

Interaction of the Retinol/Cellular Retinol-binding Protein Complex with Isolated Nuclei and Nuclear Components

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ABSTRACT Retinol (vitamin A alcohol) is involved in the proper differentiation of epithelia. The mechanism of this involvement is unknown. We have previously reported that purified cellular retinol-binding protein (CRBP) will mediate specific binding of retinol to nuclei isolated from rat liver. We now report that pure CRBP delivers retinol to the specific nuclear binding sites without itself remaining bound. Triton X-100-treated nuclei retain the majority of these binding sites. CRBP is also capable of delivering retinol specifically to isolated chromatin with no apparent loss of binding sites, as compared to whole nuclei. CRBP again does not remain bound after transferring retinol to the chromatin binding sites. When isolated nuclei are incubated with [³H]retinol-CRBP, sectioned, and autoradiographed, specifically bound retinol is found distributed throughout the nuclei. Thus, CRBP delivers retinol to the interior of the nucleus, to specific binding sites which are primarily, if not solely, on the chromatin. The binding of retinol to these sites may affect gene expression.

Early histological studies have clearly shown that when animals become vitamin A deficient various epithelial tissues of these animals lose the ability to maintain proper differentiation (1). However, providing retinol (vitamin A alcohol) to the animal permits tissue repair, with improperly differentiated cells rapidly replaced by normal cells (2). This indicates that vitamin A has an essential role in cellular differentiation. The action of retinol appears to be mediated by a specific intracellular protein called cellular retinol-binding protein (CRBP). CRBP binds retinol with great avidity and specificity and has been detected in a number of tissues (3, 4). Recently, CRBP was purified and partially characterized (5, 6). It is distinct from the well-known serum retinol transport protein called retinol-binding protein (5, 7). That CRBP plays an important role in the action of vitamin A is suggested by the following observations: It is found complexed with retinol *in vivo* (4, 8). It binds *cis*-isomers of retinol with a specificity that parallels the *in vivo* activity of these isomers (9). Finally, if retinol is first complexed with CRBP, the retinol can bind to the nucleus in a specific and saturable manner (10). In this study we compare the interaction of the CRBP-retinol complex with isolated nuclei to its interaction with isolated chromatin and follow the fate of both the protein and the ligand. The nuclear binding sites for retinol were localized using autoradiography.

MATERIALS AND METHODS

Materials

All-*trans*-retinol was purchased from the Sigma Chemical Co. (St. Louis, Mo.). NaB[³H]₄ (48 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). Spectrafluor and NCS tissue solubilizer were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). CRBP (rat liver) was purified as previously described (5). Radioactive CRBP ([³H]methyl-CRBP, 10 or 24 Ci/mmol) was prepared by reductive methylation as previously described (11). Radioactive retinol was bound to CRBP or [³H]methyl-CRBP as previously described (10).

Animals

Male weanling albino rats (Holtzman strain) were fed *ad libitum* with a control (vitamin A-supplemented) diet. Rats were made vitamin A-deficient by the procedure of Lamb et al. (12). Vitamin A status of the rats was assessed by examining vitamin A content in the liver and serum as previously described (13).

Preparation of [³H]Retinol

6 mg (2 μmol) of retinal was dissolved in 3 ml of ethanol. This was added directly to 1 μmol NaB[³H]₄ (48 Ci/mmol). After a 30-min incubation, equal volumes of petroleum ether (boiling point 30–60°C) and 0.5 M Tris buffer (pH 7) were added and mixed well. The petroleum ether layer was then removed and taken to dryness under a stream of N₂. The resulting residue was dissolved in cyclohexane/chloroform (1:1, vol:vol) with 50 μg butylated hydroxytoluene/ml and passed through a Sephadex LH-20 column (1.5 × 5 cm), equilibrated in the

same solvent mixture. This column separates unreacted retinol from retinol. Fractions containing [³H]retinol were pooled, taken to dryness under a stream of N₂, and dissolved in isopropanol containing 2 mg butylated hydroxytoluene/ml. The solution was stored at -20°C until used. The spectrum of [³H]retinol (13.4 Ci/mmol) was identical to that of authentic retinol.

