Mechanism of Vitamin A Action

GENE EXPRESSION IN RETINOL-DEFICIENT RATS*

(Received for publication, May 24, 1982)

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The effects of retinol (vitamin A alcohol) and retinoic acid (vitamin A acid) on genomic expression was studied in rat testis, intestinal mucosa, and liver by determining total cytoplasmic poly(A)-containing RNA, by using techniques of molecular hybridization and by in vitro translation. Experiments were designed to elucidate whether the effects of vitamin A or differentiation of epithelia involves genomic expression. Dietary retinol deficiency induces loss of poly(A)-containing RNA in all tissues studied. Homologous and heterologous hybridization of poly(A)-containing RNAs from testes of retinol-deficient and control rats to cDNAs showed differences, especially in the fast annealing sequences, which are expressed in smaller quantities in retinol-deficient rats. Polyacrylamide gel electrophoresis and subsequent fluorography of in vitro translation of cytoplasmic poly(A)-containing RNA revealed additional and more intensive bands representing polypeptides in the region of 15,000 and 25,000 daltons when testicular preparations from deficient rats were compared with those from controls. One hour after feeding retinol-deficient rats with retinyl acetate as source of retinol, a loss in testicular and intestinal mucosa in poly(A)-containing RNA is observed. Subsequently, this RNA accumulates but does not reach control levels even after refeeding with the vitamin for two weeks. The fast reaction of the genomic expression is also evident when patterns of in vitro translation products are analyzed in testicular preparations. One hour after dosing deficient animals with retinyl acetate, retinoic acid, or a synthetic derivative of retinoic acid, a polypeptide band of 22,000 daltons disappears. When retinoic acid, or a synthetic derivative of retinoic acid is administered in contrast to retinyl acetate, a band of 55,000 daltons intensifies. The data indicate that retinol and retinoic acid influence genomic expression in vivo by activation as well as suppression of the genome. Moreover, it appears that the effects of retinol and retinoic acid are rapid and not identical.

Early histological studies demonstrated that lack of retinol (vitamin A alcohol) in the diet influences the differentiation of epithelia of many tissues. In many instances, depending on severity of the deficiency of retinol, the normal epithelium disappears and is replaced by keratinizing epithelium (1). For instance, in the rat intestinal mucosa, the number of goblet cells decreases (2). In the testis, spermatogenesis is halted and the germinal epithelium disappears (1). The effects of retinol deficiency are multiple, but reversible. Refeeding the deficient animal by a retinol-containing diet for several months eventually returns the testicular morphology to normal (3). Diet containing retinol as esters or its precursors, carotenes, covers all physiological functions of the animal. However, retinoic acid (vitamin A acid) formed from retinol in vivo (4) appears to be a minor dietary component. When animals are fed retinol-deficient diet supplemented with retinoic acid, vision (5) and testicular functions are impaired, while other epithelia appear normal (6). The molecular mechanism(s) responsible for the effect of retinol and retinoic acid on the differentiation of epithelial cells is not known.

Presently, there are two major hypotheses concerning the molecular mode of retinol action. The first puts primary emphasis on retinol as a coenzyme in membrane-mediated synthesis of glycoproteins (7). The second one predicts existence of a specific interaction of retinol or retinoic acid with cell nucleus mediated by their specific cellular binding proteins (8, 9). Such interaction of retinol or retinoic acid with the genome should lead to alterations in genomic expression. Although the two hypotheses of vitamin A action are mutually not exclusive, the latter one appears to us more plausible, especially when effects of retinol on cell differentiation are to be explained. To test this hypothesis, we have studied some parameters of gene expression in retinol-deficient and control rats.

Previously, several observations suggested that retinol may be influencing nuclear nucleic acid metabolism. Administration of retinol to deficient animals results in increased incorporation of radioactive precursors into nuclear RNA (10-12). Nuclear RNA extracted from livers of deficient and normal rats differs (13), as does RNA synthesized in vitro by hamster tracheal epithelium (14) or by isolated rat liver nuclei (15).

