INTERFERON INHIBITS THE IN VITRO ACCUMULATION OF VIRUS SPECIFIC RNA IN NUCLEI ISOLATED FROM SV40 INFECTED CELLS

D.H. Metz, M.N. Oxman*, and M.J. Levin**

National Institute for Medical Research
Mill Hill, London NW7 1AA, England

*Departments of Medicine and Pathology
University of California, San Diego
Virology Laboratory
San Diego Veterans Administration Hospital
San Diego, California 92161, U.S.A.

**Clinical Microbiology Division
Sidney Farber Cancer Institute
Boston, Massachusetts 02115, U.S.A.

Received January 25,1977

Summary. Nuclei prepared from Vero cells infected with SV40 in the presence of cytosine arabinoside synthesize [^{3}H]RNA in vitro, a small proportion of which is virus specific. This synthesis is sensitive to low doses of α-amanitin. The accumulation of viral RNA is blocked in nuclei prepared from cells pretreated with interferon prior to infection and incubated in a reaction mixture containing 100 mM KCl. This resembles the situation found in intact cells. However, when the reaction mixture contains 300 mM KCl, the interferon-induced block in viral RNA accumulation is reversed.

Introduction. Interferon is an inhibitor of virus replication which is produced by virus infected cells. When applied to cells prior to infection it blocks the subsequent growth of virus. The locus of action of this interferon-induced inhibition is at an early stage in the virus replication cycle. The primary site of action appears to be either viral RNA or protein synthesis, depending upon the virus in question (1). In the case of the small, DNA containing, simian virus 40 (SV40), interferon pretreatment of monkey cells results in a reduction in the amount of early virus specific RNA detectable during lytic infection (2,3). We have recently presented evidence suggesting that this is due either to reduced transcription of the viral genome, or to enhanced degradation of newly synthesized viral RNA within the nucleus (4). To explore further the mechanism by which interferon inhibits SV40 replication we have

ISSN 0006-291X

prepared an in vitro system, consisting of nuclei from infected cells which synthesize early SV40 RNA. We find that under specified conditions the accumulation of viral RNA is blocked in nuclei obtained from interferon treated cells.

Methods. Vero cell monolayers were pretreated with human leukocyte interferon and infected with SV40 in the presence of cytosine arabinoside as described (4). At 27 hr post-infection cells were removed from the roller bottles by trypsin treatment and scraping. Samples of cells were allowed to attach to glass coverslips for subsequent assay of SV40 T antigen (4). Nuclei were prepared from saline washed cells essentially according to the procedure of Marzluff et al. (5). The nuclear pellet was resuspended in 25% glycerol, 5 mM $Mg(Ac)_2$, 50 mM Tris (pH 8), 5 mM dithiothreitol, 0.1 mM EDTA at 3-4 mg DNA per ml. This preparation was used immediately for RNA synthesis, the reaction mixture for which contained: 12.5% glycerol, 5 mM $Mg(Ac)_2$, 1 mM $MnCl_2$, 25 mM Tris (pH 8), 0.05 mM EDTA, 5 mM dithiothreitol, 100 mM KCl (unless otherwise specified), 0.4 mM each of ATP, CTP and GTP, 100 µCi [5,6-^{3}H] UTP (50 Ci/mmole) and nuclei equivalent to 0.75-1.0 mg DNA in a total volume of 0.5 ml. The highest available specific activity [^{3}H] UTP was employed and no non-radioactive UTP was added. The mixture was incubated at 25° for 2-3 hr.

RNA was extracted from the reaction mixture as follows. 50 µg DNase (Worthington, electrophoretically purified) was added and the mixture incubated at room temperature for 1 to 2 minutes. The mixture was made 1% in Sarkosyl (Ciba-Geigy, NL97) and 10 mM in EDTA. Wheat germ RNA (1 mg, Calbiochem) as carrier and Protease K (0.5 mg/ml, Merck) were then added. After 30 min incubation at room temperature the mixture was diluted to 3.0 ml with 1% Sarkosyl in 10 mM Tris (pH 8), and 3.0 g CsCl was added and disolved (6). The resulting solution was layered over 1.2 ml CsCl in 100 mM EDTA (1.707 g/ml) in a SW 50.1 centrifuge tube, overlaid with paraffin oil and centrifuged at 35,000 rpm for 16 hr at 25°. The supernatant was decanted and the pellet of purified RNA recovered as described (6) by dissolving in 10 mM Tris (pH 8) and precipitating with 0.1 volume of 5 M NaCl and 2 volumes ethanol. The RNA was collected by centrifugation and dissolved in 2 x SSC plus 0.05% SDS. Samples were assayed for acid insoluble radioactivity (i.e. Total [^{3}H] RNA) and for SV40 specific [^{3}H] RNA by hybridization to SV40 DNA on filters as described (4). The DNA content of the reaction mixtures was determined (4) and the results of in vitro RNA synthesis experiments are expressed in the form: cpm [^{3}H] RNA per mg DNA in the reaction mixture.

