Interferon Action III. The Rate of Primary Transcription of Vesicular Stomatitis Virus is Inhibited by Interferon Action

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(Accepted 20 August 1977)

SUMMARY

Transcription of vesicular stomatitis virus (VSV) in Vero cells was confined to the synthesis of parentally-derived mRNA (primary transcription) by the use of cycloheximide and/or a ts mutant, G41(IV), at a non-permissive temperature (40 °C). More transcripts accumulated in the presence of cycloheximide than in its absence. This so-called ‘cycloheximide effect’ results from higher rates of virus transcription sustained for longer periods of time. The rate of VSV transcription initially increases linearly for 1 to 2 h after infection. Interferon reduces this rate (≈ fourfold with 50 units/ml interferon) irrespective of the presence or absence of cycloheximide. The VSV mRNA transcripts synthesized in mock- or interferon-treated cells were equal in size and had an equivalent half-life of 17 h at 40 °C. It seems likely that once transcription is initiated in interferon-treated cells, it is completed successfully.

Since interferon reduces the rate of early VSV primary transcript synthesis to below that achieved in the presence of cycloheximide, we conclude that interferon has an effect on transcription beyond that attributable solely to protein synthesis inhibition. We postulate that interferon decreases the probability of initiating virus transcription. Virus mRNA escaping this facet of interferon action may then encounter other facets such as post-transcriptional modification and/or inhibition of translation. However, the mandatory sequence of primary transcription → primary translation for negative-strand viruses like VSV dictates that the overall inhibitory effect of interferon on translation would derive in part from this prior inhibition of transcription. Thus, to apply the term ‘primary effect’ to one particular facet of interferon action may not always be meaningful.

INTRODUCTION

Following the discovery of a virion-associated transcriptase in vesicular stomatitis virus (VSV) and its demonstration in vitro by Baltimore, Huang & Stampfer (1970), we described an assay for the in vivo accumulation of transcription products in which inhibitors of protein synthesis, such as cycloheximide, were used to restrict transcripts to those originating from the parental genome, i.e. primary transcripts (Marcus et al. 1971). We demonstrated that chick embryo cells manifesting interferon-mediated interference accumulated, in a dose-dependent manner, fewer virus transcripts than mock-treated cells also infected in the presence of cycloheximide. These observations were confirmed and extended by Manders, Tilles & Huang (1972) using VSV in human muscle skin fibroblasts.
Again with VSV, Repik, Flamand & Bishop (1974; their Fig. 1D) observed a significant decline in the rate of virus transcript accumulation in mouse L cells treated with interferon, as did Baxt, Sonnabend & Bablanian (1977, see their Fig. 9) in LLC-MK2 monkey cells and U human amnion cells. Results similar to our earlier study with chick embryo cells also have been obtained by P. B. Sehgal and I. Tamm in the FS-4 line of human cells and by H. R. Thacore in human tonsil cells (personal communications). Thus, in four different cell species and four separate laboratories, interferon action was shown to have an adverse effect on the accumulation of VSV-derived transcripts in vivo as measured in the presence of cycloheximide. However, the use of protein synthesis inhibitors, while successful in isolating primary transcription from subsequent replicative or transcriptive events, might have introduced complications. For example, several studies have reported aberrations in the regulation of virus transcription in different virus-cell systems under conditions where translation of virus (or cellular) mRNA was adversely affected (Kates & McAuslan, 1967; Watanabe, Kudo & Graham, 1967; Esteban & Metz, 1973; Wertz & Levine, 1973; Kiley & Payne, 1974; Woodson, 1976). Furthermore, cycloheximide and other inhibitors of protein synthesis enhance VSV transcription in vivo under certain conditions—the so-called ‘cycloheximide effect’ (Wong, Holloway & Cormack, 1972; Perlman & Huang, 1973; Wertz & Levine, 1973; Unger & Reichmann, 1973; Combard et al. 1974; Marcus & Sekellick, 1975).

In addition, cycloheximide might affect intracellular processes other than inhibition of protein synthesis (McMahon, 1975).

In order to obviate aberrant effects attributable to the use of inhibitors of protein synthesis, we used a temperature-sensitive (ts) mutant which produces only parentally-derived transcripts at non-permissive temperatures. This communication describes the use of this mutant, tsG41(IV) (Pringle, 1970; Unger & Reichmann, 1973), to examine the effect of interferon action on primary transcription of VSV in green monkey kidney (Vero) cells. We compare the rates of virus transcription and transcript accumulation in the presence and absence of cycloheximide, and present evidence that (i) cycloheximide prolongs the initial increase in rate of primary transcription, creating the so-called ‘cycloheximide effect’, (ii) interferon acts to reduce the rate of primary transcription, and (iii) virus transcripts synthesized in both mock- and interferon-treated cells are equally long-lived.

METHODS

Cells. The Vero line, clone M51, of green monkey kidney cells was used in all experiments reported here. The growth conditions and characteristics of these cells as maintained in our laboratory have been described (Marcus & Sekellick, 1974).

Vesicular stomatitis virus (VSV). The temperature-sensitive (ts) mutants G114(V) and G41(IV) of Pringle, (1970), and the Wlo(IV) mutant of Holloway, Wong & Cormack (1970) were used throughout this study. Our procedures for preparing stocks of ts mutants at 30 °C (permissive temperature) in monolayers of Vero cells have been described (Marcus & Sekellick, 1975). Low multiplicity (m) infections with plaque forming particles (p.f.p.; m_p.f.p. = 0.0001 to 0.001) were used to obtain preparations free or low in defective interfering particles. At 30 °C the stocks usually assayed at 5 to 50 × 10^8 p.f.p./ml. Revertant titres for ts mutants, G41 and W10, measured at 40 °C (non-permissive temperature) were usually around 10^4 to 10^5 p.f.p./ml. We have never detected revertants of G114 (Marcus & Sekellick, 1975, footnote 2).

