Retinoic acid receptor γ2 gene expression is up-regulated by retinoic acid in 3T3-L1 preadipocytes

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Retinoids, especially all-*trans* retinoic acid (RA), have been shown to inhibit the differentiation of preadipose cells. In the present study, the expression of retinoic acid receptors (RAR α, β and γ) and retinoid X receptors (RXR α, β and γ) was examined by Northern blot analysis in rat adipose tissue and mouse 3T3-L1 adipose cells. The adipose tissue and/or 3T3-L1 cells expressed mRNAs for a number of nuclear retinoid receptors, including RAR α, β and γ, and RXR α, β and γ. RAR α, RAR γ, RXR α and RXR β mRNAs were abundant in adipose tissue and 3T3-L1 cells. RXR γ mRNA was detected in adipose tissue but not in 3T3-L1 cells. Treatment of 3T3-L1 cells with 1 μM RA led to a 4–5-fold increase in the RAR γ mRNA level, but only a trace amount of RAR β mRNA was detected. RAR γ mRNA expression was rapidly (within 2 h) induced by physiological concentrations of RA in a dose-dependent manner. The response of RAR γ mRNA expression to RA was reversible; rapid disappearance of RAR γ mRNA occurred on RA removal. In addition, the induction of RAR γ expression did not require *de novo* protein synthesis, but was completely abolished by an inhibitor of RNA synthesis. Using RAR γ1 and γ2 isoform-specific probes, the patterns of RAR γ1 and γ2 mRNA expression in 3T3-L1 cells in the presence and absence of RA were examined,. RAR γ1 mRNA was detected in 3T3-L1 cells but was not affected by RA treatment; however, RAR γ2 mRNA was strongly induced by RA.

INTRODUCTION

Vitamin A exerts a broad range of effects on many essential life processes (e.g. development, differentiation, reproduction, vision, immunity and homeostasis) [1–3]. The effects of vitamin A have been widely assumed to be mediated by retinoic acid (RA). In turn, the effects of RA are believed to be mediated by specific nuclear receptor proteins {retinoic acid receptor (RAR); designated as subtypes α [4,5], β [6] and γ [7]} which are members of the steroid and thyroid receptor superfamily of ligand-dependent transcriptional regulators [8]. Distinct nuclear receptors that participate in retinoid signal transduction in response to 9-*cis* RA [9,10], referred to as retinoid X receptor (RXR) α [11], β [12] and γ [13,14] subtypes, have also been identified. RXRs also function to augment RARs and other receptors, such as the vitamin D receptor and the thyroid hormone receptor [13,15–19]. Distinct patterns of tissue distribution and developmental stage specificity in expression of the RARs/RXRs have been demonstrated [7,14,20,21]. In addition, multiple isoforms have been found for RARs α, β and γ [22–24]. The expression of the RAR α2 [22] and RAR β2 [21,23,25,26] isoforms is controlled by RA, through an RA-responsive *cis*-acting element (RARE) located in the 5′ upstream region of their genes [27–30]. Recently, Lehmann et al. [31] showed that the promoter of RAR γ2 contains an RARE, which can trans-activate the receptor gene *in vitro*. The tissue- and developmental stage-specific expression patterns of the RARs/RXRs or differences in the mechanisms underlying autoregulation by RA of RAR isoforms could indicate distinct physiological roles for RARs/RXRs. These RAR/RXR subtypes and isoforms may exert specific functions, perhaps by regulating the transcription of different genes in specific cells. Moreover, the existence of multiple subtypes and their isoforms may in part account for the pleiotropic effects of RA. The various subtypes and isoforms are believed to respond differentially to ligands, thereby facilitating the pleiotropic effects of RA.

Adipose tissue plays an important role in the storage and metabolism of lipids [32], and is known to be a target organ for RA. Adipose tissue plays an important role in retinol uptake and storage and in retinol-binding protein synthesis [33,34]. The adipogenic differentiation of preadipocyte lines was prevented and/or interrupted at an early stage by RA [35–37]. It has been reported that RA can down-regulate the gene for adipsin, which belongs to the serine proteinase family and is suggested to be a systemic regulator of lipid metabolism and energy balance; its expression in 3T3-F442A adipocytes is regulated post-transcriptionally through a mechanism involving mRNA stability [38]. We have shown that RA decreases the activity of lipoprotein lipase, the key enzyme implicated in adipocyte lipid storage, in 3T3-L1 adipocytes [39]. The mechanisms by which RA elicits such cellular responses are unclear at present, but presumably involve the regulation of the expression of specific genes via RARs/RXRs. Riaz-ul-Haq and Chytil [40] showed that, in rat adipose tissue, RAR α and RAR γ are expressed strongly and RAR β is expressed weakly. RAR α but not RAR β was detected in BFC-1β preadipocytes and adipocytes [34]. However, the expression of RARs/RXRs in adipose tissue and adipocytes has not been systematically investigated. In this study, we have examined the expression of RARs/RXRs in adipose tissue and 3T3-L1 cells. Moreover, to determine whether or not RA modulates the expression of its own receptors, we also examined the effects of RA on the expression of RARs/RXRs in 3T3-L1 cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and streptomycin

Abbreviations used: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RARE, RA responsive element; FCS, fetal calf serum; DEX, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's modified Eagle's medium.

