Mechanism of Interferon Action: Further Evidence for Transcription as the Primary Site of Action in Simian Virus 40 Infection

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SUMMARY

Interferon inhibits the replication of simian virus 40 (SV40) in monkey cells and reduces markedly the formation of both early virus protein (i.e. SV40 T antigen) and early SV40 RNA. This suggests that in SV40 infection interferon acts primarily by inhibiting transcription. To test this conclusion further, we examined alternative mechanisms which might explain these results and made the following observations. (1) The quantity of input SV40 DNA in the nucleus 24 h post infection (p.i.) was the same in interferon-treated and control cells. Thus interferon does not appear to diminish the quantity of SV40 DNA template available for transcription. (2) Chemical inhibitors of protein synthesis did not mimic the selective inhibition of early SV40 RNA formation induced by interferon, indicating that the transcription of early SV40 RNA is not dependent upon the prior synthesis of any virus-induced protein. Thus a block in translation cannot readily explain the reduced formation of early SV40 RNA in interferon-treated cells. (3) Fractionation of SV40 infected cells after a one-hour labelling period showed that interferon produced a comparable reduction in the quantity of early SV40 RNA in the nucleus and the cytoplasm. Thus the observed inhibition of early SV40 RNA is not due solely (if at all) to enhanced cytoplasmic degradation. These results indicate that the primary effect of interferon in SV40 infected monkey cells is either to inhibit the transcription of early virus RNA or to enhance its turnover in the nucleus.

INTRODUCTION

Interferons inhibit the replication of a wide variety of DNA and RNA viruses by mechanisms which remain obscure. With poxviruses, interferon has been shown to act by inhibiting virus protein synthesis (Joklik & Merigan, 1966; Bodo et al. 1972; Jungwirth et al. 1972; Metz & Esteban, 1972; Metz, Esteban & Danielescu, 1975). With certain other viruses, the primary mechanism of interferon action appears to be inhibition of virus RNA synthesis, although the evidence for this is less secure. Thus, in the case of vesicular stomatitis virus (VSV), it has been reported that primary transcription of the virus genome by the virion polymerase is inhibited in interferon-treated cells (Marcus et al. 1971; Manders, Tilles & Huang, 1972). Recently, however, this observation has been both disputed (Repik, Flamand & Bishop, 1974) and reinterpreted in terms of enhanced degradation of the newly synthesized virus RNA in interferon-treated cells (Marcus, Terry & Levine, 1975). Similarly, with influenza virus, evidence that primary virus transcription is inhibited in interferon-treated
cells has been reported (Bean & Simpson, 1973) and subsequently disputed (Repik et al. 1974). With two DNA viruses, frog polyhedral cytoplasmic deoxyribovirus (Gravell & Cromeans, 1972) and vaccinia virus (Bialy & Colby, 1972), interferon treatment has been reported to reduce the amount of virus-specific RNA in infected cells, although this observation could not be confirmed in the case of vaccinia virus (Esteban & Metz, 1973). Finally, interferon inhibits the replication of mouse leukaemia viruses, even though it does not block the synthesis of the group-specific virus antigens, apparently by interfering with some late step(s) in the virus replicative cycle (Billiau, Sobis & De Somer, 1973; Friedman & Ramseur, 1974; P. M. Pitha, W. P. Rowe & M. N. Oxman, unpublished data).

Simian virus 40 (SV40) is another DNA virus which is sensitive to interferon. Synthesis of SV40 T antigen, an early virus protein, is markedly inhibited in interferon-treated cells (Oxman & Black, 1966; Oxman, Rowe & Black, 1967), and there is a comparable reduction in the amount of early SV40 RNA (SV40-specific RNA transcribed from the DNA of input virions in the absence of SV40 DNA replication) detectable during lytic infection (Oxman & Levin, 1970). This suggests that the primary action of interferon is to inhibit transcription of the SV40 genome, but certain other possibilities have not been excluded. Among these are: (1) interferon might affect SV40 replication at a step which precedes the transcription of early SV40 RNA (e.g. it might block the adsorption, penetration or uncoating of virions, or increase the rate at which uncoated SV40 DNA is degraded); (2) interferon might, by inhibiting translation, secondarily inhibit the bulk of early virus RNA synthesis. This would occur, for example, if the product of one of the early SV40 genes was essential for the transcription of the remaining early genes, or if interferon blocked the synthesis of a virus-induced cellular protein required for the efficient transcription of the SV40 genome; (3) interferon might reduce the quantity of early SV40 RNA in infected cells by increasing the rate at which it is degraded rather than by decreasing the rate at which it is transcribed. This might occur as a secondary consequence of an interferon-mediated inhibition of virus messenger RNA (mRNA) translation. For example, in vaccinia virus infection, interferon treatment inhibits the association of virus mRNA with cellular ribosomes and thus blocks the formation of virus polyribosomes (Joklik & Merigan, 1966; Metz et al. 1975). If, as in the case of cellular mRNA (Singer & Penman, 1972), displacement from polyribosomes results in a significant increase in the rate of mRNA degradation, this could lead to a reduction in the net amount of virus RNA present in infected cells.

The results of the experiments reported here provide evidence against these alternative possibilities and suggest, instead, that interferon acts upon a step in SV40 replication which precedes the translation of early SV40 RNA and which takes place in the nucleus of the infected cell.

