Rat testes interstitial cell nuclei exhibit three distinct receptors for retinoic acid[1]

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Summary. Purified nuclei from rat testes interstitial cells were incubated with an equimolar complex of [^{3}H]retinoic acid and purified cellular retinoic acid-binding protein (cRABP) and with ATP. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and radiofluorographic analysis of the nuclear fractions indicated the presence of 3 highly labeled receptors for retinoic acid which were distinct from cRABP. These data demonstrate that retinoic acid binds to 3 novel nuclear acceptors of which cRABP does not appear to be a part.

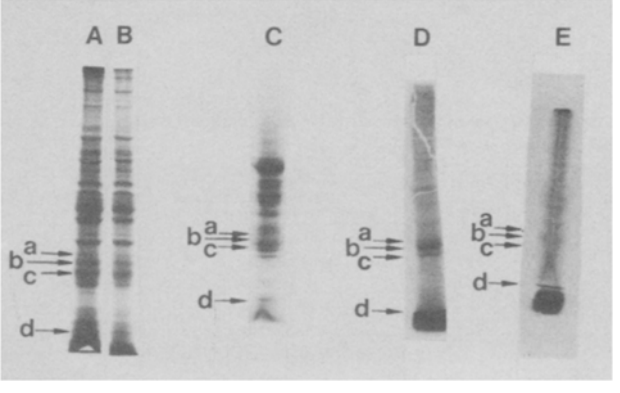
Vitamin A compounds (retinoids) are essential for the maintenance of terminal differentiation in epithelial cells[3]. Two major roles for retinoids have evolved regarding this function. First, it has been demonstrated that retinoids are major components in glycosylation and post-translational processing of proteins[4,5]. Second, it has been reported that retinol specifically binds to rat liver nuclei only in the presence of its cytosolic receptor, cellular retinol-binding protein (cRBP)[6] and that retinol is transferred from cRBP to an uncharacterized chromatin-associated acceptor[7]. Mancino et al.[8] have demonstrated that the acceptors in the HeLa cell nucleus are composed of 2 binding components for retinol, one being distinct from cRBP. Thus, we questioned whether retinoic acid might also be transferred from its cellular receptor, cellular retinoic acid-binding protein (cRABP), to a nuclear acceptor(s) in testes cells which normally express both cRBP and cRABP. This investigation deals with the binding of retinoic acid to purified rat testes interstitial cell nuclear components in the presence of exogenous cRABP.

Material and methods. Male, weanling Sprague-Dawley rats (Holtzman) were made retinol-deficient and sacrificed at their weight-gain plateau. Retinol deficiency was characterized by a serum retinol of < 2 µg/dl, and hepatic retinol of < 40 ng/g. These rats also exhibited a $> 50\%$ incidence of nasolacrimal squamous metaplasia and $> 90\%$ incidence of elevated cerebrospinal fluid pressure ($\gtrsim 95$ mm saline; normal = 65 mm saline), further documenting their deficient status. Testes were removed and placed in ice-cold Hank's balanced salt solution (HBSS). The testes were then decapsulated and interstitial cells prepared according to the nonenzymatic method of Steinberger[9]. The cell suspension was centrifuged at $800 \times g$; the resulting pellet was resuspended in HBSS and flagellated cells were removed ($< 0.5\%$) according to the method of Salhanick and Terner[10]. Microsomes and Triton X100-washed nuclei were prepared from nonflagellated cells. These cell fractions were characterized enzymatically as previously described and by transmission electron microscopy[11-13].

cRABP was purified > 5000-fold from a $100{,}000 \times g$ testes supernatant. Holo-cRABP containing the [^{3}H]retinoic acid ligand was prepared as an equimolar complex as previously described[6]. The final [^{3}H]retinoic acid-cRABP complex ([^{3}H]RA-cRABP) had a specific activity of 1.86 Ci/mmol.

Nuclei (1×10^7) were incubated at 37 °C for 35 min with [^{3}H]RA-cRABP (150–180 k DPM) in 50 mM Tris-HCl buffer, pH 7.8 containing 25 mM KCl, 0.1 mM dithiothreitol, 5 mM Mn as $MnCl_2$, 7 mM NaF, 25 mM GDP-mannose, 50 µM NADP, 3 mM ATP, 5 mM AMP, 5 µM K_3PO_4, and 0.01% phenylmethylsulfonyl fluoride; the final incubation volume was 0.3 ml. After incubation, the nuclei were sedimented at $60{,}000 \times g$ for 20 min and solubilized in electrophoresis sample buffer[14]. The nuclei were subjected to one-dimensional sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) electrophoresis and stained as previously described[14]. The resulting gels were soaked in Enhance (New England Nuclear), dried, and exposed to preflashed Kodak X-Omat AR film for 24 days at −70 °C. Incubations containing a 350-fold excess of unlabeled retinoic acid-cRABP were processed in parallel while aliquots of the interstitial cell microsomal fraction were also subjected to SDS-PAG electrophoresis.

Results and discussion. Figure A indicates the Coomassie blue-binding components of the rat testes interstitial cell nuclei. At least 41 peptides are indicated as constituents of the Triton-washed membrane-free nuclei on this one dimensional pattern. Figure D, a radiofluorograph of A, demonstrates that nuclear components a-c (designated nA-a; nA-b and nA-c) preferentially bind [^{3}H]retinoic acid (RA). Apparent binding of [^{3}H]RA is greatest in component $b > c > a$. Other minor bands also bind RA. However, the specificity of binding in the nA components is demonstrated by the inhibition of detectable radiofluorescence in the presence of 350-fold unlabeled retinoic acid-cRABP (fig. E). Assuming that the Coomassie blue-binding sensitivity of nA a–c reflects the amount of protein in those bands and further, that they represent single peptides, it is apparent that the specific binding activity for [^{3}H]RA is highest in $b > a > c$.