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were purchased from Flow Laboratories Inc. (Irvine, Scotland, U.K.). Fetal calf serum (FCS), from M. A. Bioproducts (Walkersville, MD, U.S.A.), was used for cell culture. Insulin, dexamethasone (DEX) and all-*trans* retinoic acid (RA) were obtained from Sigma (St. Louis, MA, U.S.A.), 3-Isobutyl-1-methylxanthine (IBMX) was from Nacalai Tesque (Kyoto, Japan), [α-^{32}P]CTP was from ICN Biomedicals, Inc. (Costa Mesa, FL, U.S.A.), [γ-^{32}P]ATP was from Amersham, nylon membranes were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.), a random primer labelling kit and Oligotex-dT30 were from Takara Shuzo (Kyoto, Japan), and Fuji AIF X-ray film was from Fuji (Tokyo, Japan). All other products used for cell culture and biochemical analysis were of the highest quality commercially available.

Cell culture

3T3-L1 cells [41] were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Ob1771 cells [42] were kindly provided by Professor Gérard Ailhaud (C.N.R.S., Nice, France). The cells were routinely cultured in 90 mm plastic culture dishes in DMEM containing 10% FCS and 500 μg/ml streptomycin. The cells were maintained at 37 °C under a humidified 5% CO_2 atmosphere. For adipose conversion, confluent cultures were treated with DMEM containing 10% FCS, 0.25 μM DEX, 0.5 mM IBMX and 10 μg/ml insulin [43]; 2 days later, the medium was changed to DMEM containing 10% FCS. RA was dissolved in ethanol and then diluted with DMEM containing 10% FCS to an ethanol concentration of 0.1%, which did not affect the proliferation or differentiation of 3T3-L1 cells. Control cells received an equal volume of the vehicle. For examination of RA-induced RAR γ mRNA regulation, 3T3-L1 cells, having reached confluency, were placed in DMEM containing 10% FCS which had been treated with dextran-coated charcoal to remove endogenous retinoids (charcoal-stripped FCS) [44]. The cells were maintained in DMEM containing 10% charcoal-stripped FCS for 1 day, and then treated with RA in DMEM containing 10% charcoal-stripped FCS. For the use of protein and RNA synthesis inhibitors, 3T3-L1 cells were allowed to become confluent for 1 day and then cultured in DMEM containing 10% charcoal-stripped FCS for 1 day. Cells were exposed to DMEM/10% charcoal-stripped FCS containing 5 μg/ml actinomycin D or 10 μg/ml cycloheximide in the absence or presence of 1 μM RA for 3 h. Actinomycin D and cycloheximide were dissolved in ethanol and then diluted with DMEM containing 10% charcoal-stripped FCS to an ethanol concentration of 0.1%. Control cells were exposed to DMEM/10% charcoal-stripped FCS containing 0.1% ethanol for 3 h. The concentrations of actinomycin D and cycloheximide were as in [25].

RNA analysis

Total RNA was isolated from cells and rat adipose tissue by extraction with guanidine isothiocyanate, followed by centrifugation through 5.7 M CsCl [45]. The RNA was ethanol-precipitated, pelleted by centrifugation and resuspended in water. Total RNA was quantified by measuring the absorbance at 260/280 nm. Poly $(A)^+$-containing RNA was prepared from total RNA using Oligotex-dT30 latex. Samples of 4 μg of poly$(A)^+$ RNA or 20 μg of total RNA were separated by electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel [45] and then transferred to a nylon membrane by capillary transfer in 20 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate). After baking for 1 h at 80 °C in an oven, the membrane was prehybridized at 42 °C for 6 h in 50% formamide containing 0.65 M NaCl, 0.1 M sodium Pipes, pH 7.4, 5 mM EDTA, 0.1% each of SDS, BSA, poly(vinylpyrrolidone) and Ficoll, and 100 μg of calf thymus DNA/ml. Hybridization was carried out overnight under the same conditions in the presence of a random-primer-labelled (about 10^7 c.p.m./ml) mouse RAR/RXR cDNA probe (kindly provided by Professor Pierre Chambon, Strasbourg, France) [4,6,13] or a rat β-actin cDNA probe (Y. Kamei, T. Kawada, R. Kazuki and E. Sugimoto, unpublished work) as a control. After hybridization, the membrane was washed in a washing buffer (2 × SSC, 0.2% sodium pyrophosphate, 0.1% SDS) for 2 h at 50 °C with constant agitation, air-dried and then subjected to autoradiography with Fuji AIF X-ray film at −70 °C. Each probe was removed from the membrane by treatment with water at 100 °C for 15 min, followed by hybridization with the next cDNA probe. The relative intensities of the bands on the autoradiogram were integrated with a densitometer. Isoform-specific oligonucleotides complementary to RAR γ1 and RAR γ2 with the following sequences were prepared: RAR γ1, 5′-AAAGAGTCTCTCCTTATTGGTGGC-3′ (24-mer); RAR γ2, 5′-GACAAACGATTCCATGCAGTCGTA-3′ (24-mer). These are portions of the 5′ region, but include the ATG initiation codon of the cDNAs for mouse RAR γ1 and RAR γ2, as described [24]. For the use of oligonucleotides, prehybridization was performed at 47 °C for 6 h in 2 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.4), 0.1% SDS and 100 μg of calf thymus DNA/ml. Hybridization was performed under the same conditions in the presence of a 3′-end-labelled oligonucleotide (about 10^5 c.p.m./ml). The membranes were washed in 2 × SSPE and 0.1% SDS for 30 min at 47 °C. The transcript sizes of RARs/RXRs were estimated by comparison with 28 S (4.7 kb) [46] and 18 S (1.8 kb) [47] rRNAs.