METHODS

Buffers. Phosphate-buffered saline (PBS): 137 mM-NaCl, 2·7 mM-KCl, 12·2 mM-Na$_2$HPO$_4$, 1·54 mM-KH$_2$PO$_4$, 0·49 mM-MgCl$_2$, 0·9 mM-CaCl$_2$, pH 7·2. Saline-sodium citrate (SSC): 1·5 mM-Na$_2$HPO$_4$, 1·5 mM-MgCl$_2$, 10 mM-tris HCl, pH 7·5. Lysis buffer (LB): 1% SDS, 1 mM-EDTA, 100 mM-NaCl, 0·1% polyvinylsulphate, 10 mM-tris HCl, pH 7·4. High salt buffer (HSB): 500 mM-NaCl, 50 mM-MgCl$_2$, 10 mM-tris HCl, pH 7·4.

Tissue cultures. Three lines of African green monkey kidney cells, BSC-1, Vero, and CV-1, were grown in roller bottles as previously described (Oxman & Levin, 1971) with Dulbecco’s modified Eagle’s medium (DUL) containing penicillin (250 U/ml), streptomycin (250 µg/ml) and 2 mM-glutamine, supplemented with either 2% (DUL-2) or 10% (DUL-10) foetal calf
serum. All cell lines were repeatedly shown to be free of mycoplasmas by anaerobic culture on Hayflick's medium.

**Viruses.** SV40 (strain 777) was propagated in BSC-1 or CV-1 cells by low multiplicity (10⁻⁵ p.f.u./cell) infection as previously described (Oxman, Levin & Lewis, 1974). The resulting virus pools (10⁶ to 10⁷ p.f.u./ml in CV-1 cells) were stored at -70 °C. The Indiana strain of VSV was propagated in BSC-1 cells.

**Virus purification.** SV40 virions were precipitated with polyethylene glycol in the presence of 0.5 M-NaCl, eluted from the pellet as described by Friedman & Haas (1970), and enzyme treated (Oxman et al. 1971). Virus was then centrifuged into a cushion of CsCl (density = 1.40 g/ml) and purified by two cycles of equilibrium density gradient centrifugation in CsCl (density = 1.34 g/ml).

**Interferon.** Sendai virus induced human leukocyte interferon (Strander & Cantell, 1967), partially purified by selective precipitation, was the generous gift of Dr K. Cantell (State Serum Institute, Helsinki). The preparation employed contained 2 × 10⁶ 'reference' units/mg protein (assayed in parallel with the Research Standard of human interferon, code 69/19; National Institute for Biological Standards and Control, Hampstead, London). This preparation of human interferon was less active in the Vero (monkey) cells employed in our experiments than in diploid human cells. Consequently, a unit of interferon is defined in this paper as the amount required to produce a 50 % reduction in VSV plaque formation in the Vero cells employed. This amount, which corresponds to approx. 25 'reference' units, also reduced by 50 % the proportion of Vero cells in monolayer cultures which exhibited T antigen following SV40 infection.

**SV40 T antigen.** This was assayed by an indirect immunofluorescence procedure as previously described (Pope & Rowe, 1964).

**DNA extraction.** Form I SV40 virus DNA was used for RNA–DNA hybridization experiments, and as template for the in vitro synthesis of SV40 complementary RNA (SV40 cRNA). It was extracted from purified virions, further purified by two cycles of equilibrium density gradient centrifugation in CsCl-ethidium bromide as previously described (Oxman et al. 1974) and stored at -70 °C in 0.01 × SSC. This SV40 DNA was shown to be free of detectable host cell (monkey) nucleotide sequences by sensitive hybridization assays (Oxman et al. 1971). Escherichia coli and Vero cell DNA were extracted by the method of Marmur (1961), except that when DNA was extracted from interferon-treated and control cells infected with SV40 and from fractions thereof, the procedure for spooling DNA was omitted. DNA concentrations were determined by a modified diphenylamine reaction (Burton, 1956) with calf thymus DNA (Calbiochem) as a standard.

**Synthesis of SV40 complementary RNA.** Radioactive RNA complementary to purified Form I SV40 DNA (³H-SV40 cRNA) was synthesized in vitro as previously described (Levine et al. 1970).

**SV40 DNA in the nuclei of interferon-treated and control cells following SV40 infection.** Confluent roller bottle cultures of Vero cells were treated for 18 h with DUL-2 medium containing interferon or with DUL-2 alone, and then washed and infected with SV40 at a multiplicity of 50 to 100 p.f.u./cell in the presence of 30 μg/ml of cytosine arabinoside (CA). After 4 h, the cultures were washed 3 times with serum-free medium to remove unadsorbed virus and refed with DUL-2 containing interferon or with DUL-2 alone. CA was added to all cultures to a final concentration of 30 μg/ml. Twenty-four hours p.i., the cultures were washed and the cells were trypsinized, washed again and resuspended in IB for subsequent fractionation, DNA extraction, and hybridization with ³H-SV40 RNA.