Interferon and induction of virus interference. The human leucocyte interferon used throughout was supplied by T. C. Merigan. Its titre was 7.2 × 10^6 reference interferon units/ml
and its specific activity $2.8 \times 10^6$ reference interferon units/mg protein (based on the British human leucocyte standard 69/19). In a plaque reduction assay with VSV in our Vero cells, this preparation led to a 50% reduction in plaque numbers at a concentration of 13.9 reference interferon units/ml. In some preliminary experiments, which are not reported, a much cruder preparation of interferon was used, produced by inoculating the M29L line of human fibroblasts (from D. H. Carver) with u.v.-irradiated Newcastle disease virus, and partial purification by perchloric acid treatment. This preparation gave results comparable to those described for the more purified material. Mock-interferon preparations were obtained by perchloric acid treatment of medium from mock-infected fibroblasts; these had no antiviral or anticellular activities, and had no adverse effect on cell-killing particle activity or on in vivo transcription (Marcus & Sekellick, 1976). Interferon-mediated interference was accomplished by exposing Vero cell monolayers to various concentrations of human interferon for 20 to 24 h at 37 °C. Mock-treated cells received mock interferon and were manipulated similarly.

**Analyses of virus transcripts.**

**Cumulative synthesis.** The in vivo assay for VSV-associated transcript accumulation in Vero cells was carried out basically as previously described (Marcus & Sekellick, 1975, 1976). Virus ($m_{p.t.s}$ $\approx 500$) was adsorbed at 37-5 °C for 30 min, unadsorbed virus removed and 1.0 ml of pre-warmed standard reaction mixture (Marcus & Sekellick, 1975: actinomycin D, 10 µg/ml; cycloheximide, 50 µg/ml and 5-3H-uridine, 5 to 10 µCi/ml, sp. act. about 29 Ci/mol) in NCI medium (Marcus & Carver, 1965) was added to each 35 mm dish. In some experiments, cycloheximide was omitted from the reaction mixture. Plates were incubated at 40 °C for appropriate periods before determining the amount of 3H-uridine incorporated into acid-insoluble material as previously described (Marcus et al. 1971). Annealing of VSV virion RNA with the 3H-labelled product has established the radioactive material as virion-derived [+] strand mRNA – the product of primary transcription (Marcus et al. 1971; Huang & Manders, 1972).

**Rate determination (pulse-label).** The rate of tsG41(IV) RNA transcription at non-permissive temperature (40 °C) – conditions which allow only the synthesis of primary virus transcripts (Unger & Reichmann, 1973) – was examined in mock- and interferon-treated Vero cells. Confluent monolayers of cells in 35 mm dishes were treated with 1.0 ml (50 units/ml) of interferon (mock interferon was diluted similarly) at 37-5 °C for 20 h. Cell monolayers were pre-treated for 60 min with actinomycin D (10 µg/ml) at 37-5 °C and then infected with the ts mutant ($m_{p.t.s.} \approx 1000$), always in the presence of DEAE-dextran (10 µg/ml), at 37-5 °C for 30 min. Unadsorbed virus was removed by aspiration and 0.5 ml of pre-warmed 40 °C nutrient medium (NCI) with actinomycin D (10 µg/ml) and, when appropriate, cycloheximide (50 µg/ml), was added to each dish. Infected cell monolayers were incubated at 40 °C in a water-jacketed CO2 incubator. At appropriate intervals, 0.5 ml of radioactive medium was added to each plate giving a final concentration of 30 µCi of 5-3H-uridine (sp. act. 29 Ci/mmol) per plate, and incubation was continued at 40 °C for either 5 or 20 min. At the end of the pulse labelling period, the radioactive medium was removed by aspiration and monolayers were solubilized and assayed for TCA-precipitable radioactivity as previously described (Marcus et al. 1971).

**Sucrose gradient analysis.** Confluent monolayers of Vero cells in 100 mm dishes were treated with 4.0 ml of appropriately diluted human interferon (0, 12.5, 25 or 50 units/ml in NCI medium with 6% calf serum). Following incubation at 37-5 °C for 20 h, medium was removed, monolayers were washed twice with cold medium and 1.0 ml of stock tsG41
(m_{p.t.p.} \approx 400) in DEAE-dextran (10 \mu g/ml) was adsorbed to each plate for 30 min at 4 °C. Unattached virus was removed by aspiration and 3-0 ml of pre-warmed reaction mix containing 10 \mu g/ml actinomycin D and 40 \mu Ci 5^{3}H-uridine (sp. act. 29 Ci/mmol) was added. In some experiments, the reaction mixture also included 50 \mu g/ml cycloheximide. Cell monolayers were incubated at 37.5 °C for 20 min and then shifted to 40 °C for 3 h.

At the end of the labelling period, radioactive medium was removed by aspiration and the cell monolayers were washed once with warm NT-5 \times E (0.1 M-NaCl, 0.01 M-tris, pH 7.4, 0.005 M-EDTA). Cells were readily removed from each plate into 3-0 ml of NT-5 \times E by gentle pipetting with a Pasteur pipette. The high concentration of EDTA in this buffer facilitated the rapid removal from the plate of totally intact cells and obviated the need for scraping or trypsin treatment.