RESULTS

Expression patterns of RARs/RXRs in rat adipose tissue and 3T3-L1 adipose cells

To study the expression patterns of the RAR/RXR genes, we performed Northern blot analysis using 4 μg samples of poly$(A)^+$ RNA extracted from rat adipose tissue and mouse 3T3-L1 cells. We also examined the effect of RA on the expression of RARs/RXRs in preadipocytes and adipocytes. 3T3-L1 cells were induced to differentiate into adipocytes by treating them with DEX, IBMX and insulin [43]. 3T3-L1 cells that had been allowed to differentiate for 9 days were treated with medium containing 1 μM RA and then prepared for RNA extraction at 24 h after treatment. Confluent 3T3-L1 cells (preadipocytes, before differentiation into adipocytes) were also treated with medium containing 1 μM RA and then prepared for RNA extraction at 24 h after treatment. For Northern blot analysis, full-length cDNAs of mouse RARs α, β and γ and RXRs α, β and γ were used as probes. The spatial distribution patterns were clearly distinct for the six receptors (Figure 1). Adipose tissue (Figure 1, lane 5) and 3T3-L1 cells (lanes 1–4) expressed mRNAs for a number of nuclear retinoid receptors, including RARs α, β and γ and RXRs α, β and γ. The Northern blot probed with RAR α revealed two transcripts of approximate size 3.8 kb and 2.8 kb in all samples. The levels of RAR α expression in 3T3-L1 adipocytes (Figure 1, lanes 3 and 4) were higher than in the preadipocytes (lanes 1 and 2). A faint band (~ 3.4 kb) was detected for RAR β in RA-treated 3T3-L1 cells. Treatment of 3T3-L1 preadipocytes and adipocytes with RA led to a 4–5-fold

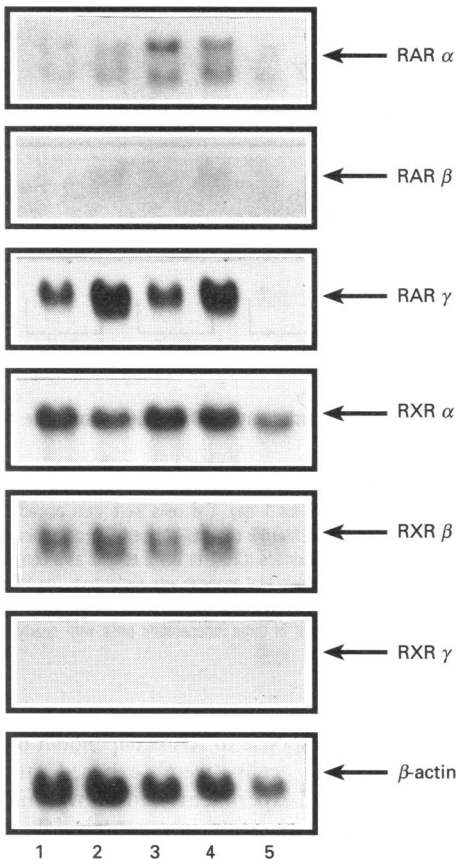

Figure 1 Northern blot analysis of RAR/RXR mRNAs in adipose tissue and 3T3-L1 cells

Lanes 1 and 2 contain poly(A)$^+$ RNA from 3T3-L1 preadipocytes allowed to reach confluency for 1 day and then maintained for 24 h in DMEM containing 10% FCS (lane 1) or supplemented with 1 μM RA (lane 2). Lanes 3 and 4 contain RNA from 3T3-L1 differentiated adipocytes maintained for 24 h in DMEM containing 10% FCS (lane 3) or supplemented with 1 μM RA (lane 4). Lane 5 contains RNA from rat (Wistar male, about 200 g body weight) perirenal adipose tissue. A 4 μg sample of poly(A)$^+$ RNA was analysed in each lane as described in the Materials and methods section. The exposure times for each of the blots at −70 °C with an intensifying screen were as follows: RAR α, 4 h; RAR β, 5 days; RAR γ, 30 min; RXR α, 1 h; RXR β, 14 h; RXR γ, 7 days; β-actin, 1 h. The approximate sizes of the respective mRNA species were as follows: RAR α, 3.8 and 2.8 kb; RAR β, 3.4 kb; RAR γ, 3.2 kb; RXR α, 5.6 kb; RXR β, 3.0 kb and 2.7 kb; RXR γ, 2.0 kb; 28 S and 18 S rRNAs were used as markers. These data are from one representative experiment out of two independent ones with nearly identical results.