Preparation of Nuclei

Isolation of nuclei was performed by the method of Chauveau et al. (14) as modified (15). Briefly, the livers were minced and homogenized in a solution (10 ml/g of liver) containing 2.2 M sucrose in buffer A (50 mM Tris HCl [pH 7.5], 25 mM KCl, 0.5 mM dithiothreitol, 4 mM MnCl₂) using a glass/Teflon homogenizer. The homogenates were filtered through four layers of cheesecloth and centrifuged in a Beckman 45Ti rotor at 70,000 g for 60 min at 4°C. The pellets were gently resuspended in buffer A containing 1.8 M sucrose (4 ml/g of liver). The nuclei were pelleted by centrifugation at 15,000 g for 20 min at 4°C. The pellets were gently resuspended in buffer B (the same as buffer A, also containing 0.1 mM EDTA and 0.3 M sucrose). The purity of isolated nuclei was assessed by microscopic examination. The DNA content of the purified nuclei suspension was determined according to Burton (16). Before determination of DNA, nuclei were washed by suspension and centrifugation in 0.4 M HClO₄ at 0°C to remove sucrose.

Preparation of Chromatin

Isolated nuclei were resuspended in hypotonic sucrose solution (0.3 M sucrose, 5 mM Tris-HCl, pH 7.5) for 1 h at 0°C, then sonicated with a Sonifier Cell Disruptor Heat Systems Ultrasonics, Inc. (Plain View, N.Y.) for 2 min at 0°C. The solution was centrifuged at 2,000 g for 10 min to sediment undisturbed nuclei. An equal volume of buffer B was then added to the supernate solution dropwise to precipitate the chromatin. The chromatin was sedimented by centrifugation at 2,000 g for 15 min. The pellet was then gently rehomogenized in buffer B containing 100 µg/ml ovalbumin and centrifuged at 2,000 g for 10 min and resuspended in the same buffer for binding studies. DNA content of chromatin was determined as described for nuclei.

Determination of Nuclear and Chromatin Binding of [³H]Retinol

Binding was determined as previously (10). Freshly prepared nuclei and chromatin were used. Briefly, a mixture (200 µl) containing nuclei or chromatin, the appropriately labeled complex of retinol-CRBP, plus or minus nonradioactive complex, was incubated at 25°C for 15 min in buffer B containing 100 µg/ml ovalbumin. Binding was terminated by chilling tubes in ice water for 3 min followed by centrifugation at 2,000 g for 5 min at 4°C. Nuclei or chromatin were washed twice by resuspending in 400 µl of buffer B containing ovalbumin and centrifuging as above. The pellets were resuspended in 200 µl of buffer B, and 100-µl aliquots were placed in scintillation vials for digestion at 45°C for 2 h with 1 ml of NCS tissue solubilizer. The samples were neutralized with 0.034 ml of glacial acetic acid and 10 ml of liquid scintillation solution (47 ml of Spectrafluor in 1,000 ml of toluene) was added. The samples were kept in the dark overnight before determination of radioactivity. The counting efficiency was ~40%.

Phospholipid Extraction and Analysis

Phospholipid was extracted from nuclei and chromatin by a method essentially that of Folch et al. (17). Suspensions of nuclei or chromatin were homogenized in 20 vol of chloroform-methanol (2:1), then passed through sintered glass funnels. The residue was extracted twice more with the same solvent mixture. The combined filtrate was then washed with 0.22 vol of 0.003 M MgCl₂ solution. After siphoning off the upper phase, the surface of the lower phase was gently rinsed with 0.003 M MgCl₂ solution previously equilibrated with chloroform-methanol (2:1). Sufficient methanol was then added to the lower phase to form one phase. The samples were taken to dryness, redissolved in chloroform-methanol (2:1), and aliquoted for ashing and phosphorus analysis by the method of Chen et al. (18). KH₂PO₄ was used as a standard. Phospholipid was calculated by assuming 25 µg of phospholipid per µg of lipid phosphorus.

