Analyses of the Interactions between Retinoid-binding Proteins and Embryonal Carcinoma Cells

Uriel Barkai and Michael I. Sherman
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Abstract. [3H]Retinoic acid (RA) and [3H]retinol bind in an unsaturable manner to isolated nuclei from Nulli-SCC1 and PCC4.azaIR embryonal carcinoma (EC) cells. When nuclei are challenged with the same labeled retinoids on their respective binding proteins (CRABP and CRBP), much less binding is observed and the binding is saturable. RA-CRABP does not compete with [3H]retinol-CRBP for binding to specific Nulli-SCC1 nuclear sites, whereas retinol-CRBP (but not apo-CRBP) actually potentiates the binding of [3H]RA-CRABP to these nuclei. The binding of [3H]RA-CRABP and [3H]retinol-CRBP is not dramatically affected by prior removal of the outer nuclear membrane with Triton X-100. However, treatment with the detergent after the binding reaction is complete removes about half of the bound [3H]RA-CRABP and almost all of the bound [3H]retinol-CRBP.

We measured specific retinoid-binding activities in nucleoplasmic extracts of Nulli-SCC1 and PCC4.azaIR cells. The only readily detectable specific binding activity in nucleoplasmic extracts from untreated cells was for [3H]retinol in PCC4.azaIR preparations. Nucleoplasmic extracts from Nulli-SCC1 and PCC4.azaIR cells pretreated with RA had considerable levels of specific [3H]RA-binding activity with little or no increase in [3H]retinol binding. By contrast, similar extracts from Nulli-SCC1 cells treated with retinol bound large amounts of both [3H]retinol and [3H]RA. Under the same conditions, PCC4.azaIR extracts also contained [3H]RA-binding activity with no increase in [3H]retinol binding above the high endogenous levels.

Although these results might reflect translocation of binding proteins from cytoplasm to nucleus, other interpretations must be considered since we often observed an increase, rather than the expected reduction, in cytoplasmic retinoid-binding protein levels.

Several studies support the view that retinoic acid (RA) promotes differentiation of embryonal carcinoma (EC) cells is mediated by cellular RA-binding protein (CRABP) (Jetten and Jetten, 1979; Sherman et al., 1983a; Schindler et al., 1981; McCabe et al., 1983, 1984a, b; Wang and Gudas, 1984). Evidence has been presented from studies with [3H]RA that the RA-CRABP holoprotein interacts specifically with nuclei in vivo (Wiggert et al., 1977; Jetten and Jetten, 1979) and Bok et al. (1984) have detected CRABP with specific antiserum in nuclei of freshly dissected retinal cells. Mehta et al. (1982), Cope et al. (1984a), and Takase et al. (1986) have described the interaction in vitro between CRABP holoprotein and nuclei from mammary carcinoma and testicular cells. In an abstract McCormick et al. (1984) stated that nuclei from F9 EC cells could also bind [3H]RA-CRABP. In this study, we have undertaken further characterization of the interaction between RA-CRABP and EC cell nuclei. Because RA can promote differentiation of HL-60 promyelocytic leukemia cells even though these cells have little or no CRABP (Douer and Koelffer, 1982), and because Chou et al. (1983) have claimed that RA interacts specifically with HL-60 nuclear components, we have also examined the interaction of free RA with EC nuclei.

Retinol promotes differentiation of some EC cells but it is substantially less potent than RA (Eglicht and Sherman, 1983; Jetten and De Luca, 1983). In addition to CRABP, EC cells possess CRBP, a binding protein for retinol (Matthaei et al., 1983). Chytil and his colleagues (Takase et al., 1979; Liau et al., 1981) and Cope et al. (1984a) have thoroughly described the interaction between retinol-CRBP and nuclei from rat testis and liver. Liau et al. (1981, 1985) have provided evidence that the retinol-CRBP complex interacts specifically with chromatin components and that at some point the complex dissociates, leaving the ligand, but not the apoprotein, tightly adherent to some nuclear site(s). Since both RA and retinol might, therefore, be translocated to nuclei via their binding proteins, we have compared the interaction between RA-CRABP and retinol-CRBP with EC...
nuclei as a first step in determining whether differences in binding characteristics could explain the marked margin of potency between retinol and RA as inducers of differentiation.

