopoietic target cells (1, 6). Therefore, we performed Northern blot analysis to compare the relative mRNA levels of the retroviral vector-transduced RA receptor genes in G418-resistant clones of infected HL-60R cells. In RAR-β- and RAR-γ-infected HL-60R cells, we found considerable variation in the steady-state level of Mo-MuLV LTR-driven transcripts harboring RA receptor cDNA; some infected G418-resistant clones exhibited readily detectable levels of the appropriate-size mRNA, while in other clones this mRNA was undetectable (Fig. 2A and B). As expected, all of these G418-resistant clones exhibited the SV40 promoter-driven 1.7-kb *neo* transcript (blot not shown). Less variation in transduced receptor mRNA expression was noted in the G418-resistant subclones isolated from LRXRαSN-infected HL-60R cells (Fig. 2C), although one clone (clone 5) exhibited significantly less transduced RXR-α mRNA than did the others. On these Northern blots (Fig. 2), we observed no endogenous RAR-β, RAR-γ, or RXR-α mRNA transcripts in either the uninfected or retroviral vector-infected HL-60R cells, although endogenous RAR-α mRNA transcripts are readily detectable in HL-60R cells (10).

We chose infected HL-60R clones displaying relatively high amounts of the appropriate transduced RA receptor retroviral mRNA (i.e., LRARβSN clone 1, LRARγSN clone 2, and LRXRαSN clone 1; Fig. 2) for further analysis as described below.

RA-induced morphological differentiation of RA receptor retroviral vector-transduced cells. RA-induced granulocytic differentiation of parental HL-60 cells is associated with prominent morphological changes characteristic of maturing myelocytes, metamyelocytes, and banded and segmented neutrophils (7) (Fig. 3A). In contrast, the RA-resistant

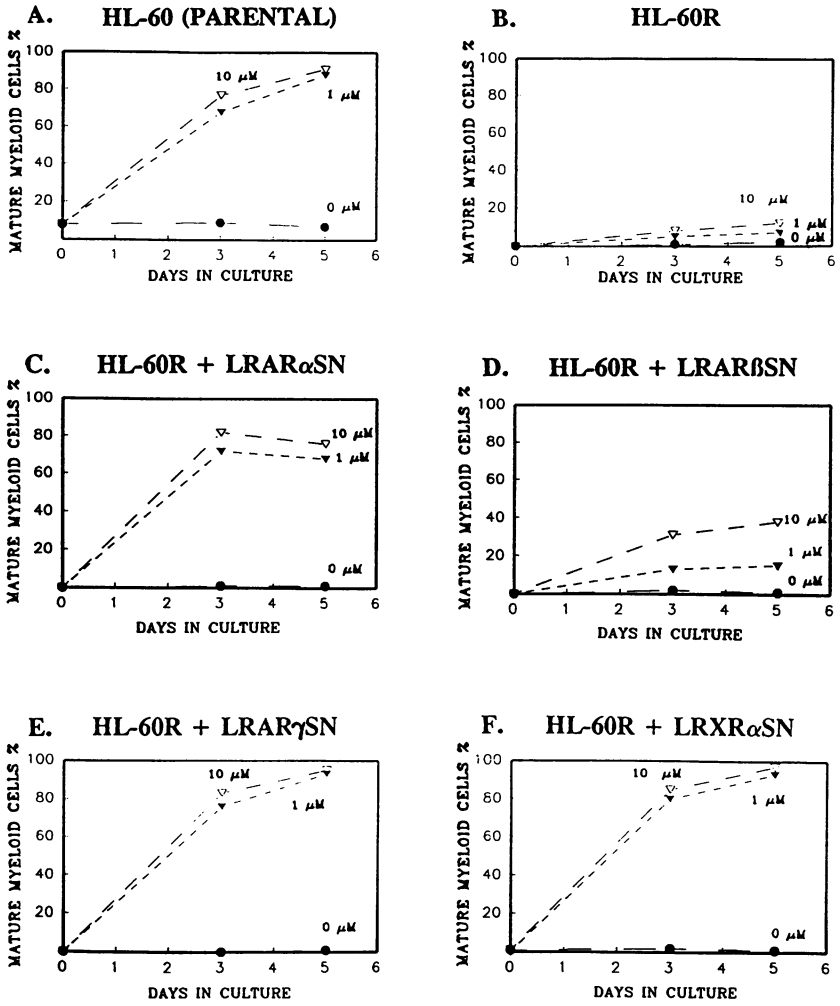

FIG. 3. RA-induced morphological differentiation of retroviral vector-infected HL-60R cells. HL-60R cells infected with the designated retroviral vectors were incubated in liquid suspension in the indicated concentration of RA. Differential counts were performed on Wright-Giemsa-stained cells at the indicated intervals after RA exposure. "Mature myeloid cells" refers to myelocytes, metamyelocytes, and banded and segmented neutrophils.