Electron Microscopy

Nuclei were fixed for 2 h in freshly prepared 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After rinsing three times in phosphate buffer containing 5% sucrose, the nuclei were postfixed in 1% OsO₄ in the same buffer. The pellets were dehydrated in a graded series of ethanol solutions and transferred to propylene oxide before embedding in Epon (19). Thin sections were placed on copper grids and stained with uranyl acetate and lead citrate (20). Specimens

were examined in a Hitachi HU-11B electron microscope at 75 kV accelerating voltage.

Autoradiography

Nuclear pellets were suspended in Bouin's solution (saturated aqueous picric acid, 37% formalin, glacial acetic acid 15:5:1) for 4 h at 4°C, then centrifuged at 1,000 g for 5 min. The pellets were then embedded in paraffin, and 6 µm sections were mounted on slides. Slides were deparaffinized and dipped in premelted "Kodak nuclear track emulsion" and allowed to dry in the dark. Slides were stored at 4°C under CO₂ atmosphere for ~4 wk, then they were developed and stained with Mayer's hematoxylin.

RESULTS

CRBP Allows Specific Binding of Retinol to the Nucleus But Does Not Itself Remain Bound

To examine the question of whether the complex of retinol-CRBP, or only retinol, binds to the nucleus, CRBP was radioactively labeled. We chose to label the protein by reductive methylation using sodium borotritide and formaldehyde. This insured a large enough quantity of stable protein for physical characterization. The modified radioactive CRBP ([³H]methyl-CRBP) was able to bind retinol with high affinity and was capable of interacting with antibody directed against CRBP (11). [³H]Methyl-CRBP complexed with cold retinol was then incubated with isolated liver nuclei as previously described (10). Assuming that the complex remains bound, one would expect ~4 pmol of [³H]methyl-CRBP to be bound per 100 µg DNA (10). However, <4% of the expected radioactivity was found associated with the nucleus, and this radioactivity was not reduced in the presence of excess native retinol-CRBP (Table I).

It was possible that the modified protein, although able to bind retinol, might have been rendered incapable of binding to the nucleus. However, when [³H]methyl-CRBP was complexed with [³H]retinol and incubated with liver nuclei, and total radioactivity bound greatly increased and this increase was considerably reduced by the presence of a 20-fold excess of nonradioactive retinol-CRBP.

It should be stressed that since the reduction in binding was caused by retinol bound to unmodified CRBP, this binding of [³H]retinol must be to the same sites as when [³H]retinol is presented bound to native CRBP. Under saturating conditions,

TABLE I
Interaction of Retinol-[³H]Methyl-CRBP and [³H]Retinol-[³H]Methyl-CRBP with Liver Nuclei or Chromatin Isolated from Normal and Vitamin A-deficient Rats

Additions	Specific binding	
	Normal	Vitamin A-deficient
	dpm bound/100 µg DNA	
Retinol-[³ H]methyl-CRBP + nuclei or chromatin	Not detected	Not detected
[³ H]Retinol-[³ H]methyl-CRBP + nuclei	3.6 × 10 ³	7.4 × 10 ³
[³ H]Retinol-[³ H]methyl-CRBP + chromatin	7.0 × 10 ³	7.9 × 10 ³

Binding was determined using 28-30 pmol of labeled protein-ligand complex with or without a 20-fold excess of unlabeled retinol-CRBP and 40-60 µg of DNA. Binding and DNA analysis were performed as described in Materials and Methods. Specific dpm bound/100 µg DNA was determined by subtracting radioactivity bound when a 20-fold excess of retinol-CRBP was also present from total radioactivity bound.

we observed $\sim 1.34 \times 10^5$ molecules of retinol bound specifically per nucleus. This is very similar to the amount of specific binding observed when [^3H]retinol bound to native CRBP was used. When [^3H]retinol-[^3H]methyl-CRBP was presented to liver nuclei, the binding of [^3H]retinol reached saturation by 10 min and remained stable for at least 60 min. This is indistinguishable from the kinetics observed when [^3H]retinol bound to unmodified CRBP was used (10). Finally, nuclei from deficient animals bound more retinol than nuclei from control animals (see Table I). This also agrees with the result obtained previously using unmodified CRBP (10). Thus, the modified protein is capable of mediating the specific and saturable binding of retinol to the nucleus in a manner indistinguishable from that of native CRBP. The binding involves recognition of the complex, but a retinol must be transferring to the nuclear binding sites, with CRBP leaving after this transfer.

