

Retinoic acid (vitamin A acid) induced transcriptional control of interferon production

[cycloheximide/L-929 cells/poly(I)poly(C)/Newcastle disease virus/repressors]

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ABSTRACT The production of interferon was used to study the site and mechanism of action of retinoic acid (vitamin A). The data are consistent with a site of action at the gene level, because it appears that interferon production is blocked at the transcriptional step by a retinoic acid-induced protein. (i) The effect of retinoic acid is probably on an early cellular function associated with interferon production rather than an effect on the inducer [virus or poly(I)poly(C)]. (ii) The suppression of interferon production by retinoic acid is blocked by cycloheximide, indicating that a newly synthesized protein (repressor) mediates the suppression. (iii) When allowances are made for the time required for the synthesis of the retinoic acid-induced protein, the time course of retinoic acid suppression of interferon production is superimposable on the time course of actinomycin D suppression because the slopes are parallel. These data provide evidence for transcriptional control of a specific protein (interferon) by retinoic acid. Additionally, they support the existence of transcriptional control of interferon production after addition of inducer.

Vitamin A is known to induce and control cellular differentiation in many tissues (1). Recently, vitamin A was shown to suppress the induction and development of tumors in response to chemical carcinogens (2) and to have an antitumor action in mice inoculated with murine melanoma cells (3). The bases for these actions are unknown, but the ability to control cellular differentiation is undoubtedly involved in the suppression of carcinogenesis. We have shown that vitamin A acid (retinoic acid) restores density-dependent growth control to a transformed cell line (mouse L-929 cells) (4). This finding suggests that the antitumor action of vitamin A may result from a direct effect of the vitamin that restores normal functions to transformed cells. It is important to understand the mechanism(s) by which vitamin A regulates gene expression. One approach to this problem is to study the effect of vitamin A on a cellular molecule that is inducible *in vitro*.

For a number of reasons, interferon synthesis offers a model for the study of mammalian gene expression. First, it is a molecule that is easily inducible in almost every nucleated mammalian cell. Second, after induction, interferon synthesis is thought to be controlled by specific regulatory protein(s) (repressors) (5-7). Interestingly, these putative repressors are almost exclusively demonstrable in normal (diploid) cells rather than in cell lines and might be thought of as a marker of normalcy. Finally, interferon action results from the induction of another cellular protein (8, 9). Because interferon action as well as interferon production results from a derepression event, the comparative effect on the two derepressions could be used to monitor the specificity of the control mechanism(s).

We have previously shown that retinoic acid suppresses in-

terferon production (10). The ring portion of the vitamin A molecule seemed to be the most important part of the molecule for this suppression (11). The inhibited event was early and suggested that transcription of the interferon gene was blocked. This current study is concerned with the site and mechanism of inhibition of interferon production by retinoic acid. Evidence is presented that interferon mRNA transcription is blocked by a retinoic acid-induced protein.

MATERIALS AND METHODS

Chemicals. All-*trans* form of retinoic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (Me₂SO) (Fisher Scientific Co.) and stored at -20°; the stock solution contained 2 mg/ml. A stock solution of the homopolymer pair polyribinosinate-polyribocytidylate [poly(I)poly(C)] (Sigma Chemical Co.) at a concentration of 1 mg/ml was prepared in Eagle's minimal essential medium (EMEM) and stored at -20°. DEAE-Dextran (Pharmacia, Uppsala, Sweden), cycloheximide (Grand Island Biological Co.), and actinomycin D (Grand Island Biological Co.) were dissolved at 10 mg/ml, 1 mg/ml, and 100 µg/ml, respectively, in EMEM and stored at -20°.

Cells. Mouse L-929 cells were maintained by weekly passage in EMEM supplemented with 10% calf serum. This medium contained 125 µg of streptomycin and 150 units of penicillin per ml.