The effect of inhibitors of protein synthesis upon the synthesis of early SV40 RNA. Early
\( ^3 \text{H-SV} 40 \) RNA was prepared by infecting confluent roller bottle cultures of Vero cells with 30 to 100 p.f.u. of SV40 per cell in the presence of 30 \( \mu \text{g/ml} \) of CA as previously described (Oxman et al. 1971). Inhibitors of protein synthesis (cycloheximide, 7.5 to 15 \( \mu \text{g/ml} \); \( p \)-fluorophenylalanine, 25 \( \mu \text{g/ml} \)) were added 1 h before or at the time of virus infection. At 4 or 18 h p.i., \( ^5 \text{H-uridine} \) (New England Nuclear Corp., 25 to 30 Ci/mmol) was added to a final concentration of 200 \( \mu \text{Ci/ml} \). To monitor protein synthesis, \( ^{14} \text{C}-\text{amino acids} \) (leucine, isoleucine, valine and phenylalanine; New England Nuclear Corp., 200 to 400 mCi/mmol; each at a final concentration of 0.1 \( \mu \text{Ci/ml} \)) were added at the time of infection and were present throughout the experiment. Cells were harvested 20 to 26 h p.i.; the cultures were drained, washed twice with serum-free medium, and the cells detached with trypsin and centrifuged (300 g for 10 min at 4 °C). The cells were resuspended in cold 0.85% saline and a 5% sample removed for determination of the incorporation of \( ^{14} \text{C}-\text{amino acids} \) into protein. The remaining cells were recentrifuged and their RNA extracted.

**Measurement of protein synthesis.** Proteins in harvested cells were separated from nucleic acids by a modification of the method of Schneider (1945). The cells were precipitated with cold 10% trichloroacetic acid (TCA). The precipitate was washed twice with cold 5% TCA, incubated for 15 min at 90 °C in 5% TCA, and centrifuged again. The resulting pellet was washed twice with cold 5% TCA and dissolved in 1 ml of 1 M-NaOH, and the \( ^{14} \text{C}-\text{radioactivity} \) was determined by scintillation spectrometry. The \( ^{14} \text{C}-\text{ct/min} \) present, minus the \( ^{14} \text{C}-\text{ct/min} \) in comparable samples from cells to which the \( ^{14} \text{C}-\text{amino acids} \) were added at the moment of harvesting, was taken as a measure of overall protein synthesis. Determination of total protein was by the method of Lowry et al. (1951).

**Effect of interferon upon the synthesis of early SV40 RNA.** Confluent monolayer cultures of Vero cells in glass roller bottles were incubated with interferon in DUL-2 or with DUL-2 alone for 18 h at 37 °C. The medium was then decanted (and retained) and the cultures infected with SV40 at a multiplicity of 50 to 100 p.f.u./cell in 10 ml DUL-2 containing 30 \( \mu \text{g/ml} \) of CA. The cultures were incubated for 4 h at 37 °C, and then the corresponding previously decanted medium, with or without interferon as appropriate, was returned and CA added to a final concentration of 30 \( \mu \text{g/ml} \). At the beginning of the labelling period, this medium was discarded and replaced with 100 or 12.5 ml of pre-warmed DUL-2 containing 30 \( \mu \text{g per ml} \) of CA and 5 mCi of \( ^{5} \text{H-uridine} \). In some experiments \( ^{14} \text{C}-\text{amino acids} \) (leucine, isoleucine, phenylalanine and valine; 5 \( \mu \text{Ci each} \)) were also present in the labelling mixture. The addition of medium with radioactive label was performed in the warm room in all experiments involving short labelling periods.

When the labelling period was 3 h or longer, the cultures were washed with PBS and the cells harvested with trypsin, centrifuged (450 g at 4 °C for 5 min), washed with 30 ml of ice cold IB, recentrifuged, and resuspended in 20 ml of ice-cold IB. All subsequent steps were performed at 4 °C with ice-cold reagents. When the labelling period was 1 h, the bottles were cooled rapidly in ice water and the cell monolayers were rinsed with 50 ml of ice-cold PBS and then with 20 ml of ice-cold IB. The washed cells were scraped into 30 ml of IB, centrifuged (450 g for 5 min) and resuspended in 20 ml of IB. One ml samples were taken for measurement of total protein content and \( ^{14} \text{C}-\text{amino acid incorporation} \), and small samples of the harvested cells were allowed to attach to glass coverslips in the continued presence of CA for subsequent assay of SV40 T antigen. The remaining cells were recentrifuged for RNA extraction or cell fractionation.

**Preparation of cell fractions.** Washed cell pellets from each roller bottle culture were immediately resuspended in 3 ml of IB with 0.15 ml of 10% Triton X-100, and clumps were dispersed with 5 strokes in a Dounce homogenizer. The cell suspension was held at 0 °C
until 90% or more of the cells were lysed as determined by phase contrast microscopy (5 to 10 min) and then centrifuged (800 g at 4°C for 5 min). The supernatant fluid was retained as the 'cytoplasmic fraction'. The pellet was resuspended in 2 ml of IB to which was added 0.2 ml of 10% Triton X-100 (in some experiments 0.1 ml of 10% sodium deoxycholate was also added). The mixture was agitated on a vortex mixer for 5 s and then layered onto 5 ml of 20% glycerol in IB. After centrifugation (800 g at 4°C for 10 min) in a swinging-bucket rotor, the layer on top of the glycerol was removed and added to the previously retained 'cytoplasmic fraction'. The glycerol layer was discarded and the pellet of washed nuclei retained.

