Enhancement of Transcription of the SV40 Genome in Mouse Embryo Cells by Pretreatment with 5-Iodo-2'-deoxyuridine

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SUMMARY

Treatment of mouse embryo (ME) cells with 5-iodo-2'-deoxyuridine (IdUrd) before infection with SV40 virus, enhances T-antigen (T-Ag) production as detected by immunofluorescence and complement fixation. Cellular DNA and RNA synthesis are inhibited in both SV40 and mock-infected cells after IdUrd treatment. The analogue pretreatment significantly increases the amount of radiolabelled nuclear and cytoplasmic SV40-specific RNA and the RNA polymerase activity of the viral transcriptional complexes of the Sarkosyl supernatants, suggesting that the enhancement of SV40 T-Ag production in infected pretreated ME cells results from an increased synthesis of early virus RNA.

It has been reported previously (Suárez et al., 1976, 1977) that treatment of fully, as well as semi-permissive, cells with 5-iodo-2'-deoxyuridine (IdUrd) before treatment with SV40 virus or virus DNA, resulted in a marked increase of T-antigen (T-Ag) production and virus yield. No effect of the drug on the replication of the virus was found when the experiments were carried out with non-permissive (mouse or Syrian hamster embryo) cells (Suárez et al., 1976). Experiments performed to determine whether IdUrd affects adsorption, penetration or decapsidation of SV40 in permissive or semi-permissive cells indicated that the analogue acts between the arrival of the virus DNA in the nucleus and T-Ag synthesis. Moreover, the enhanced production of T-Ag persisted in permissive cells even if cytosine arabinoside (Ara-C) was immediately added after infection (Suárez et al., 1977). These results suggest that the halogenated pyrimidine acts at an early step of the SV40 cycle, perhaps at the level of early virus transcription or translation.

Mouse primary or secondary cultures are non-permissive for SV40. Infection with the virus does not lead to synthesis of virus DNA or production of late virus polypeptides, but substantial amounts of SV40 T-Ag and the early SV40 RNA which encodes this protein(s) can usually be detected. This infection leads, within a few weeks, to the appearance of SV40-transformed cell colonies (Smith et al., 1979). Mouse cells infected with SV40 are therefore particularly useful for investigating the effects of IdUrd pretreatment on the expression of SV40 early functions.

Mouse embryo (ME) fibroblasts were prepared from 12- to 14-day-old embryos of Swiss albino mice. Cultures were routinely grown in Eagle's minimal essential medium (MEM, Eurobio, Paris, France) supplemented with 10% calf serum. Secondary ME cells suspended in growth medium supplemented with 10% calf serum, were seeded at 4 \times 10^6 or 2 \times 10^6 cells/culture in 10 cm diam. plastic dishes, in the presence or absence of 100 \mu g/ml IdUrd respectively, and incubated at 37 °C. Four days later, the medium was removed from the cultures and after trypsinization, the number of cells was determined. It was virtually the same in treated and untreated cultures. The cells were then infected with SV40 virus at 50 p.f.u./cell. After 2 h adsorption at 37 °C, excess virus inoculum was removed by three washings with serum-free medium, and drug-free medium with 2% calf serum was added. Cultures were incubated at 37 °C. All experiments were carried out in the dark.
Table 1. *Effect of IdUrd on SV40 T-Ag in ME cells infected with the virus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after infection (h)</th>
<th>Immunofluorescence*</th>
<th>Complement fixation titre†</th>
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<tbody>
<tr>
<td>None</td>
<td>9 ND‡</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>12 1</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>18 20</td>
<td>ND</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>24 25</td>
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<td></td>
<td>30 30</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>36 34</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>48 20</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>IdUrd</td>
<td>9 ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>12 5</td>
<td>ND</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>18 34</td>
<td>ND</td>
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<td>30 51</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>36 62</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>48 57</td>
<td>ND</td>
<td>64</td>
</tr>
</tbody>
</table>

* Values represent percentage of cells containing the antigen; each point was determined by counting 2000 cells. The coverslips were stained by indirect immunofluorescence.
† Titres represent reciprocal of highest dilution of antigen yielding less than 50% haemolysis against 1:10 hamster serum (from SV40 tumour-bearing animals).
‡ ND, Not done.

Table 1 shows that IdUrd treatment increased the synthesis of T-Ag as determined by immunofluorescence and complement fixation (Tournier et al., 1967).

Data on incorporation of \(^3\)H-thymidine into cellular DNA confirmed earlier reports using semi- and fully permissive cells (Suárez et al., 1976, 1977). The activation of the cellular DNA-synthesizing apparatus took place after the analogue treatment, but it was reduced and delayed compared to the untreated cells. After the removal of the drug, the treated cells progressively resumed a normal cellular DNA synthesis and growth rate, culminating in a complete reversal of the effect of the analogue (data not shown).

