

# Regulation of adipocyte gene expression by retinoic acid and hormones: effects on the gene encoding cellular retinol-binding protein

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**Abstract** Our laboratory has reported that adipose tissue and adipocytes are importantly involved in retinoid storage and metabolism. To gain further insight, we examined factors that may regulate CRBP mRNA levels in primary cultures of rat epididymal and murine BFC-1 $\beta$  adipocytes. Northern blot analysis revealed that retinoic acid is a potent inducer of CRBP mRNA, causing a 7.5-fold rise in mRNA levels in primary adipocytes and a 9.5-fold rise in BFC-1 $\beta$  adipocytes. This induction of CRBP mRNA was dose-dependent at retinoic acid concentrations ranging between 10<sup>-8</sup> and 10<sup>-5</sup> M. Retinoic acid induction of CRBP mRNA levels showed a short lag period (6 h) and reached a maximal level of induction by 12 h in BFC-1 $\beta$  adipocytes and by 48 h in primary epididymal adipocytes. Nuclear run-on transcription assays of retinoic acid-induced BFC-1 $\beta$  adipocytes indicated that the rate of CRBP gene transcription is enhanced 3.6- to 4.3-fold by retinoic acid. In contrast, dexamethasone markedly down-regulated CRBP expression in a dose-dependent manner at concentrations ranging between 10<sup>-9</sup> and 10<sup>-6</sup> M. CRBP mRNA levels in primary and BFC-1 $\beta$  adipocytes declined, respectively, by 90% and 80% when adipocytes were exposed to 10<sup>-6</sup> M dexamethasone for 24 h. Studies of mRNA half-life indicated that dexamethasone acts to lessen CRBP expression through the specific destabilization of CRBP mRNA. Treatment of both primary and BFC-1 $\beta$  adipocytes with triiodothyronine alone had no effect on CRBP mRNA levels; however, when adipocytes were treated with a mixture of triiodothyronine and retinoic acid, the induction of CRBP mRNA levels by retinoic acid was reduced. ■ In summary, these studies indicate that CRBP gene expression is regulated by retinoic acid, dexamethasone, and triiodothyronine; thus suggesting that retinol uptake, intracellular transport, and metabolism are dynamically regulated in adipocytes.—Okuno, M., V. E. Caraveo, D. S. Goodman, and W. S. Blaner. Regulation of adipocyte gene expression by retinoic acid and hormones: effects on the gene encoding cellular retinol-binding protein. *J. Lipid Res.* 1995. 36: 137-147.

**Supplementary key words** retinoid • vitamin A • retinoid-binding protein • retinol-binding protein • dexamethasone • gene expression

Retinoids (vitamin A) are essentially involved in the induction and maintenance of cellular differentiation (1, 2). All retinoids in the body originate from the diet (3). After uptake by the intestine, dietary retinoid, as retinyl ester in

chylomicron remnants, is taken up by the liver, where the majority of the body's retinoid reserves are stored (3, 4). In order to meet body needs for retinoid, retinol is secreted from the liver bound to its specific plasma transport protein, retinol-binding protein (RBP) (5-7). In the circulation, RBP forms a one to one protein complex with another plasma protein, transthyretin (TTR) (5-7). Retinol is taken up by cells from the circulating retinol-RBP-TTR complex through a process that has been proposed to be mediated by cellular retinol-binding protein (CRBP) (8, 9). Within cells, the retinol may be esterified by the action of lecithin:retinol acyltransferase (LRAT) for storage as retinyl ester or enzymatically oxidized to retinoic acid (10-13). Both the enzymatic esterification of retinol catalyzed by LRAT and the enzymatic oxidation of retinol to retinoic acid have been shown to be facilitated when retinol is provided bound to CRBP (10-13), thus suggesting that the retinol-CRBP complex is the physiological substrate for these reactions. Retinoic acid functions in regulating the expression of retinoid-responsive genes by binding to its specific nuclear receptors, retinoic acid receptor  $\alpha$ ,  $\beta$ ,  $\gamma$  (RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ ) and retinoid X receptor  $\alpha$ ,  $\beta$ ,  $\gamma$  (RXR $\alpha$ , RXR $\beta$ , and RXR $\gamma$ ) (14). These retinoic acid nuclear receptors function as ligand-dependent transcription factors, which recognize specific response elements on genes and, upon binding to these response elements, modulate the expression of the genes (14).

Recent studies from our laboratory have demonstrated that adipose tissue and adipocytes are importantly involved in retinoid storage and metabolism (15, 16). These

Abbreviations: CRBP, cellular retinol-binding protein; RBP, retinol-binding protein; TTR, transthyretin; LPL, lipoprotein lipase; LRAT, lecithin:retinol acyltransferase; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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studies in rats indicate that adipose tissue stores of retinoid could account for as much as 15–20% of the total retinoid present in the body and that adipose tissue synthesizes a substantial portion of the RBP present in the rat (15). In addition, we have shown that retinol storage and RBP synthesis and secretion are properties acquired by adipocytes as they differentiate from preadipocytes to adipocytes (16). Like retinol accumulation and RBP expression, CRBP expression was also found to be modulated during adipocyte differentiation (16). No information, however, is available regarding the regulation of retinoid storage and metabolism in mature adipocytes. As the storage and metabolism of triglycerides by adipocytes are acutely responsive to hormonal status, it would seem reasonable that adipocyte retinoid metabolism and CRBP expression may also be hormonally regulated. The studies reported in this manuscript were designed to explore the regulation in adipocytes of CRBP expression by glucocorticoids and thyroid hormone. We also report studies on the effects of retinoic acid on adipocyte expression of CRBP.

## MATERIALS AND METHODS

### Cell isolation and primary culture

Primary cultures of rat adipocytes were prepared according to the method originally described by Rodbell (17), with some modifications. Briefly, epididymal fat pads were removed from nonfasted male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing approximately 350 g. The fat pads were minced and digested at 37°C for 45–60 min with 0.1% collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) in Medium M199 (Sigma Chemical, St. Louis, MO) containing 2% fatty acid-free bovine serum albumin (Sigma) and 10 mM glucose. The digest was filtered through a 250- $\mu$ m nylon mesh to separate the cells from undigested pieces and the filtrate was centrifuged for 1 min at 100 *g* and 25°C. The floating layer of adipocytes was washed 3 times by flotation through Medium M199 containing 10% fetal calf serum (Intergen, Inc., New York, NY), 10 mM glucose, 100 mg/l gabexate mesilate (Ono Pharmaceuticals, Osaka, Japan), 200 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, 100  $\mu$ g/ml gentamicin sulfate, and 2.5  $\mu$ g/ml amphotericin B. After incubation overnight in a metabolic shaker set at 60 cycles/min, 2–3  $\times 10^6$  cells were placed in each flask and treated with retinoic acid and/or hormones for the indicated time intervals. Each experiment was performed in triplicate. The stock solutions of all-*trans*-retinoic acid (Sigma) and dexamethasone (Sigma) were 5 mM in absolute ethanol. Control cells received an equal volume of vehicle.