Viruses. A large-plaque variant of vesicular stomatitis virus, Indiana strain, and a lentogenic strain of Newcastle disease virus (NDV) were grown in the allantoic cavity of developing chick embryos. Semliki Forest virus (SFV), Kumba strain, was propagated in the brains of newborn mice. When assayed on L-929 cells, the pools of vesicular stomatitis virus and SFV contained 3×10^9 and 2×10^8 plaque-forming units/ml, respectively. The pool of NDV contained about 640 hemagglutination units/ml.

Production and Assay of Interferon. Mouse interferon was produced by infection of L-929 cells with NDV or SFV. After adsorption for 1 hr at room temperature, residual virus was removed and the cultures were washed and re-fed with fresh medium. Triplicate cultures were used for each determination. Culture fluids were collected 24 hr after infection, pooled, and clarified by low-speed centrifugation. They were dialyzed against pH 2 buffer (10 µM HCl/0.9% NaCl) for 5 days at 9° and then against Gey's balanced salt solution to restore the pH to neutrality. Interferon was also induced by treatment of L-929 cells with medium containing 10 µg of poly(I)poly(C) and 100

Abbreviations: Me₂SO, dimethyl sulfoxide; EMEM, Eagle's minimal essential medium; NDV, Newcastle disease virus; SFV, Semliki Forest virus; PDD₅₀ units, interferon titer expressed as µl of preparation required to depress plaque numbers to 50% of control.

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Table 1. Effect of retinoic acid on NDV, SFV, and poly(I)-poly(C) induction of interferon

Interferon inducer	Retinoic acid (20 $\mu\text{g}/\text{ml}$)	Interferon yield, PDD ₅₀ /ml	% inhibition of interferon yield	Virus yield, PFU* $\times 10^8$
NDV	+	2,600	74	†
	-	10,000		
SFV	+	260	71	1.70 \pm 0.45
	-	900		1.65 \pm 0.40
Poly(I)-poly(C)	+	100	86	—
	-	700		

* PFU, plaque-forming units.

† NDV abortively infects L-929 cells and therefore no virus is produced.

μg of DEAE-dextran per ml. After 2 hr, the medium was removed and the cells were washed and re-fed with fresh medium. Culture fluids were harvested 24 hr after poly(I)-poly(C) treatment and assayed for interferon content.

Interferon was assayed by a plaque reduction assay using vesicular stomatitis virus for challenge as described (12). Four dilutions of each sample were each assayed in triplicate or quadruplicate. Interferon titers are expressed as the amount of an interferon preparation, in μl , that depressed plaque numbers by 50% (PDD₅₀) as compared to the controls. In our assay system, the National Institutes of Health mouse reference reagent interferon preparation (code G002-902-026; assigned activity, 6000 units) had a titer of 3500 PDD₅₀ units.

RESULTS

Effect of Retinoic Acid on NDV, SFV, and Poly(I)-Poly(C) Induction of Interferon. That retinoic acid suppresses an early event in the production of interferon was suggested by the same degree of suppression (74 or 76%) of interferon yield when retinoic acid remained in contact with cells for 24 or 2 hr (10). After virus infection, the earliest event in the interferon production cycle is thought to be the presence of the inducer which is presumably RNA (13). If retinoic acid interferes with virus replication, the generation of the interferon inducer would probably also be affected. Because NDV abortively infects L cells (14), we could not detect an effect of retinoic acid on NDV replication. We therefore investigated the effect of retinoic acid on interferon production in response to other inducers.

Table 1 shows that 3-hr treatment with retinoic acid (20 $\mu\text{g}/\text{ml}$) immediately after SFV adsorption to L-929 cells caused a marked suppression (71%) in the yield of interferon without affecting the yield of virus. The degree of suppression of interferon yield was similar with both SFV (71%) and NDV (74%). These data show that retinoic acid did not suppress interferon production by interfering with virus replication.

Similarly, in L cells, induction of interferon production by poly(I)-poly(C) was also inhibited (84%) by 3-hr treatment with retinoic acid (20 $\mu\text{g}/\text{ml}$) (Table 1). Because there is almost certainly no viral function associated with poly(I)-poly(C) induction of interferon, these data indicate that the effect of retinoic acid is probably on an early cellular function associated with interferon production.