In this paper, we report that dietary retinol deficiency results in lower levels of cytoplasmic poly(A)-containing RNA in testis, intestinal mucosa, and liver. Lack of retinol alters the quantity of abundance sequences of testicular cytoplasmic poly(A)-containing RNA and patterns of proteins synthesized in vitro. The genomic expression appears to be very sensitive to the vitamin since 1 h after feeding retinyl acetate as the source of retinol to retinol-deficient rats, levels of poly(A)-containing RNA and in vitro translation pattern change.

EXPERIMENTAL PROCEDURES

Materials

Oligo(dT)-cellulose type T-3 and oligo(dT)12-18 and poly(rA) were purchased from Collaborative Research Inc., Waltham MA. [3H]dTTP (specific activity, 14 or 21 Ci/mmol) and [3H]TTP (specific activity, 62 Ci/mmol) were obtained from P-L Biochemicals. [3H]TTP (specific activity, 62 Ci/mmol) and [3H]leucine (187 Ci/mmol) were produced of Amersham-Searle. ENHANCE was from New England Nuclear. Avian myeloblastosis

*This work was supported by Grants HD-9195, HL-14214, and HL-1534 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Gene Expression in Retinol Deficiency

TABLE I

Levels of cytoplasmic poly(A)-containing RNAs in testes, intestinal mucosa, and liver of control and retinol-deficient rats

|                      | Testes     | Intestinal mucosa | Liver       |
|----------------------|------------|-------------------|-------------|
| **µg RNA/mg DNA**    | **%**      | **µg RNA/mg DNA**  | **%**       |
| Control              | 17.83 ± 0.56 * | 2.53 ± 0.08    | 4.05 ± 0.31 | 2.07 ± 0.16 | 15.70 ± 1.2 | 1.58 ± 0.12 |
| Retinol-deficient    | 10.23 ± 0.39 | 1.78 ± 0.05     | 3.45 ± 0.39 | 1.90 ± 0.17 | 10.20 ± 0.6 | 1.26 ± 0.07 |

* Expressed as percent of total cytoplasmic RNA.

Values indicate an average ± S.E. determined from at least 5 rats.

Methods

Virus reverse transcriptase was a gift from the National Cancer Institute (Dr. J. Beard). Calf thymus and Escherichia coli type VI11 DNAs, ATP (K salt), creatine phosphate, creatine phosphokinase, all-trans-retinyl acetate, and all-trans-retinoic acid were supplied by Sigma. S1 nuclease (Aspergillus oryzae) was a product of Miles Laboratories. Wheat germ was provided by General Mills. RNase inhibitor purified from human placenta by the method of Blackburn (16) was generously provided by Dr. T. Inagaki, Vanderbilt University School of Medicine. Synthetic retinoic acid derivative 5,7,11,15-tetramethyl-2,4,6,10-hexadecapentaenoic acid (17) was supplied by Eisai Co., Ltd. (Tokyo, Japan).

**Methods**

**Animals**—To obtain retinol-deficient rats, 21-day-old Sprague Dawley rats "suitable for vitamin A assay," purchased from Holtzman Co., Madison, WI, were fed a retinol-free diet described previously (18) for a 3-week period or until their body weight plateaued. The rats were then fed the same diet but supplemented with retinoic acid for 18 days and then returned to the deficient diet for 10 days (19). The cycling was repeated for at least two more times to insure depletion of stored retinol. Rats were used only at the end of the 10-day deficient portion of a cycle (20). In another series of experiments, 21-day-old rats were fed retinol-deficient diet for 30 days (18). The mode of inducing retinol deficiency did not influence the outcome of results reported here. The control animals received a normal chow diet from day 21 postnatally with no deficient period. To alleviate the possible effects of lower food intake by retinol-deficient animals, the control animals received a normal chow diet from day 21 postnatally with no deficient period. To alleviate the possible effects of lower food intake by retinol-deficient animals, the control animals received a normal chow diet from day 21 postnatally with no deficient period. To alleviate the possible effects of lower food intake by retinol-deficient animals, the control animals received a normal chow diet from day 21 postnatally with no deficient period.