**Materials and Methods**

**Cell Cultures**

Nulli-SCC1 and PCC4.azalR are EC cell lines that differentiate readily in response to RA (Jetten et al., 1979). Nulli-SCC1 cells differentiate to a modest extent in response to retinol, whereas this retinoid fails to induce differentiation of PCC4.azalR cells and is toxic to the cells during treatment for more than a few days at concentrations at or above 8 × 10^(-6) M (Eglicht and Sherman, 1983). Cells were maintained in DME supplemented with 10% heat-inactivated FCS, extra glucose and glutamine, and antibiotics; Nulli-SCC1 cells were cultured on gelatin-coated substrata (see Jetten et al., 1979 for further details of the characteristics and culture conditions of these cells). To obtain nuclei for in vitro retinoid-binding protein assays, cells were cultured in 600-cm^2 Nunclon plates (Gibco, Grand Island, NY) and harvested when the cell density reached 5 × 10^5 to 1 × 10^6 per plate. To evaluate the effects of retinoids on nuclear retinoid-binding activity, cells were cultured for at least 5 d in medium containing 10% FCS depleted of diisopropyl ether (Cham and Knowles, 1976). We have previously determined that this procedure removes at least 95% of added labeled retinol or RA (Sherman et al., 1983a). Retinoids were added to the culture medium at 2 × 10^(-6) M in 0.1% ethanol 2 h before harvesting the cells. Control cultures were treated with ethanol alone.

**Preparation of Nuclei**

Nuclei were isolated by the method of Liau et al. (1981). Briefly, cells were washed with PBS, trypsinized, collected by low speed centrifugation, and washed once more with cold buffered saline. All subsequent procedures were carried out at 4°C and in the presence of 0.25 μM phenylmethylsulfonyl fluoride to minimize protein degradation. Cells were homogenized in 0.87 M sucrose in TKDM (50 mM HEPES, pH 7.5, 25 mM KCl, 0.5 mM DTT, and 4 mM MnCl_2) and centrifuged at 12,000 g for 30 min. The supernatants were centrifuged at 105,000 g for 90 min and extensively dialyzed against TPN25 (25 mM Tris HCl pH 7.5, 25 mM Na_2HPO_4, 25 mM NaCl, 300 mM sucrose). For in vitro studies, the resultant nuclear pellet was resuspended by gentle homogenization in 0.87 M sucrose in TKDM, and centrifuged at 95,000 g for 75 min in a swinging bucket rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA) between a pad of 2 M sucrose and an overlay of 0.3 M sucrose, both in TKDM buffer. The pellet was resuspended in 1.8 M sucrose in TKDM, centrifuged at 16,000 g for 30 min, and dialyzed with 0.3 M sucrose in TKDM. For in vitro studies, the resultant purified nuclei were resuspended in 0.3 M sucrose in TKDM and used immediately. For determination of intrinsic retinoid-binding activity, the nuclei were centrifuged at 800 g for 10 min, resuspended in TPN25, and frozen. After thawing, the extracts were sonicated for 3 min and centrifuged at 14,700 g for 90 min (model SW50 rotor; Beckman Instruments, Inc.) to generate nucleosolic extracts. The purity of isolated nuclei was confirmed by light and electron microscopy.

**Preparation of Retinoid-binding Proteins**

Tumor tissue generated in mice by the injection of PCC4.azalR EC cells was prepared as described by Ong and Chytil (1981). Pooled tumor tissue (250–300 g) was homogenized in 2 vol of 50 mM Hepes buffer, pH 7.5. The homogenate was cleared by centrifugation for 30 min at 30,000 g. The supernate was brought to pH 5.0 by dropwise addition of glacial acetic acid, centrifuged, and the resultant supernate was readjusted to pH 7.5 with 1 N NaOH. A 40–75% ammonium sulphate cut was obtained and dialyzed against 50 mM Tris, pH 7.5, plus 200 mM NaCl. 10 μCi each of [3H]RA and [3H]retinol (both at 20–30 Ci/mmol) were added and the preparation was kept overnight at 4°C. The labeled material was then applied to a Sephadex G-75 column (12 × 75 cm) and eluted with the same buffer. The two retinoid-binding proteins coeluted between 880 and 1,020 ml. After regeneration of apoproteins by exposure to fluorescent light (5 h at 4°C), the crude binding protein preparation was dialyzed against 25 mM Tris, pH 7.5, plus 25 mM NaCl, labeled again with 5 μ Ci each of [3H]retinol and [3H]RA, and applied to a Bio-Gel HTP hydroxylapatite column (2.5 × 40 cm). The column was eluted with a gradient of 0-120 mM sodium phosphate in the same buffer. This chromatographic procedure successfully separated the two binding proteins, as could be demonstrated by light inactivating the bound retinoids and, after dialysis, recharging with either [3H]retinol or [3H]RA. CRBP activity was eluted at 70 mM phosphate whereas CRABP activity was recovered at 90 mM phosphate. As demonstrated in Table I, no cross-binding of labeled retinoids was observed. The CRBP and CRABP preparations were dialyzed against TPN25, concentrated to ~1 mg/ml, and stored at ~70°C. When the binding protein preparations were analyzed by SDS PAGE, both contained prominent peaks at ~45 Kd as expected. Unidentified bands were observed at ~20 and 30 Kd (not shown); the latter might represent dimerized binding protein. Subsequent purification steps were not implemented because the partially purified preparations showed complete discrimination of ligand binding and because attempts to further purify the proteins (e.g., by HPLC) resulted in preparations that had little or no capacity for holoprotein regeneration after ligand removal by exposure to light. For final charging, the binding proteins (1.5 mg) were incubated in 9 ml of TPN25 in the presence of 200 μCi of the appropriate [3H]retinoid or 0.1 μmol unlabeled retinoid. After incubation overnight at 4°C, 1 ml of dextran-coated charcoal (20 mg charcoal and 2 mg dextran-F70 per ml TPN25) was added, and the solutions were mixed and centrifuged at 1,500 g for 20 min. The supernatants were dialyzed in the cold against TPN25. The resultant specific activities of the labeled holoproteins were in the range of 1.5–3.0 × 10^5 dpm/mg protein.