Triton X-100-washed Nuclei Retain Binding Sites for Retinol

Triton X-100 has been used previously to remove nuclear membrane while leaving the nucleus intact (21, 22). In an attempt to localize the specific binding sites for retinol, isolated nuclei from rat liver were treated with 0.5% Triton X-100.

Because the majority of the lipids in nuclear membranes are phospholipids (23), we performed phospholipid analyses to estimate the amount of nuclear membrane removed by Triton. We found that, in agreement with the literature (24, 25), untreated nuclei had a phospholipid to DNA ratio of 0.141 (wt/wt), whereas Triton-washed nuclei had a ratio of 0.016 (wt/wt). Thus, $\sim 90\%$ of the lipid present was removed by the detergent treatment. Electron micrography (Fig. 1) indicated that the double laminar membrane structure of normal nuclei was no longer evident after Triton treatment. Both preparations of nuclei contained little contamination by cellular debris.

The ability of the Triton-treated nuclei to bind retinol when

incubated with [^3H]retinol-CRBP was then compared to the ability of untreated nuclei. We found that the Triton-treated nuclei consistently retained $>70\%$ of the specific binding sites for retinol (data not shown). Yet these Triton-washed nuclei contain only 10% of the phospholipids found in untreated nuclei, indicating that not only the outer membrane but also much of the lipids of the inner membrane had been removed as well. This strongly suggests that most, if not all, of the specific binding sites are not associated with the nuclear membrane.

Retinol-CRBP Interacts with Isolated Chromatin as It Does with Isolated Nuclei

Because the majority of the binding sites were retained on Triton-treated nuclei, we examined the interaction of the protein-ligand complex with chromatin. The procedure used to isolate chromatin gave a preparation which had a phospholipid to DNA ratio of 0.077 (wt/wt). This preparation showed a 50% reduction of the phospholipids compared to our nuclei preparations, indicating a substantial removal of nuclear membrane during chromatin isolation. Isolated chromatin was capable of binding [^3H]retinol specifically when incubated with [^3H]retinol-CRBP, as shown in Fig. 2. When increasing concentrations of [^3H]retinol-CRBP were incubated with a constant amount of chromatin, a saturation curve was obtained, shown in Fig. 3. Saturation occurred at ~ 140 nM [^3H]retinol-CRBP. Thus the concentration at saturation is similar to that found with isolated nuclei (10). The saturation curve indicates there are $\sim 1.6 \times 10^5$ molecules of retinol bound per 10.7 pg chromatin DNA (assumed to be the DNA content of one nucleus) (26). This is again similar to values obtained using isolated nuclei, indicating that the binding sites have been retained.

As was found with isolated nuclei, when chromatin was incubated with retinol-[^3H]methyl-CRBP, we observed no specific binding of the protein (Table I). However, when [^3H]-