Comparison of the Kinetics of Suppression of Interferon Production by Retinoic Acid and Actinomycin D. Actinomycin D, a DNA-dependent RNA synthesis inhibitor (15), has been used by numerous investigators to show that interferon mRNA synthesis is completed within a few hours after RNA virus infection (16-18). Specific mRNA synthesis therefore is an early event in the production of interferon that could be blocked by retinoic acid. This hypothesis was investigated by comparing the temporal relationship of suppression of inter-

feron production by retinoic acid and by actinomycin D. At intervals after NDV adsorption (1 hr at 24°), retinoic acid (20 $\mu\text{g}/\text{ml}$) or actinomycin D (0.5 $\mu\text{g}/\text{ml}$) was added to the L cell cultures and remained in the medium throughout the incubation period. This level of actinomycin D was sufficient to inhibit [³H]uridine incorporation by 80% in 15 min (data not shown). Fig. 1 shows that the 24-hr yields of interferon were maximally decreased when retinoic acid was added at or before 2 hr; with addition 4 hr after NDV adsorption, cells were less sensitive to the suppressive action, and by 8 hr they were resistant to suppression by retinoic acid. It appears that, regardless of whether poly(I)-poly(C) or NDV is the inducer, retinoic acid has the same time course of action. This result indicates that retinoic acid probably acts on the same early event in interferon induction by either agent. This is in contrast to actinomycin D, in which case for 6 hr after NDV adsorption interferon production was maximally suppressed and resistance to this inhibitor occurred after 14 hr.

If it is assumed that retinoic acid acts on the cell shortly after its addition, the earlier time course of retinoic acid action compared to actinomycin D indicates that retinoic acid suppresses interferon production prior to transcription of interferon mRNA. Because a transcriptional block would have to occur before or during transcription, the results suggest that retinoic acid acts by inhibiting interferon mRNA transcription either by directly blocking the transcription or by indirectly affecting the regulation of the transcription. This idea is supported by the striking similarity in the slopes of the curves for retinoic acid and actinomycin D as inhibitors of interferon synthesis.

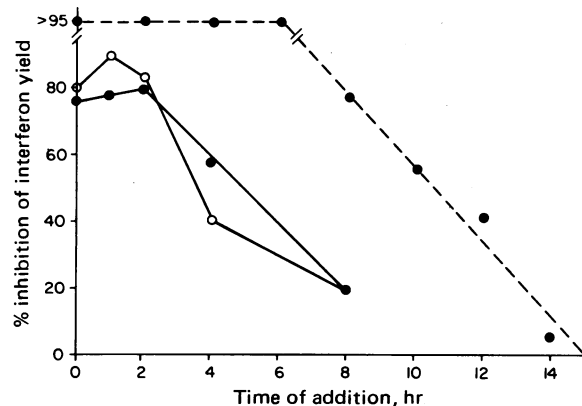


FIG. 1. Kinetics of suppression of interferon production by retinoic acid (—) or actinomycin D (---). Retinoic acid (20 $\mu\text{g}/\text{ml}$) or actinomycin D (0.5 $\mu\text{g}/\text{ml}$) was added to the L cell cultures at intervals after NDV (●) or poly(I)-poly(C) treatment (○) (1 hr at room temperature) and remained in the medium throughout the incubation period. After 24 hr, supernatant fluids were dialyzed at pH 2 for 5 days to inactivate virus and assayed for interferon content.