**Association of [3H]Retinoid-binding Proteins with Nuclei**

Freshly isolated nuclei were incubated at 20°C for 2 h in an assay mixture (1.25 ml) containing TPN25 plus 250 mM sucrose and 0.5–1 × 10^6 dpm of [3H]retinoid holoprotein-binding protein. Unlabeled retinoid holoprotein-binding proteins were added at the indicated molar ratios. At the end of the incubation period, nuclei complexed with [3H]retinol-CRBP were trapped on GVWP hydrophilic filters (0.22 μm; Millipore/Continental Water Systems, Bedford, MA) and washed twice with 10 ml cold PBS. Nuclei complexed with [3H]RA-CRABP were collected on cellulose acetate filters (Metricel membrane, GA-G; Gelman Services, Inc., Ann Arbor, Ml) and similarly washed. These conditions were found to produce the lowest background values (<1% for [3H]retinol-CRBP and <5% for [3H]RA-CRABP). The filters were air dried and counted.

**Binding Protein Measurements**

Retinoid-binding protein contents were measured by a procedure adapted from a combination of a previously published Sephadex assay (Sherman et al., 1983a) and a method used for measuring progesterone receptor (Barkai et al., 1981). Samples containing 50–200 μg of protein in TPN25 buffer were pipetted onto the disk from a Tetrulate kit (Arnes-Tissum Ltd., Jerusalem, Israel) that was placed on top of a 1 × 2 cm Sephadex G-25 column. For CRBP measurements [3H]RA (3–4 × 10^4 cpmm; 20–30 Ci/mmole), in the absence or presence of a 100-fold molar excess of unlabeled RA, was added in 10 μl ethanol. The reaction, in a total volume of 300 μl, was carried out for 90 min at 4°C. Column outlets were then opened, the void volume drained, and the bound fraction eluted with 1 ml of TPN25 directly into minitabs. CRBP was assayed in the same way except that the labeled retinoid was [3H]retinol and 0.3 M sucrose was included in the assay mixture.

| Table I. Retinoid–binding Specificity of Partially Purified CRABP and CRBP Preparations* |
|-----------------|-----------------|-----------------|
| Binding protein | Specific [3H]RA binding | Specific [3H]retinol binding |
| CRABP | 43,240 ± 1,110 | 240 ± 140 |
| CRBP | 900 ± 900 | 46,110 ± 1,130 |

* Binding-protein preparations were generated as described in Materials and Methods. After separation by Bio-Gel HTP hydroxylapatite chromatography, 25 μg protein from each preparation was challenged with the indicated [3H]retinoid without and with a 100-fold molar excess of the homologous unlabeled retinoid. Assay conditions and procedures for measuring specific binding-protein activities are provided in Materials and Methods. Data are averaged from four independent measurements and are expressed as cpm ± SEM.
to reduce background. Each sample condition was measured in triplicate pairs (i.e., with and without unlabeled retinoid). Sephadex columns were regenerated by washing sequentially with 0.1 N HCl, distilled water, 0.1 N NaOH, distilled water, and TPN25.

This assay procedure was reproducible and sensitive. Variation among triplicates was on the order of 10% and day-to-day reproducibility was excellent. Incubation on the disks instead of in tubes did not result in increased amounts of [3H]retinoid binding but led to accelerated reaction rates. The use of fewer counts per minute of labeled retinoid (about one-fifth that in previous studies; e.g., Sherman et al., 1983a) reduced background, thus obviating the need for dextran-charcoal treatment that can lead to displacement of labeled retinoid from its binding protein (unpublished results). As a result, we consistently obtained binding protein specific activities 5-10-fold greater than those observed previously (e.g., Matthaei et al., 1983).