Cells were pelleted by centrifugation at 1000 rev/min for 5 min in an International Centrifuge (Model PR6). The supernatant fluid was decanted and the pelleted cells were washed once with ice-cold NT-5 \times E. Each pellet was then resuspended in 0-5 ml of 1/10 NT-5 \times E and the cells were allowed to swell for 20 min at 4 °C. Each cell suspension was taken up and expressed 12 times through a 25 gauge needle attached to a 1 ml syringe. Microscopic examination showed that virtually all the cells had been disrupted leaving the nuclei intact. This preparation was centrifuged at 2000 rev/min for 5 min, and the pellets were discarded. SDS was added to each sample to a final concentration of 0.5 % prior to rapid freezing and storage at -70 °C.

Samples were layered on to 15 to 30 % sucrose-SDS-NTE gradients (0.1 M-NaCl, 0.01 M-tris, pH 7.4, 0.001 M-EDTA, 0.5 % SDS) and centrifuged in a Beckman SW40 rotor for 17 h at 78000 g and 20 °C. Gradients were fractionated from the top, collected dropwise (20 drops per tube) and frozen at -20 °C.

For assay of radioactivity, 200 \mu g of yeast RNA was added to each tube, and then precipitated with an equal volume of 10 % TCA. The mixture was held on ice for 10 min. Radioactive precipitates were collected by filtration on to Whatman GF/A filters and counted in a Packard Scintillation Spectrometer.

Use of a temperature-labile mutant, tsG114(I) to achieve ‘pulse-chase’ conditions. VSV tsG114(I) with its thermolabile transcriptase (Pringle, 1970; Hunt & Wagner, 1974) was used to achieve a pulse-chase of virus mRNA synthesized in mock- and interferon-treated Vero cells. Following pre-treatment of confluent monolayers of cells in 35 mm dishes with 50 units of interferon or appropriately diluted mock-interferon (in NCI medium with 6 % calf serum) at 37.5 °C for 20 h, cells were treated with 10 \mu g/ml of actinomycin D for 60 min at 37.5 °C, and then infected with tsG114(I) at m_{p.t.p.} \approx 500 (in the presence of DEAE-dextran, 10 \mu g/ml) for 30 min at 37.5 °C. Unadsorbed virus was removed by aspiration, and 0.5 ml of a reaction mixture of NCI containing 10 \mu g/ml actinomycin D and 50 \mu g/ml cycloheximide was added to each plate. Primary transcription was allowed to proceed for 2 h at a permissive temperature (30 °C). A 5 min pulse of ongoing RNA synthesis (primary transcription) was accomplished by adding 0.5 ml of this reaction mixture (at 30 °C) to each plate such that the final radioisotope concentration was 30 \mu Ci of 5,6^{3}H-uridine (sp. act. 37-6 Ci/mol) per ml per plate. After incubation at 30 °C for 5 min, medium was removed and the plates were set on to a large aluminium block in a 45 °C water bath while 1-0 ml of chase medium at 45 °C (NCI with 10 \mu g/ml actinomycin D and 1 \times 10^{-4} M-uridine) was added to each dish. The plates were then immediately placed in a 40 °C water-jacketed, CO_{2} incubator for the chase period. Plates were removed at appropriate intervals and the cells solubilized and processed for TCA-precipitable radioactivity as previously described (Marcus et al. 1971).
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RESULTS

Both of the human interferon preparations used in our experiments were relatively crude, and consequently, we cannot ignore the possibility that they contain an activity, other than interferon, responsible for the effects described herein. However, the crude fibroblast interferon used in the preliminary experiments and the 'purified' leucocyte interferon used in the reported experiments produced comparable results when made equivalent on the basis of antiviral units or in terms of the inhibition of cell-killing particle activity (Marcus & Sekellick, 1976).

Virus transcript accumulation by tsG41(IV)

Transcript accumulation in the presence of cycloheximide

The time-course of transcript accumulation by VSV tsG41(IV) at 40 °C in mock- and interferon-treated Vero cells in the presence of cycloheximide is illustrated in Fig. 1. In mock-treated cells, virus transcripts accumulated at a nearly constant rate for 6 h (Fig. 1),
Fig. 2. Sucrose gradient pattern of the virus RNA species synthesized in interferon-treated Vero cells by VSV tsG41 (IV) at 40 °C in the presence of cycloheximide (50 μg/ml). The RNA represents primary transcripts of VSV, and was extracted from the cells 3 h after infection and continuous labelling with 3H-uridine, as described in Methods. Cells treated for 24 h prior to infection with mock interferon (O—O), or with interferon at 12.5 (□—□), 25 (×—×), or 50 (○—○) units/ml. demonstrating the stability and function of the virion-transcriptase complex of this ts mutant under our experimental conditions. In three separate experiments where cycloheximide was included in the reaction mixture, the amount of 3H-uridine incorporated from UTP into acid-precipitable virus transcripts, over a 6-h period of labelling in mock-treated cells, averaged 1.4-fold (mean ± standard deviation = 1.4 ± 0.4) greater than the amount incorporated in the absence of the drug. These data are in good agreement with those of Unger & Reichmann (1973, their Fig. 4), who studied this same ts mutant in BHK-21 cells. When cycloheximide was present, 3H-virus RNA accumulation at 40 °C always exceeded that which accumulated at 30 °C. In its absence, virus RNA accumulation at 40 °C was about 90% of that observed at 30 °C, but consisted solely of primary transcripts.

When GMK Vero cells were treated with interferon and infected with tsG41(IV) in the presence of cycloheximide, virus transcripts accumulated for a much shorter period of time than in mock-treated cells (Fig. 1). About 2 to 3 h after infection, the amount of virus
transcripts which could be isolated as $^3$H-acid insoluble material increased very little. As illustrated in Fig. 1, the time-course of transcript accumulation and its apparent inhibition by interferon action is quite similar to that reported earlier by Marcus et al. (1971, their Fig. 2) for wild type VSV (Indiana) in chick embryo cell cultures, by Manders et al. (1972, their Fig. 4) in human cells and by Repik et al. (1974, their Fig. 1D) in mouse L cells. (In this last paper this relationship is revealed more clearly when the data which represent accumulated virus transcripts are plotted on a linear rather than logarithmic scale.)