It may be noted that this method of RNA purification, employing Protease K and CsCl, yields a product comparable to that obtained with extensive phenol extraction (4) and requires significantly less effort (unpublished results).

Results. Nuclei prepared from Vero cells infected with SV40 by the procedure of Marzluff et al. (5) incorporated [^{3}H] UTP into RNA for up to 3 hours at a rate which was initially linear with time and which remained so for at least 30 minutes before decreasing. The conditions described in Methods are approximately optimal for the incorporation of

Table I. Synthesis of SV40 specific [^{3}H] RNA in nuclei isolated from SV40 infected cells.

Expt.	SV40	Additions	SV40 specific [^{3}H]RNA*	inhibition (%)	Total [^{3}H]RNA**	inhibition (%)
1	+	-	2200	-	4.0	-
	+	α-amanitin (1 μg/ml)	137	94	1.8	56
	-	-	4	-	5.0	-
	-	α-amanitin (1 μg/ml)	0	-	2.1	58
2	+	-	177	-	3.9	-
	+	α-amanitin (5 μg/ml)	8	95	2.0	49
	+	DNase (20 μg/ml)	32	82	0.44	89
	+	RNase (10 μg/ml)	0	100	0	100

* cpm per mg DNA
** cpm per mg DNA x 10^{-6}

Nuclei from SV40 infected or uninfected cells were incubated as described in Methods with the addition, as indicated, of α-amanitin, electrophoretically-purified DNase or pancreatic RNase.

[^{3}H] UTP into acid insoluble materia. Nuclei were prepared from cells infected in the presence of cytosine arabinoside, which blocks viral DNA replication and inhibits the synthesis of late, but not of early, SV40 RNA (4,7). Under these conditions a small proportion of the incorporation was into SV40 specific [^{3}H] RNA as judged by hybridization to SV40 DNA bound to filters (Table I). This proportion (of the order of 0.01%) is comparable, though on average somewhat higher, than the proportion of early viral RNA made in intact infected cells (2,4). Nuclei were routinely prepared from cells 27 hr after infection; in

Table II. Effect of interferon on the synthesis of SV40 specific $[^3H]$RNA in nuclei isolated from SV40 infected cells.

Interferon	SV40	T * Antigen	100 mM KCl		300 mM KCl	
			Total $[^3H]$RNA**	SV40 RNA*** / Total RNA	Total $[^3H]$RNA**	SV40 RNA*** / Total RNA
-	+	86	3.4	77	3.2	192
+	+	6	4.2	9	4.4	187
-	-	0	4.4	0	4.2	0

* % of cells staining positively for T antigen

** cpm per mg DNA x 10^{-6}

*** $\frac{\text{SV40 } [^3H]\text{RNA}}{\text{Total } [^3H]\text{RNA}} \times 10^6$

Cells were pretreated with or without interferon (150 units/ml) prior to infection. Nuclei were prepared at 27 hr post-infection and were incubated with $[^3H]$UTP and the other components of the reaction mixture in the presence of either 100 mM or 300 mM KCl.

vitro viral RNA synthesis was difficult to detect prior to about 20 hr post-infection.

The in vitro synthesis of SV40 specific RNA is almost completely blocked by low doses of α-amanitin while total RNA synthesis is only partially inhibited (Table I). The inclusion of either deoxyribonuclease or pancreatic ribonuclease in the reaction mixture markedly inhibited RNA synthesis.

Interferon pretreatment of Vero cells prior to SV40 infection inhibits the synthesis of SV40 T antigen and the accumulation of viral RNA without affecting either cellular protein or RNA synthesis (4). In the presence of 100 mM KCl the net in vitro synthesis of RNA by nuclei prepared from such cells appears to reflect the in vivo situation (Table II). While total $[^3H]$ RNA synthesis is not affected by interferon

pretreatment, the synthesis of SV40 specific [^{3}H] RNA is markedly inhibited. Ionic strength is, in general, an important variable in the behavior of in vitro transcriptional systems and this is the case here. Increasing the concentration of KCl from 100 mM to 300 mM does not alter total [^{3}H] RNA synthesis, but enhances SV40-specific RNA synthesis by more than two-fold (Table II). However, the most striking result of increasing the ionic strength of the reaction mixture is the abolition of the interferon induced inhibition of viral RNA synthesis (Table II).

Discussion. The synthesis of viral RNA within isolated nuclei prepared from infected cells has been demonstrated for adenovirus (8,9,10), herpes virus (11,12) and for SV40 late in infection in the absence of inhibitors of DNA synthesis (13,14). We have now shown that early SV40 RNA can be synthesized in vitro in nuclei prepared from cells infected in the presence of sufficient cytosine arabinoside to inhibit viral DNA synthesis by more than 99% (7). In this circumstance the synthesis of late viral RNA is also blocked (7). This early SV40 RNA synthesis is sensitive to low doses of α-amanitin which indicates that, like the synthesis of late SV40 RNA (13), it probably results from transcription of viral DNA by cellular RNA polymerase II, the enzyme believed to be responsible for the synthesis of cellular messenger RNA.