To measure retinoid-binding activities in nuclei and cytosols of cells pretreated with retinoids, extracts were maintained at 4°C under fluorescent light for 7 h. In previous studies with both crude and purified retinoid-binding proteins, we determined that these conditions were sufficient to remove all previously bound [3H]retinol or [3H]RA from their respective binding protein without detectable loss of binding activity when the samples were rechallenged with appropriate [3H]retinoid. Specific retinoid-binding activities were then determined as described above.

Results

Characterization of the Association of Retinoid-binding Proteins with Nuclei

Binding of [3H]RA-CRABP to PCC4.azalR and Nulli-SCCI nuclei was maximal after 30 min at 20°C and was stable for at least 2 h. Specific binding of [3H]retinol-CRBP to the same nuclei appeared to be slower, reaching a maximum by 2 h. Specific binding was linear in the range of 1-3 × 10^6 nuclei per assay. In subsequent studies we used 2 × 10^6 nuclei per assay and an incubation period of 2 h at room temperature.

As reported previously by us (Sherman et al., 1985) and by others (Takase et al., 1979; Cope et al., 1984a), when isolated nuclei were incubated with [3H]RA or [3H]retinol, binding was unsaturable. Although binding was apparently nonspecific, nuclei bound more RA than retinol at similar concentrations of retinoid (Fig. 1 A). When the same nuclear preparations were incubated with radioactive [3H]RA-CRABP or [3H]retinol-CRBP, trapped on filters, and extensively washed, less retinoid was associated with the nuclei (inset, Fig. 1 B). Furthermore, under these conditions, binding reached a plateau and there was more retinol than RA bound. The amount of radioactive retinoid bound could be reduced by including cold homologous holoprotein in the incubation mixture (Fig. 1 B). Similar results were obtained when PCC4.azalR nuclei were used as targets for both binding proteins (not shown). We calculate from our data that the total numbers of specific binding sites in or on Nulli-SCCI nuclei for RA-CRABP and retinol-CRBP were 1.7 ± 0.54 × 10^5 and 2.4 ± 0.71 × 10^5, respectively. The corresponding numbers for holo-binding protein association with PCC4.azalR nuclei were calculated to be 2.8 ± 0.91 × 10^5 and 5.7 ± 1.17 × 10^5, respectively.

Effects of Free Ligand on Retinoid Holo-binding Protein Association with Nuclei

Takase et al. (1979) have reported that free ligand failed to compete with [3H]retinol-CRBP for specific sites in liver nuclei, though data were not presented. We have extended these experiments with EC nuclei to include both retinoids and their binding proteins. The results are presented in Fig. 2. The data illustrate that the heterologous ligand had no effect upon holo-binding protein interaction with Nulli-SCCI nuclei. The homologous ligand did not reduce [3H]retinoid interaction with nuclei, indicating that free ligand does not compete for specific holo-binding protein binding sites. To the contrary, large excesses of the homologous ligands actually augmented the association of label with nuclei. The significance of this observation will be discussed below (see Discussion).

Effects of Heterologous Retinoid Holo-binding Protein on Association with Nuclei

To determine whether CRBP and CRABP binding sites on EC nuclei are shared or unique, we carried out competitive binding studies as described in Fig. 1 B except that the unlabeled retinoid holo-binding protein was heterologous. Fig. 3 illustrates that the binding of [3H]retinol-CRBP to Nulli-
CRBP markedly reduced [3H]retinol-CRBP association with labeled RA-CRABP. Since equal amounts of unlabeled retinol-binding proteins with Nulli-SCC1 nuclei. Binding experiments not effectively competed for retinol-CRBP sites on Nulli-SCC1 nuclei. Reciprocal experiments illustrate that retinol plus RA;

Values with heterologous ligand are averages of triplicate determinations from triplicate determinations in four independent experiments. Values with homologous ligand are shown as the mean + SEM from triplicate determinations in two independent experiments. The extent of increase in label associated with nuclei varied from three- to ninefold. Similar potentiation was observed with nuclei from PCC4.azalR but not by the apoprotein. As mentioned above and demonstrated in Fig. 2, free retinol failed to influence the interaction of [3H]RA-CRABP with nuclei; neither was there any CRBP not only failed to reduce [3H]RA-CRABP binding to nuclei but actually potentiated the interaction (Fig. 3). Qualitatively, this potentiation was reproducibly observed in several independent experiments. The extent of increase in label associated with nuclei varied from three- to ninefold. Similar potentiation was observed with nuclei from PCC4.azalR cells (not shown). The data in Fig. 4 illustrate that the potentiation could be elicited by the retinol holo-binding protein, but not by the apoprotein. As mentioned above and demonstrated in Fig. 2, free retinol failed to influence the interaction of [3H]RA-CRABP with nuclei; neither was there any