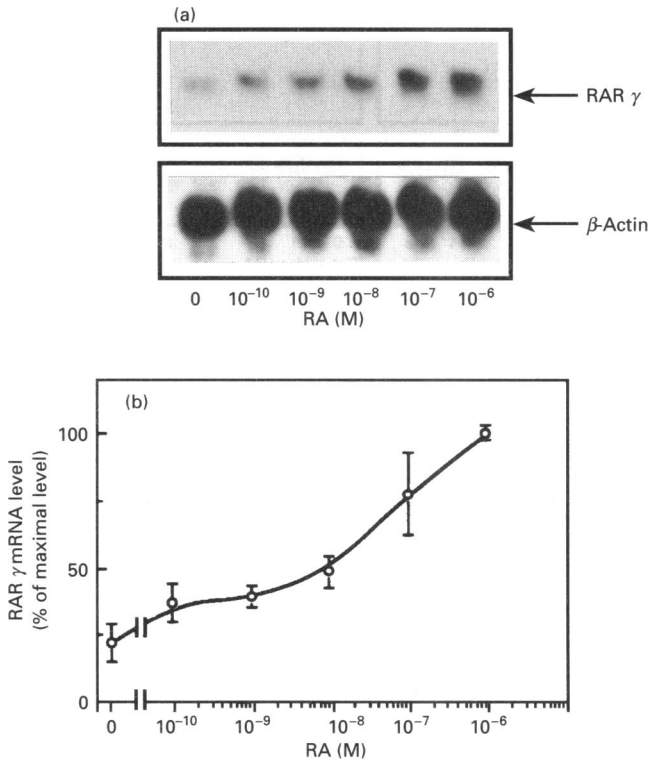

Figure 2 Response of the RAR γ mRNA level to increasing concentrations of RA

3T3-L1 cells were allowed to reach confluency for 1 day and then cultured in DMEM containing 10% charcoal-stripped FCS for 1 day. The cells were then placed in DMEM containing 10% charcoal-stripped FCS plus the indicated concentrations of RA. After 6 h, total RNA was isolated and Northern blot analysis was performed (**a**). The indicated mRNAs were detected using radiolabelled cDNA probes for RAR γ and β-actin. These data are for one representative experiment out of three independent ones with nearly identical results. The blots were scanned by densitometry and the highest level of RAR γ expression found on each blot was taken as 100% (**b**). Results are means ± S.E.M. of triplicate determinations at each dose.

increase in RAR γ mRNA levels (~ 3.2 kb). We examined this in detail (see below). RXR α mRNA (~ 5.6 kb) was abundant in all samples, and RXR β mRNAs (~ 3.0 kb and 2.7 kb) were relatively abundant in all samples. A very faint band (~ 2.0 kb) was detected for RXR γ in adipose tissue only, i.e. not in 3T3-L1 cells. Our transcript sizes for RARs/RXRs in adipose tissue and 3T3-L1 cells are similar to data reported previously [7,14,20,40].

RA-induced regulation of RAR γ mRNA

The expression of RAR γ in adult rodents is highly restricted to skin, lung [7] and adipose tissue [40]. Treatment of 3T3-L1 cells with RA led to a 4–5-fold increase in the RAR γ mRNA level (Figure 1, lanes 2 and 4). The up-regulation of the RAR γ gene by RA in 3T3-L1 cells seems especially important in analysis of the physiological functions of RA in adipose tissue. 3T3-L1 cells, having reached confluency, were placed in DMEM containing 10% FCS that had been treated with dextran-coated charcoal (charcoal-stripped FCS) to remove endogenous retinoids [44]. The cells were maintained in the DMEM containing 10% charcoal-stripped FCS for 1 day, and then treated with various concentrations of RA in DMEM containing 10% charcoal-stripped FCS. Then, 6 h after the addition of RA, the RAR γ mRNA level was measured by Northern blot analysis. The induction of RAR γ mRNA expression by RA was dose-dependent, showing a maximal increase at 1 μM RA in 3T3-L1 cells (Figure 2) (at 10 μM RA the RAR γ mRNA content was not further increased; results not shown). The concentration of RA required to produce a half-maximal effect (ED_{50}) was approx. 50 nM. As observed with densitometry, the magnitude of the RA-induced up-regulation was 5-fold at 1 μM. We repeated the experiment using Ob1771 cells [42], another preadipocyte cell line, and observed a greater (10-fold at 1 μM RA) RA-induced stimulation of RAR γ mRNA expression (results not shown). The time course for the up-regulation of RAR γ mRNA expression was investigated (Figure 3). When 3T3-L1 cells were treated with RA at 1 μM, a rapid (within 1 h) increase in RAR

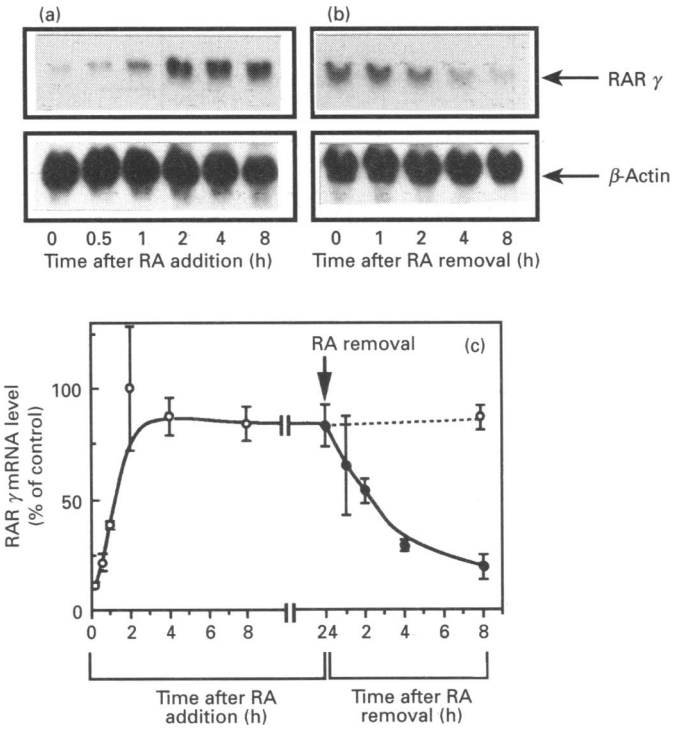

Figure 3 Time course of the RAR γ mRNA level in response to RA and following RA removal