**Tissues**—Mucosa of the small intestine was separated as described previously (2). Testes were first decapitated before use.

**Extraction of Total Cytoplasmic RNA**—The method described by Miller and McCarthty (23) was used. No pH 6 was used for extraction instead of pH 7.5. The RNA precipitated by adding absolute ethanol was suspended in 1-2 ml of 3 M sodium acetate, pH 6.0, containing 5 mM Na2EDTA and centrifuged at 30,000 × g for 5 min at 4 °C as described previously by Palmiter (24).

**Isolation of Poly(A)-containing RNA**—The method of Aviv and Leder (25) was used.

**Determination of Poly(A)-containing RNA**—The amount of poly(A) present in RNA preparations was determined as described previously (26). Content of poly(A)-containing RNA in total RNA was calculated as the average amount of poly(A) in the poly(A)-containing RNA molecule in the respective organ. The following values were used: 7.5% for liver preparations from control and retinol-deficient rats, 8.2% for testicular preparations from control rats, 10.5% for preparations from retinol-deficient rats. For intestinal mucosa RNA isolated from control and retinol-deficient rats, the value of 8.4% poly(A) was used.

**Size Analysis of Poly(A)-containing RNA**—Methods previously described were used (15, 26).

**Synthesis of cDNA and [3H]poly(dT)-cDNAs to poly(A)-containing RNA preparations from testes of control and retinol-deficient rats**

The abbreviations used are: Na2EDTA, disodium ethylenediaminetetraacetic acid; NaDodSO4, sodium dodecyl sulfate; Rd, RNA concentration in moles of nucleotides per liter × time in seconds.

Effects of Retinol Deficiency on Levels of Poly(A)-containing RNA—A considerable decrease in the amount of poly(A)-containing RNA was found in testis, intestinal mucosa, and liver of retinol-deficient rats when compared to controls (Table I). Values for control rat intestinal poly(A)-containing RNA, which accounted for about 2% of the total RNA, and for rat liver, which accounted for about 1.5%, are in agreement with the reports of Morrison and Porteous (34) and Towle et al. (26), respectively. Size analysis of poly(A)-containing RNAs showed values slightly lower for preparations from deficient animals (about 16 S) in comparison with those obtained from controls (about 18 S). The lengths of poly(A) segments of the poly(A)-containing RNAs did not differ in preparations from control and retinol-deficient rats. In both

**Size Analysis of DNA**—Alkaline sucrose density gradient centrifugation method described earlier was used (23).

**Hybridization Conditions**—The method according to Miller and McCarthy was used (23). All hybridization reactions were carried out in a volume of 20 µl in 50-µl capillary pipettes in a buffer containing 0.12 M sodium phosphate and 1 mM Na2EDTA, pH 6.8. Approximately 1,000 cpm of CDNA was mixed with the appropriate amount of RNA, lyophilized to dryness, taken up in the above buffer, and sealed in the capillary pipettes. Pipettes were put in a boiling water bath for 30 s, incubated at 70 °C from 15 min to 72 h, and then chilled rapidly, and stored at -20 °C until further analysis.

For determination of poly(A) chains, 30,000 cpm of [3H]poly(dT) and RNA containing 0.5-1 ng of poly(A) was used. Reaction mixtures were incubated at 47 °C overnight.

After incubation, contents of capillaries was blown into 2.0 ml of buffer (0.5 M NaCl, 0.02 mM sodium acetate, 3 mM ZnCl2, pH 4.5) containing 5 mM Na2EDTA and centrifuged at 30,000 × g for 5 min at 4 °C as described previously. Size analysis of poly(A)-containing RNAs was performed as described previously (31). The mixture was incubated at 25 °C for 2 h and electrophoresed using 11% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and collected on Millipore filters and the radioactivity was determined.

**Determination of the Length of Poly(A) Chain in Poly(A)-containing RNA**—The method of Kaufman and Gross was used (28).

**Determination of DNA and RNA**—DNA was determined colorimetrically according to Burton (29), whereas RNA was determined spectrophotometrically (30).