The effect of interferon pretreatment of cells on the accumulation of viral RNA by isolated nuclei assayed in the presence of 100 mM KCl parallels the effect seen in intact cells (4). In both situations the synthesis of SV40 specific RNA is inhibited, while that of total cellular RNA is unaffected. However, when the KCl concentration is increased to 300 mM, this in vitro inhibitory effect is abolished and viral RNA is synthesized to an extent comparable to that seen in the controls untreated with interferon. The equivalent rate of accumulation of SV40-specific RNA in nuclei from interferon treated and control cells in the presence of 300 mM KCl supports our previous conclusion (4) that interferon treat-

ment does not reduce the quantity of SV40 DNA template potentially available for transcription in the nucleus of the cell.

There are at least two possible explanations for the effect of interferon treatment on the accumulation of early viral transcripts in SV40 infected cells which would be consistent with the present results (4). First, interferon treatment may induce the formation of an intracellular inhibitor which blocks viral transcription, perhaps by recognizing and binding to specific sites on the viral DNA. Elevated salt concentrations in vitro might then displace the inhibitor and permit transcription of the viral genome. It is well known that, in general, proteins associated with DNA can be displaced by increasing the ionic strength of the millieu. An alternative explanation is that the reduced accumulation of early viral RNA may be due to enhanced degradation, within the nucleus of the interferon treated cell, effected by a ribonuclease specific for viral (but not for most cellular) RNA. Conceivably this putative nuclease might be inactivated at elevated ionic strength, thus resulting in the increased level of SV40 specific RNA found in nuclei incubated with 300 mM KCl in vitro. These two possibilities cannot be distinguished at present. Further investigation of RNA metabolism in such isolated nuclei and further characterization of the viral RNA with respect to the portion of the SV40 genome that is transcribed will be necessary to achieve a better understanding of the nature of the interferon mediated inhibition of virus function. Nevertheless, in the presence of 100 mM KCl, the in vitro system described appears to accurately reflect the behavior of the intact cells. It represents the first in vitro system in which the action of interferon on nuclear functions has been demonstrated.

In addition to their potential use in connection with the elucidation of the mechanism of interferon action, the present findings may also be helpful in providing a criterion for the proper behavior of an in vitro

transcriptional system prepared from eukaryotic cells. Since, in general, the precise nature of the primary RNA transcript made in vivo remains uncertain, the preparation of an in vitro system known to behave correctly is a difficult task. The present demonstration of the apparent maintenance of a regulatory process (the interferon induced inhibition of viral RNA accumulation) in an in vitro transcriptional system under one set of conditions (100 mM KCl) but not under another (300 mM KCl) suggests that the former conditions are the more physiological. In contrast, in the absence of interferon treatment, the mere optimization of the in vitro system for maximum viral RNA synthesis would have led us to choose the latter (presumably less physiological) conditions. The maintenance of the interferon effect during studies designed to fractionate this transcriptional system should provide a useful criterion for its proper behavior.

Acknowledgement. We thank Alan Douglas and Margareta Simpson for skilled assistance. This investigation was supported by Research Grants PCM 76-22342 from the National Science Foundation and AI-13872 from the National Institute of Allergy and Infectious Diseases.

References

1. Metz, D.H. (1975) Cell, 6, 429-440.
2. Oxman, M.N. and Levin, M.J. (1971) Proc. Nat. Acad. Sci. USA, 68, 299-302.
3. Yamamoto, K., Yamaguchi, N. and Oda, K. (1975) Virology, 68, 58-70.
4. Metz, D.H., Levin, M.J. and Oxman, M.N. (1976) J. Gen. Virol., 32, 222-240.
5. Marzluff, W.F., Murphy, E.C. and Huang, R.C. (1973) Biochemistry, 12, 3440-3446.
6. Glisin, V., Crkvenjakov, R. and Byus, C. (1974) Biochemistry, 13, 2633-2637
7. Oxman, M.N., Levine, A.S., Crumpacker, C.S., Levin, M.J., Henry, P.H. and Lewis, A.M. (1971) J. Virol., 8, 215-224.
8. Price, R. and Penman, S. (1972) J. Virol., 9, 621-626.
9. Wallace, R.D. and Kates, J. (1972) J. Virol., 9, 627-635.
10. Weinman, R., Raskas, H. and Roeder, R.G. (1974) Proc. Nat. Acad. Sci. USA, 71, 3426-3430.
11. Alwine, J.C., Steinhart, W.L. and Hill, C.W. (1974) Virology, 60, 302-307.
12. Ben-Zeev, A., Asher, Y. and Becker, Y. (1976) Virology, 71, 302-311.
13. Jackson, A.H. and Sugden, B. (1972) J. Virol., 10, 1086-1089.
14. Gilboa, E. and Aviv, H. (1976) Cell, 7, 567-574.