Similar cultures of Vero cells treated with the same amount of interferon (50 units/ml) and challenged at 40 °C with wild type VSV ($N_{p.e.}$ = 10 or 100), produced 10- to 15-fold less virus than mock-treated cells, whether actinomycin D (10 µg/ml) was present or not. Virtually all cells can be protected from the lethal action of wild type virus at this dose of interferon (Marcus & Sekellick, 1976). Fig. 2, illustrates the effect of the interferon dose on the quantity and size distribution of the virus transcripts which accumulated during 3 h of continuous labelling with $^3$H-uridine. The RNA profile from mock-treated cells shows that large amounts of 12 to 18S mRNA accumulated, but very little of the 28S species (cf. Unger & Reichmann, 1973). This RNA profile resembles that produced by two other mutants of complementation group $IV$, as reported by Combard et al. (1974) in HeLa cells. In Fig. 2, the curves formed by dashed lines represent, reading from the top down, RNA from cells treated with 12·5, 25, and 50 PR$_{50}$ units of interferon, respectively. These data demonstrate that the amount of virus transcripts which accumulate in cells over a 3-h period in the presence of cycloheximide decreases with increasing doses of interferon,
Table 1. Effect of cycloheximide on the accumulation of VSV tsW10(IV) transcripts at 30 and 40 °C in mock- and interferon-treated Vero cells*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VSV transcript accumulation (cpm/5 × 10⁶ cells/6 h)</th>
<th>Cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Mock</td>
<td>30 °C 33020 650</td>
<td>30 °C 9750</td>
</tr>
<tr>
<td>Interferon</td>
<td>30 °C 7370 740</td>
<td>40 °C 4220</td>
</tr>
</tbody>
</table>

* Virion-associated transcriptase activity was measured *in vivo* in mock- and interferon-treated (50 units/ml) Vero cells as described in Methods. In this experiment, cpm/min represent cumulative incorporation of ³H-uridine over a 6-h period at the permissive (30 °C) or non-permissive (40 °C) temperature and in the presence or absence of cycloheximide (50 μg/ml). All values have been corrected by subtracting the background radioactivity, i.e., between 500 and 1000 cpm/min, from appropriate uninfected cell cultures. The actual background levels may have been less because of virus-induced inhibition of cellular RNA synthesis (Marcus *et al.* 1971, Note no. 8).

confirming an earlier observation in the VSV-chick embryo cell system (Marcus *et al.* 1971) and a similar study on VSV transcript accumulation in human muscle skin fibroblasts (Manders *et al.* 1972). Although not illustrated, doses of interferon in excess of those used here were disproportionately less effective in reducing the accumulation of virus transcripts, and hence appeared to reveal a fraction of virus transcripts refractory to the action of interferon (cf. Fig. 3 and 4 in Marcus *et al.* 1971, and Table 1 in Manders *et al.* 1972). The smallest species of RNA shown in Fig. 2 (and in Fig. 4) were not observed consistently.

Transcript accumulation in the absence of inhibitors of protein synthesis

The experiments described above also were carried out in replicate monolayer cultures, but with cycloheximide omitted from the reaction mixture during virus transcription. Fig. 3 shows that when virus transcription by tsG41(IV) took place at 40 °C under conditions which permit protein synthesis, the rates at which virus transcripts accumulated in mock- and interferon-treated cells were similar for up to about 4 h after infection. These results are in contrast with those observed when cycloheximide is present (Fig. 1). We note that under these conditions, where protein synthesis was allowed, the high multiplicity of infection (m.p.i. ≈ 500) brought about a significant cytopathic effect by 5 to 6 h in both mock- and interferon-treated cells (cf. Yamazaki & Wagner, 1970).

Fig. 4 illustrates the profiles of RNA species that were isolated from Vero cells exposed to different doses of interferon, and infected with tsG41(IV) for 3 h at 40 °C in the absence of cycloheximide. Under these conditions interferon treatment had little or no effect on the size distribution or quantity of virus transcripts which accumulated (contrast the interferon dose dependent reduction observed when cycloheximide was present — Fig. 2). As noted above, the smaller RNA species seen in the gradients representing two of the doses of interferon in Fig. 4 (and the mock sample in Fig. 2) were not always observed, and hence could not be considered significant.
Interferon action on virus transcription

Virus transcript accumulation by tsW10(IV) in the presence and absence of cycloheximide: the 'cycloheximide effect'

The results obtained with ts mutant G41(IV) were not unique. The data in Table 1 show that tsW10, another mutant from complementation group IV, behaved similarly. Thus, in the presence of cycloheximide, Vero cells treated with 50 PR50 units of interferon and infected with tsW10(IV) accumulated about threefold fewer virus transcripts than mock-treated cells, whether at a non-permissive (40 °C) or permissive (30 °C) temperature. More transcripts accumulated at the lower temperature, presumably because of increased stability of the virion-associated transcriptase complex. However, in the absence of cycloheximide, interferon action did not appear to affect the already low level at which virus transcripts accumulated at 40 °C. At a permissive temperature and in the absence of cycloheximide, i.e. when transcription and replication proceeded normally, virus RNA accumulated to high levels. Not surprisingly, under these conditions interferon action produced a significant inhibitory effect.