3T3-L1 cells were allowed to reach confluency for 1 day and then cultured in DMEM containing 10% charcoal-stripped FCS for 1 day. The cells were then placed in DMEM containing 10% charcoal-stripped FCS plus 1 μM RA at time zero. At the indicated times, total RNA was isolated and Northern blot analysis was performed (**a**). After 24 h the cells were placed in DMEM containing 10% charcoal-stripped FCS at time zero (no RA). At the indicated times, total RNA was again isolated and Northern blot analysis was performed (**b**). The indicated species of mRNA were detected with radiolabelled cDNA probes for RAR γ and β-actin. These data are for one representative experiment out of three independent ones with nearly identical results. The blots were scanned by densitometry and the highest level of RAR γ expression found on each blot was taken as 100% (**c**). ○, Samples obtained at the indicated times after RA addition; ●, values after RA removal. Values are means ± S.E.M. of triplicate determination for each time point.

γ mRNA was observed (Figure 3). The effect of RA removal, following exposure of 3T3-L1 cells to RA for 24 h, was also investigated; the results in Figure 3 show that 3T3-L1 cells respond to RA in a reversible manner; the rapid disappearance of RAR γ mRNA occurred within 4 h of the removal of RA.

Effects of inhibitors

To investigate the mechanism underlying activation of the RAR γ gene by RA, experiments with 3T3-L1 cells were performed in the presence and absence of an inhibitor of protein synthesis (cycloheximide) or RNA synthesis (actinomycin D). The increase in the steady-state level of RAR γ mRNA resulting from treatment with RA could be due to an increase in either the rate of transcription or the stability of RAR γ mRNA. As shown in Figure 4, no RA-induced increase in RAR γ mRNA occurred when actinomycin D (5 μg/ml) was added to 3T3-L1 cells. Thus the induction of RAR γ mRNA in 3T3-L1 cells was completely blocked by actinomycin D, suggesting that the induction is transcription-dependent. To determine whether or not induction

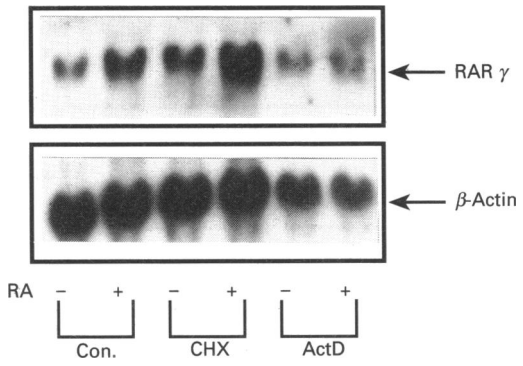

Figure 4 Effects of actinomycin D and cycloheximide on induction of RAR γ mRNA by RA

3T3-L1 cells were allowed to reach confluency for 1 day and then cultured in DMEM containing 10% charcoal-stripped FCS for 1 day. The cells were then placed in the absence (−) or presence (+) of RA in DMEM/10% charcoal-stripped FCS containing 5 μg/ml actinomycin D (ActD) or 10 μg/ml cycloheximide (CHX), or 0.1% ethanol as a control (Con.). After 3 h, total RNA was isolated and Northern blot analysis was performed. The indicated species of mRNA were detected with radiolabelled cDNA probes for RAR γ and β-actin. These data are from one representative experiment out of three independent ones with nearly identical results.

of the RAR γ in response to RA is dependent on *de novo* protein synthesis, 3T3-L1 cells were exposed to cycloheximide (10 μg/ml). Control experiments had shown that a 97% decrease in [^{3}H]leucine incorporation into protein [48] occurred in cells treated with cycloheximide (results not shown). As shown in Figure 4, cycloheximide treatment for 3 h did not affect the basal expression of RAR γ mRNA, i.e. the effects of RA on RAR γ mRNA induction were unaffected by inhibition of protein synthesis. These observations clearly demonstrate that the induction of the RAR γ gene by RA is a primary response, independent of ongoing protein synthesis. The RNA synthesis inhibitor, actinomycin D, abolished the RA-induced increases in the levels of RAR γ transcripts, whereas the protein synthesis inhibitor, cycloheximide, did not. These findings suggest that RA induction of the RAR γ gene is a direct transcription effect.

