Ultraviolet Irradiation of Herpes Simplex Virus (Type 1): Delayed Transcription and Comparative Sensitivities of Virus Functions

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SUMMARY

The delay in the replication of herpes simplex virus surviving u.v. irradiation occurs after the uncoating of virus, as judged by sensitivity to DNase. It occurs before translation, judged by the kinetics of appearance of various virus-specific proteins, and before transcription, judged by the detection of virus-specific RNA by in situ hybridization. Since the delays in both transcription and translation are reversed by photoreactivation, the simplest hypothesis is that pyrimidine dimers directly obstruct transcription; unless these are broken by photoreactivating enzymes, there will be transcriptional delay until reactivating processes have repaired the lesion. The u.v. sensitivities of the abilities to induce various enzymes (thymidine kinase, DNase and DNA polymerase) were only about four times less than that of infectivity. The ability to induce the three enzymes was three times less sensitive than that of the structural antigen (Band II).

INTRODUCTION

When irradiated with ultraviolet (u.v.) light, herpes simplex virus shows three notable features. First, the survival of infectivity gives a multicomponent curve (Lytle, 1971; Ogino & Rapp, 1972; Ross et al. 1972). Secondly, the survival of infectivity is greatly influenced by host-cell reactivation and to some extent by post-replication repair (Lytle, 1972). Thirdly, there is so marked a delay in the replicative cycle that plaques are considerably reduced in size (Ross et al. 1971, 1972).

We have previously obtained firm evidence that the delay in virus replication cannot be attributed to compromised adsorption or penetration of virus and that it must occur before the appearance of virus-specific antigens (judged by immunofluorescence; Ross et al. 1971, 1972). By using various inhibitors of reactivation processes and photoreactivation in avian cells, we obtained indirect evidence that the major cause of the delay was probably at the transcriptional level (Ross et al. 1972). However, Miyamoto & Morgan (1971) found that large doses of u.v. irradiation inhibited the uncoating of virus, suggesting that this might also partially account for the delay.

We therefore set out to pinpoint the cause of the delay in greater detail between the time of penetration (i.e., after virus becomes inaccessible to neutralizing antibody) and until several specific functions become translated; the results are reported here. In the course of the work, it became plain that the u.v. sensitivities of various virus functions were greater than expected; this too is reported.

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METHODS

**Virus.** A twice plaque-purified clone of the HFEM strain of herpes simplex virus type I was used after one pass in baby hamster kidney (BHK) cells. Stocks were produced in BHK cells and stored at -70 °C (Watson et al. 1966).

**Cells** were grown in a modified Eagle’s medium (Vantsis & Wildy, 1962) containing 10% tryptose phosphate broth and 10% calf serum (ETC). The following lines were used: (a) BHK21 (C13) (Macpherson & Stoker, 1962); (b) BHK21 cells resistant to 100 μg/ml BrdUrd (Sigma Chemical Co. Ltd, St Louis, Mo., U.S.A.) which were used for the evaluation of virus-specific functions; (c) human epidermoid carcinoma number 2 (HEp-2) cells (Fjelde, 1966); (d) primary chick embryo cells were prepared by mincing and trypsinizing 14-day-old chick embryos from which the head, viscera and legs were removed. The chick embryo cells used in the photoreactivation experiments were at the second pass.

**Standard experimental procedure.** Cell monolayers were grown in 20 oz bottles, infected with irradiated or unirradiated virus suitably diluted in 5 ml of ETC. After 1 h the monolayers were washed twice with warm PBS and overlaid with 50 ml warm ETC. At appropriate times after incubation at 37 °C cells were scraped off the glass, concentrated by slow centrifugation and washed in ice-cold PBS. Cells from a control bottle taken at the same time were trypsinized and counted.

**Assays.** For each time point duplicate cultures were assayed independently for the various functions. Results were expressed as the geometric mean of the values obtained. All assays were performed on cell extracts made by disrupting 1 × 10⁷ cells/ml in distilled water by sonication (M.S.E. 150 W Ultrasonic Probe Disintegrator). Virus infectivity was assayed in plastic Petri dishes by plaque formation in BHK cells using the suspension method of Russell (1962) with a CMC overlay and incubation at 37 °C for 72 h in a humidified atmosphere containing about 5% CO₂. Thymidine kinase was measured as described by Klemperer et al. (1967). Alkaline deoxyribonuclease was assayed by a method based on that of Morrison & Keir (1968). DNA polymerase was assayed by a method based on that of Keir & Shepherd (1965). Complement fixation assays were performed as described by Sim & Watson (1973) using anti-Band II antiserum prepared as described by Watson & Wildy (1969).

**Evaluation of virus-specific functions.** Preliminary experiments using BHK cells, employing a range of multiplicities from 0.1 to 100 and three independently assayed cultures per point, indicated a linear relation between enzyme activity and input multiplicity over the range 0.1 to 5 (slope = 0.94, r = +0.97, P = 0.01) the standard deviation on particular points lay between ±8%.

To obtain valid estimates of the u.v.-sensitivity of the ability to express enzyme functions and Band II antigen, care was taken to ensure (a) that the products of singly infected cells were analysed and (b) that the products of second and later cycles of infection were eliminated. The virus was therefore inoculated on to monolayers at low multiplicity (0.1 to 1 p.f.u./cell) and the products harvested before the onset of the second cycle of infection (12 h). In calculating multiplicities the infectivity titre before irradiation has been used.