The data in Table 1 also demonstrate that tsW10(IV) manifests a significant so-called 'cycloheximide effect'. Other inhibitors of protein synthesis, e.g., puromycin, pactamycin, and fluorophenylalanine produce a similar effect. In the experiment illustrated here, the addition of cycloheximide (50 μg/ml) to mock-treated Vero cells infected at 40 °C resulted in a 6.5-fold increase in tsW10 transcript accumulation over the 6-h incubation period. In an experiment reported previously, the cycloheximide present:absent ratio in tsW10(IV) infected Vero cells was 7:4 (Table 1, in Marcus & Sekellick, 1975). Accumulation of virus transcripts via the 'cycloheximide effect' does not lead to a more rapid rate of virus production upon removal of the drug and incubation of the virus-cell complexes at 30 °C (P. I. Marcus & M. J. Sekellick, unpublished observations).

In contrast to tsW10(IV), tsG42(IV) produces only a minimal 'cycloheximide effect'. For example, after 6 h of transcription in mock-treated cells the amount of 3H-labelled VSV transcripts which accumulated with cycloheximide present (Fig. 1), or absent (Fig. 3), differed by only 15 % from the mean value of 19050 ct/min. At 4 h, this difference was 41 % from the mean value of 12600 ct/min. Differences in transcript accumulation in mock-treated cells in the presence and absence of cycloheximide as calculated from the gradients of Fig. 2 and 4 (for 3 h) and similar data (not shown) for 6 h, were, respectively, 54 % and 37 % of the mean value of total acid-precipitable 3H-ct/min. These results agree well with a similar calculation we have made, based on data of Unger & Reichmann (1973, their Fig. 4A and B) with the same mutant (tsG41) in BHK-21 cells, and the results of Flamand & Bishop (1974, their Fig. 6b and c) with a different group IV mutant, tsO194.

Uridine uptake

The reduction in virus transcript accumulation observed in Vero cells treated with interferon and challenged with VSV in the presence of cycloheximide (Fig. 1 and 2, Table 1) might simply reflect a reduction in cell membrane permeability to ribonucleosides. Consequently, we determined the uptake of 3H-uridine into acid-soluble material in both mock- and interferon-treated cells with conditions identical to those under which we measured virus transcript accumulation. Cells were infected at high multiplicity with tsG41(IV), incubated at 40 °C in the presence or absence of cycloheximide and pulse-labelled with 3H-uridine for 5 min at hourly intervals for 5 h. The results of a typical experiment are displayed in Table 2, and represent the mean value and standard deviation of the 5 individual hourly determinations for each of the experimental conditions tested. The narrow range of
Table 2. Effect of cycloheximide, interferon and VSV infection on $^3$H-uridine uptake by Vero cells into acid-soluble $^3$H-ct/min*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptake of $^3$H-uridine (acid-soluble $^3$H-ct/min/5 x $10^6$ cells per 5 min pulse)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>1161 ± 148</td>
</tr>
<tr>
<td>Interferon</td>
<td>1238 ± 176</td>
</tr>
<tr>
<td>Mock + cycloheximide§</td>
<td>849 ± 97</td>
</tr>
<tr>
<td>Interferon + cycloheximide</td>
<td>927 ± 69</td>
</tr>
<tr>
<td>Mock + tsG41</td>
<td>1303 ± 141</td>
</tr>
<tr>
<td>Interferon + tsG41</td>
<td>1447 ± 203</td>
</tr>
<tr>
<td>Mock + cycloheximide + tsG41</td>
<td>885 ± 127</td>
</tr>
<tr>
<td>Interferon + cycloheximide + tsG41</td>
<td>1084 ± 164</td>
</tr>
</tbody>
</table>

* Uptake was measured under conditions identical to those used for measuring the rate of virus transcription (see Methods). Cells infected at high multiplicity with tsG41 (IV) at 40 °C and pulse-labelled for 5 min at hourly intervals for 5 h.
† These values represent the mean and standard deviation of the five individual hourly determinations for each experimental condition tested.
§ 50 units/ml. ¶ 50 μg/ml.

The standard deviations (± 7 to 15 %) indicates that there was little variation in the uptake of $^3$H-uridine for any single experimental condition during the 0 to 5 h test period. The differences between the mean values of appropriately paired samples are considered within the range for sampling error except that in the last set of treatments, the 20 % difference borders on being significant. This, however, represents a change that would tend to minimize rather than maximize any inhibitor effect of interferon action on virus transcript accumulation due to a decrease in membrane permeability to the ribonucleoside. Our results agree with those of Genty (1975), who observed that uridine entry into HeLa or L-cells was little affected by infection with VSV.

The rate of virus transcription in the presence of cycloheximide

Using the pulse-labelling procedures described in Methods, we determined first the amount of virus transcripts which accumulated at 40 °C during a 5 min pulse of $^3$H-uridine at various times after infection with tsG41 (IV) in the presence of cycloheximide. The amount of acid-precipitable $^3$H-ct/min was taken to represent the apparent rate of transcription – a term which takes into consideration the sum of synthetic and degradative reactions extant during the time of labelling. Since it requires about 4 min or less to transcribe the entire genome of VSV in vivo (Flamand & Bishop, 1974), a 5 min pulse-interval should provide a close approximation to the absolute rate of transcription by minimizing the time available for degradation. Fig. 5 demonstrates that when cycloheximide was present during virus infection of mock-treated cells, the rate of transcription increased linearly for almost 1 h, reached a plateau, and then remained relatively constant and maximal over the next 4 h (1 to 5 h post-infection). In the interferon-treated cells the rate of virus transcription initially also increased linearly, but at a significantly reduced rate. (In two 5-min and 20-min pulse experiments, there was an average fourfold reduction.) The maximal rate of transcription in interferon-treated cells was observed at about 2 h post-infection, and declined steadily over the next 3 h (2 to 5 h post-infection). In effect, with cycloheximide present, and com-
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Fig. 5. Rate-time course of primary transcription in mock-treated (○—○) and interferon (50 units/ml)-treated (●—●) Vero cells infected with VSV tsG4I (IV) at 40 °C and measured in the presence of cycloheximide (50 μg/ml). Rate determinations were carried out as described in Methods. The \(^{3}H\)-ct/min represent incorporation of \(^{3}H\)-uridine into acid-precipitable material by infected cells during exposure for the 5-min periods of labelling indicated on the abscissa by the arrows and bars.

pared to mock-treated cells, fewer transcripts of VSV were synthesized per unit time in the interferon-treated cell. These results are consistent with the observations recorded in Fig. 1 and 2. Furthermore, a 20 min pulse experiment produced similar results, suggesting that degradation of virus transcripts may be minimal in both mock- and interferon-treated cells in the presence of cycloheximide (see below).