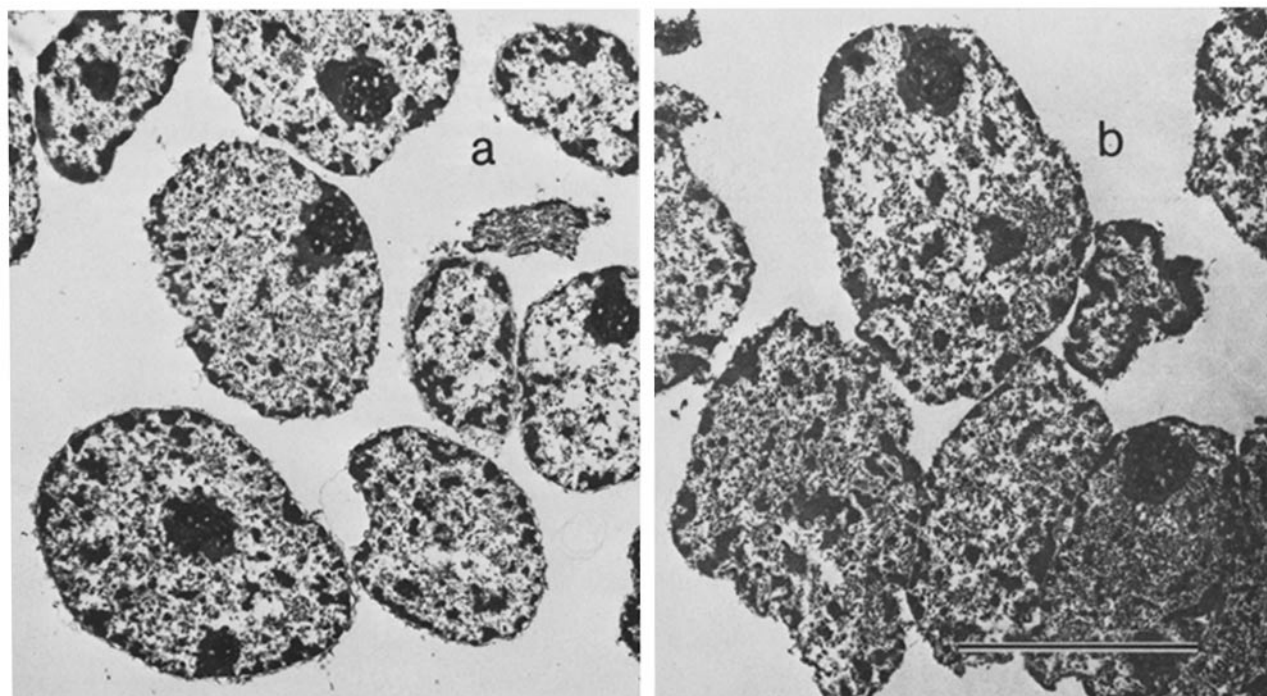


FIGURE 1 Electron micrographs of rat liver nuclei fixed in 2.5% glutaraldehyde and postfixed in 1% OsO_4 . a, without Triton X-100 treatment. b, with Triton X-100 treatment. Bar, 5 μm . $\times 6,700$.

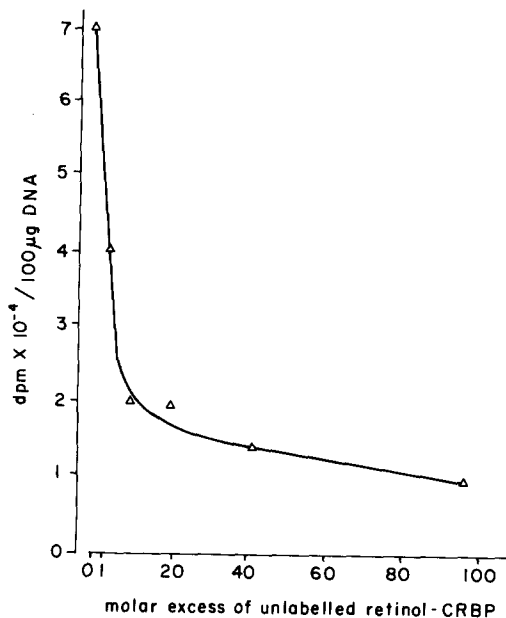


FIGURE 2 Competition for chromatin binding of [³H]retinol supplied as [³H]retinol-CRBP complex by increasing concentrations of unlabelled retinol-CRBP. Chromatin (64 μg) was incubated with [³H]retinol-CRBP (28 pmol) and unlabelled complex retinol-CRBP when appropriate for 15 min at 25°C.