HL-60R cells exhibit minimal morphological changes despite exposure to relatively high concentrations of RA (10) (Fig. 3B). We previously noted that infection of HL-60R cells with a retroviral vector expressing the RAR-α gene restored the sensitivity of these cells to RA (10) (Fig. 3C). In the present study, we wished to determine whether introducing the RAR-β, RAR-γ, and RXR-α genes into HL-60R cells would similarly restore sensitivity to this RA-induced granulocytic differentiation. Indeed, we found that HL-60R cells infected with the retroviral vectors harboring the RAR-β, RAR-γ, and RXR-α genes all display enhanced morphological differentiation when cultured with RA (Fig. 3D to F). This differentiative response is particularly prominent in HL-60R cells infected with the LRARγSN and LRXRαSN retroviral vectors; over 90% of these cells exhibit granulocytic differentiation after 5 days of exposure to RA (Fig. 3E and F). As previously noted (7, 11), we found that this morphological differentiation is associated with growth inhibition of the RA-treated cells (data not shown), indicating that such cells are terminally differentiating. In contrast, HL-60R cells infected with the LRARβSN vector exhibited significantly less RA-induced morphological differentiation (Fig. 3D). Nevertheless, these LRARβSN-infected cells displayed a greater degree of RA-induced differentiation than did the uninfected HL-60R cells (Fig. 3B).

Differential gene expression in RA receptor retroviral vector-transduced HL-60R cells. The RA-induced morphological differentiation of HL-60 cells is associated with the modulation of expression of specific genes. For example, the c-*myc* proto-oncogene is transcriptionally down-regulated during RA-induced HL-60 differentiation (4), while the leukocyte adherence receptor β subunit (CD18) is transcriptionally up-regulated in these same RA-treated cells (18). Assessing the level of mRNA expression of these two genes provides a convenient and sensitive means of comparing the responses of the different retrovirus-infected HL-60R clones to RA (10).

Parental RA-sensitive HL-60 cells exhibit a marked decrease in steady-state levels of c-*myc* mRNA and a prominent increase in CD18 mRNA when cultured in 0.1 to 10 μM RA (Fig. 4A). In contrast, little change in steady-state levels of c-*myc* and CD18 mRNAs are noted in the RA-resistant

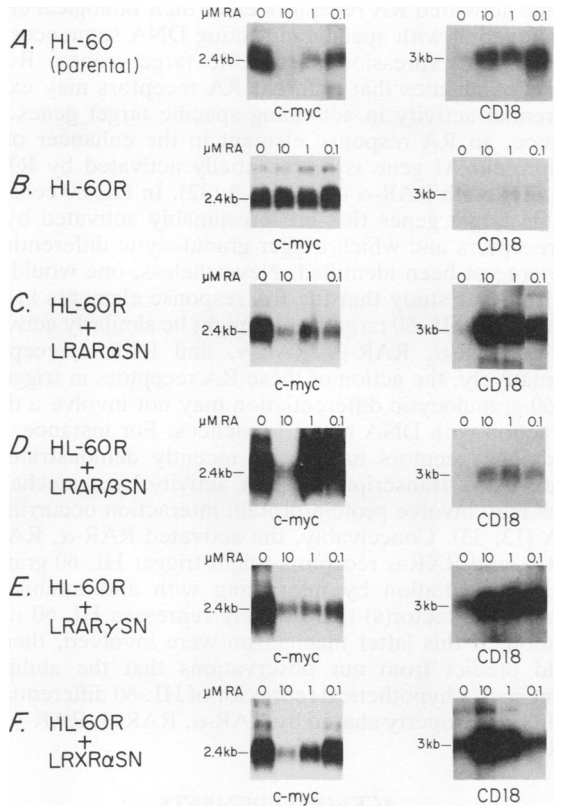

FIG. 4. Differential gene expression in RA-treated, retroviral vector-infected HL-60R cells. Different retroviral vector-infected HL-60R cell lines were incubated with the indicated concentrations of RA for 3 days. Total RNA was then extracted and subjected to Northern blot hybridization (5 μg per lane) with the indicated probes.