### BFC-1 $\beta$ cell culture

The BFC-1 preadipocyte line from mouse brown fat tissue was established and characterized by Forest et al. (18, 19). BFC-1 $\beta$  preadipocytes, a subclone of the BFC-1 line, were used in this study and exhibited >90% conversion into mature adipocytes (20, 21). Cells were normally plated in 25-cm<sup>2</sup> flasks containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Intergen), 8  $\mu$ g/ml biotin, 4  $\mu$ g/ml pantothenate, 200 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin. At confluence (approximately 2  $\times 10^6$  cells per flask) 20 nM insulin (Sigma) and 2 nM triiodothyronine (T<sub>3</sub>) (Sigma) were added to the culture medium to induce differentiation. All experiments were performed on mature adipocytes grown for 14 days after confluence and the responses of the cells were studied in triplicate flasks 3 days after removal of T<sub>3</sub> from the media and 1 day after a media change. The treatments with retinoic acid and/or hormones were carried out the same as was described for the primary adipocyte cultures.

### RNA isolation and Northern analysis

Total RNA was isolated from primary or BFC-1 $\beta$  adipocytes by the method of Chomczynski and Sacchi (22). The RNA (7  $\mu$ g/lane for primary adipocytes and 20  $\mu$ g/lane for BFC-1 $\beta$  adipocytes) was electrophoresed on a 1% agarose gel in the presence of formaldehyde, and transferred to nylon membranes (Stratagene, La Jolla, CA) by capillary action. Immediately after transfer, the ratios of intensities of 28 S and 18 S ribosomal RNA bands in the total RNA preparations were assessed by ethidium bromide staining. This ratio was found to be approximately 2 for all RNA samples examined in our studies. The membranes were then prehybridized at 65°C for 1 h in a buffer containing 6 $\times$  SSC (1 $\times$  SSC; 0.15 M sodium chloride and 0.015 M sodium citrate), 5 $\times$  Denhardt's solution (1 $\times$  solution, 0.02% polyvinylpyrrolidone, 0.02% ficoll, and 0.02% bovine serum albumin), 10% dextran sulfate, and 1% sodium dodecyl sulfate (SDS) in the presence of 100  $\mu$ g/ml denatured salmon testes DNA (Sigma), and hybridized at 65°C overnight in the above buffer with <sup>32</sup>P-labeled probes (2  $\times 10^6$  cpm/ml) specific for rat RBP, rat CRBP, murine LPL, murine adipsin, murine adipocyte P2 (aP2), and murine  $\beta$ -actin mRNAs prepared by nick translation of cDNA clones (23). The cDNA clones for rat RBP (24) and rat CRBP (25) have been previously described, a cDNA clone for murine LPL was kindly provided by Dr. Ira Goldberg (Columbia University, New York, NY), and cDNA clones for murine adipsin and aP2 were kindly provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute,

Boston, MA). The membranes were washed in  $2\times$  SSC-0.5% SDS for 10 min at room temperature, two times in  $1\times$  SSC-1% SDS for 15 min at  $65^{\circ}\text{C}$ , and finally in  $0.1\times$  SSC-1% SDS for 15 min at  $65^{\circ}\text{C}$ . The hybridized blots were then exposed to XAR-2 films at  $-80^{\circ}\text{C}$  using intensifying screens. The bands on the autoradiographs were quantitated by scanning laser densitometry (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

#### Determination of mRNA half-life

Two different methods for assessing the half-life of CRBP mRNA were used for these studies. For one method of assessing the half-life of CRBP mRNA (Method 1), BFC-1 $\beta$  adipocytes were incubated for 24 h in the presence or absence of either  $1\ \mu\text{M}$  retinoic acid or  $1\ \mu\text{M}$  dexamethasone. Subsequently,  $5\ \mu\text{g}/\text{ml}$  actinomycin D was added to the adipocyte cultures and the cells were incubated for time intervals up to 48 h (in the continued presence or absence of retinoic acid or dexamethasone). The treated adipocytes were taken for total RNA isolation and determination of CRBP and  $\beta$ -actin mRNA levels by Northern blot analysis using the procedures described above. CRBP mRNA half-life was also assessed using a procedure described by Hod and Hanson (26) which does not use potentially toxic agents. For these studies (Method 2), BFC-1 $\beta$  adipocytes were incubated for 24 h in  $1\ \mu\text{M}$  retinoic acid,  $1\ \mu\text{M}$  dexamethasone, or vehicle (absolute ethanol). After incubation with these factors,  $1.0\ \text{mCi}$  of [ $^3\text{H}$ ]uridine was added to the plates for 2 h to label newly synthesized cellular RNA. The adipocytes were washed 2 times with fresh media containing  $5\ \text{mM}$  each of unlabeled uridine and cytosine and then cultured for up to 48 h in the media supplemented with  $5\ \text{mM}$  uridine and cytosine, to dilute out residual [ $^3\text{H}$ ]uridine. At predetermined time intervals, the BFC-1 $\beta$  adipocytes were taken for RNA isolation using the procedures described above. The different treatments did not seem to influence the rates of total RNA synthesis as evidenced by incorporation of [ $^3\text{H}$ ]uridine into the total RNA (for each treatment approximately  $40,000\ \text{cpm } ^3\text{H}/\mu\text{g}$  isolated RNA). Adipocyte levels of [ $^3\text{H}$ ]uridine-labeled CRBP mRNA were assessed essentially as described below for the nuclear run-on transcription assays.