Table 2. Effect of cycloheximide on suppression of interferon production by retinoic acid

Treatment	Interferon yield, PDD ₅₀ units/ml	% inhibition of interferon yield
Retinoic acid (20 μ g/ml)	2,800	75
Retinoic acid (20 μ g/ml) + cycloheximide (1 μ g/ml)	11,200	0
Me ₂ SO (1%) + cycloheximide (1 μ g/ml)	11,200	0
Me ₂ SO (1%)	11,200	0

Effect of Cycloheximide on Suppression of Interferon Production by Retinoic Acid. Retinoic acid could suppress interferon production either directly or indirectly through induction of another molecule. A paradoxical effect of inhibitors of RNA and protein synthesis on interferon production by rabbit kidney cells stimulated with poly(I)-poly(C) has been described (19). In the continuous presence of cycloheximide these cells produced 3 to 10 times more interferon than did control cultures. It was concluded that this effect was most likely explained by a preferential inhibition by cycloheximide of a cellular regulatory protein (repressor) that controls interferon production. To determine if retinoic acid induces the synthesis of a regulatory protein, we studied the effect of cycloheximide on suppression of interferon production by retinoic acid. In preliminary experiments we found that, although cycloheximide at 10 and 5 μ g/ml inhibited interferon production by 99 and 72%, respectively, at 1 μ g/ml it caused no inhibition. That a cycloheximide concentration of 1 μ g/ml inhibited 70% of protein synthesis (see below) but did not suppress interferon production is not surprising because interferon is induced by double-stranded RNA which itself inhibits protein synthesis (20). Therefore, cycloheximide at 1 μ g/ml was used in the following series of experiments.

After adsorption of NDV (1 hr at room temperature), cultures were washed and replenished with retinoic acid, 20 μ g/ml in EMEM with 10% calf serum, with or without cycloheximide. Controls received 1% Me₂SO in medium with or without cycloheximide. Twenty-four hours later, supernatant fluids were dialyzed and assayed for interferon. Table 2 shows that the suppressive effect (75% inhibition) of retinoic acid on interferon production was completely prevented by cycloheximide. Because cycloheximide at 1 μ g/ml inhibited ³H-labeled amino acid incorporation by 70% in 15 min (data not shown), these data suggest that retinoic acid acts indirectly through synthesis of a protein repressor that inhibits interferon production. The synthesis of this protein must, therefore, be more sensitive to cycloheximide than is interferon synthesis. This differential effect of cycloheximide has been reported (19).

Kinetics of Cycloheximide Prevention of Retinoic Acid Suppression of Interferon Production. If time were required for synthesis of an intermediary protein, the action of retinoic acid probably would be slower than if it acted directly. Therefore, the determination of the probable site of retinoic acid suppression of interferon production required a kinetic study of the preventive action of cycloheximide.

Addition of cycloheximide 2 hr after retinoic acid completely prevented the inhibitory action of the vitamin (Fig. 2), by 4 hr the retinoic acid-treated cells had lost about 50% of their sensitivity to cycloheximide, and by 8 hr they were resistant to the action of cycloheximide. If one assumes that retinoic acid induces a mRNA for the putative repressor, these data suggest that its translation begins by at least 2 hr after retinoic acid addition and is completed by 8 hr or earlier. Furthermore, these data

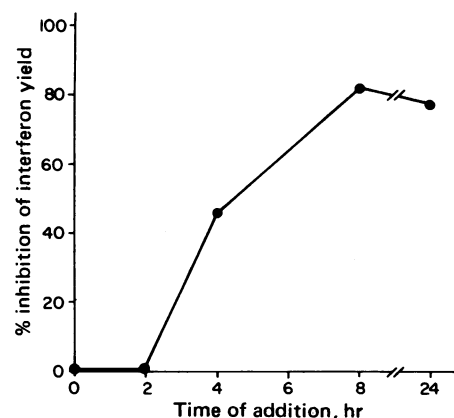


FIG. 2. Effect of time of addition of cycloheximide on suppression of interferon production by retinoic acid. L-929 cells were exposed to NDV for 1 hr at room temperature, washed, and replenished with EMEM containing 10% calf-serum and retinoic acid (20 μ g/ml) or 1% Me₂SO (controls). At the indicated intervals after retinoic acid or Me₂SO addition, cycloheximide was added to a concentration of 1 μ g/ml. Twenty-four hours later, supernatant fluids were dialyzed and assayed for interferon content.

indicate that 4–6 hr are required for complete translation of the putative repressor and 6–8 hr for maximal induction and translation.