A Coomassie blue R-250 staining pattern of rat testes interstitial cell nuclear proteins subjected to electrophoresis on a 7.85% SDS-polyacrylamide gel in the presence of [^{3}H]retinoic acid-cRABP according to the method of Dewald et al.[14]. Molecular weight (mol.wt) standards (RNase, 13 kd; chymotrypsinogen A, 25 kd; ovalbumin, 46 kd; and aldolase, 158 kd) were run in a parallel lane. The equation derived from the standard Rf values and applied to sample constituents was: $Rf = 3.101 - (0.526)$ log mol.wt. The regression correlation coefficient (r^2) for the standards was -0.986. *B* The Coomassie blue R-250 staining pattern of nuclear proteins in the absence of the [^{3}H]retinoic acid-cRABP complex. *C* Coomassie blue R-250 staining pattern of microsomal proteins derived from retinol-deficient rat testes interstitial cells. *D* Fluorogram of the rat testes interstitial cell nuclear proteins preincubated with [^{3}H]retinoic acid-cRABP. Note the appearance of at least three peptides having molecular weights of (a) 49.0 kd, (b) 44.5 kd and (c) 39.0 kd which bind [^{3}H]retinoic acid. These are distinct from the exogenous cRABP (d). *E* Fluorogram of the rat testes interstitial cell nuclear proteins preincubated with [^{3}H]retinoic acid-cRABP + 350-fold unlabeled retinoic acid-cRABP. Note the loss of bands a, b, and c which were apparent in Lane B and the retention of fluorescing band d (exogenous cRABP).

Liau et al.[7] have demonstrated that retinol is transferred from its soluble receptor, cellular retinol-binding protein (cRBP) to an undescribed chromatin acceptor(s). This results in the release of apo-cRBP and retention of retinol by a nuclear acceptor(s). Our data here suggest that such a phenomenon also occurs with retinoic acid. In support of this, figure B shows that the pattern of Coomassie blue-binding components of nuclei not preincubated with cRABP exhibited only marginal shifts in the nA a–c Rf values. No 15.0 kd shift, which might be expected if [^{3}H]RA-cRABP were a nuclear complex component, was observed.

In another aspect of this experiment we questioned whether the preferential binding of the 3 nA bands resulted from cRABP aggregates. If this were the case, first, we would not expect to see correlative bands in figures A and B in the area of the nA a–c peptides. Second, the loss of radiofluorescence demonstrated in figure E would not have been apparent even in the presence of excess unlabeled RA-cRABP. Thus, we concluded that the nA a–c components were not cRABP aggregates.

Finally, we suggest that the nA peptides are unique to the nucleus. Figure C indicates that the Coomassie blue-binding in the nA a–c peptide area associated with the microsomal fraction is significantly less than that of the nuclear fraction suggesting that the nuclear peptides were not derived from contaminating endoplasmic reticulum (ER). Further, during isolation of the cellular fractions we did add a protease inhibitor and therefore it is our contention that nA a–c peptides are distinct and did not arise as fragments of high molecular weight ER or nuclear peptides. However, given the closeness of the Mr of the nA peptides it is possible that they are, in fact, the same protein which has been subjected to limited post-translational proteolysis or glycosylation, resulting in altered or multiple ligand specificity for RA.

Thus, we conclude that rat testes interstitial cell nuclei contain at least 3 forms of RA acceptor distinct from cRABP. These are apparently native constituents of the nuclei. However, it is not possible to draw any specific conclusions regarding the role of these peptides in mediating the nuclear activity of retinoic acid.

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Egg pigment is accumulated in the tadpole's brain

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Summary. Four different crosses with wild-type and albino mutants of *Xenopus laevis* show 1. that the occurrence of the pigmented cell clusters in the tadpole's brain is independent of the presence of pigmentation of the body and 2. that there is a strict correlation between a pigmentation of the egg and the appearance of pigmented cells in the brain of the developing larva. The data strongly support the idea that the egg pigment appears in the brain later in development.

In a tadpole of *Xenopus* there are conspicuous darkly pigmented clusters of cells floating in the brain cavities. They are visible in a living animal when inspected with a stereomicroscope (fig. 1) and can also be examined on cut slices (fig. 2). They occur in other anuran species and seem to be common in the tadpoles[3]. Nevertheless, up to the present a reasonable explanation of their origin and functions has not yet been provided[4]. The supposition has been made that they are melanophores circulating in the cerebro-spinal fluid[3-5] since they appear as early as the other melanophores in the developing tadpole, about stage 33/34[6]. Moreover, as in the melanophores, their content of melanin increases with age. Morphological data indicate, however, that the cells that build the clusters cannot be melanophores[7-10]. It has been shown by electron microscopy that they contain exclusively spherical melanosomes of uniform size (0.6–1.1 µm), while the melanophores of the same animal are filled in addition with smaller, usually elongated, newly-formed pigment granules[9]. Spherical melanosomes are characteristic in the cytoplasm of the egg[11]. It has been previously supposed[7] that the egg melanin, which is distributed among the cells of the tadpole's body, is next discharged (mainly from the ectodermal tissues) into the external space[12,13]. Since the deposits of free melanosomes in the enclosed neural tube lumen increase with age, the clusters under observation were supposed to be aggregates of scavenger cells that keep the cerebro-spinal fluid free of the excreted melanosomes[7,8].