**RNA extraction.** In early experiments RNA was extracted from whole infected cells by a hot phenol-SDS procedure (Levin et al. 1969). For later experiments, including those involving cell fractionation, a modified phenol-chloroform-isoamyl alcohol procedure was utilized (Penman, 1969; Weinberg, Warnaar & Winocour, 1972). In this procedure whole cell pellets were resuspended in 10 ml of 10% IB. Cytoplasmic fractions were processed by adding an equal vol. of 2 x LB and sufficient LB to bring the total vol. to 10 ml. Washed nuclei were resuspended in 1 ml of HSB and digested with 40 μg of deoxyribonuclease (Worthington; electrophoretically purified) at 37°C until the viscosity had dropped markedly (3 to 5 min). One mg of wheat germ RNA carrier was then added to the nuclear extract, which was brought to a vol. of 10 ml by the addition of 0.5 ml of 0.1 M-EDTA, 1.5 ml of 2 x LB, and 7 ml of LB. Nuclear, cytoplasmic and whole cell RNA were then extracted by the addition of 10 ml of pre-warmed, water-saturated phenol-m-cresol (7:9:1) containing 1 mg of 8-hydroxyquinoline/ml and 10 ml of chloroform-isoamyl alcohol (99:1) to each 10 ml of extract. The mixture was vigorously shaken in a 60°C water bath for 2 min, cooled in ice, and centrifuged (300 g at 4°C for 5 min). The lower phase was discarded and the combined upper phase and interface re-extracted at 60°C. The lower phase was again discarded and the remainder re-extracted with 20 ml of chloroform-isoamyl alcohol (99:1) at room temperature. The upper (aqueous) phase was retained, combined with an equal vol. of 4 M-LiCl, and held overnight at 4°C. The RNA was then collected by centrifugation (20000 g for 30 min at 4°C), and redissolved in 5 ml of 10 mM-tris HCl-7.5 mM-MgCl₂, pH 7.4, containing 200 μg of deoxyribonuclease. After incubation at 37°C for 15 min, 100 μg of pronase (pre-digested at 37°C for 2 h) was added and the incubation continued for 10 min. The reaction was halted by the addition of 0.5 ml of 1% SDS, 0.2 ml of 0.1 M-EDTA, 1 ml of 5 M-NaCl and 5 ml of tris-HCl, pH 7.4. This solution was then extracted once with phenol-m-cresol and chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol alone. The aqueous phase was then removed, combined with 2 vol. of ethanol, held at -20°C overnight, and centrifuged (800 g for 20 min at 4°C). The RNA pellet was dissolved in 2 x SSC plus 0.05% SDS, a sample removed for the measurement of TCA-insoluble radioactivity, and the remainder stored at -70°C. RNA concentrations were determined by an orcinol reaction (Brown, 1946) using yeast soluble RNA (Calbiochem) as a standard.

**RNA-DNA hybridization.** ³H-RNA was hybridized with single-stranded SV40 DNA immobilized on nitrocellulose membrane filters by the method of Gillespie & Spiegelman (1965) as modified by Levin et al. (1969). Reactions were performed for 18 to 20 h at 60°C with 13 mm filters in a volume of 0.25 ml of 2 x SSC with 0.05% SDS. The amount of DNA on the filters was in sufficient excess so that the radioactivity bound was proportional to the amount of RNA present in the reaction mixture. All RNA-DNA hybrids were washed, treated with pancreatic ribonuclease (type XII-A, Sigma; 20 μg/ml) and ribonuclease T₁ (B grade, Calbiochem; 10 units/ml) for 1 h at room temperature, and washed again.
Hybridization of cellular $^3$H-RNA with an excess of Vero cell DNA was performed in liquid by the method of Nygaard & Hall (1963) in 100 µl volumes at 67 °C for 20 h. All hybrids were trapped by filtration on Millipore filters, washed extensively, and treated with ribonuclease as described (Levine et al. 1970). The same method, but with an incubation temperature of 70 °C, was used for the hybridization of $^3$H-SV40 cRNA with the DNA extracted from the nuclei of interferon-treated and control cells which had been infected with SV40. SV40-specific or host cell–specific radioactivity is defined as the radioactivity bound to SV40 or host cell DNA minus the radioactivity bound (non-specifically) to E. coli DNA.

RESULTS

The effect of interferon on the early stages of SV40 infection

In initial experiments (data not shown) we attempted to determine the effect of interferon on the adsorption, penetration and uncoating of SV40 virions directly, by infecting Vero cells with purified virus which had its DNA radiolabelled with $^3$H- or $^{14}$C-thymidine. However, despite the use of 3 to 5 replicate cultures for each point, the inclusion of CA, the oxidation of samples prior to scintillation counting, and the use of virus purified by two different techniques, the proportion of the inoculated virus adsorbed to the cells and the distribution of acid-precipitable radioactivity among the various cell fractions varied markedly from experiment to experiment. From 4 to 21% of the TCA-precipitable radioactivity in the inoculum was cell-associated by 4 h p.i., and there was little change during the subsequent 20 h. However, the proportion of the cell-associated radioactivity that was in the nucleus increased with time, so that by 24 h p.i. it comprised an average of 37% of the total. Moreover, at 24 h p.i. approx. 90% of the TCA-precipitable radioactivity in the nuclear fractions was DNase-sensitive. In spite of the variability from experiment to experiment, there was no consistent difference between the results obtained in interferon-treated and control cultures. In particular, in the interferon-treated cultures at 24 h p.i. (at which time the amount of early SV40 RNA was decreased by 90 to 95%) there was no consistent reduction in either the total cell-associated TCA-precipitable radioactivity or the amount of DNase-sensitive TCA-precipitable radioactivity (corresponding to uncoated SV40 DNA) present in the nucleus.