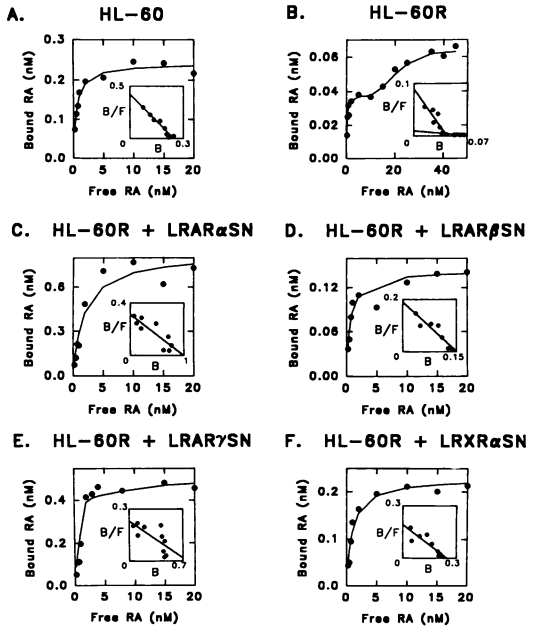

FIG. 5. Equilibrium binding of [^{3}H]RA to retroviral vector-infected HL-60R cells. Increasing concentrations of [^{3}H]RA were incubated with nuclear extracts from the indicated uninfected and retroviral vector-infected HL-60R cells, and specific [^{3}H]RA binding was then determined by size exclusion HPLC as described in Materials and Methods. Scatchard plots of the equilibrium binding data are represented in the insets; a summary of this analysis is presented in Table 1.

HL-60R cells after exposure to RA (Fig. 4B). HL-60R cells infected with the LRARγSN and LRXRαSN retroviral vectors display the expected pattern of c-*myc* down-regulation and CD18 up-regulation after exposure to RA (Fig. 4E and F). This modulation in gene expression appears to be significantly less in HL-60R cells infected with the LRARβSN vector but nevertheless is greater than that displayed by uninfected HL-60R cells (compare Fig. 4D with Fig. 4B).

Transduced RA receptor number and K_d in retrovirus-infected cells. The observations noted above indicate that the transduced RAR-β, RAR-γ, and RXR-α genes all to some degree restore sensitivity of HL-60R cells to RA. Nevertheless, the RA-induced granulocytic differentiation in the RAR-γ- and RXR-α-transduced HL-60R cells appears to be more pronounced than in the RAR-β-transduced cells. To determine whether differences in transduced RA receptor number might correlate with this differential sensitivity, we subjected nuclear extracts from retrovirus-transduced HL-60R cells to a [^{3}H]RA-specific binding assay as detailed in Materials and Methods. This technique generates an equilibrium binding curve which together with Scatchard plot analysis provides an assessment of the number and relative RA binding affinity (K_d) of the transduced RA receptors in retrovirus-infected HL-60R cells (10). The equilibrium binding curves and Scatchard plots generated through this analysis are displayed in Fig. 5, and the results are summarized in Table 1. RA-sensitive parental HL-60 cells display approximately 1,000 high-affinity (K_d of approximately 0.6 nM) RA receptors per cell. In contrast, uninfected RA-resistant HL-60R cells display relatively low numbers of two distinct RA receptor populations, including approximately 125 high-affinity (K_d of 0.6 nM) receptors per cell and approximately 100 low-affinity (K_d 12 nM) receptors per cell. These same HL-60R cells infected with the RAR-α and RAR-γ vectors harbor a relatively high number of high-affinity RA receptors per cell (2,300 and 2,100, respectively; Table 1). In contrast, only 310 high-affinity RA receptors per cell are noted in HL-60R cells infected with the RAR-β vector, which are the retroviral vector-transduced cells displaying lower sensitivity to RA. RXR-α-infected HL-60R cells (clone 1) display a significant response to RA, particularly at 1 and 10 μM concentrations (Fig. 3F and 4F), and these transduced cells harbor approximately 700 high-affinity RA receptors per cell (Table 1).

DISCUSSION

The biological effects of RA are diverse and complex and appear to be mediated not through a single receptor but through multiple receptors which are structurally similar to other members of the steroid receptor superfamily of nuclear transcription factors. The RAR-α, RAR-β, and RAR-γ receptors exhibit considerable sequence homology in their RA- and DNA-binding domains but markedly diverge in their NH_2-terminal activator domains (25). In contrast, the RXR-α receptor appears to define a separate family of RA receptors which exhibit little overall homology to RAR-α, RAR-β, and RAR-γ (28). An intriguing but perplexing question is why so many different RA receptors have evolved to