SCC1 nuclei was unaffected by a large molar excess of unlabeled RA-CRABP. Since equal amounts of unlabeled retinol-CRBP markedly reduced [3H]retinol-CRBP association with nuclei (Fig. 1 B), it can be concluded that RA-CRABP cannot effectively compete for retinol-CRBP sites on Nulli-SCC1 nuclei. Reciprocal experiments illustrate that retinol-

Figure 2. Effects of free retinoid on interaction of retinoid holo-binding proteins with Nulli-SCC1 nuclei. Binding experiments were carried out as described in Fig. 1 except that where indicated unlabeled free retinoid was added in the indicated molar excess. Values with homologous ligand are shown as the mean ± SEM from triplicate determinations in four independent experiments. Values with heterologous ligand are averages of triplicate determinations from a single experiment. (Open circles) [3H]RA-CRABP plus RA; (open squares) [3H]RA-CRABP plus retinol; (solid circles) [3H]retinol-CRBP plus retinol; (solid squares) [3H]retinol-CRBP plus RA.

Figure 3. Potentiation of the association of RA-CRABP with Nulli-SCC1 nuclei by retinol-CRBP. Binding studies were carried out as described in Fig. 1 b except that where indicated unlabeled holo-binding proteins were used as competitors in molar excess as indicated. (Open circles) [3H]RA-CRABP plus retinol-CRBP; (solid circles) [3H]retinol-CRBP plus RA-CRABP. Data are pooled from duplicate determinations in at least two independent experiments.

Figure 4. Comparison of effects of apo-CRBP vs. retinol-CRBP on the interaction of [3H]RA-CRABP with Nulli-SCC1 nuclei. Binding studies were carried out as described in Fig. 1 b except that the interaction of [3H]RA-CRABP with nuclei was measured in the presence of retinol-CRBP (open circles) or apo-CRBP (solid circles) at the indicated molar excess. Data are averaged from triplicate determinations.

Table II. Effects of Triton X-100 upon the Interaction of Holo-retinoid-binding Proteins with Nuclei*

<table>
<thead>
<tr>
<th>Binding protein</th>
<th>Time of Triton-X treatment</th>
<th>Counts per minute bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nulli-SCC1</td>
<td>PCC4.azalR</td>
</tr>
<tr>
<td>[3H]Retinol-CRBP</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Before binding</td>
<td>83 ± 13.2</td>
</tr>
<tr>
<td></td>
<td>After binding</td>
<td>107 ± 7.6</td>
</tr>
<tr>
<td>[3H]RA-CRABP</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Before binding</td>
<td>109 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>After binding</td>
<td>125 ± 3.8†</td>
</tr>
<tr>
<td>[3H]RA-CRABP + retinol-CRBP</td>
<td>None</td>
<td>219 ± 9.2†</td>
</tr>
<tr>
<td></td>
<td>Before binding</td>
<td>125 ± 3.8†</td>
</tr>
</tbody>
</table>

* EC nuclei (2 × 10⁶) were preincubated for 30 min at room temperature without or with 0.4% Triton X-100 in TKDM buffer plus 0.3 M sucrose. They were washed by centrifugation in the same buffer and incubated for 2 h at 25°C with 6 × 10⁶ cpm [3H]retinol-CRBP, 3 × 10⁶ cpm [3H]CRABP, or 3 × 10⁶ cpm [3H]RA-CRABP plus a fivefold molar excess of retinol-CRBP. The nuclei were then trapped on filters (see Materials and Methods) and washed either with three 10-ml vol of PBS or, as indicated, 2 vol of saline plus 0.4% Triton X-100. Values are expressed as percent of radioactivity relative to that associated with nuclei challenged with [3H]retinol or [3H]RA-CRABP without added unlabeled holoprotein or detergent (6-7 × 10⁶ cpm for Nulli EC nuclei, 10-11 × 10⁶ cpm for PCC4.azalR nuclei). Values are averaged from triplicate determinations in two independent experiments for [3H]retinol-CRBP and [3H]RA-CRABP binding to Nulli-SCC1 nuclei, and from triplicate determinations in a single experiment for [3H]RA-CRABP plus retinol-CRBP binding to Nulli-SCC1 nuclei and for binding assays with PCC4.azalR nuclei.† Values are relative to those obtained simultaneously for [3H]RA-CRABP binding to nuclei in the absence of detergent treatment or retinol-CRBP.
Effect when retinol was added complexed to BSA or ovalbumin (not shown).