To examine the effect of IdUrd on virus DNA replication in these experiments, within the limits of the sensitivity of filter hybridization, two sets of cultures (IdUrd-treated and control) infected with SV40 were incubated for 24 h at 37 °C. At this time, cells were pulse-labelled for 1 h with \(^3\)H-thymidine at 37 °C and the Hirt supernatant fractions were purified (Hirt, 1967). These DNA preparations were denatured and annealed exhaustively with SV40 DNA on filters (Shih & Khoury, 1976). No newly synthesized DNA was detectable by hybridization in either treated or control cultures. Both IdUrd-treated and control cells were negative for V-antigen by immunofluorescence (data not shown).

To study the metabolism of cellular RNAs in ME cells pretreated with the analogue or not, cultures were either infected with SV40 virus or mock-infected. At different times after infection, the cells were labelled for 3 h with 20 \(\mu\)Ci/ml \(^3\)H-uridine, washed and after lysis with 1% Nonidet P-40 (Shell), the homogenate was scraped off and the nuclear and cytoplasmic fractions separated by centrifugation at 2500 rev/min for 5 min at 4 °C. DNA and RNA from the nuclear and cytoplasmic fractions were then extracted according to the Schmidt and Thannhauser procedure (Munro & Fleck, 1966). The incorporation of \(^3\)H-uridine into both nuclear or cytoplasmic total RNAs was expressed as ct/min/\(\mu\)g DNA. The contribution of ribosomal RNA to the labelling of nuclear RNA was suppressed by treating the cells with 0.05 \(\mu\)g/ml actinomycin D. This was added to the culture medium 30 min before labelling with \(^3\)H-uridine. As shown for permissive cells (Suárez et al., 1977), in IdUrd-pretreated ME cells, cellular RNA synthesis was reduced 9 h after removal of the drug and infection with SV40, but there was a recovery at 24 h. Similar results were obtained in mock-infected IdUrd-treated ME cells (data not shown).
Table 2. Incorporation of $^3$H-uridine into newly synthesized SV40 RNA and enzyme activities of the SV40 transcriptional complexes present in the Sarkosyl supernatant, in IdUrd pretreated and control infected cells

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>$^3$H-uridine labelling (h p.i.)</th>
<th>IdUrd</th>
<th>SV40 RNA*</th>
<th>Enzyme activity†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nuclear (hybridizable ct/min $\times 10^{-3}$/10 Petri dishes)</td>
<td>Poly(A)$^+$ cytoplasmic (hybridizable ct/min $\times 10^{-3}$/100 µg RNA)</td>
</tr>
<tr>
<td>1</td>
<td>6-9</td>
<td>-</td>
<td>0.570</td>
<td>0.160</td>
</tr>
<tr>
<td></td>
<td>21-24</td>
<td>+</td>
<td>1.360</td>
<td>0.400</td>
</tr>
<tr>
<td>2</td>
<td>6-9</td>
<td>-</td>
<td>0.720</td>
<td>0.400</td>
</tr>
<tr>
<td></td>
<td>21-24</td>
<td>+</td>
<td>2.700</td>
<td>42.121</td>
</tr>
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</table>

* ME cell cultures were pretreated with IdUrd or not and labelled for 3 h with $^3$H-uridine (expt. 1, 250 µCi/ml; expt. 2, 300 µCi/ml) at the indicated times after infection. SV40 RNA was extracted from the nuclear and cytoplasmic fractions and hybridized to 0.2 µg SV40 DNA immobilized on minifilters, as described in the text.
† Treated and untreated cultures were harvested 24 h after infection and processed for the isolation of viral transcriptional complexes with Sarkosyl. The Sarkosyl supernatants were incubated in vitro in the standard polymerase reaction mixture containing α-$^3$P-UTP and after 2 h at 21°C, the radioactivity incorporated in the synthesized RNA was determined by acid precipitation (Shani et al., 1977). The synthesized $^3$P-RNA was hybridized to SV40 DNA immobilized on minifilters. The enzyme activity (input) was expressed as $^3$P-UMP ct/min/mg protein in the Sarkosyl supernatant.