#### Nuclear run-on transcription assays

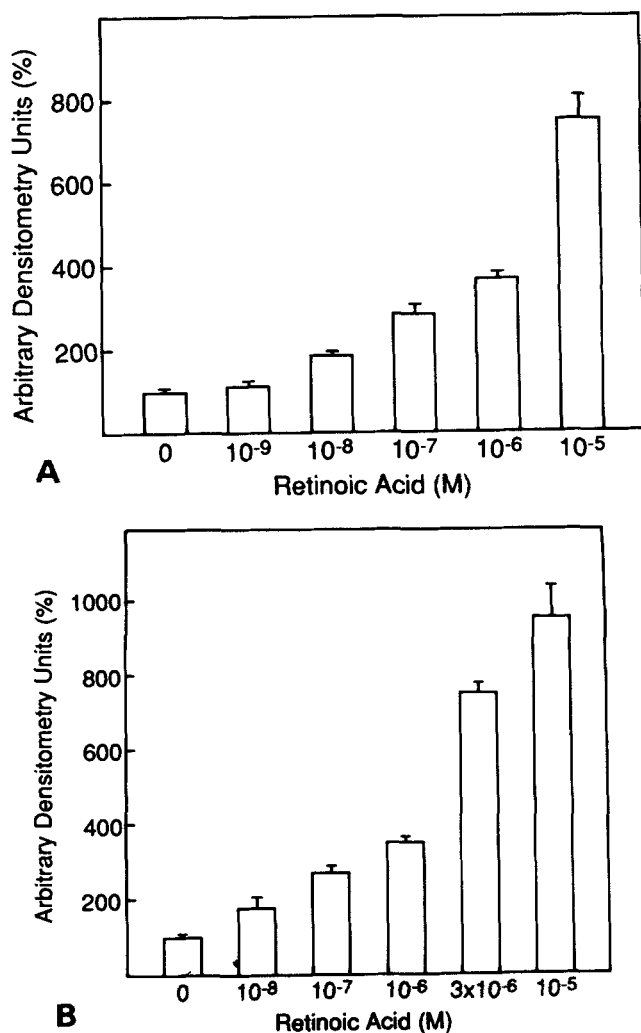
To measure the effects of retinoic acid and dexamethasone on the rate of transcription of the gene for CRBP, we used a procedure which was originally used by Zechner et al. (27) for the study of lipoprotein lipase gene expression in 3T3-L1 adipocytes. Briefly, ten  $150\text{-cm}^2$  flasks, each containing approximately  $6 \times 10^6$  BFC-1 $\beta$  adipocytes, were treated with media containing either  $1\ \mu\text{M}$  retinoic acid,  $1\ \mu\text{M}$  dexamethasone, or vehicle (absolute ethanol) for 24 h at  $37^{\circ}\text{C}$  under the standard adipocyte culture conditions described above. For isolation of nuclei, the

culture dishes were placed on ice immediately after incubation, the media was removed, the cells were washed 3 times with ice-cold PBS, and 5 ml of ice-cold lysis buffer consisting of  $10\ \text{mM}$  Tris-HCl, pH 7.5,  $5\ \text{mM}$   $\text{MgCl}_2$ ,  $25\ \text{mM}$  KCl,  $0.1\ \text{mM}$  EDTA,  $1\ \text{mM}$  dithiothreitol, and 0.5% Nonidet P-40. The lysis buffer was pipetted up and down over the adipocytes at least 10 times, and the lysates from equally treated dishes were combined. Nuclei were separated from the remaining cellular contents by centrifugation at  $4000\ g$  for 5 min. The pelleted nuclei were resuspended in 5 ml lysis buffer, carefully layered over storage buffer consisting of  $50\ \text{mM}$  Tris-HCl,  $5\ \text{mM}$   $\text{MgCl}_2$ , and  $0.1\ \text{mM}$  EDTA, and centrifuged at  $2000\ g$  for 10 min. The pelleted nuclei were resuspended in 1.0 ml of storage buffer and immediately frozen at  $-70^{\circ}\text{C}$  for up to 1 week prior to use. Nuclei preparations for the different treatments contained approximately the same levels of total protein.

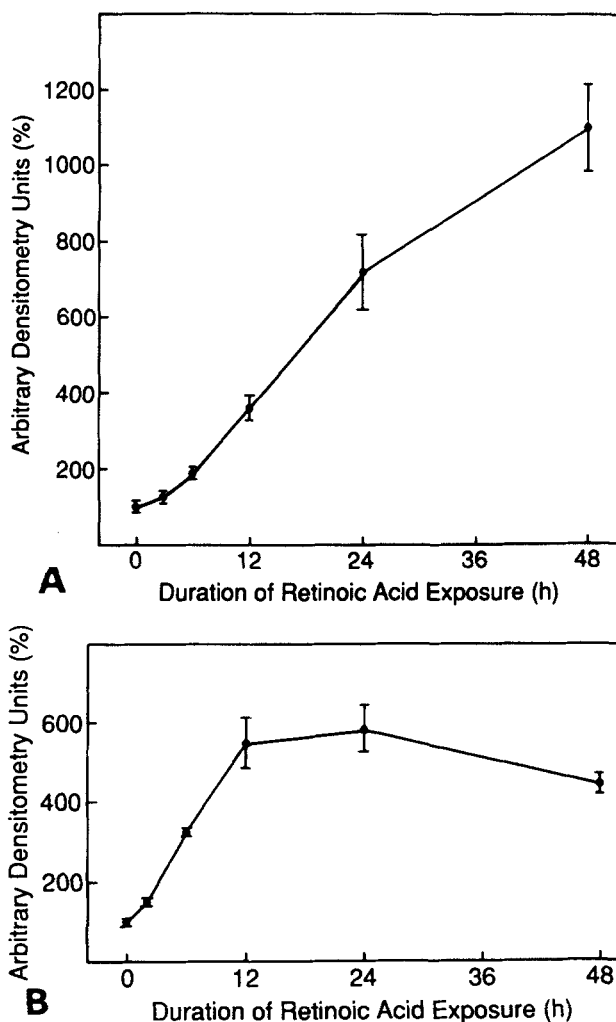
For the nuclear run-on transcription assay,  $40\ \mu\text{l}$  of the nuclear suspension was incubated with  $20\ \mu\text{l}$  of  $5\times$  transcription buffer ( $120\ \text{mM}$  Tris-HCl, pH 7.8,  $50\ \text{mM}$  NaCl,  $350\ \text{mM}$   $(\text{NH}_4)_2\text{SO}_4$ ,  $4\ \text{mM}$   $\text{MnCl}_2$ ,  $0.24\ \text{mM}$  EDTA),  $5\ \mu\text{l}$  of heparin ( $20\ \text{mg}/\text{ml}$ ),  $5\ \mu\text{l}$  of a mixture of nucleotide triphosphates ( $20\ \text{mM}$  ATP,  $20\ \text{mM}$  UTP,  $20\ \text{mM}$  GTP), and  $500\ \mu\text{Ci}$  [ $\alpha$ - $^{32}\text{P}$ ]CTP in a total volume of  $100\ \mu\text{l}$  for 45 min at  $32^{\circ}\text{C}$ . The reaction was stopped by adding  $0.4\ \text{ml}$  of solution that was prepared by adding  $25\ \mu\text{l}$  of DNase I ( $1\ \mu\text{g}/\text{ml}$ ) and  $25\ \mu\text{l}$  of proteinase K ( $1\ \mu\text{g}/\text{ml}$ ) to  $0.35\ \text{ml}$  of  $20\ \text{mM}$  Tris-HCl, pH 7.8, containing  $10\ \mu\text{g}$  yeast tRNA. After incubating this mixture at  $37^{\circ}\text{C}$  for 30 min,  $50\ \mu\text{l}$  of  $0.2\ \text{M}$  EDTA,  $25\ \mu\text{l}$  of 20% SDS, and  $25\ \mu\text{l}$  of  $\text{H}_2\text{O}$  were added. The whole mixture was incubated for 15 min at  $37^{\circ}\text{C}$ , extracted with  $0.5\ \text{ml}$  of phenol, and precipitated with  $600\ \mu\text{l}$  of ice-cold 20% trichloroacetic acid for 15 min. The nuclear RNA was pelleted, rinsed twice with ice-cold trichloroacetic acid, dissolved in TE buffer ( $10\ \text{mM}$  Tris-HCl, pH 7.5,  $1\ \text{mM}$  EDTA), and precipitated with ethanol. To measure levels of newly synthesized CRBP mRNA,  $5\ \mu\text{g}$  of plasmid DNA containing a rat CRBP cDNA insert (25), a rat  $\beta$ -actin cDNA insert, or the same plasmid with no insert (pGEM I) (to assess nonspecific hybridization) were slot-blotted onto nylon membranes and crosslinked by UV-irradiation. The cDNA-containing filters were first prehybridized in the buffer described above for Northern blot analysis. The filters were hybridized with equal counts of radiolabeled RNA ( $5 \times 10^6\ \text{cpm}$ ) from each nuclear run-on transcription assay for 24 h. After hybridization, blots were washed 2 times in  $2\times$  SSC at  $37^{\circ}\text{C}$  and treated with RNase ( $20\ \mu\text{g}$  of RNase A per ml). The remainder of the wash procedures were identical to those described above for Northern blot analysis. The washed blots were exposed to XAR-2 films at  $-80^{\circ}\text{C}$  using intensifying screens. The intensity of each signal was quantitated by scanning laser densitometry.