To further test the possibility that interferon might act on an early stage of SV40 infection, we infected interferon-treated and control cells with unlabelled SV40, extracted the total DNA from their isolated nuclei and, by hybridizing this DNA with radiolabelled SV40 cRNA, assessed the relative amount of SV40 DNA that was present. These experiments did not reveal a consistent or significant effect of interferon on the content of SV40 DNA in the nucleus at 24 h p.i. (Table 1). Although the first experiment was not performed with a saturating amount of SV40 cRNA, the same result was obtained in the second experiment in which the ratio of SV40 cRNA to nuclear DNA was varied over a 30-fold range.

Taken together, these results indicate that the observed reduction in the quantity of early SV40 RNA in interferon-treated cells is not due to: (1) any inhibition of the entry of SV40 into the cell or of its subsequent transport to and uncoating in the nucleus, or (2) to any increase in the rate of degradation of uncoated SV40 DNA template in the nucleus.

Attempts to mimic the effect of interferon with inhibitors of protein synthesis

The reduction of early SV40 RNA observed in interferon-treated cells could be secondary to an inhibitory effect on the translation of one species of early SV40 RNA if the product of this is essential for the transcription of the remaining early SV40 genes. Alternatively,
Table 1. Relative amounts of SV40 DNA in the nuclei of interferon-treated and control cells 24 h after infection

<table>
<thead>
<tr>
<th>Tritiated SV40 complementary RNA</th>
<th>Amount hybridized* (ct/min/100 μg nuclear DNA)</th>
<th>Interferon-treated cells†</th>
<th>Control cells</th>
<th>Significance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interferon DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.22</td>
<td>1,151 ± 29§</td>
<td>927 ± 29</td>
<td>0.001 &lt; P &lt; 0.02</td>
</tr>
<tr>
<td>2 A</td>
<td>1.56</td>
<td>9,801 ± 589</td>
<td>9,557 ± 772</td>
<td>0.1 &lt; P &lt; 0.4</td>
</tr>
<tr>
<td>2 B</td>
<td>2.31</td>
<td>26,049 ± 7,347</td>
<td>20,405 ± 2,604</td>
<td>P &gt; 0.4</td>
</tr>
<tr>
<td>2 C</td>
<td>11.5</td>
<td>45,536 ± 6,759</td>
<td>49,166 ± 15,442</td>
<td>P &gt; 0.4</td>
</tr>
<tr>
<td>2 D</td>
<td>49.9</td>
<td>37,060 ± 16,035</td>
<td>46,767 ± 24,604</td>
<td>P &gt; 0.4</td>
</tr>
</tbody>
</table>

* Samples of nuclear DNA were hybridized with 3H-SV40 cRNA (4.4 x 10^7 ct/min/μg) in a volume of 100 μl at 70 °C for 20 h as described in Methods. Each value represents the mean of triplicate determinations. The DNA in Expt. 1 was prepared from samples of washed nuclei from Expt. 3, Table 4.  
† 200 units/ml.  
‡ Probabilities are calculated using the t test for small samples (and a table of the distribution of t for certain probability levels; Snedecor & Cochran, 1973).  
§ Standard deviation.

...interferon might block the synthesis of a virus-induced cellular protein required for the efficient transcription of the SV40 genome. In either case, it should be possible to mimic the effect of interferon by inhibiting protein synthesis. We therefore examined the effect of cycloheximide, or of a mixture of cycloheximide and p-fluorophenylalanine, on the incorporation of 3H-uridine into early SV40 RNA. Inhibition of protein synthesis in these experiments was monitored by measuring the incorporation of radiolabelled amino acids into protein. The effect of the inhibitors on cellular RNA synthesis was determined by measuring the incorporation of 3H-uridine into total cell RNA and into that fraction of cellular RNA which hybridized rapidly with cellular DNA. It should be emphasized that these and all subsequent experiments (see below) were performed in the presence of CA, an efficient inhibitor of SV40 DNA replication in Vero cells, to ensure that the SV40 RNA which was synthesized was transcribed only from the early region of input virus genomes (Oxman et al. 1971). While inhibitors of DNA synthesis might conceivably alter the transcription or processing of papovavirus RNA (Kamen et al. 1974; Beard, Acheson & Maxwell, 1976), we have shown that in Vero cells this concentration of CA effectively blocks both SV40 DNA and late RNA synthesis without preventing the formation of SV40 early RNA and T antigen (Oxman et al. 1971). Moreover, the presence of CA does not interfere with the inhibition of SV40 early RNA and T antigen formation produced by interferon treatment (Oxman & Levin, 1971).