**Effects of Triton X-100 on Retinoid Holo-binding Protein Interaction with Nuclei**

Treatment of isolated nuclei with 0.25–0.5% Triton X-100 removes the outer nuclear membrane and a substantial proportion of nuclear phospholipids (e.g., Knowles et al., 1973; Liau et al., 1981). Liau et al. (1981) have demonstrated that such treatment does not dramatically reduce retinol-CRBP interaction with liver nuclei. We studied the effects of Triton X-100 on the association of [3H]retinol-CRBP and [3H]RA-CRABP with Nulli-SCC1 and PCC4.azalR nuclei. Table II illustrates that Triton X-100-treated nuclei specifically bound both [3H]RA-CRABP and [3H]retinol-CRBP at levels similar to that of untreated nuclei. On the other hand, when nuclei were first challenged with holo-binding proteins, trapped on filters, and then treated with the same concentration of detergent during washing, about half of the [3H]RA and almost all of the [3H]retinol were displaced (Table II). An initial series of studies suggested that pretreatment of nuclei with Triton X-100 prevented the potentiation of [3H]RA-CRABP by retinol-CRBP, but that once this potentiation had occurred, the stability of the extra [3H]RA-CRABP bound was as resistant to detergent treatment as basal [3H]RA-CRABP binding (Table II).

**Effects of Retinoid Treatment of Intact Cells upon Nuclear and Cytosolic Retinoid-binding Activities**

It has been shown that a 2-h exposure is adequate for movement of [3H]RA or [3H]retinol into the nuclear compartment in EC cells (Jetten and Jetten, 1979; Sherman et al., 1985). To evaluate the effects of exposing cells to retinoids upon specific retinoid-binding activities, we cultured Nulli-SCC1 or PCC4.azalR cells for 5 d or more in medium depleted of retinoids and then treated them with unlabeled retinol or RA for 2 or 5 h. We collected and fractionated the cells and tested cytosolic and nucleosolic extracts for specific retinoid-binding activities. To do this, we exposed the cellular fractions to light under conditions that strip retinoids from their binding protein sites and then incubated aliquots of the extracts with [3H]RA or [3H]retinol with or without a large molar excess of the homologous unlabeled retinoid.

As the data in Table III illustrate, nucleosolic preparations from untreated Nulli-SCC1 cells contained little if any specific RA- or retinol-binding activity. After 2-h exposure of these cells to RA, there was a large (more than 20-fold) increase in specific RA-binding capacity in the nucleosolic extracts. After the cells were treated with retinol, the specific binding of retinol by nucleosols was elevated by more than 40-fold (Table III). There was, however, a notable difference between the effects of RA and retinol pretreatment on heterogeneous specific binding activity in nucleosolic extracts: RA had only minimal effects (not statistically significant) on retinol-binding capacity, whereas nucleosols from retinol-treated cells possessed considerably increased RA-binding activity (Table III). The same qualitative patterns were observed after cells were preincubated with retinoids for 5 h except that the amount of increase in specific binding activities was in every instance lower than that observed after 2 h retinoid pretreatments (Table III). Subnuclear fractions other than the nucleosol were found to possess little or no specific retinoid-binding activity, with or without retinoid pretreatment of the intact cells (not shown).

The above experiments were repeated with PCC4.azalR cells (Table IV). RA pretreatments affected RA-binding activities in PCC4.azalR nucleosolic extracts in the same way as those in Nulli-SCC1 preparations. The specific retinol-binding ability of PCC4.azalR nucleosols was markedly different from that of Nulli-SCC1 nucleosols: the former preparations contained readily detectable, and essentially unchanged, binding capacities regardless of the retinoid content of the medium in the 2- or 5-h period before collection of the cells (Table IV). Despite the lack of effect of retinol pretreatment on retinol-binding activity in PCC4.azalR nucleosols, specific RA binding was markedly elevated in response to retinol.

In a series of experiments, we attempted to characterize this specific nucleosolic binding of [3H]RA by sucrose density gradient centrifugation. We consistently observed a heterodisperse radioactivity profile; although there was a clear labeled peak in the 2S region of the gradient, as would be expected for the [3H]RA-CRABP complex, radioactive material also cosedimented with faster moving components. There was no obvious qualitative difference in the radioactivity profiles from extracts of cells pretreated with unlabeled RA or retinol (data not shown).