Patterns of expression of RAR γ isoforms in 3T3-L1 cells

Kastner et al. [24] characterized several mouse RAR γ cDNA isoforms (RAR γ1–γ7). RAR γ2 and RAR γ1 are considered to be the predominant RAR γ forms in tissues and embryos. Though the predominant expression of the RAR γ subtype has been demonstrated in adipose tissue [40], and in 3T3-L1 cells in this study, RAR γ isoform expression levels have not yet been analysed in adipose tissue. Thus we compared RAR γ1 and RAR γ2 expression in 3T3-L1 cells. Since the probe used in Figures 1–4 (full-length cDNA of mouse RAR γ1) hybridizes with both RAR γ1 and RAR γ2, we used oligonucleotides specific for either RAR γ1 or RAR γ2 in this experiment. The patterns of RAR γ1 and RAR γ2 mRNA expression were studied by Northern blot analysis using ^{32}P-labelled isoform-specific probes. Two blots were hybridized separately with either the RAR γ1 or γ2 specific probe. We confirmed that the two blots gave identical autoradiographic patterns after subsequent rehybridization with the β-actin cDNA probe. Figure 5 shows that RAR γ1 mRNA was expressed in both RA-treated and non-treated 3T3-L1 cells, and was not affected by RA treatment. On the other hand, RA treatment strongly increased the amount of RAR γ2 mRNA in 3T3-L1 cells.

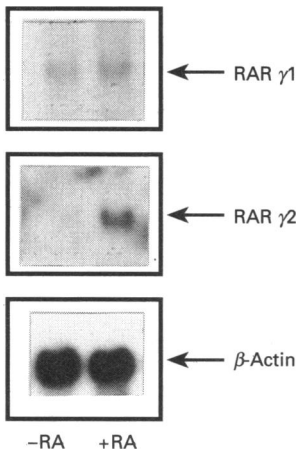

Figure 5 Patterns of expression of the RAR γ1 and γ2 isoforms in 3T3-L1 cells treated with RA

3T3-L1 cells were prepared and treated with RA as described in the legend to Figure 2. The cells were incubated in the absence (−) or presence (+) or RA (1 μM) in DMEM containing 10% charcoal-stripped FCS for 6 h. Then RNA was isolated and Northern blot analysis was performed. Poly(A)$^+$ RNA (4 μg per lane) was analysed using a ^{32}P-labelled RAR γ1 or γ2 oligonucleotide probe specific for either, as indicated. A β-actin cDNA probe was used to check the integrity of RNAs. These data are from one representative experiment out of two independent ones with nearly identical results.

DISCUSSION

RAR α, RAR γ, RXR α and RXR β mRNAs are abundant in adipose tissue, 3T3-L1 preadipocytes and adipocytes. Because of nearly equivalent hybridization conditions, comparison of the exposure time of autoradiography (see the legends to Figure 1) and the intensity of hybridization signals (Figure 1) could reflect the relative abundances of RARs/RXRs mRNAs in the given samples. Thus RAR γ and RXR α are the most abundant subtypes in adipose cells, with RAR α and RXR β the next most abundant. Clearly, compared with those of other receptor species, the RAR β and RXR γ mRNA levels were quite low. If a direct relationship exists between the levels of a receptor mRNA and its protein, the RAR α, RAR γ, RXR α and RXR β proteins would predominate in adipose tissue. The expression of RAR/RXR mRNAs, and of β-actin mRNA, appeared to be lower in rat adipose tissue than in 3T3-L1 adipocytes. Adipose tissue consists of several different types of cells, whereas cultured 3T3-L1 cells consist mainly of adipocytes, and the different abundances of mRNAs in adipose tissue and 3T3-L1 cells are probably caused by this adipose tissue heterogeneity.

It has been shown that RAR α and RXR β are expressed in most tissues [7,14,20], and these receptors have been suggested to be involved in the regulation of general cellular functions. In contrast, the expression of RAR γ in adult rodents is restricted to skin, lung [7] and adipose tissue [40] only. The up-regulation of the RAR γ gene by RA in 3T3-L1 cells seems to be especially important in analysis of the physiological functions of RA in adipose tissue. Our results are supported by the report that the intragastric administration of RA to retinol-deficient rats moderately increased (2-fold) the RAR γ mRNA level *in vivo* [49]. RXR α is highly expressed in the liver [11], and has also been shown to be expressed in the absorptive cells of the small intestine [50]. The liver is known to be the major organ in the body involved in vitamin A/lipid storage and mobilization [51]. From its expression pattern, RXR α is thus suggested to play an important role in vitamin A/lipid metabolism [11,50,52]. This hypothesis was supported further by the demonstration that the cellular retinol binding protein II and apolipoprotein AI genes, both of which are involved in vitamin A/lipid metabolism, are RXR-responsive [50,53]. Moreover, we have noted (Y. Kamei, T. Kawada and E. Sugimoto, unpublished work) that there are possible RXR-responsive elements [52] in the genes coding for malic enzyme [55], intestinal fatty acid binding protein [56], S14 protein [57] and Clara cell 10 kDa protein [58] all of which are involved in lipid metabolism. Since adipose tissue is considered to play an important role in vitamin A/lipid metabolism and storage [32–34], RXR α is likely to be involved in regulating vitamin A/lipid metabolism-related gene expression in adipose tissue. In addition, it has been reported that RXR forms heterodimers with RAR, the thyroid hormone receptor and the vitamin D receptor, preferentially increasing their DNA binding and the transcriptional function of the respective response elements [13,15–19]. These observations suggest a combined and potentially interactive role for RAR α, RAR γ, RXR α and RXR β in adipose tissue.