The inhibition of protein synthesis observed in these experiments varied from 86% to 95% (Table 2) and was consistently associated with a decrease in overall RNA synthesis. The amounts of 3H-early SV40 and 3H-host cell RNA per culture, and thus the ratio of SV40 RNA to host cell RNA, varied markedly from experiment to experiment due to the independent variation of factors affecting their synthesis. However, there was no selective inhibition of SV40 early RNA synthesis, and consequently in no individual experiment was there a significant reduction in the ratio of SV40 RNA to host cell RNA. In marked contrast, interferon greatly reduces the synthesis of SV40 early RNA without affecting cellular RNA or protein synthesis and, therefore, significantly reduces the ratio of SV40 RNA to host cell RNA.
Table 2. Effect of inhibitors on the synthesis of early SV40 RNA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibitor*</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition of 14C-amino acid incorporation (%)</th>
<th>Total ct/min (× 10^-4)†</th>
<th>SV40-specific ct/min (× 10^-4)§</th>
<th>Host-specific ct/min (× 10^-4)§</th>
<th>Ratio SV40 to host</th>
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<tr>
<td>Vero‡</td>
<td>CHX</td>
<td>7.5</td>
<td>92</td>
<td>26.5</td>
<td>460</td>
<td>200</td>
<td>23.0</td>
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<td>49.5</td>
<td>1298</td>
<td>46.5</td>
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<td>88.1</td>
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<tr>
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<td>CHX</td>
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<td>612</td>
<td>144.0</td>
<td>5.4</td>
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<tr>
<td></td>
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<td>200.0</td>
<td>913</td>
<td>129.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Vero</td>
<td>CHX</td>
<td>8.0</td>
<td>93</td>
<td>57.2</td>
<td>1297.1</td>
<td>63.4</td>
<td>205.0</td>
</tr>
<tr>
<td></td>
<td>+ FPA</td>
<td>25.0</td>
<td>—</td>
<td>94.3</td>
<td>10810</td>
<td>75.2</td>
<td>144.0</td>
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<tr>
<td>Vero</td>
<td>Interferon</td>
<td>50 units/ml</td>
<td>—</td>
<td>93.4</td>
<td>39</td>
<td>83.0</td>
<td>0.47</td>
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<td></td>
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<td>—</td>
<td>89.8</td>
<td>527</td>
<td>76.8</td>
<td>6.9</td>
</tr>
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</table>

* Inhibitors of protein synthesis (cycloheximide = CHX; p-fluorophenylalanine = FPA) were added 1 h before or at the time of infection. Each number represents the mean of values from duplicate roller bottle cultures. A previous experiment with interferon (added 18 h before infection) is included for comparison. The concentration of 3H-uridine in this experiment was only 100 µCi/ml.
† TCA-precipitable ct/min/roller bottle.
‡ In the first experiment, amino acid incorporation was measured in parallel cultures. In all subsequent experiments, amino acid incorporation, and SV40 and cellular RNA synthesis were measured in the same cultures.
§ Determined by RNA–DNA hybridization as described in Methods. The RNA extracted from each roller bottle culture was hybridized in duplicate or triplicate. Each number represents the mean of values from duplicate cultures.

The effect of variation in the length of the labelling period on the interferon-mediated reduction in SV40 early RNA in infected Vero cells

In previous experiments (Oxman & Levin, 1971), the amount of radiolabelled early SV40 RNA in infected Vero cells was determined after a relatively long (12 to 18 h) labelling period. Since the observed reduction in this RNA in interferon-treated cells might be due to its accelerated degradation, we examined the effect of labelling for shorter periods on the magnitude of the interferon-mediated reduction. The results of these experiments (summarized in Table 3) show that the extent of reduction in radiolabelled early SV40 RNA is independent of the duration of the labelling period over the range of 1 to 24 h.

The effect of interferon treatment on the distribution of SV40 early RNA between the nucleus and the cytoplasm

If there is less SV40 early RNA in interferon-treated cells primarily because of enhanced turnover of this RNA in the cytoplasm (and not because its synthesis is inhibited), there should be no alteration in the amount of SV40 early RNA present in the nucleus. We therefore labelled interferon-treated and control cells with 5-3H-uridine at 27 h after SV40 infection, harvested these cells 1 h later, and prepared nuclear and cytoplasmic fractions. The RNA extracted from these fractions was assayed for its content of 3H-SV40 RNA by hybridization with an excess of single-stranded SV40 DNA immobilized on nitrocellulose filters.
Table 3. The effect of variation in the length of the labelling period on the interferon-mediated reduction in early SV40 RNA in Vero cells

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Interferon period* (h)</th>
<th>SV40-specific RNA (ct/min)†</th>
<th>Cells with SV40 T antigen (%)</th>
<th>Inhibition(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85 4-28</td>
<td>1090</td>
<td>6</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>0 4-28</td>
<td>11700</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>85 25-28</td>
<td>1425</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0 25-28</td>
<td>9765</td>
<td>71</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>85 27-28</td>
<td>155</td>
<td>ND</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>0 27-28</td>
<td>2745</td>
<td>ND</td>
<td>—</td>
</tr>
</tbody>
</table>

* Labelled with 3H-uridine; the interval refers to hours p.i.
† Total SV40-specific RNA ct/min/roller bottle culture, i.e. 3H-RNA hybridizable to SV40 DNA minus 3H-RNA hybridizable to E. coli DNA. The average total E. coli DNA blank was: Expt. 1, 520 ct/min (s.e. 90); Expt. 2, 230 ct/min (s.e. 231).