## RESULTS

In order to explore the effects of hormones and retinoic acid on CRBP mRNA levels in adipocytes, we used two *in vitro* cell culture systems. One consisted of primary cultures of rat epididymal adipocytes and the other consisted of BFC-1 $\beta$  adipocytes, one of the established murine preadipocyte cell lines (18-21). Both primary and BFC-1 $\beta$  adipocytes accumulate retinol and express the RBP and CRBP genes (15, 16). The viability of our primary rat adipocytes, after the cells were cultured overnight, was routinely characterized by measurement of basal and insulin-stimulated rates of glucose oxidation



**Fig. 1.** The dose-dependent effects of retinoic acid on CRBP mRNA levels in primary rat epididymal and BFC-1 $\beta$  adipocytes. Panel A shows the effects of retinoic acid in increasing CRBP mRNA levels for primary epididymal adipocytes and panel B shows these effects for BFC-1 $\beta$  adipocytes. The adipocytes were exposed for 24 h to the indicated concentrations of retinoic acid. Northern blot analysis was performed as described in Materials and Methods. Data from densitometric scans were normalized for  $\beta$ -actin signal and are given as the percentage of CRBP mRNA present in adipocytes treated only with vehicle. Values for each treatment represent the mean  $\pm$  SD for triplicate determinations.



**Fig. 2.** The kinetics of retinoic acid effects on CRBP mRNA levels in rat epididymal adipocytes (panel A) and BFC-1 $\beta$  adipocytes (panel B). For the experiments using epididymal adipocytes (panel A), freshly isolated adipocytes were cultured overnight in media M199 and then exposed to 1  $\mu$ M retinoic acid for the indicated times. For the experiments using BFC-1 $\beta$  adipocytes (panel B), the adipocytes were cultured for 14 days after induction to undergo adipose conversion and were then exposed, for the indicated times, to 1  $\mu$ M retinoic acid. Northern blot analysis was performed as described in Materials and Methods. Data from densitometric scans were normalized for  $\beta$ -actin signal and are given as the percentage of CRBP mRNA present in the vehicle treated controls. Values for each treatment represent the mean  $\pm$  SD for triplicate determinations.

(28) and by the enhancement of LPL mRNA levels upon insulin treatment (29). For all of our experiments, the glucose oxidation rates were stimulated by greater than 2-fold in the presence of 10 nM insulin and LPL mRNA levels were enhanced by greater than 3-fold after a 2-h incubation with 50 nM insulin. Thus, for all of our experiments involving primary adipocytes, we were using hormonally responsive adipocyte cultures.

**Figures 1A and 1B** show the effects of different concentrations of retinoic acid on CRBP mRNA levels in pri-



primary rat adipocytes (Fig. 1A) and BFC-1 $\beta$  adipocytes (Fig. 1B). For both types of adipocyte cultures, retinoic acid enhanced CRBP mRNA levels in a dose-dependent manner. In primary culture, treatment with 10 nM retinoic acid (a physiologic dose of retinoic acid) for 24 h increased adipocyte CRBP mRNA levels to 186% of control levels and maximal stimulation (750% of control levels) was observed at a retinoic acid concentration of 10  $\mu$ M (Fig. 1A). A similar enhancement of CRBP mRNA levels by retinoic acid was observed in BFC-1 $\beta$  adipocytes, where the maximal level of induction was found to be 960% over control levels, at 10  $\mu$ M retinoic acid (Fig. 1B). The kinetics of retinoic acid-induced increases in CRBP mRNA levels were examined in both primary and BFC-1 $\beta$  adipocytes (Fig. 2). The rate of retinoic acid induction of CRBP mRNA showed a short lag (6 h) and reached the maximal induction at 48 h in primary adipocyte cultures (Fig. 2A). After 48 h exposure to 1  $\mu$ M retinoic acid, CRBP mRNA levels showed a 1100% increase in primary adipocytes. For the BFC-1 $\beta$  adipocytes, the mRNA levels of CRBP reached a maximal level of expression between 12 and 24 h, followed by a slight decline in expression at 48 h (Fig. 2B). After 12 h exposure to 1  $\mu$ M retinoic acid, a 580% increase in CRBP mRNA levels, over those of control cells, was observed in BFC-1 $\beta$  adipocytes.

In contrast to the effect observed for retinoic acid treatment, dexamethasone down-regulated CRBP mRNA levels in a dose-dependent manner in primary adipocytes. When primary adipocytes were treated with 1 nM dexamethasone, CRBP mRNA levels fell to 39% of control CRBP mRNA levels (Fig. 3A). When exposed to 1  $\mu$ M

dexamethasone, primary adipocyte levels of CRBP mRNA were found to fall to approximately 10% of control levels (Fig. 3A). A similar effect on CRBP mRNA levels was also observed in BFC-1 $\beta$  adipocytes (Fig. 3B). The time dependence of the dexamethasone-mediated reduction of CRBP mRNA levels in BFC-1 $\beta$  adipocytes is given in Fig. 4. As can be seen in Fig. 4, the rate of decrease in CRBP mRNA levels upon dexamethasone treatment showed a short lag (6 h) followed by a marked decline continuing through 48 h of exposure (Fig. 4). CRBP mRNA levels were found to recover to control levels 12 h after the removal of dexamethasone from the culture medium of the BFC-1 $\beta$  adipocytes (Fig. 4).

The effect of T<sub>3</sub> on CRBP mRNA levels was also explored in primary and BFC-1 $\beta$  adipocytes (Figs. 3A and 3B). For both concentrations examined (0.1 and 1  $\mu$ M), T<sub>3</sub> had little effect on the steady-state levels of CRBP mRNA in both primary and BFC-1 $\beta$  adipocytes.