The rate of virus transcription in the absence of added inhibitors of protein synthesis

Experiments identical to those described above were performed in parallel, but without cycloheximide in the reaction mixture. Fig. 6 (which may be compared directly with Fig. 5) shows that in mock-treated cells the rate of virus transcription initially increased rapidly for about 1 h, then decreased slowly over the next 4 h. In interferon-treated cells, and in the absence of cycloheximide, the initial rate of increase of virus transcription was slower than in mock-treated cells, reached its peak rate at about 2 h, and declined only slightly over the last 3 h of the experiment, i.e. 2 to 5 h post-infection. In three different experiments we observed that 50 units/ml of interferon produced an average threefold (extremes of two- to sixfold) reduction in the initial slope (rate-change) of the transcription rate-time curves. A 20 min pulse experiment produced similar results.

VSV transcription rate curves generated in mock- and interferon-treated cells in the absence of inhibitors of protein synthesis differed from each other much less than those generated with cycloheximide present (cf. Fig. 5 and 6). Thus, cumulative yield curves based on these data might as a first approximation, account for the results recorded in Fig. 3 and 4.
The half-life of virus transcripts: ‘pulse-chase’ experiments with tsG114(I)

We have taken advantage of the heat lability of the virion-associated L-protein (transcriptase) of VSV mutant tsG114(I) (Szilágyi & Pringle, 1972; Perlman & Huang, 1973; Hunt & Wagner, 1974; Marcus & Sekellick, 1975) to develop a ‘pulse-chase’ procedure to determine the half-life of virus transcripts synthesized in mock- and interferon-treated cells. Monolayers were infected with tsG114 at a permissive temperature (30 °C) in the presence of cycloheximide, and primary transcription (Marcus et al. 1971; Manders et al. 1972; Flamand & Bishop, 1973) was allowed to proceed for 2 h in order to reach a high rate of synthesis (cf. Fig. 5). The infected cell monolayers were then labelled with 3H-uridine for 5 min (the pulse) while still at 30 °C. Upon completion of the pulse, medium containing radioisotope and cycloheximide was removed, and a new reaction mixture (containing 10⁻⁴ M-uridine, but without cycloheximide) at 45 °C was added and incubation immediately carried out at a non-permissive temperature, 40 °C (the chase). The virion-associated transcriptase quickly ceases to function at 40 °C and incorporation of radioisotope from the precursor pool is concomitantly arrested, creating conditions for an effective chase. The half-life of the virus transcripts synthesized under these conditions was measured by determining the amount of acid-precipitable ³H-ct/min extractable from the cells as a function of time after the pulse. Fig. 7 shows that in both mock- and interferon-treated cells, an increase in incorporation of labelled precursor continued for 30 min after termination of the 5 min pulse with ³H-uridine – presumably reflecting the time required to inactivate the transcriptase in all transcribing complexes. After the first 30 min, no further increase in acid-precipitable material was observed. Assuming little change in the specific activity of the UTP pool (Metz & Esteban, 1972), almost half of the virus transcripts were labelled during the 5 min pulse, and the other half during the first 30 min of the ‘chase’ period. Continued incubation of the infected cells for 3 h at 40 °C revealed a slight decrease in
Interferon action on virus transcription

Fig. 7. Virus transcript stability in mock-treated (○) and interferon (50 units/ml)-treated (●) Vero cells infected with VSV tsG114 (I) determined under the conditions of a "pulse-chase" experiment as described in Methods.

Our data show that in mock-treated cells, larger amounts of primary transcripts accumulated in the presence of cycloheximide than in its absence. We have shown that this is due to higher rates of virus transcription sustained for longer periods of time. These results provide an explanation for the so-called 'cycloheximide effect', i.e. the enhanced accumulation of virus transcripts in the presence of inhibitors of protein synthesis. This effect may be large, as in cells infected with tsW10(IV) (Table 1), smaller as with tsG41(IV) (compare the mock-treated cells in the presence of cycloheximide in Fig. 1, 2 and 5 with those in the absence of cycloheximide in Fig. 3, 4 and 6), or nonexistent when the mutant defect is a heat-labile virion-transcriptase as with tsG114(I) (Table 1 in Marcus & Sekellick, 1975).

Basically, our data demonstrate that the rate of primary transcription of VSV initially increases linearly for about 1 to 2 h after infection, and that interferon acts to reduce this rate significantly (about fourfold for 50 units/ml interferon), irrespective of the presence or absence of cycloheximide (cf. Fig. 5 and 6). While the initial linear increase in the rate
of primary transcription in mock-treated cells is essentially the same whether cycloheximide is present or absent, the action of the drug results in a higher and sustained rate of mRNA synthesis (cf. Fig. 5 and 6). The end result is to exaggerate the inhibition of virus transcription by interferon action when the drug is present (cf. Fig. 1, 2 and 5 with 2, 3 and 6, respectively).