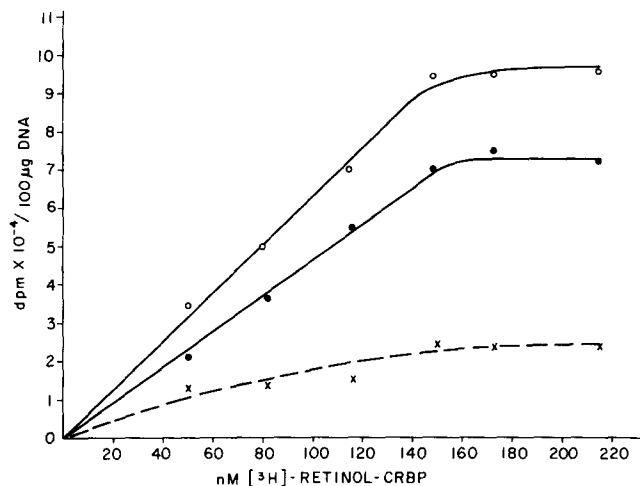


FIGURE 3 Concentration dependence of specific binding of [³H]retinol to isolated chromatin. Isolated chromatin (60 μg) was incubated with increasing concentration of [³H]retinol-CRBP in the absence (O) or in the presence (x) of 20-fold excess of retinol-CRBP. (●) specific binding.

methyl-CRBP was charged with [³H]retinol, we observed specific binding of retinol equivalent to that seen previously using [³H]retinol bound to native CRBP. This demonstrated that the specific binding of retinol involves the transfer of retinol from CRBP to binding sites on the chromatin and that the binding protein does not remain associated with the binding sites.

Nuclei isolated from vitamin A-deficient liver can bind more retinol than nuclei from normal liver. However, as shown in Table I, this difference in binding is no longer apparent in isolated chromatin. When presented as a complex, the percentage of [³H]retinol which can bind to isolated chromatin depends on the amount of the complex, the amount of chromatin, and their relative abundance. Up to 18% of the retinol

in the presented complex can be bound specifically to isolated chromatin. This is comparable to the 25% bound previously demonstrated with isolated nuclei (10).

Free retinol has been shown to associate with isolated nuclei but not in a specific and saturable manner (10). However, since the CRBP-mediated binding is ultimately of retinol alone, it was of interest to explore whether the specific binding sites on isolated chromatin would be accessible to free retinol. When free [³H]retinol was incubated with isolated chromatin, a large amount of radioactivity remained with the pellet after three washes. However, the presence of up to a 1,000-fold excess of unlabelled retinol did not reduce the amount of [³H]retinol bound. The data, summarized in Fig. 4, indicate that one cannot demonstrate saturable binding sites on chromatin when retinol is provided as the free ligand not complexed with CRBP. Additionally, free retinol (up to 1,000-fold excess) did not reduce the CRBP-mediated specific binding of [³H]retinol. This is consistent within the essential role of CRBP in the specific binding of retinol to chromatin.

Autoradiographic Demonstration of the Binding of Retinol to the Nucleus

When nuclei were incubated with [³H]retinol-CRBP or with [³H]retinol and sections were examined by autoradiography, two distinctive patterns emerged. As can be seen in Fig. 5 a, in nuclei previously incubated with [³H]retinol-CRBP the silver grains were distributed evenly throughout the sectioned nuclei. Nuclei incubated with [³H]retinol-CRBP in the presence of a 20-fold excess of unlabelled complex (Fig. 5 b) contained few grains, and these were confined mostly to the nuclear periphery. When nuclei were incubated with free [³H]retinol (Fig. 5 c), the grains appeared to be almost exclusively on the periphery of the nucleus. Since free retinol is hydrophobic, this might indicate a preferential association with the nuclear membrane. Also, this indicated that the internal localization of [³H]retinol when it was presented to nuclei bound to CRBP was not due to redistribution of retinol during the processing of nuclei for