**Translation of Poly(A)-containing RNAs in Vitro**—Wheat germ extract was prepared and a translation mixture used as described previously (31). The mixture was incubated at 25 °C for 2 h and electrophoresed using 11% polyacrylamide slab gel containing 0.1% NaDodSO4 for about 7 h (32). The gels were fixed, treated with ENHANCE (33), and dried. The translational products were detected by fluorography using Kodak X-Omat AR film.

**RESULTS**

Effects of Retinol Deficiency on Levels of Poly(A)-containing RNA—A considerable decrease in the amount of poly(A)-containing RNA was found in testis, intestinal mucosa, and liver of retinol-deficient rats when compared to controls (Table I). Values for control rat intestinal poly(A)-containing RNA, which accounted for about 2% of the total RNA, and for rat liver, which accounted for about 1.5%, are in agreement with the reports of Morrison and Porteous (34) and Towle et al. (26), respectively. Size analysis of poly(A)-containing RNAs showed values slightly lower for preparations from deficient animals (about 16 S) in comparison with those obtained from controls (about 18 S). The lengths of poly(A) segments of the poly(A)-containing RNAs did not differ in preparations from control and retinol-deficient rats. In both
Effect of Retinol Deficiency on Base Sequences of Poly(A)-containing RNA—To test further whether genomic expression is altered in retinol deficiency, hybridization studies were undertaken to determine whether the cytoplasmic poly(A)-containing RNA sequences in retinol-deficient animals differ from those present in control animals. Labeled cDNA was transcribed from poly(A)-containing RNA isolated from testes of control rats. This cDNA was then hybridized to its homologous (control) poly(A)-containing RNA and similar preparation from deficient animals. The hybridization kinetics observed (Fig. 1) show striking differences in the more abundant sequences which are present in higher quantities in the preparations from the control animals. Since the poly(A)-containing RNA from deficient animals appears to contain less of the abundant sequences, this should be reflected in cDNA prepared from it. Indeed, when such a probe was synthesized and hybridized to a homologous preparation (deficient) and to poly(A)-containing RNA from control rats (Fig. 2), it did not detect abundant sequences present in the control animals.

In Vitro Translation of Poly(A)-containing RNAs—When poly(A)-containing RNAs isolated from retinol-deficient and control animals were translated in vitro by the wheat germ system, subsequent analysis of the products by NaDodSO4-polyacrylamide electrophoresis revealed differences. Additional and more intensive bands in the region of 15,000- and 18,000-dalton molecular weights were observed in the preparations from the retinol-deficient animals.

FIG. 1. Comparative hybridization kinetics of rat testicular cytoplasmic poly(A)-containing RNAs and cDNA transcribed from poly(A)-containing RNA of control animals hybridized with the homologous preparation (○) and with preparation from deficient animals (●). Cytoplasmic poly(A)-containing RNAs (see Table I) were isolated from testes of control and retinol-deficient rats, and 3H-labeled cDNA synthesized from both preparations using avian myeloblastosis virus reverse transcriptase. Hybridizations were performed in RNA excess and the degree of hybridization determined using S1 nuclease. R2 values were not corrected for values with mRNA standard. Best line fit was determined by eye.

FIG. 2. Comparative hybridization kinetics of testicular cytoplasmic poly(A)-containing RNAs and cDNAs transcribed from poly(A)-containing RNA of retinol-deficient rats hybridized with the homologous preparation (○) and with preparation from controls (●). For details see Fig. 1.

FIG. 3. Polyacrylamide gel electrophoresis of in vitro translation products of rat testicular poly(A)-containing RNAs. Poly(A)-containing RNA from control (lane 1), retinol-deficient (lane 2), retinol-deficient 1 h after oral dosing with 100 μg of retinyl acetate (lane 3), retinol-deficient 1 h after dosing with 100 μg of retinoic acid (lane 4), retinol-deficient 1 h after dosing with 100 μg of synthetic retinoic acid derivative (lane 5), retinol-deficient fed 2 weeks with retinol supplemented diet (lane 6), and retinol-deficient fed 2 weeks with retinoic acid supplemented diet (lane 7). Conditions of in vitro translation, NaDodSO4-polyacrylamide gel electrophoresis and fluorography are described under "Experimental Procedures." The molecular weight markers indicated on the left are myosin (200K), β-galactosidase (116.5K), phosphorylase b (94K), bovine serum albumin (68K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (21K), and lysozyme (14.3K). The lines on the right indicate bands where major alterations occur.