To determine whether SV40 early transcription is affected by IdUrd, the specific radioactivity of virus mRNA was determined in treated and untreated infected ME cells. At different times after infection (6 and 21 h), the pretreated or untreated cultures were labelled for 3 h with $^3$H-uridine (250 to 300 µCi/ml) and nuclear and cytoplasmic fractions separated as indicated above. Nuclear RNA was extracted as described by Scherrer (1969), including a DNase treatment. Cytoplasmic RNA was extracted by the phenol–chloroform method and fractionated by oligo(dT)-cellulose chromatography (Rosenthal, 1976) in order to obtain poly(A)$^+$ RNA. $^3$H-labelled SV40 RNA was detected by hybridization to denatured SV40 DNA (present in excess) fixed on membrane filters (0-2 µg DNA/filter), as described by May et al. (1973). Results presented in Table 2 show that IdUrd enhanced significantly the specific radioactivity of SV40 mRNA in the pretreated cells.

It has been observed (Khoury et al., 1972; M. Lange, E. May & P. May, personal communication) that in SV40-infected mouse cells, the SV40-specific RNA consists mainly of early mRNA, but that a minor amount of late mRNA can be detected. To compare the nature of the SV40-specific RNA synthesized in IdUrd-treated and untreated cells, $^3$H-labelled RNA from infected ME cultures was purified and hybridized to separated strands (early, E; late, L) of the early (A) and late (B) regions of the SV40 genome, produced by cleavage with BamHI and HpaII restriction enzymes (Birkenmeier et al., 1979). The results showed that both early and late SV40 RNAs were synthesized in larger amounts in pretreated cells, as compared to control cells. They suggest that in IdUrd-pretreated cells, the synthesis of early and late SV40 RNAs was stimulated in parallel (data not shown).

In these experiments we examined the accumulation of SV40 RNAs in the nuclei and cytoplasm of infected cells. The increased amount of SV40 RNA observed in IdUrd-pretreated cells could reflect a higher rate of initiation of virus RNA synthesis. To test this possibility we used viral transcriptional complexes isolated by the Sarkosyl extraction procedure, which are capable of synthesizing virus RNA in vitro and are believed to be derived from in vivo transcriptional intermediates of the virus (Gariglio & Mousset, 1975). It has been shown (Shani et al., 1977; Birkenmeier et al., 1979) that SV40 RNA chain...
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elongation in vitro, measured by incorporation of labelled nucleotides, reflects primarily the frequency at which the transcription of the early and late strands is initiated in vivo. Virus RNA polymerase activity was estimated as described by Shani et al. (1977) in the Sarkosyl supernatant isolated from either IdUrd-pretreated or untreated cells, 24 h after infection. Since transcriptional activity was expected to be very low it was determined by hybridization of in vitro synthesized RNA to SV40 DNA immobilized on minifilters (May et al., 1973). The activity was expressed as $^{32}$P-UMP ct/min incorporated into hybridizable RNA per mg protein of Sarkosyl supernatant. The results (Table 2) show that IdUrd pretreatment caused an increase in the rate of SV40 transcription of the same order as that observed in the in vivo experiments. These data suggest that the in vivo stimulation was the consequence of an effect of the IdUrd pretreatment on the SV40 transcriptional activity.

Few experiments have been done to study the action of thymidine analogues on the level of transcription. Some authors (Case et al., 1975; Colbert et al., 1976; Price, 1976) have reported that the effects of exposure to 5-bromodeoxyuridine (BrdUrd) in various systems appears to involve alterations in patterns of RNA synthesis. Lin et al. (1976) more precisely suggested that the analogue acts by affecting both histone–DNA interactions and non-histone protein–DNA interactions.

Present and previous data (Suárez et al., 1976, 1977) showed that (i) IdUrd treatment markedly suppressed the synthesis of cellular DNA and RNA in ME and other cellular systems and (ii) the virus functions in IdUrd-pretreated permissive cultures were preferentially expressed in cells not synthesizing DNA. Using IdUrd-pretreated SV40-infected 3T3-4E cells, a thymidine kinase-deficient (TK−) Swiss mouse cell line propagated in the presence of 100 µg/ml BrdUrd, we have been able to show that the cells were resistant to the stimulating effect of the drug on T-Ag synthesis (preliminary results).

In the present study, we have employed a high dose of IdUrd in order to obtain heavy substitution of the halogenated analogue into cellular DNA. The fact that (i) infection takes place after IdUrd treatment and (ii) no SV40 replication was detectable in the pretreated or control cultures, suggests that there is no substitution of thymidine in the input virus DNA. However, this possibility cannot be completely excluded until methods more sensitive than filter hybridization have been used.

In conclusion, our present data suggest that the enhancement of SV40 T-Ag production in ME-infected pretreated cells results from an increased synthesis of virus early RNA. The reasons for this enhancement of the early virus transcription are still unknown. It can be assumed that, as cellular RNA synthesis is inhibited, the infecting virus does not have to compete for precursors and, therefore, enhanced viral synthetic activity can take place. Experiments are still being carried out to establish whether or not there is a relationship between the stimulation of virus transcription and the modification by IdUrd of cell physiology.

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