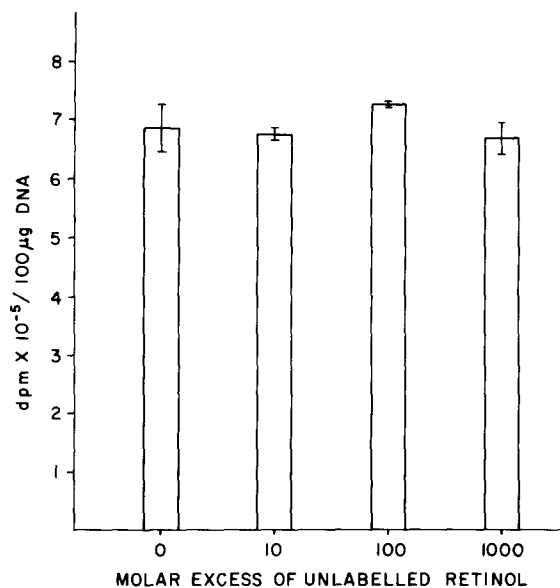


FIGURE 4 Binding of free [³H]retinol to chromatin. Chromatin (60 μg) was incubated with [³H]retinol (30 pmol) and unlabelled retinol when appropriate for 15 min at 25°C. Bar represents SD of two measurements.

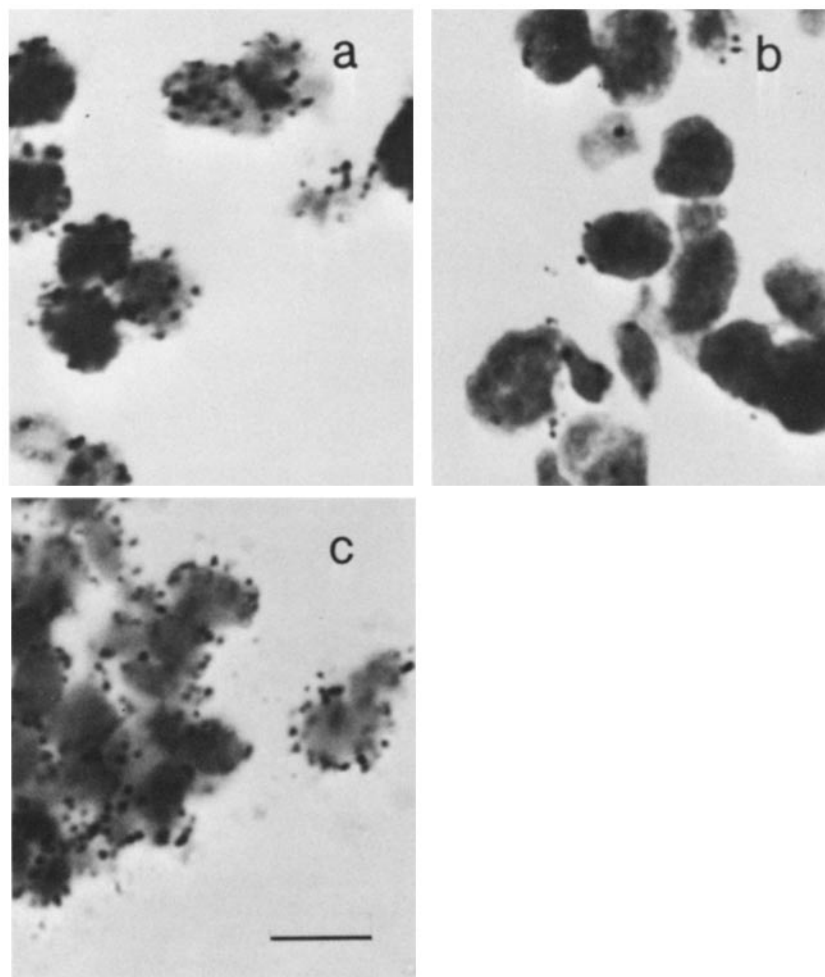


FIGURE 5 Autoradiographs of isolated nuclei previously incubated with [^3H]retinol-CRBP (a), [^3H]retinol-CRBP plus 20-fold excess of unlabeled retinol-CRBP (b), and [^3H]retinol (c), each for 15 min at 25°C. Nuclei were then chilled and washed three times with buffer before being subjected to autoradiography as described. Bar, 10 μm . $\times 1,400$.

autoradiography. Thus most, if not all, of the specific binding sites for retinol in nuclei are located internally. This is consistent with these sites being on the chromatin.