Pre-treatment with interferon produced a comparable reduction in the quantity of 3H-early SV40 RNA in both the nucleus and cytoplasm (Table 4). In contrast, there was no reduction in the incorporation of 3H-uridine into total RNA in either cell fraction. In Expt. 3, the quantity of 3H-RNA hybridizable to cellular DNA was also determined in order to provide an internal control for possible non-specific effects of the interferon preparation used (e.g. on uptake of 3H-uridine or precursor pool size) and for variations in the efficiency of the RNA extraction procedure. The results (Table 4) also show that there was no reduction in the quantity of 3H-RNA hybridizable to Vero cell DNA in either the nuclei or the cytoplasm of the interferon-treated cells. Thus interferon treatment did not produce any general inhibition of cellular RNA synthesis or reduce the overall incorporation of 3H-uridine into RNA. Moreover, interferon treatment did not affect the total protein content of the cells or reduce the incorporation of 14C-amino acids into protein: in Expt. 3, the cells were also labelled with 14C-amino acids and the total protein content and the incorporation of 14C-amino acids into TCA-insoluble material was measured. The total protein per culture was 2.6 mg in both the treated and control cultures. The mean total TCA-insoluble 14C-ct/min were 125000 and 121000 in two cultures treated with interferon and 127000 and 111000 in two control cultures. Thus, the inhibitory effect of interferon on the formation of 3H-early SV40 RNA was highly selective, and was not associated with any general inhibition of cellular macromolecular synthesis.

**DISCUSSION**

Pre-treatment with interferon markedly reduces the amount of SV40 early RNA which can be detected in monkey cells infected with SV40. Although this observation is consistent with direct inhibition of the transcription of early SV40 RNA, several other possible mechanisms have been proposed (see Introduction). Our experiments examined the possibility that one or more of these alternate explanations might, indeed, be correct.

The nuclei of interferon-treated and control cells were found to contain comparable amounts of hybridizable SV40 DNA at 24 h p.i. (Table 1). Since at that time most of the SV40 DNA in the nuclei of both interferon-treated and control cells is sensitive to deoxyribonuclease, this suggests that the interferon-induced inhibition of SV40 replication is
The amounts of early SV40 RNA in the nucleus and cytoplasm of interferon-treated and control Vero cells 27 h after infection

<table>
<thead>
<tr>
<th>Interferon (units/ml)</th>
<th>Cell fraction</th>
<th>Total per roller bottle*</th>
<th>% of input RNA hybridizing with:</th>
<th>Ratio inhibition of early SV40 RNA±</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3H-RNA specific SV40 RNA (ct/min)</td>
<td>SV40 DNA (ct/min)</td>
<td>Host DNA (ct/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(x 10^4)</td>
<td>(x 10^4)</td>
<td></td>
</tr>
<tr>
<td>1 85</td>
<td>Nuclear</td>
<td>105</td>
<td>60</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>9.7</td>
<td>85</td>
<td>9.8</td>
</tr>
<tr>
<td>2 70</td>
<td>Nuclear</td>
<td>75</td>
<td>205</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>25</td>
<td>755</td>
<td>30.2</td>
</tr>
<tr>
<td>3 140</td>
<td>Nuclear</td>
<td>65 (3)</td>
<td>255 (55)</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>3.7 (0.6)</td>
<td>270 (15)</td>
<td>73.0</td>
</tr>
</tbody>
</table>

* Expt. 1, cells from 1 roller bottle; Expt. 2, pooled cells from 2 roller bottles; Expt. 3, 2 roller bottle cultures handled separately (figures in parentheses are the standard deviations).

† 3H-RNA hybridizable to SV40 DNA minus 3H-RNA hybridizable to E. coli DNA. Each sample was hybridized in duplicate or triplicate. The average total E. coli DNA blank was: Expt. 1, 121 ct/min (s.e. 17); Expt. 2, 242 ct/min (s.e. 57); Expt. 3 nuclear fraction, 145 ct/min (s.e. 11); Expt. 3 cytoplasmic fraction, 51 ct/min (s.e. 14).

‡ Percentage of input 3H-RNA hybridizable to Vero cell DNA minus percentage of input 3H-RNA hybridizable to E. coli DNA. Each sample was hybridized in triplicate. The average E. coli DNA blank was 0.06 %.

§ $\frac{\text{SV40-specific ct/min}}{\text{Total ct/min}}_{\text{control}} - \frac{\text{SV40-specific ct/min}}{\text{Total ct/min}}_{\text{interferon}}$

∥ ND = not done.