FIG. 4. Changes in the amount of cytoplasmic poly(A)-containing RNA in testes (○) and intestinal mucosa (●) after oral administration of 100 μg of retinyl acetate to retinol-deficient rats. The animals were killed at times as indicated; some animals 24 h after dosing with retinyl acetate were fed with retinol-containing diet and killed 2 weeks later. Values indicate average ± S.E. from 3-5 rats.
25,000-dalton polypeptides were consistently detected when poly(A)-containing RNAs from retinol-deficient rats were used. (Fig. 9).

**Effect of Refeeding Vitamin A Like Compounds to Retinol-deficient Rats on Poly(A)-containing RNA and on In Vitro Translation Patterns**—The amounts of cytoplasmic poly(A)-containing RNA in testis as well as in intestinal mucosa undergo pronounced alterations even after oral administration of retinyl acetate as source of retinol. Results in Fig. 4 show a substantial fall of the values 1 and 2 h after administration of vitamin A. Thereafter, the values rise but do not reach the control values even after two weeks of refeeding. Furthermore, the rapid reaction of the genome to refeeding with vitamin A is indicated by the change in the pattern of polypeptides translated in vitro from poly(A)-containing RNA isolated from retinol-deficient rats 1 h and 2 weeks after refeeding with retinyl acetate, retinoic acid, and a synthetic derivative of retinoic acid. As shown in Fig. 3, as early as 1 h after refeeding, a polypeptide band of 22,000 daltons is not present among the translation products and does not appear even after 2 weeks of refeeding. Also after refeeding, slight alterations in banding pattern are observed in the 15,000- and 27,000-dalton region. Consistently, intensification of the peptide of 55,000 daltons is observed (Fig. 3) when retinoic acid in contrast to retinyl acetate is administered for 1 h as well as for 2 weeks. At this time, the difference is more apparent. Administration of the synthetic derivative of retinoic acid had almost the same effect as retinoic acid.

**DISCUSSION**

The data presented here support the hypothesis that retinol and retinoic acid influence genomic expression in vivo. In retinol-deficient rats, the level of poly(A)-containing RNA per DNA decreases in the testis, intestinal mucosa, and liver. It could be concluded that this decrease is due to the change in cell population which occurs in tissues affected by retinol deficiency. Such a conclusion is certainly tenable for the testes, which show pronounced morphological alterations since retinol deficiency results in the loss of the cells of the germinal epithelium (1). The differences in hybridization data revealing that the preparations of poly(A)-containing RNA from testes of retinol-deficient rats contain much lesser amounts of fast annealing sequences than those from the controls could also be due to the loss of the cells of the germinal epithelium. On the other hand, a decrease in the poly(A)-containing RNA was observed also in the intestinal mucosa and liver. Very small morphological changes occur in retinol-deficient animals in the intestinal mucosa (2) and no changes are observed in the liver (1). Thus, it appears that the decrease in poly(A)-containing RNA does not always correlate with the extent of the alteration in cell population.