That retinol, complexed to CRBP, binds in a manner distinct from the nonspecific association of free retinol is further suggested by the observation that, when nuclei were incubated with equal molar amounts of free [^3H]retinol-CRBP or free [^3H]retinol, it was found that the washed nuclei that had been incubated with [^3H]retinol contained approximately five times more radioactivity. However, when these nuclei were subjected to autoradiography, significantly less silver grains were observed, indicating that [^3H]retinol, when presented free to nuclei, was lost much more readily during processing for autoradiography. Thus retinol, when presented as the complex, binds less to apparently different sites than when presented free, and this retinol is not removed to the same degree during the autoradiographic processing.

DISCUSSION

The experiments described here were designed to gain insight concerning the still unknown molecular mechanisms by which retinol exerts its effects on the differentiation of epithelia. Alterations in genomic expression appear to be induced in animals fed a retinol-deficient diet, as shown by changes in

nuclear RNA synthesis observed *in vivo* (27–30) as well as *in vitro* (13). A working hypothesis has been used that retinol, being a small molecule, might exert its action in a way similar to the accepted model for the mode of action of steroid hormones in differentiation. This model involves binding of the steroid hormone inside the target cell to a specific binding protein called a receptor. The resulting cytoplasmic ligand-receptor complex, after undergoing a not fully understood conformational change, translocates to the nucleus. The receptor protein can then be detected in nuclear extracts by its ability to bind specifically the steroid hormone. The receptor-steroid complex has been shown to interact with chromatin. Such interaction is believed to lead to an altered expression of the genome, which is the basis for the steroid hormone-induced differentiation (31).

The steroid hormone model has been used profitably to investigate the mode of retinol action. Indeed, a specific binding protein for retinol, CRBP, was discovered to be present in many tissues (3). Moreover, after purifying this protein to homogeneity, it was demonstrated that CRBP is able to deliver retinol to the nucleus in a specific manner (10).

However, we report here a unique feature which appears to be distinct from the steroid hormone model. Using retinol-CRBP complex in which the radioactive label is on the protein, we find that CRBP delivers retinol in a specific manner to the nucleus; the retinol associates with chromatin, but the protein

itself does not remain bound. This conclusion is based on the observation that the radioactively-labeled protein is still able to deliver retinol inside the nucleus, but it cannot be recovered with the nucleus, in contrast to steroid hormone receptors.

The interaction of the specifically delivered retinol appears to be primarily with chromatin. The outer nuclear envelope is apparently not significantly involved in the interaction as Triton X-100-treated nuclei retain 70% of the retinol binding sites found in intact nuclei. It is still possible that the isolated chromatin and the Triton-treated nuclei contain some of the nuclear matrix and that it is actually the matrix which contains the specific binding sites for retinol. However, preliminary evidence indicates that the specific binding sites remain with a soluble chromatin preparation prepared by mild nuclease digest of nuclei rather than with the nuclear matrix. That the CRBP is necessary for delivering retinol to the nucleus is clearly documented by autoradiography. Free retinol, not bound to CRBP, binds nonspecifically to the nuclei, and to chromatin, and autoradiography shows indiscriminate localization of retinol in the lipid-rich nuclear membrane areas.

The data presented here invite the proposal that the retinol-CRBP complex enters the nucleus in some manner which is apparently not dependent on the nuclear membrane. The complex then recognizes a limited number (generally an order of magnitude greater than for steroid hormones) of specific sites on the chromatin where the transfer of retinol from CRBP to these sites takes place. The sites were not detectable and may not be accessible if the retinol is free from CRBP. After the transfer CRBP does not remain associated with the specific sites. The functional significance of the specific interaction between retinol and chromatin remains to be demonstrated.

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