Figure 5 shows the Northern blots for CRBP, RBP, LPL, adipsin, and aP2 mRNAs in primary adipocyte cultures that had been exposed to either retinoic acid, dexamethasone, or a combination of the two agents. Figure 5 shows that retinoic acid did not influence adipocyte expression of RBP. Similarly, as can be seen in this figure, retinoic acid did not affect either the steady-state levels of LPL mRNA, a key enzyme involved in adipocyte lipid storage, or of adipsin or aP2, two adipose specific proteins that are expressed in mature adipocytes. Thus, the effects of retinoic acid on CRBP expression in adipocytes appear to be specific for this cellular retinoid-binding protein. Both LPL and aP2 mRNA levels were influenced by dexamethasone presence in the media. Unlike CRBP ex-

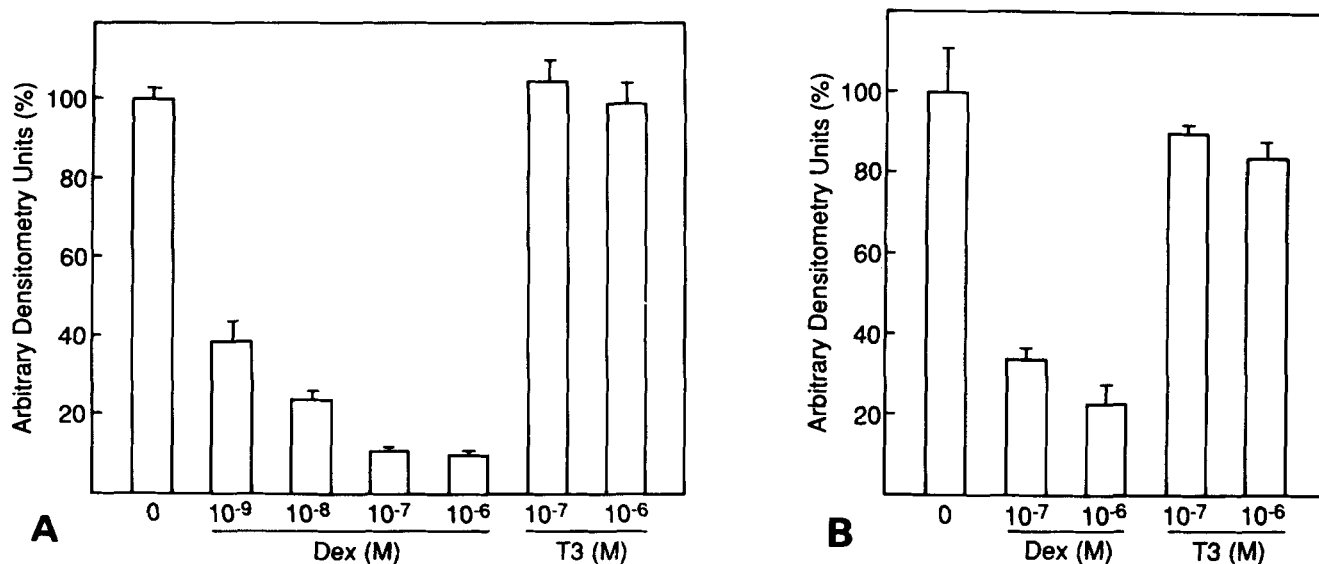
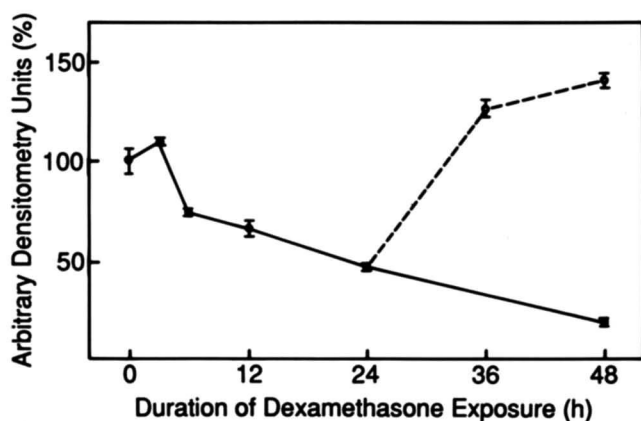


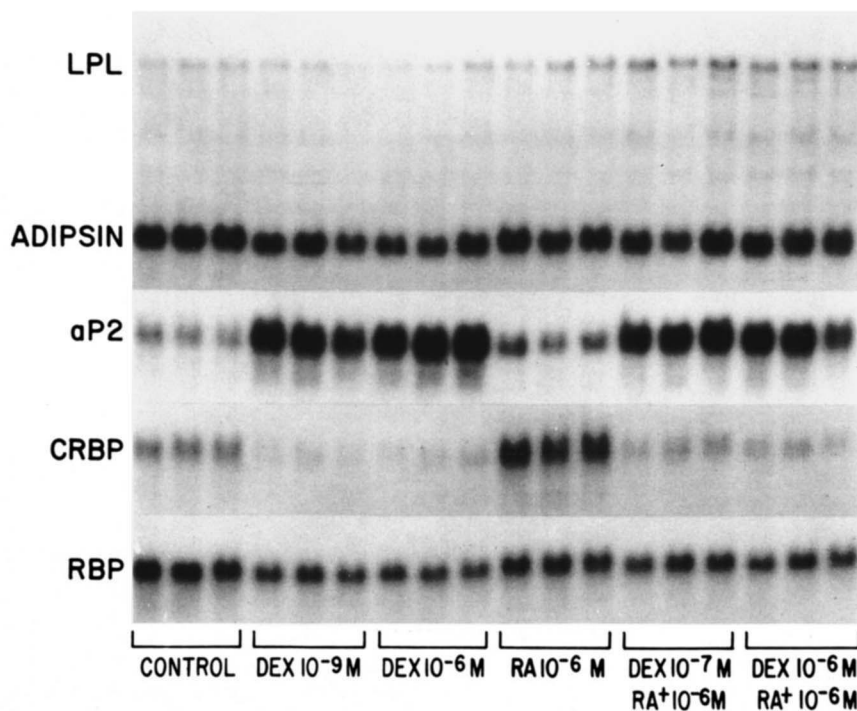
Fig. 3. The dose-dependent effects of dexamethasone (Dex) and T<sub>3</sub> on CRBP mRNA levels in primary rat epididymal adipocytes (panel A) and BFC-1 $\beta$  adipocytes (panel B). The adipocytes were exposed for 24 h to the indicated concentrations of Dex or T<sub>3</sub>. Northern blot analysis was performed as described in Materials and Methods. Data from densitometric scans were normalized for  $\beta$ -actin signal and are given as the percentage of CRBP mRNA present in adipocytes treated only with vehicle. Values for each treatment represent the mean  $\pm$  SD for triplicate determinations.