RA regulates the expression of the RAR γ gene in 3T3-L1 cells quite rapidly. An increase in RAR γ mRNA was observed within 1 h, with the maximum being reached within 2 h of RA addition (Figure 3), as reported previously for expression of the RAR β gene induced by RA in human hepatoma cells [25]. The ED_{50} value for the action of RA observed in the present study (approx. 50 nM) is consistent with that of 50 nM obtained for RA-dependent activation of transglutaminase expression in HL-60 cells [59]. Likewise, the ED_{50} value for the RA-associated increase in RAR β mRNA levels in F9 teratocarcinoma cells is around 50–80 nM [60]. The regulation of the RAR γ gene by RA is different in skin, embryocarcinoma cells and 3T3-L1 cells: the RAR γ gene was not RA-inducible in embryocarcinoma cells [7,24] or in skin [61]. The mechanism of autoregulated amplification of RAR γ by RA may be an important component of the RA response in adipose tissue, and our results show for the first time the rapid regulation of RAR γ gene expression. 3T3-L1 cells should be helpful in gaining some insight into the promoter function of the RAR γ gene involved in its regulation.

Kastner et al. [24] characterized several mouse RAR γ cDNA isoforms (RAR γ1 to RAR γ7). RAR γ1 and RAR γ2 are considered to be the predominant RAR γ forms in tissues and embryos. Skin contains almost exclusively RAR γ1 transcripts, whereas lung, embryo and embryocarcinoma cells contain both RAR γ1 and RAR γ2 [24]. Although the predominant expression of the RAR γ subtype has been demonstrated in adipose tissue [40], and in 3T3-L1 cells in the present study, RAR γ isoform expression levels have not previously been analysed in adipose tissue. We compared the expression of the two major RAR γ isoforms, RAR γ1 and RAR γ2, in 3T3-L1 cells. As shown in Figure 5, RA treatment greatly increased the amount of RAR γ2 mRNA, while RAR γ1 mRNA was not affected. Our results supported the study of Lehmann et al. [31], which showed that the promoter of RAR γ2, but not that of RAR γ1, contains an RARE which can trans-activate the reporter gene *in vitro*, i.e. RA can trans-activate the RAR γ2 gene, but not the RAR γ1 gene, in 3T3-L1 cells.

As described in the Introduction, adipose tissue is a target organ for RA. RA prevents the differentiation of 3T3-L1 cells from preadipocytes into adipocytes, and also decreases adipsin mRNA levels and lipoprotein lipase activity in 3T3 adipocytes. As observed in our study, the presence of many RARs/RXRs in 3T3-L1 preadipocytes, adipocytes and adipose tissue would indicate that retinoids must play a role in regulating gene

expression in adipose tissue. Further studies should clarify the relationship between induction of RAR $\gamma 2$ expression by RA and the phenomena elicited by RA in 3T3-L1 cells, and provide an insight into the mechanisms underlying the actions of RA in adipose tissue. Moreover, understanding the roles played by retinoids and RARs/RXRs in regulating gene expression in adipose tissue and during adipocyte differentiation should prove to be important for understanding the process of adipogenesis.