Because cycloheximide was present continuously, the preceding experiment provides no information as to when the putative retinoic acid-induced protein acted after its synthesis. The following experiment was designed to determine how long a cycloheximide block was required to prevent the action of retinoic acid. This experiment was made possible by the reversibility of cycloheximide inhibition of protein synthesis (7).

Table 3 shows that a maximal degree of suppression occurred if the cycloheximide block was reversed at or before 4 hr after retinoic acid addition; no suppression was observed if cycloheximide was removed after 8 hr. That reversal of the cycloheximide block at 4 hr resulted in the same amount of suppression as reversal at earlier times could be interpreted as reflecting the accumulation of a mRNA (for a repressor) that is translated and acts after removal of the cycloheximide, because addition of retinoic acid at 4 hr only partially suppressed interferon production (compare Fig. 1 and Table 3). Because

Table 3. Effect of time of removal of cycloheximide on suppression of interferon production by retinoic acid

Treatment with cycloheximide (1 μ g/ml),* hr	Retinoic acid (20 μ g/ml for 24 hr)	Interferon yield [†]	
		PDD ₅₀ units/ml	% of control
0–0.5	+	1600	20
	–	8000	
0–1	+	1400	18
	–	8000	
0–2	+	1600	21
	–	7700	
0–4	+	1100	15
	–	7100	
0–8	+	7100	100
	–	7100	

* 0 time is immediately after adsorption of NDV for 1 hr at room temperature. Retinoic acid was added at 0 time.

[†] Interferon was determined in the pooled supernates from triplicate cultures at 24 hr after adsorption of NDV.

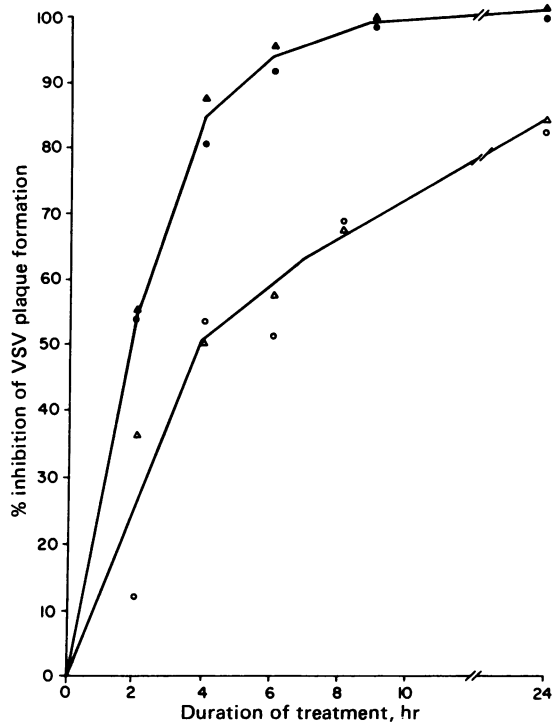


FIG. 3. Kinetics of interferon action in the presence of retinoic acid. L-929 cell cultures were treated for varying times with mouse interferon 4 [(open symbols) or 16 (solid symbols) PDD₅₀ units/ml] in EMEM containing 10% calf serum and either retinoic acid (triangles) at 20 μ g/ml or 1% Me₂SO (circles). Controls without interferon received retinoic acid or Me₂SO in medium. After interferon treatment, cultures were washed three times and immediately infected with about 150 plaque-forming units of vesicular stomatitis virus (VSV).

removal of the cycloheximide at 8 hr was too late to allow suppression of interferon production by retinoic acid and maximal translation of the putative repressor requires 4–6 hr (Fig. 2), these data indicate that the retinoic acid-sensitive step occurs prior to 12–14 hr after NDV adsorption. This interpretation assumes that the mRNA for the putative repressor is partially stable for 8 hr.