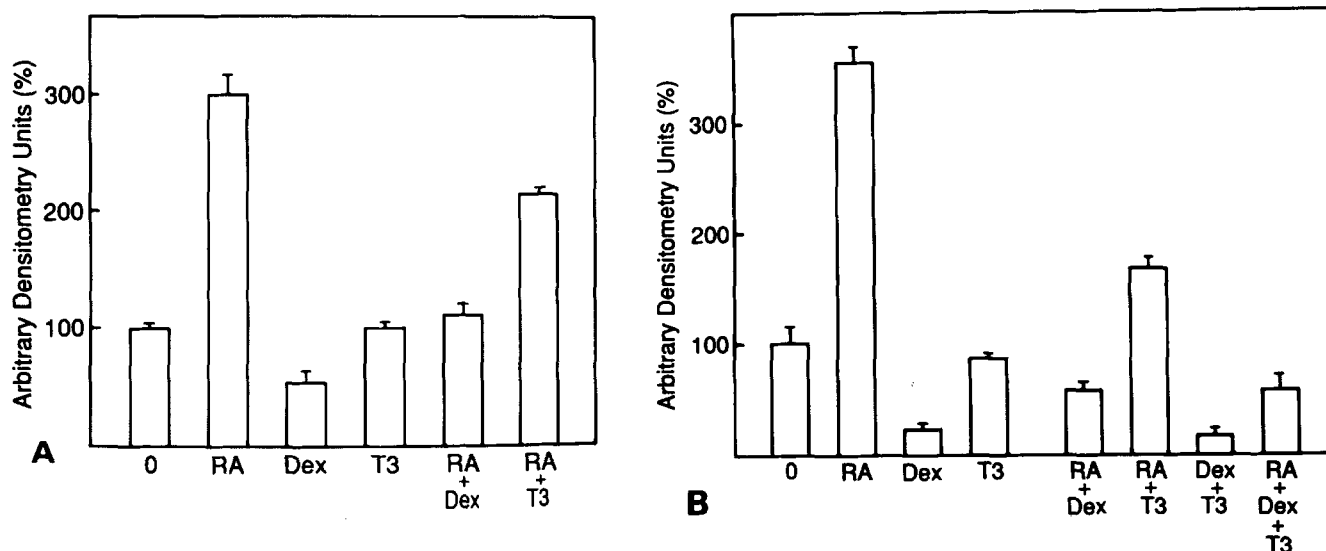
**Fig. 4.** The kinetics of the dexamethasone effect on CRBP mRNA levels in BFC-1 $\beta$  adipocytes. On day 14 after induction to undergo adipose conversion, BFC-1 $\beta$  adipocytes were exposed for 24–48 h to the complete differentiation medium supplemented with 1  $\mu$ M dexamethasone. For some cultures, the dexamethasone-containing media were removed after 24 h and replaced with fresh medium lacking dexamethasone (indicated by the dashed line). Northern blot analysis was performed as described in Materials and Methods. Data from densitometric scans were normalized for  $\beta$ -actin signal and are given as the percentage of CRBP mRNA present in the controls. Values for each treatment represent the mean  $\pm$  SD for triplicate determinations.

pression, which was markedly decreased by dexamethasone, aP2 mRNA levels were found to increase in dexamethasone-treated adipocytes. LPL mRNA seemed to be slightly down-regulated by dexamethasone. Dexamethasone had no effect on the expression of either RBP or adipsin. When the adipocytes were treated with combinations of retinoic acid and dexamethasone, the retinoic acid effect in increasing CRBP mRNA levels was not observed.

The combined effects of retinoic acid, dexamethasone, and T<sub>3</sub> on CRBP expression were further examined in both primary (Fig. 6A) and BFC-1 $\beta$  adipocytes (Fig. 6B). Figure 6A shows that dexamethasone inhibited the induction of CRBP mRNA by retinoic acid in primary culture. T<sub>3</sub> seemed to have a slight suppressing effect when given in combination with retinoic acid to primary adipocytes (Fig. 6A). As was observed with primary adipocytes, dexamethasone also inhibited the up-regulation of CRBP mRNA by retinoic acid in BFC-1 $\beta$  adipocytes (Fig. 6B). Although T<sub>3</sub> had no effect on CRBP mRNA levels when provided to the cells alone, T<sub>3</sub> had a suppressing effect on the retinoic acid-induced enhancement of CRBP mRNA, which was most pronounced in BFC-1 $\beta$  adipocytes (Fig. 6B). These data may suggest that retinoic acid and T<sub>3</sub>



**Fig. 5.** Northern blot analyses for CRBP, RBP, adipsin, aP2, and LPL mRNAs in total RNA prepared from primary rat epididymal adipocytes. The adipocytes were cultured overnight in medium M199 and then exposed for 24 h to retinoic acid and/or dexamethasone at the indicated concentrations. Each lane contains 7  $\mu$ g of total RNA. Northern blot analyses were carried out as described in Materials and Methods. After hybridization with the probe for CRBP, the blots were washed and hybridized sequentially with probe for RBP, adipsin, aP2, and LPL. Thus, the same total RNA samples were hybridized for CRBP, RBP, adipsin, aP2, and LPL.



**Fig. 6.** The effects of combinations of retinoic acid, dexamethasone, and/or T<sub>3</sub> on CRBP mRNA levels in rat epididymal adipocytes (panel A) and BFC-1 $\beta$  adipocytes (panel B). For these studies, the concentrations of all agents used were 1  $\mu$ M. Northern blot analysis was performed as described in Materials and Methods. Data from densitometric scans were normalized for  $\beta$ -actin signal and are given as the percentage of CRBP mRNA present in the controls. Values for each treatment represent the mean  $\pm$  SD for triplicate determinations.

function together to regulate CRBP expression in adipocytes but, before such a conclusion can be reached, more extensive mechanistic investigations of T<sub>3</sub> effects on CRBP gene expression will first be required. When T<sub>3</sub> was provided in combination with dexamethasone to BFC-1 $\beta$  adipocytes, the effect observed when dexamethasone was provided alone was found to predominate.

The effects of different steroid hormones on CRBP expression were studied in primary adipocytes. For this purpose, the relative effects of 1  $\mu$ M concentrations of corticosterone, aldosterone, deoxycorticosterone, progesterone,  $\beta$ -estradiol, and dexamethasone on CRBP expression were examined. Dexamethasone was found to most strongly down-regulate CRBP expression (to 24% of control levels in 24 h), followed closely by hydrocortisone and corticosterone (both 42% of control) and aldosterone (52% of control). Both progesterone and  $\beta$ -estradiol were found to have no effect on CRBP mRNA levels in primary adipocytes. These data would suggest that the effect of dexamethasone on regulating CRBP gene expression in adipocytes is not a generalized property of all steroid hormones.