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REFERENCES

1 Dowling, J. E. and Wald, G. (1960) Proc. Natl. Acad. Sci. U.S.A. **46**, 587–608
2 Sporn, M. B., Roberts, A. B. and Goodman, D. S. (1984) The Retinoids, Academic Press, New York
3 Saurat, J.-H. (1990) Retinoids: 10 years on, Karger, Geneva
4 Petkovich, M., Brand, N. J., Krust, A. and Chambon, P. (1987) Nature (London) **330**, 444–450
5 Giguere, V., Ong, E. S., Segui, P. and Evans, R. M. (1987) Nature (London) **330**, 624–629
6 Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tilollais, P. and Dejean, A. (1988) Nature (London) **332**, 850–853
7 Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989) Nature (London) **339**, 714–717
8 Green, S. and Chambon, P. (1988) Trends Genet. **4**, 309–314
9 Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J. F. (1992) Nature (London) **355**, 359–361
10 Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C. (1992) Cell **68**, 397–406
11 Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. and Evans, R. M. (1990) Nature (London) **345**, 224–229
12 Hamada, K., Gleason, S. L., Levi, B.-Z., Hirschfeld, S., Appella, E. and Ozato, K. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8289–8293
13 Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) Cell **68**, 377–395
14 Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A. and Evans, R. M. (1992) Genes Dev. **6**, 329–344
15 Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Näär, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. and Rosenfeld, M. G. (1991) Cell **67**, 1251–1266
16 Zhang, X.-K., Hoffmann, B., Tran, P. B.-V., Graupner, G. and Pfahl, M. (1992) Nature (London) **355**, 441–445
17 Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. and Evans, R. M. (1992) Nature (London) **355**, 446–449
18 Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) EMBO J. **11**, 1409–1418
19 Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M. and Ozato, K. (1992) EMBO J. **11**, 1419–1435
20 Rees, J. L., Daly, A. K. and Redfern, C. P. F. (1989) Biochem. J. **259**, 917–919
21 Kato, S., Mano, H., Kumazawa, T., Yoshizawa, Y., Kojima, R. and Masushige, S. (1992) Biochem. J. **286**, 755–760
22 Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J.-M., Kastner, P., Dierich, A. and Chambon, P. (1991) EMBO J. **10**, 59–69
23 Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J.-M., Ruffenach, F., Leroy, P. and Chambon, P. (1991) EMBO J. **10**, 71–81
24 Kastner, P., Krust, A., Mendelsohn, C., Garnier, J. M., Zelent, A., Leroy, P., Staub, A. and Chambon, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2700–2704
25 de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1989) EMBO J. **8**, 429–433
26 Martin, C., Ziegler, L. M. and Napoli, J. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4804–4808
27 Leroy, P., Nakshatri, H. and Chambon, P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10138–10142
28 de Thé, H., Vivanco-Ruiz, M. d. M., Tollais, P., Stunnenberg, H. and Dejean, A. (1990) Nature (London) **343**, 177–180
29 Sucov, H. M., Murakami, K. K. and Evans, R. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5392–5396
30 Hoffmann, B., Lehmann, J. M., Zhang, X.-K., Hermann, T., Husmann, M., Graupner, G. and Pfahl, M. (1990) Mol. Endocrinol. **4**, 1727–1736
31 Lehmann, J. M., Zhang, X.-K. and Pfahl, M. (1992) Mol. Cell. Biol. **12**, 2976–2985
32 Cryer, A. and Van, R. L. R. (1984) New Perspectives in Adipose Tissue, Butterworths, London
33 Tsutsumi, C., Okuno, M., Tannous, L., Piantedosi, R., Allan, M., Goodman, D. S. and Blaner, W. S. (1992) J. Biol. Chem. **267**, 1805–1810
34 Zovich, D. C., Orologa, A., Okuno, M., Kong, L. W. Y., Talmage, D. A., Piantedosi, R., Goodman, D. S. and Blaner, W. S. (1992) J. Biol. Chem. **267**, 13884–13889
35 Sato, M., Hiragun, A. and Mitsui, H. (1980) Biochem. Biophys. Res. Commun. **95**, 1839–1845
36 Stone, R. L. and Bernlohr, D. A. (1990) Differentiation **45**, 119–127
37 Kawada, T., Aoki, N., Kamei, Y., Maeshige, K., Nishiu, S. and Sugimoto, E. (1990) Comp. Biochem. Physiol. **96A**, 323–326
38 Antras, J., Lasnier, F. and Pairault, J. (1991) J. Biol. Chem. **266**, 1157–1161
39 Kamei, Y., Fujita, A., Kawada, T. and Sugimoto, E. (1992) Biochem. Int. **26**, 923–934
40 Riaz-ul-Haq and Chytil, F. (1991) Biochem. Biophys. Res. Commun. **176**, 1539–1544
41 Green, H. and Kehinde, O. (1974) Cell **1**, 113–116
42 Négrel, R., Grimaldi, P. and Ailhaud, G. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 6054–6058
43 Rubin, C. S., Hirsch, A., Fung, C. and Rosen, O. M. (1978) J. Biol. Chem. **253**, 7570–7578
44 Dierich, A., Gaub, M.-P., LePennec, J.-P., Astinotti, D. and Chambon, P. (1987) EMBO J. **6**, 2305–2312
45 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
46 Hassouna, N., Michot, B. and Bachellerie, J.-P. (1984) Nucleic Acids Res. **12**, 3563–3583
47 Raynal, F., Michot, B. and Bachellerie, J.-P. (1984) FEBS Lett. **167**, 263–268
48 Freshney, R. I. (1991) Culture of Animal Cells, Alan R. Liss, Inc., New York
49 Riaz-ul-Haq and Chytil, F. (1992) J. Lipid Res. **33**, 381–384
50 Mangelsdorf, D. J., Umesone, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. and Evans, R. M. (1991) Cell **66**, 555–561
51 Morré, D. M. (1992) Int. Rev. Cytol. **135**, 1–38
52 Kleiwer, S. A., Umesono, K., Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A. and Evans, R. M. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1448–1452
53 Rottman, J. N., Widom, R. L., Nadal-Ginard, B., Mahdavi, V. and Karathanasis, S. K. (1991) Mol. Cell. Biol. **11**, 3814–3820
54 Reference deleted
55 Morioka, H., Tennyson, G. E. and Nikodem, V. M. (1988) Mol. Cell. Biol. **8**, 3542–3545
56 Lowe, J. B., Sacchettini, J. C., Laposata, M., McQuillan, J. J. and Gordon, J. I. (1987) J. Biol. Chem. **262**, 5931–5937
57 Jump, D. B., Bell, A. and Santiago, V. (1990) J. Biol. Chem. **265**, 3474–3478
58 Singh, G., Katyal, S. L., Brown, W. E., Kennedy, A. L., Singh, U. and Wong-Chong, M.-L. (1990) Biochim. Biophys. Acta **1039**, 348–355
59 Davies, P. J. A., Murtaugh, M. P., Moore, J. W. T., Jhonson, G. S. and Lucas, D. (1985) J. Biol. Chem. **260**, 5166–5174
60 Hu, L. and Gudas, L. J. (1990) Mol. Cell. Biol. **10**, 391–396
61 Elder, J. T., Fisher, G. J., Zhang, Q.-Y., Eisen, D., Krust, A., Kastner, P., Chambon, P. and Voorhees, J. J. (1991) J. Invest. Dermatol. **96**, 425–433

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