Together, these results (Fig. 2 and Table 3) support the view that the postulated retinoic acid-induced protein suppresses interferon mRNA transcription directly or indirectly. This interpretation is strengthened by the apparent necessity to shift the kinetics of retinoic acid suppression 6–8 hr (Fig. 2) to the right because this amount of time is required for synthesis of the putative retinoic acid-induced repressor. This makes the time course of retinoic acid suppression almost superimposable on the time course of actinomycin D suppression because the slopes are parallel (Fig. 1). Additionally, the retinoic acid-sensitive step occurs before the end of the interferon mRNA (14 hr) (Fig. 1).

Kinetics of Interferon Action in the Presence of Retinoic Acid. The above data indicate that retinoic acid induces a regulatory protein that inhibits interferon mRNA transcription. This putative repressor might be expected to demonstrate some specificity in the number of inducible genes it may affect. Interferon is thought to inhibit virus replication by inducing the formation of the antiviral protein (8, 9). If the retinoic acid-induced protein nonspecifically repressed all inducible cell functions, one would expect it to inhibit induction of the antiviral protein. Contrary to this, retinoic acid caused no difference in the rate or degree of development of viral resistance

as compared to controls treated with 4 or 16 PDD₅₀ units of interferon per ml (Fig. 3). These data indicate that the proposed repressor protein induced by retinoic acid did not inhibit interferon induction of the antiviral protein and is therefore somewhat specific in its action. The idea of inhibition of transcription of only one or a few mRNA species is supported by the absence of an effect of retinoic acid on total [³H]uridine incorporation (data not shown).

DISCUSSION

The recent observations of the therapeutic and preventive (2, 3) effects of vitamin A on many tumors have intensified interest in the mechanism(s) by which vitamin A regulates gene expression. Numerous studies of the *in vitro* action of vitamin A have yielded much information. For instance, vitamin A can stimulate growth of chick heart fibroblasts (21), alter differentiation of cells (22–24), and alter glycoprotein synthesis (25). Although all of these studies suggest a key role for vitamin A in gene expression, none of them has shown a specific site of action. An intracellular retinoic acid-binding protein has recently been discovered, leading to speculation that retinoic acid may bind this protein and act at the gene level (26, 27), similar to a steroid hormone–steroid hormone receptor complex (28). Our data are consistent with a site of action of retinoic acid at the gene level, because it appears that interferon production is blocked at the transcriptional level by a retinoic acid-induced protein.

Any substance, such as vitamin A, involved in the control of cellular differentiation should demonstrate some specificity in the number of genes it regulates. Consistent with this idea is the observation that retinoic acid did not alter total [³H]uridine incorporation in our system (data not shown). Further evidence for the specificity of retinoic acid action was the demonstration that, although interferon production was inhibited by retinoic acid, the induction of the antiviral state in interferon-treated cells was not altered by it.

The control of interferon production by specific protein repressors has been postulated (5–7) on the basis of the observation that inhibitors of protein or RNA synthesis enhance the production of interferon in cells induced with double-stranded RNA or viruses (termed “superinduction”). Interestingly, superinduction of interferon has almost exclusively been observed in normal (diploid) cell cultures (5–7). Superinduction was not observed in L-929 cells (transformed cells) (Table 2) which implies that they either do not have or do not express a normal regulatory mechanism for interferon production. The retinoic acid induction of a putative repressor for interferon production may, therefore, represent the restoration of a normal regulatory process for interferon production to a transformed cell line. This hypothesis is consistent with the retinoic acid restoration of growth control to L-929 cells (which is another normal characteristic) (4).

Finally, this study has provided evidence for transcriptional control of the production of a specific protein (interferon) by retinoic acid. Thus, this system may provide a model for the study of the action of retinoic acid on gene expression, particularly as it relates to transformed cells. Additionally, evidence for transcriptional control of interferon production after inducer addition was presented.

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