In order to better understand the biochemical basis for the effect of retinoic acid and dexamethasone on adipocyte CRBP mRNA levels, we carried out studies exploring the effects of vehicle, retinoic acid, and dexamethasone on CRBP mRNA half-life in BFC-1 $\beta$  adipocytes. For this purpose, we used two different experimental approaches. The first approach (Method 1) used actinomycin A to inhibit new cellular RNA synthesis and the second (Method 2) used [<sup>3</sup>H]uridine in a pulse-chase experiment for determining CRBP mRNA half-life. The

results of this study are given in **Table 1**. As can be seen in Table 1, both experimental approaches provided estimations of CRBP mRNA half-life that agreed well. These data indicate that dexamethasone acts on CRBP mRNA to decrease its half-life in BFC-1 $\beta$  adipocytes. The half-life of CRBP mRNA in dexamethasone-treated adipocytes was approximately 25% of that observed in the vehicle-treated adipocytes. Retinoic acid was found to have no effect on half-life of CRBP mRNA in BFC-1 $\beta$  adipocytes. The half-life of  $\beta$ -actin mRNA in BFC-1 $\beta$  adipocytes was not affected by either retinoic acid or dexamethasone treatment.

As the observed effects of retinoic acid and dexamethasone on CRBP mRNA levels in BFC-1 $\beta$  adipocytes might also arise through changes in the rate of transcription of

**TABLE 1.** Effect of retinoic acid and dexamethasone on CRBP mRNA half-life in BFC-1 $\beta$  adipocytes<sup>a</sup>

	Half-life in Hours		
	Vehicle	Retinoic Acid	Dexamethasone
Method 1 <sup>b</sup>	23.8	23.5	5.9
Method 2 <sup>c</sup>	27.2	26.8	7.2

<sup>a</sup>CRBP mRNA half-life in BFC-1 $\beta$  adipocytes was determined graphically using two different experimental approaches. For all treatments, the half-life of  $\beta$ -actin mRNA was not observed to change.

<sup>b</sup>For Method 1, actinomycin A was used to inhibit cellular RNA synthesis. Details of this method are provided in Materials and Methods.

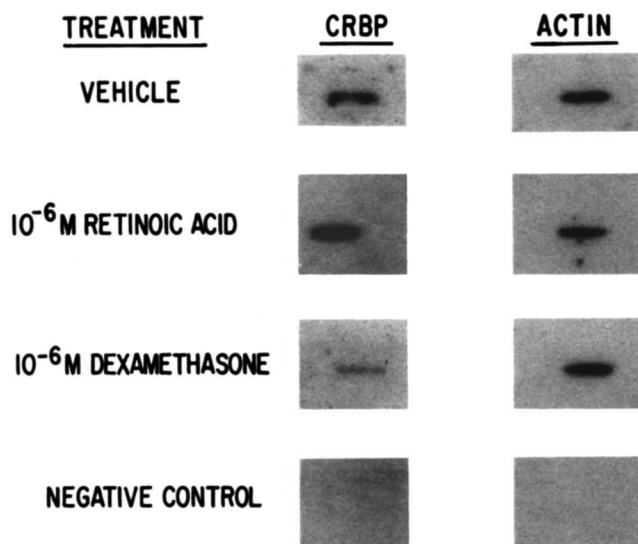
<sup>c</sup>For Method 2, CRBP mRNA half-life was measured through administration of a pulse of [<sup>3</sup>H]uridine followed by a chase with unlabeled uridine. The values presented are means of duplicate determinations. Details of this method are provided in Materials and Methods.



the gene for CRBP, we also carried out nuclear run-on transcription assays to assess the effects of retinoic acid and dexamethasone on CRBP gene transcription. The results of one representative nuclear run-on transcription assay are given in Fig. 7. For this experiment, treatment of BFC-1 $\beta$  adipocytes with 1  $\mu$ M retinoic acid was found to increase the rate of CRBP gene transcription by 3.6-fold over the vehicle-treated rate. As seen in Fig. 7, treatment with 1  $\mu$ M dexamethasone decreased the rate of CRBP gene transcription (by 32% for the experiment presented in Fig. 7). Triplicate nuclear run-on transcription assays indicated that retinoic acid increases CRBP gene transcription rate (between 3.6- to 4.3-fold compared to vehicle-treated controls) and that dexamethasone decreases CRBP gene transcription rate (by 20 to 32% compared to vehicle-treated controls). Thus, both retinoic acid and dexamethasone influence the rate of transcription of the CRBP gene.

## DISCUSSION

We have reported previously that adipose tissue and adipocytes are importantly involved in RBP synthesis and secretion, and in retinoid uptake, accumulation, and metabolism. The present study was designed to address two important questions regarding retinoid and retinoid-



**Fig. 7.** Nuclear run-on transcription assays exploring the effects of retinoic acid, dexamethasone, and vehicle on the rate of CRBP gene transcription in BFC-1 $\beta$  adipocytes. For these studies, BFC-1 $\beta$  adipocytes were incubated for 24 h with 1  $\mu$ M retinoic acid or 1  $\mu$ M dexamethasone or with vehicle prior to isolation of the nuclei. The negative control was vector (pGem I) DNA which did not contain the CRBP cDNA insert. The determination of transcription rates was carried out as described in Materials and Methods. The relative transcription rates were determined by scanning laser densitometry.

binding protein metabolism and its regulation in adipocytes. First, we wanted to gain insight into the factors that regulate the expression of the gene for CRBP, a retinoid-binding protein that plays a central role in cellular retinol uptake and processing and is synthesized in adipocytes (15). Secondly, we wanted to determine whether BFC-1 $\beta$  adipocytes are a useful model for the study of the tissue-specific regulation of the gene for this retinoid-binding protein. Our studies, as reported in this manuscript, clearly indicate that CRBP gene expression in adipocytes is markedly influenced by retinoic acid, dexamethasone, and T<sub>3</sub>. In addition, our studies demonstrate that BFC-1 $\beta$  adipocytes show the same pattern of regulation in response to retinoic acid, dexamethasone, and T<sub>3</sub>, for CRBP gene expression as do primary rat adipocytes. Thus, our data suggest that the gene for CRBP is regulated similarly in primary rat epididymal adipocytes and BFC-1 $\beta$  adipocytes.

The data reported in this manuscript demonstrate that retinoic acid, when given at physiological concentrations (30), induces the expression of the CRBP gene in adipocytes. Other studies have reported that retinoic acid induces CRBP gene expression in the murine embryonal carcinoma cell lines F9 (31) and P19 (32) and that retinoic acid induces CRBP expression in intact rat lung (25, 33), testis (25), and liver (25, 34). The 5'-regulatory region of the murine CRBP gene has been described in the literature (35). This report describes the presence of a retinoic acid response element in the 5'-flanking region of the murine gene for CRBP. Thus, the expression of CRBP within cells could, in part, be under the control of the nuclear receptors for retinoic acid. We and others have previously reported the presence of RAR $\alpha$  and RAR $\beta$  in rat adipocytes (15, 36) and the presence of RAR $\alpha$  in BFC-1 $\beta$  adipocytes (16). Our data clearly indicate that the induction of CRBP mRNA in adipocytes after exposure to retinoic acid is rapid and that this induction of mRNA levels results through an increase in the rate of transcription of the CRBP gene (Fig. 7) and not an increase in half-life of CRBP mRNA (Table 1). Exposure of BFC-1 $\beta$  adipocytes to 1  $\mu$ M retinoic acid for 24 h results in approximately a 4-fold increase in the rate of CRBP gene transcription. This increase in CRBP gene transcription rate correlates well with our observed increases in steady state CRBP mRNA levels in BFC-1 $\beta$  adipocytes, which upon exposure to 1  $\mu$ M retinoic acid for 24 h range between 3.6- to 5.8-fold over control levels (Figs. 1A, 2B, and 6B). Considering these observations, it seems very likely that the induction of adipocyte CRBP mRNA levels by retinoic acid arises from the direct transcriptional activation of the CRBP gene through its retinoic acid response element.