mediate the biological activities of RA. In general, these different RA receptors exhibit tissue-specific expression, leading to the suggestion that each of these different receptors may exhibit tissue-specific biological activity (23). To directly test this hypothesis in hematopoietic cells, we have used retrovirus-mediated gene transduction to introduce structurally distinct RA receptors into a subclone of RA-sensitive HL-60 cells that harbors an aberrant RA receptor. We previously noted that introducing the gene for RAR-α, which is the RA receptor primarily expressed in hematopoietic cells, into this mutant subclone restored the sensitivity of these cells to RA (10). In the present study, we have noted that introducing the RAR-β, RAR-γ, and RXR-α genes into the RA-resistant HL-60R cells also restores RA sensitivity to these cells. We consistently found that RAR-β-transduced HL-60R cells exhibit less response to RA than do RAR-γ- and RXR-α-transduced cells (Fig. 3 and 4), perhaps reflecting the relatively low number of transduced RA receptors in LRARβSN-infected cells (Table 1). Despite these relatively low numbers, the response of LRARβSN-infected HL-60R cells to RA is consistently greater than that exhibited by uninfected HL-60R cells (Fig. 3 and 4). Thus, in the hematopoietic HL-60 cells, these structurally distinct RA receptors exhibit similar biological activities with respect to mediating terminal granulocytic differentiation, and we have observed no evidence for tissue-specific function of these different RA receptors in this study.

Our study indicates that one important variable determining the biological response to RA is the number of transduced receptors that are introduced by the retroviral vectors into the target HL-60R cells. For example, HL-60R cells infected with the RAR-β vector exhibited fewer high-affinity RA receptors and was less sensitive to RA than were the same target cells infected with the RAR-α, RAR-γ, and RXR-α vectors (Table 1). In addition, one clone of HL-60R cells infected with the RXR-α retroviral vector (clone 1; Fig. 2C) harbored approximately 700 RA receptors per cell and exhibited a marked response to RA, while another HL-60R clone infected with the same RXR-α vector (clone 5; Fig. 2C) displayed approximately 250 receptors per cell and exhibited significantly less response to RA (Table 1). This latter observation suggests that a critical threshold number of RA receptors must be present to mediate the biological activities of RA. Moreover, it appears that relatively small changes in target cell RA receptor number may have significant effects on the target cell response to RA.

Since the initiation of our studies, several more isoforms of the RA receptors have been identified. These differ primarily in the NH_2-terminal region and result from either alternative promoter usage or alternative splicing at a specific receptor locus. They include two RAR-α isoforms (26), three RAR-β isoforms (37), and two RAR-γ isoforms (17). It is interesting that these isoforms are distinguished primarily by divergence at the NH_2-terminal activator domain, a region which in steroid hormone receptors has been related to tissue-specific activity (35). Since we have not constructed retroviral vectors corresponding to each of these different isoforms, we cannot rule out the possibility that some of these RA receptors exhibit differential activity in HL-60 cells. Nevertheless, it should be emphasized that the RA receptors that we have studied to date all exhibit marked divergence in this NH_2-terminal domain and yet appear to display similar functional activities in mediating granulocytic differentiation of HL-60R cells.

The modular structure of the RA receptors with their distinct ligand (RA)- and DNA-binding domains suggests that the activated RA receptors exert their biological effects by interacting with specific *cis*-acting DNA sequences that regulate the expression of specific target genes. Recent evidence indicates that different RA receptors may exhibit differential activity in activating specific target genes. For instance, an RA response element in the enhancer of the apoliprotein AI gene is preferentially activated by RXR-α compared with RAR-α and RAR-β (32). In HL-60 cells, the specific target genes that are presumably activated by the RA receptors and which trigger granulocytic differentiation have not yet been identified. Nevertheless, one would predict from our study that the RA response elements in such hypothetical HL-60 target genes might be similarly activated by the RAR-α, RAR-β, RAR-γ, and RXR-α receptors. Alternatively, the action of these RA receptors in triggering HL-60 granulocytic differentiation may not involve a direct interaction with DNA target sequences. For instance, activated RA receptors have been recently demonstrated to repress AP-1 transcription factor activity by a mechanism which may involve protein-protein interaction occurring off DNA (13, 33). Conceivably, the activated RAR-α, RAR-β, RAR-γ, and RXR-α receptors might trigger HL-60 granulocytic differentiation by interacting with and inhibiting a transcription factor(s) that actively represses HL-60 differentiation. If this latter mechanism were involved, then we would predict from our observations that the ability to inhibit such a hypothetical repressor of HL-60 differentiation would be a property shared by RAR-α, RAR-β, RAR-γ, and RXR-α.

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