In order to find out whether vitamin A influences the gene expression when alterations in cell population are not a factor, refeeding experiments were designed. The results show that when retinol-deficient rats are fed with an oral dose of retinyl acetate (as the source of retinol), the effect on genomic expression is dramatic and rapid. As early as 1 h after administration of the vitamin, a decrease in the level of poly(A)-containing RNA is observed in the testis as well as in the intestinal mucosa. The effects on testis were further analyzed because in this tissue the regenerative repair of the germinal epithelium requires several months (3). We have found that concurrently with the fall of the poly(A)-containing RNA levels in testis, i.e. 1 h after feeding retinol or retinoic acid, alterations in patterns of in vitro translation products of poly(A)-containing RNA take place. A band representing a polypeptide of 22,000 daltons disappears and bands of 15,000 and 27,000 daltons become greater. Further characterization of polypeptides whose level changes after refeeding is necessary. Although the mechanisms underlying this phenomenon remain to be elucidated, it appears that retinol and retinoic acid simultaneously activate as well as suppress the genomic expression. The observed effects could be due to alteration in transcription or processing of the transcripts or both. Moreover, the experiments have shown that the effects of these compounds are rapid; consequently, the reported data are not influenced by proliferation of certain cell types.

That the animal genome of the retinol-deficient animal may rapidly respond to vitamin A compounds in vivo was suggested earlier when it was found that the amount of specific binding sites for retinol delivered to the nucleus by the cellular retinol binding protein changes 2 h after feeding deficient animals with retinyl acetate (22). The rapid effect of vitamin A as described here has its precedents. Induction of avian ovalbumin or vitellogenin mRNAs has been observed 30 min after administration of estrogen to estrogen-withdrawn animals (35, 36).

Comparison of the pattern of polypeptides translated from poly(A)-containing RNA isolated from testes of control and retinol-deficient rats revealed additional and more intensive polypeptide bands in the region of 15,000 and 25,000 daltons for the deficient animals. These small differences seen in the pattern of the translation products probably do not represent all changes which could be revealed by more powerful methods for separating of translation products. Nevertheless, the method of one-dimensional electrophoresis was very useful in registering major changes which occur after refeeding.

While this work was in progress, several investigators using cells in culture have reported that retinoic acid added to the medium influences synthesis of specific proteins. Lotan and Lotan (37) and Lotan et al. (38) have found increased synthesis of melanin and tyrosinase when retinoid acid was added to medium of human HS 939 and mouse S91C2 melanoma cells. The authors also observed that after addition of retinoic acid, a glycoprotein of 230,000 daltons appears on the cell surface of HeLa S1 cells (39). Retinoic acid, or to a smaller extent, retinol, stimulates activity of alkaline phosphatase in rat urinary bladder, prostate, and Dunning R 3327 transplantable rat prostatic adenocarcinoma in culture (40, 41). Furthermore, induction of vimentin and tropomyosin (42) and laminin (43) in mouse F9 embryonal carcinoma cell line by retinoic acid has been described. Fuchs and Green (44) have shown that human keratinocytes in culture react to the addition of retinyl acetate to the culture medium by stimulation of synthesis of keratins of 40,000 and 52,000 daltons and their respective mRNAs. Moreover, these authors demonstrated that retinyl acetate causes inhibition of synthesis of the 76,000-dalton keratin and its mRNA. Taken together, it appears that vitamin A simultaneously inhibits and activates the genome in cells in culture as well as in the whole animal.

As mentioned, the repair process resulting in recovery of the morphology of the testes by feeding retinol to retinol-deficient animals requires several months (3). Furthermore, one cannot assume that morphological normalization is simultaneously accompanied by return of all physiological and biochemical functions. Therefore, it is not surprising that the levels of poly(A)-containing RNA and the in vitro translation patterns did not normalize after 2 weeks of refeeding with the vitamin. On the other hand, most recently, we have observed that when the refeeding of retinol-deficient rats with a retinol-containing diet was extended to 6 months, the 22,000-dalton polypeptide band appears again among the in vitro translation products. Interestingly enough, this does not occur when retinol-deficient animals were fed for 6 months a diet contain-
ing retinoic acid.

Finally, the in vitro translation patterns after oral administration of retinoic acid show an intensification of the band of 55,000 daltons not observed when retinol is fed. These results can be interpreted to mean that the molecular effects of retinol and retinoic acid action are not identical (20, 45). A more detailed analysis and characterization of the gene products influenced by retinol or retinoic acid is necessary in order to understand the mechanism of their molecular action.

Acknowledgments—We thank Mark Hunt for his excellent technical help in preparing retinol-deficient rats.

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