In studies with intact rats, Rush, Hag, and Chytil (34) have reported that dexamethasone treatment lowered CRBP mRNA levels in the lung. In our studies, we have



observed similar effects of dexamethasone on CRBP mRNA levels in cultures of primary rat epididymal and BFC-1 $\beta$  adipocytes. Dexamethasone treatment of adipocytes was found to decrease the level of CRBP expression by decreasing substantially the half-life of CRBP mRNA (Table 1). Dexamethasone has been reported to act in regulating mRNA levels by destabilizing specific transcripts, and thus lessening mRNA half-life. For instance, dexamethasone and cortisol are known to act in regulating type I collagen gene expression by decreasing the stability of mRNAs transcribed from this gene (37, 38). In addition to an effect on CRBP mRNA half-life, we also observed that dexamethasone had a small but reproducible effect in lowering the rate of transcription of the CRBP gene (by 20 to 32%) (Fig. 7). In our mixing experiments, where both dexamethasone and retinoic acid were provided to adipocytes (see Fig. 6), the dexamethasone-induced reduction of CRBP mRNA levels was found to predominate over the retinoic acid induction of CRBP mRNA. This marked effect of dexamethasone could arise either through the destabilization of CRBP mRNA or, alternatively, through a mechanism that results in a block of retinoic acid induction of CRBP gene expression. Taken together, these observations would suggest that glucocorticoids are very potent regulators of CRBP expression.

Treatment of adipocytes with retinoic acid or dexamethasone or combinations of these agents did not significantly influence adipocyte RBP mRNA levels. Similarly, when the rate of RBP protein synthesis was examined, using metabolically labeled adipocytes (15, 16), retinoic acid and dexamethasone both were found to exert no effect on RBP synthesis (data not shown). Hence, RBP and CRBP expression do not appear to be regulated in a coordinate manner in adipocytes. It is clear that RBP expression in adipocytes is regulated in a differentiation-dependent manner and that retinol availability to the differentiating adipocytes does not influence RBP expression during adipocyte differentiation (16). However, no other information regarding the factors that regulate RBP expression in mature adipocytes is available. Studies by Soprano, Smith, and Goodman (39) have indicated that availability of either retinol or retinoic acid in the diet does not influence RBP mRNA levels in the rat liver. These observations by Soprano et al. (39) regarding the lack of a retinoid effect on RBP expression in hepatocytes (as the hepatocyte is the site of RBP synthesis in the liver (40)) are consistent with our findings in adipocytes. In other studies using isolated and cultured rat hepatocytes, Dixon and Goodman (41) reported that treatment with dexamethasone reduced the rate of decline in hepatocyte synthesis and secretion of RBP; however, these studies provided no information regarding the effect of dexamethasone on hepatocyte RBP mRNA levels. Thus, at present, there is very little information available regard-

ing the factors that acutely regulate RBP expression in either hepatocytes or adipocytes, except that retinoic acid does not appear to influence RBP mRNA levels in either of these two cell types.

We also observed no effect of retinoic acid on the expression of LPL, adipsin, or aP2 in adipocytes (Fig. 5). LPL, adipsin, and aP2 are expressed in mature adipocytes and like CRBP and RBP are expressed in a differentiation-dependent manner during adipocyte differentiation (16). Thus, it would appear that the effects of retinoic acid on CRBP expression are specific for this gene and are not generalized effects on many genes that are expressed in adipocytes. Interestingly, other investigators have reported that retinoic acid specifically decreases adipsin mRNA levels in 3T3-F442A adipocytes by decreasing the half-life of adipsin mRNA (42). In our studies with primary rat epididymal adipocytes, we were not able to confirm such an effect of retinoic acid on adipsin mRNA levels (see Fig. 6). We can provide no insight into the basis for this discrepancy, although it is clear from our data that the murine 3T3-F442A adipocytes respond differently to retinoic acid treatment than do rat epididymal adipocytes.

It is of interest that dexamethasone increased the level of aP2 expression in adipocytes, whereas this glucocorticoid decreased adipocyte levels of CRBP mRNA. Both CRBP and aP2 are members of the same family of intracellular lipid binding proteins that are proposed to have originated from a common progenitor gene (43). Thus, as the genes for these two proteins evolved, they acquired reciprocal patterns of regulation by dexamethasone. The physiological significance of the reciprocal regulation of aP2 and CRBP expression by dexamethasone, however, remains unclear. Similar findings regarding dexamethasone regulation of aP2 expression have been reported by other laboratories (44, 45). Adipocyte adipsin mRNA levels were slightly lessened by treatment for 24 h with dexamethasone. It has been reported that corticosterone administration to mice resulted, after 2 days, in a suppression of adipsin mRNA and protein levels (46). Thus, the effect of dexamethasone on the expression of the gene for adipsin that we observed in our adipocytes seems to be the same as that observed in the intact animal.

In summary, both retinoic acid and dexamethasone have marked effects on the expression of the CRBP gene in adipocytes. CRBP has been reported to be essentially involved in the uptake of retinol by cells (8, 9), in the enzymatic esterification of retinol to retinyl esters (10–12), and in the enzymatic oxidation of retinol to retinoic acid (13). Thus, CRBP is dynamically involved in retinoid metabolism and storage. We have demonstrated that CRBP gene expression in adipocytes can be regulated by retinoic acid and dexamethasone and this could be taken to suggest, considering the important role played by CRBP in retinoid metabolism and storage, that these fac-

tors are important regulators of retinoid metabolism and storage in adipocytes. Interestingly, neither retinoic acid nor dexamethasone was found to influence the expression of the gene for RBP. As RBP functions in the mobilization of retinol from its storage depots, it would seem that neither retinoic acid nor dexamethasone can act to increase the mobilization of retinol from adipocytes through increased transcription of the gene for RBP. At present, we do not understand the metabolic significance of the marked effect of retinoic acid and dexamethasone on CRBP gene expression but no effect on RBP gene expression. It would seem reasonable to speculate that the changes in CRBP levels may reflect the need of adipocytes to take up and store retinoids in response to changing body needs; however, in order to understand retinol-RBP efflux from adipocytes, it will first be necessary to better understand the factors that regulate the transcription of the RBP gene and that regulate RBP synthesis and secretion from adipocytes. ■

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