These results account for, and extend, earlier observations that fewer primary transcripts of VSV accumulate in interferon-treated than in mock-treated cells (Marcus et al. 1971; Manders et al. 1972; Repik et al. 1974, Fig. 1D; Baxt et al. 1977), and that the size-distribution of the transcripts is unchanged by interferon action (Manders et al. 1972; Baxt et al. 1977).

Use of data from Repik et al. (1974, Fig. 1D) in support of our thesis that interferon action has an adverse effect on primary transcription of VSV may appear contradictory to their own interpretation, namely that '...interferon does not act at the level of primary transcription but rather at an intermediate step between primary and secondary transcription, such as viral protein synthesis.' However, it appears that during the determination of transcription (primary?) by VSV, cycloheximide was present in 'mock' control cells, but omitted from interferon-treated cells in several experiments (cf. their Fig. 1A, B, C). In the light of this and our present results, it would seem appropriate to view their interpretation of interferon action with reservation. As noted above, in the one experiment where cycloheximide was present in both 'mock' and interferon-treated cells (their Fig. 1D), their data are totally supportive of our own (cf. Fig. 1; also Marcus et al., 1971, Fig. 2) and those of Manders et al. (1972). Furthermore, in four of five experiments (Repik et al. 1974, Fig. 1A, 1B, 1C and 2B) where these investigators compared VSV or influenza virus transcript accumulation in the presence of cycloheximide with that in cells manifesting an antiviral effect mediated by interferon or poly(rI)-poly(rC), fewer transcripts were observed to accumulate in the cells with the specific antiviral block. Similar results were reported by Baxt et al. (1977, their Fig. 9) in VSV-infected monkey LLC-MK2 and human amnion U cells, with cycloheximide present. Thus, from four separate studies with VSV-infected cells, in addition to the present work, it is now abundantly clear that interferon action on cells reduced primary transcription to levels lower than those observed with cycloheximide alone (Marcus et al. 1971; Manders et al. 1972; Repik et al. 1974; Baxt et al. 1977). Interferon action appears to have a greater effect than cycloheximide on the synthesis and accumulation of virus transcripts – even though the latter acts as an extremely efficient inhibitor of protein synthesis. Clearly then, interferon must have an effect on primary transcription beyond that attributable solely to protein synthesis inhibition. From the results presented here we propose that this additional effect is on the rate of primary transcription. As elaborated below, it is most likely that the effect is to block the initiation of transcription. We emphasize that these results do not preclude an additional and post-transcriptional mode of interferon action operative, for example, at the level of translation; and indeed, the data of Baxt et al. (1977) indicate that this mode of inhibition may be operative.

Since the VSV transcripts which do accumulate in interferon-treated cells appear to be normal in size and shape, as deduced from their behaviour during velocity sedimentation, it seems likely that once transcription has been initiated it is completed successfully, and that transcripts are not degraded significantly. From this inference we propose, as a working hypothesis, that interferon acts to decrease the probability, P, of initiation of VSV transcription for each transcribing nucleoprotein complex entering the cell (possibly for each round of transcription), with the value of P decreasing within limits with increasing doses of interferon. It is important to recognize that since transcription rate measurements are carried
out at high multiplicities, they represent an average of the rates for all transcribing complexes in the population of infected cells. Conceivably then, at the single cell level, where infection by one virion may represent a real situation, an average reduction of, for example, fourfold in the transcription rate measured in a population of cells may actually reflect no \( (P = 0) \) transcription initiation in 75% of the cells (leading to cell survival), and normal \( (P = 1) \) transcription initiation in 25% (leading to cell death). Thus, inhibition of transcription might assume a significant role in the outcome of a virus-cell encounter when interferon-treated cells are challenged with one, or a few, virion(s), as, for example, in a plaque-reduction or cell killing particle assay (Marcus & Sekellick, 1976), or during natural infection. Experiments to test this hypothesis are planned.

Additional support for this hypothesis comes from a related study in which we observed that the dose-response curves for the loss of (i) cell-killing particle activity and (ii) virus transcript accumulation by VSV were essentially equivalent in interferon-treated cells, even though the former test measures the activity of a single virion while the latter measures that of hundreds of virions per cell (Marcus & Sekellick, 1976). Furthermore, if we assume that for a given dose of interferon each virion-cell interaction has the same low probability of initiating transcription then, predictably, increasing the multiplicity of infection would lead to successful transcription by some virus-cell complexes; this in turn would result in the accumulation of virus transcripts and, upon their successful translation into threshold amounts of minimally functional proteins, cell-killing would ensue, as was clearly demonstrated by Yamazaki & Wagner (1970). Since we know that inhibition of VSV plaque formation in GMK-Vero cells is five times more sensitive to interferon action than cell-killing particle activity or transcript accumulation, and that virus proteins N and NS need to be minimally functional to express cell-killing (Marcus & Sekellick, 1975, 1976; Marvaldi et al. 1977; Marcus et al. 1977), it seems likely that even though full-size transcripts may be available in interferon-treated cells multiply-infected with VSV, some additional facet of interferon action must be operative to inhibit virus replication at a later step, most likely inhibition of translation (Baxt et al. 1977).

If we assume that the size of ribonucleotide pools in Vero cells remains essentially unchanged following treatment with interferon, as Metz & Esteban (1972) have shown in mouse cells, our data indicate that changes in cell membrane permeability to ribonucleosides (Table 2) would not readily account for the elevated levels of transcript accumulation that constitute the cycloheximide effect in mock-treated cells, or for the reduced levels observed in interferon-treated cells.