**Table III. Binding Activities in Cytosolic and Nucleosolic Extracts of Retinoid-depleted Nulli-SCC1 Cells Following Exposure to Retinoids**

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Binding activity</th>
<th>Retinol</th>
<th>Retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>5 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>5 h</td>
</tr>
<tr>
<td>Nucleosol</td>
<td>Retinol</td>
<td>0.04 ± 0.02 (20)</td>
<td>1.88 ± 0.38 (11)†</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>0.07 ± 0.03 (22)</td>
<td>0.95 ± 0.20 (8)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>Retinol</td>
<td>5.8 ± 0.50 (5)</td>
<td>7.2 ± 0.50 (5)</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>8.0 ± 1.42 (3)</td>
<td>9.7 ± 0.69 (3)</td>
</tr>
</tbody>
</table>

* Nulli-SCC1 cells were cultured for 5 or more days in medium with delipidated serum and then exposed to unlabeled retinol or RA (2 × 10⁻⁶ M) for the indicated times. Cells were collected, fractionated, exposed to light, and specific retinoid-binding activities were measured (see Materials and Methods). Data are presented as the mean ± SEM in picomoles bound per milligram extract protein. Numbers in parentheses refer to the number of independent samples.  
† Values differed (P < 0.05) from those obtained with cytosolic extracts from control cells.  
‡ Values were significantly different (P < 0.001) from those obtained with nucleosolic extracts from control (no pretreatment) cells.
We also measured binding capacities in cytosolic fractions of the cells. We observed that RA and retinol pretreatment of Nulli-SCC1 cells resulted in increased cytosolic levels of their respective binding proteins. In fact cells exposed for 5 h with either retinoid possessed significantly elevated levels of both retinoid-binding protein activities (Table III). Some increases in retinoid-binding protein levels were also observed with cytosolic extracts of PCC4.aza1R cells, albeit not so consistently as with Nulli-SCC1 cells (Table IV).

**Discussion**

The experiments described here illustrate that both RA and retinol bind specifically to EC nuclei when introduced via their binding proteins. This finding and our estimates of the number of specific binding sites (1-3 × 10^5 per nucleus) are consistent with previous reports (Takase et al., 1979; Liau et al., 1981; Cope et al., 1984a; Mehta et al., 1982; McCormick et al., 1984). Since free retinoids do not show specific, saturable binding to EC nuclei (Fig. 1 A; see also Takase et al., 1979; McCormick et al., 1984; Sherman et al., 1985), it is logical to conclude that free and holoprotein-bound retinoids do not compete for the same nuclear sites. Fig. 2 illustrates that when the labeled ligand on the holoprotein and the competing free retinoid are the same, there is actually an increase in the amount of radioactivity bound to EC nuclei. A likely explanation is that the unlabeled retinoid can exchange with retinoid on its holoprotein, allowing the freed, labeled retinoid to bind nonspecifically to nuclei in an unsaturable manner. This would explain why excess RA has a substantially greater effect upon displaced [^3H]RA binding to nuclei than does unlabeled retinol upon displaced [^3H]retinol binding (Fig. 2), since free [^3H]RA has a greater affinity for nuclei than does free [^3H]retinol (Fig. 1 A).

Pretreatment of liver nuclei with Triton X-100 removed <30% of the specific retinol-CRBP binding sites, indicating that binding-protein-mediated interaction of retinol with these nuclei was unlikely to involve phospholipid or the outer nuclear membrane (Liau et al., 1981). Our data (Table II) confirm this result and extend it to [^3H]RA delivered to EC nuclei via its binding protein. The consequence of exposure of nuclei to Triton X-100 after interaction with[^3H]retinol-CRBP was strikingly different, since most of the label was solubilized. The complex between [^3H]RA-CRABP and nuclei was consistently more refractory to subsequent detergents treatment. Since the two holoproteins have similar affinities for untreated nuclei (Fig. 1 B), these results could reflect a different type of nuclear binding site for the two holoprotein-bound retinoids.

We have found that the two holoproteins fail to compete for the same nuclear sites, as the view (Takase et al., 1979; Cope et al., 1984a) that these specific binding sites are different. However, ours is the first observation that retinol-CRBP actually potentiates the binding of [^3H]RA-CRABP to nuclei. It is unlikely that the phenomenon we have observed has a trivial explanation, e.g., contamination of the retinol preparation with RA, since only holo-CRBP, not free retinol or apo-CRBP, elicits the potentiation. The elimination of the potentiation of [^3H]RA-CRABP binding by pretreatment of EC nuclei with Triton X-100 (Table II) suggests that these binding sites are different from the “basal” [^3H]RA-CRBP nuclear binding sites observed in the absence of retinol-CRBP.