The failure of inhibitors of protein synthesis to inhibit selectively the production of early SV40 RNA (Table 2) indicates that transcription of the early region of the SV40 genome does not require the synthesis of new protein. Thus it is unlikely that the selective inhibition of early SV40 RNA synthesis observed in interferon-treated cells is the indirect result of independent of any effect on the adsorption, penetration, or uncoating of SV40 virions. It also argues against any significant increase in the rate at which uncoated SV40 DNA is degraded in interferon-treated cells. Yamamoto, Yamaguchi & Oda (1975) have similarly concluded that pre-treatment with interferon does not significantly affect the incorporation of SV40 DNA into the nuclei of monkey cells infected with radiolabelled virions. The large inoculum of virus employed, and the fact that SV40 stocks invariably have particle-to-infectivity ratios greatly in excess of one, make it impossible to conclude that the fate of the majority of adsorbed virions, as determined in experiments such as those described here, accurately reflects the fate of that minority which actually initiate infection. Nevertheless, the results of these experiments do not reveal any general effect of interferon on the adsorption, penetration or uncoating of SV40 virions, or on the stability of uncoated SV40 DNA, a small portion of which presumably serves as the template from which early SV40 RNA is transcribed.
Interferon action in SV40 infection

inhibition of the translation of the messenger RNA transcribed from a particular early SV40 gene (e.g. a hypothetical proto-early' gene), the product of which is required for the transcription of the remaining early SV40 genes. This result is also inconsistent with the possibility that interferon might act by blocking the synthesis of a virus-induced cellular protein required for the efficient transcription of the SV40 genome.

Taken together, these observations imply that the reduced content of early SV40 RNA observed in interferon-treated cells must result either from primary inhibition of early virus RNA transcription or from accelerated degradation of early SV40 RNA.

The effect of interferon on the amount of radiolabelled early SV40 RNA in the nucleus and the cytoplasm following a 1 h labelling period was examined to assess the possibility that the observed reduction in total early SV40 RNA content might be the indirect result of enhanced degradation in the cytoplasm (possibly as a consequence of inhibition of SV40 RNA translation) rather than the direct result of diminished transcription. The finding that in interferon-treated cells the amount of early SV40 RNA is reduced to the same extent in the nucleus as in the cytoplasm (Table 4) indicates that the observed inhibition is not due solely (if at all) to enhanced cytoplasmic degradation. The invariance of this reduction with the length of the labelling period (Table 3) is consistent with this conclusion. Our results do not, however, exclude enhanced degradation of early SV40 RNA in the nucleus of the interferon-treated cell, a reasonable possibility in view of the substantial and rapid intranuclear turnover of heterogeneous nuclear RNA (Darnell, Jelinek & Molloy, 1973) and of certain SV40 RNA sequences (Aloni, 1972). Unfortunately, the low level of SV40-specific RNA synthesis occurring in interferon-treated cells has precluded the use of labelling periods of less than an hour, which would be necessary to explore this alternative.

The possibility that interferon may affect the processing of SV40 transcripts within the nucleus thus remains to be investigated. However, the decrease in early SV40 RNA in the nuclei of interferon-treated cells (Table 4) and the failure of inhibition of translation with cycloheximide to mimic the selective reduction of early SV40 RNA induced by interferon (Table 2) make it difficult to attribute the selective reduction in the early SV40 RNA content of interferon-treated cells to inhibition by interferon of the translation of that SV40 RNA. Instead, these observations indicate that interferon acts at an earlier step in the SV40 replicative cycle. Although Yamamoto et al. (1975) did not employ short labelling periods or separately analyse nuclear and cytoplasmic fractions, they also demonstrated that interferon reduced the content of early virus RNA in SV40-infected monkey cells and reached the same conclusion. The failure of interferon to exert any apparent effect on the adsorption, penetration, intracellular transport or uncoating of SV40 virions, or to enhance the degradation of uncoated SV40 DNA, leads us to conclude that the primary effect of interferon in SV40 infected monkey cells is to inhibit the synthesis of early virus RNA or to enhance its turnover in the nucleus.

It is important to recognize that in several other virus-cell systems (see Metz, 1975, for a review), inhibition of translation appears to be the primary mechanism of interferon's action. Thus, our conclusion that the reduction in the virus RNA content of interferon-treated SV40 infected cells is not due to inhibition of translation provides further evidence that there are at least two distinct mechanisms by which interferon may limit the replication of viruses. Graessman et al. (1974) observed that interferon inhibited SV40 T antigen formation when cells were micro-injected with SV40 cRNA. This procedure, which would circumvent a block in transcription, appears to have revealed the activity of an inhibitor of translation in the SV40-monkey cell system. Thus interferon treatment may induce the synthesis of two (or more) distinct intracellular inhibitors ('antivirus proteins') which inhibit,
respectively, the transcription and the translation of virus genetic information. Although both may be present in interferon-treated cells, the one which is manifest in a given experimental system may depend on whether transcription or translation is the primary event in the replication of the particular virus, and upon the accessibility of the virus nucleic acid. In the case of vaccinia virus, for example, transcription of early virus RNA is the primary event, but this occurs inside the virus core structure and may, therefore, be inaccessible to a transcription-inhibiting antivirus protein. The failure of actinomycin D to penetrate the vaccinia virus core structure and inhibit the transcription of early vaccinia RNA in mouse L cells (Metz & Esteban, 1972) is consistent with this model.

An alternative possibility is that inhibition of transcription and translation are mediated by a single interferon-induced antivirus protein. This protein may be able to distinguish virus from cellular nucleotide sequences in both DNA and RNA and, by binding tightly to the virus sequences, block their function as templates for transcription or translation. To distinguish between these two possibilities, it will be necessary to employ in vitro systems which display interferon-mediated inhibition of virus transcription and translation. It should then be possible to isolate the inhibitor from one system (e.g. Samuel & Joklik, 1974) and test it in the other.

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Interferon action in SV40 infection

239


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