For a transcribing negative-strand virus like VSV (or reovirus; Wiebe & Joklik, 1975), models of interferon action which postulate a ‘primary’ effect on translation appear to ignore the mandatory sequence of primary transcription \( \rightarrow \) primary translation which is required before there can be any amplification of virus RNA synthesis and production of new virus. Inhibition of primary transcription would reduce the number of virus messages available for subsequent translation. Thus, if interferon action inhibits translation of virus messages, the overall effect will be greatly exaggerated if there is, in addition, an effect of interferon on primary transcription: this will mean that fewer virus messages are available for translation. This important point (first expounded by J. Taylor-Papadimitriou at the 5th Katzir-Katchalsky Conference in Rehovot, Israel, on May 4, 1977) suggests that it may be prudent not to use the term ‘primary effect’ when referring to interferon action until such time as the relative contributions of transcription and translation inhibition are defined in more rigorous terms.

These data do not represent isolated observations. Interferon action has been reported to
reduce to varying degrees the accumulation of transcripts in cells infected with such transcriptase-containing viruses as (i) frog polyhedral cytoplasmic deoxyribovirus (Gravell & Cromeans, 1972), (ii) influenza virus (Bean & Simpson, 1973), (iii) reovirus (Gauntt, 1972; Wiebe & Joklik, 1975), and (iv) one virus which apparently uses cellular RNA polymerase II for transcription, i.e. SV40 (Oxman & Levin, 1971; Metz, Levin & Oxman, 1976).

We cite the study of Wiebe & Joklik (1975) in this context even though the major effect they observed appears to support their contention that ‘...the principal mechanism by which interferon inhibits reovirus replication is by inhibiting the translation of early reovirus mRNA.’ Nonetheless, data in their Fig. 6 and 7 demonstrate that interferon treatment also reduced transcription of early mRNA (primary transcription), but produced no change in the size distribution of the transcripts. (It is important to note that their studies were also carried out in the absence of inhibitors of protein synthesis: Wiebe & Joklik used a ts mutant of reovirus with a defect which restricted mRNA synthesis to virion-derived primary transcription). When, as discussed above, the consequences of virus transcription and translation as sequential events are considered, the degree of translation inhibition which they recorded may not actually represent the full significance they ascribed to it.

Though the ‘pulse-chase’ procedure developed here is restricted to the use of ts mutants with appropriate phenotypes and hence is more limited in its range of usefulness than procedures which employ biochemical means to deplete the ribonucleotide pools (Wertz, 1975), the method does provide a means to measure the stability of virus RNA in cells under conditions where the precursor-pool is not altered by biochemical manipulations.

We note that the half-life of about 17 h recorded for VSV mRNA over a 3-h period in both mock- and interferon-treated cells is consistent with the stability of VSV transcripts in chick embryo cells as measured by Wertz (1975) over a 1-h period. Furthermore, this observation is in keeping with results from recent experiments in which we failed to detect enhanced levels of ribonuclease (RNase) activity in interferon-treated Vero cells and mouse primary cultures, or in L-cells treated with interferon or poly(rI)·poly(rC) under conditions where chicken and other avian embryo cells revealed high levels of single- and double-stranded RNase activity (Marcus, Terry & Levine, 1975; Meegan, Fuller & Marcus, 1976). The absence of significant mRNA degradation under these experimental conditions also indicates that the transcription rate-curves generated by our pulse experiments provide a reasonably accurate assessment of virus transcription rate.

The evidence for in vivo stability of VSV mRNA in interferon-treated cells as reported here and by Manders et al. (1972) contrasts with the recent observations of Brown et al. (1976) who noted that reovirus mRNA was degraded in vitro by extracts from mouse Ehrlich ascites cells treated with interferon or poly(rI)·poly(rC) when double-stranded (ds) RNA was present (cf. May, Nagler & Graziadei, 1975). Since our studies with VSV were carried out under conditions which might minimize or preclude virus dsRNA formation, it is possible that insufficient dsRNA was synthesized in vivo to activate an endonucleolytic activity of this type. Further experiments should resolve this question.

Seemingly, viruses present a range of responses to the effects of the interferon system: for example, we note that in reovirus- (Wiebe & Joklik, 1975) and VSV- (Baxt et al. 1977) infected cells, a measurable reduction in transcript accumulation can be demonstrated under conditions where inhibition of translation also is manifested. In contrast, virion-associated early transcription by vaccinia virus is not sensitive to interferon action (Joklik & Merigan, 1966; Jungwirth et al. 1972; Metz & Esteban, 1972; Esteban & Metz, 1973), whereas that of VSV is sensitive (Marcus et al. 1971; Manders et al. 1972; Repik et al. 1974; Marcus & Sekellick, 1976; Baxt et al. 1977; the present paper). However, as Metz et al. (1976) have
pointed out, the insensitivity of vaccinia transcription to interferon action may simply reflect inaccessibility of the vaccinia core to an inhibitor. As we stated previously, 'There is no reason for supposing that the interferon system consists of a single molecular species with a single action' (Marcus et al. 1970), a thought still deemed appropriate, and expanded on recently by Metz et al. (1976). Thus, it seems prudent to recognize the possibility that the antiviral effects of interferon action, like those on the immune system, are multifaceted. This broader view may encourage a broader range of experimentation.

This research was aided in part by United States Public Health Service Grant CA-20882 from the National Institutes of Health, National Science Foundation Grant PCM 76-00467, Research Foundation Grant 35-773 and Damon Runyon-Walter Winchell Cancer Fund Grant 1284, and benefited from use of the Cell Culture Facility supported by National Cancer Institute Grant CA-14733.

Some aspects of this paper were presented at the 3rd International Congress for Virology on 11 September, 1975, in Madrid, Spain, at the Workshop on Interferon on 22 September, 1975, in Würzburg, West Germany, and at the 76th Annual Meeting of the American Society for Microbiology, 6 May, 1976 (American Society for Microbiology Abstracts, p. 238, 1976).

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