The interactions between retinoid-binding proteins and nuclei in vitro, including the potentiation, are paralleled by our findings with intact Nulli-SCC1 EC cells: when cells are pretreated with RA the nucleosols possess specific binding activity, but only for that retinoid; however, when the cells are preincubated with retinol, binding activities for both retinol and RA are readily detected. It must be noted, however, that neither in this study nor in previous ones (Wiggert et al., 1977; Jetten and Jetten, 1979) could ultimately be helpful in characterization of the specific retinoid-binding activities in cytosolic and nucleosolic extracts are the same. Indeed, Chou et al. (1983) and Cope et al. (1984b) have reported the presence of nuclear RA-binding proteins whose properties appear to be different from that of the well-characterized CRABP. Such proteins might also be present in EC cells and increase in response to retinoid exposure. Sucrose density gradient analyses failed to clarify this issue since the profiles obtained were heterogeneous. Antiseras specific for CRBP and CRABP (e.g., Adachi et al., 1981; Ong et al., 1982; Eriksson et al., 1984) could ultimately be helpful in characterization of the nuclear retinoid-binding activities.

Translocation of binding protein molecules from cytoplasm to nucleus in response to the uptake of retinoids by the cell should be characterized not only by detection of activity in nuclear extracts but also by diminution of cytoplasmic activity. In fact, retinoid treatments commonly resulted in modest, but statistically significant, increases in cytosolic...
binding protein activities (Tables III and IV). From the data in Tables III and IV, we calculate that EC cells possess 2-4 × 10^5 molecules each of CRBP and CRABP (similar to that reported for CRBP in liver cells by Takase et al. [1979]), and that under optimal conditions only ~10^4 specific binding sites for [3H]retinol or [3H]RA appear in the nucleosol from each cell (in several attempts we found specific retinoid binding activity only in this nuclear fraction). The loss of such a small proportion of cytoplasmic binding protein molecules in response to retinoid uptake could have escaped detection, especially if retinoid treatments lead to increased total levels of binding proteins.

The presence of constitutive retinol-binding activity in nucleosols from PCC4.azaIR cells was unexpected and is unexplained. Unlike other EC lines, PCC4.azaIR cells are killed by exposure to relatively low levels of retinol (Jetten et al., 1979; Eglitis and Sherman, 1983); we are attempting to determine whether these two observations are related. Although preincubation of PCC4.azaIR cells with retinol has no effect on nucleosolic RA-binding activity, it leads to the appearance of nucleosolic RA-binding activity, as is seen with Nulli-SCC1 nucleosols. Thus the two effects of retinol preincubation appear to be separable.

When incubated for 2 h with 10^{-9} M [3H]RA or [3H]retinol, EC cells take up ~2 × 10^7 and 8 × 10^7 retinoid molecules, respectively, and 1-2% of the label (equivalent to ~10^4-10^5 retinoid molecules) is detected in purified EC nuclei after treatment with Triton X-100 (Sherman et al., 1983b, 1985, and unpublished observations). It is therefore notable that if our in vivo studies (Tables III and IV) are detecting translocation of holo-binding proteins from cytoplasm to nucleus, many fewer binding protein molecules (~10^4) than ligands are associated with nuclei at any one time. A reasonable explanation for this is the proposal by Liu et al. (1981) and Takase et al. (1986) that the retinoid holo-binding proteins shuttle their ligands to appropriate nuclear sites and then the apoproteins return to the cytoplasm. However, our data do not eliminate the possibility that retinoids not bound to their binding proteins can also reach the nucleus in vivo.

The indication that retinol-CRBP and RA-CRABP have independent nuclear binding sites could explain why RA is considerably more potent than retinol in inducing EC cell differentiation. We find it unlikely that retinol can be converted to RA in amounts adequate to explain the potency of the former retinoid (Eglitis and Sherman, 1983; Gubler and Sherman, 1983) as has been proposed by Williams and Napoli (1985). However, the observation that retinol-CRBP can potentiate interaction of RA-CRABP with EC nuclei and that incubation of cells with retinol generates specific nucleosolic binding sites for RA suggests that in the presence of retinol very small amounts of RA can become disproportionately effective.

We wish to thank Dr. A. Liebman and his colleagues (Hoffmann-La Roche, Inc.) for providing us with [3H]labeled retinoids, and Drs. M. Harper, P. Abzurria, G. Bryce, and S. Shapiro for comments on the manuscript.

Received for publication 28 February 1986, and in revised form 4 November 1986.

Note Added in Proof. After this manuscript was submitted, Takase et al. (Takase, S., D. E. Ong, and F. Chytil, 1986, Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus, Arch. Biochem. Biophys. 247:328-334) reported that RA-CRABP bound specifically to rat testis nuclei. Contrary to our observations with EC nuclei, they stated that retinol-CRBP had no effect on the amount of [3H]RA bound to the testis nuclei when added as part of the CRABP holoprotein complex. The potentiation that we observed might, therefore, be cell-type specific.

References


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