**U.v. irradiation.** The u.v. light source was a ‘chromatolite’ 30 W low-pressure mercury discharge lamp in quartz with 90% of its energy output stated as 254 nm. The lamp gave, at a distance of 43 cm, a dose rate of 1116 ergs/mm²/min assessed by ferrioxalate actinometry (Hatchard & Parker, 1956). Virus diluted 1/10 in phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) was irradiated in 60 mm diam. Petri dishes (Ross et al. 1972).

**Photoreactivation.** Chick embryo monolayers were treated as in standard experimental procedure. Between 2 and 3 h after infection the bottles were illuminated uniformly from beneath at a distance of 30 cm by 6 white light Osram electric bulbs (100 W) at room temperature (22 °C). For controls, the under surfaces of half the bottles were shielded with
aluminium foil. A fan blew air between the lamps and bottles and limited the temperature rise to less than 0.5 °C.

\(^3\)H-labelled virus. For preparation of pure virus, HEp-2 cells were infected at a multiplicity of 1 to 2 p.f.u./cell and the medium harvested after 75 h. The virus was purified from the medium by a modification of the procedure described by Powell & Watson (1975). Preparation of \(^3\)H-labelled virus was with the ETC replaced by Eagle’s medium supplemented with 5% calf serum and methyl-\(^3\)H-thymidine at 10 μCi/ml (20 Ci/mmol; Radiochemical Centre, Amersham, Bucks).

\(^125\)I-labelled virus DNA. A virus suspension was made to 0.5% SDS, and 1 mg/ml with self-digested Protease VI (Sigma) and the mixture was incubated at 37 °C for 4 h. The solution was extracted once with phenol and once with chloroform/iso-amyl alcohol (24/1) at room temperature. The DNA was precipitated by the addition of 2 vol. of ethanol, followed by further purification by equilibrium CsCl centrifugation (Flamm et al. 1966). The subsequent \(^125\)I-labelling of the purified virus DNA followed the procedure of Minson et al. (1976) and produced DNA with a sp. act. of about 4 × 10⁷ ct/min/μg with approx. 4% of the cytosine residues labelled.

In situ hybridization. Cells were infected 24 h after seeding at low density on sterile glass slides, at which time the majority of the cells were separated. The cells were washed once with warm PBS, 0.5 ml of virus inoculum was added to each slide and allowed to adsorb at 37 °C for 1 h. The cells were washed twice with warm PBS and fresh ETC added. After appropriate incubation the cells were washed once in warm PBS then fixed at room temperature for 30 min in 70% methanol. The slides were dehydrated through an ethanol series and dried in vacuo over silica gel.

The iodinated virus DNA (sp. act. 4 × 10⁷ ct/min/μg, approx. 200 nucleotides in length) was denatured in 0.05 M-tris-HCl, pH 7.5, 0.01 M-NaCl, 0.005 M-EDTA at 100 °C for 8 min then made to 10× SSC at 2 × 10⁷ ct/min/ml. Hybridization followed the procedure of Copple & McDougall (1976) at 65 °C for 14 h. After hybridization the slides were washed with several changes of 4× SSC for 6 h, rinsed quickly in distilled water and then dehydrated and dried in vacuo. Slides were dipped in Ilford K2 emulsion (diluted 1:1 with 2% glycerol in distilled water) air dried and exposed in light-tight boxes at 4 °C for 1 week. Autoradiographs were developed in Kodak D19 for 9 min at 18 °C, washed in 3% acetic acid for 5 s and fixed in Hypam rapid fixer (diluted 1:3 in water) for 8 min, then washed for 1 h in water changed five times. The slides were stained for 10 min with Giemsa (Fisher Scientific Co., New Jersey, U.S.A.) diluted 1:30 in water. For each determination three slides were used and for each slide grains counted over 300 cells. Standard errors for each determination lay within 4%. The specificity of the hybridization reaction was established by using bacteriophage λ DNA \(^125\)I-labelled by the same method; infected cells showed no increase over background graining.

**RESULTS**

The uncoating of u.v.-irradiated virus

Ross *et al.* (1972) showed that heavily irradiated herpes virus adsorbs to and penetrates cells as efficiently as unirradiated virus. To examine uncoating, \(^3\)H-dTHd-labelled purified virus (see Methods) was irradiated (0, 2232 and 4464 ergs/mm²) and cells were infected at an added multiplicity of 4. At 2 h p.i. samples were taken, sonicated and incubated with 50 μg/ml DNase for 1 h at 37 °C. The sensitivity of the virus DNA to DNase was judged by solubility in 10% TCA solution. Even after 4464 ergs/mm², which reduces infectivity by at least 4 logs, 50% of the virus DNA was susceptible to DNase and so, presumably, uncoated, compared to 65% solubility for unirradiated virus DNA. This difference in levels of uncoating is insufficient to explain the delay in replication of u.v.-irradiated virus.
Delay in transcription

The presence of virus mRNA in infected cells was detected by in situ hybridization using $^{125}$I-labelled virus DNA probe. 10 grains/cell (s.d. = 4) was the mean for uninfected BHK cells and no cell had more than 20 grains. It was decided to take 25 grains/cell as the lower limit for the presence of virus mRNA. At the time of maximum grain counts, 18 h p.i. for unirradiated virus and 24 h p.i. for irradiated virus, the average number of grains/positive cell were respectively 98 and 121 with maximum grains/cell of 136 and 162. Judged by the number of cells with more than 25 grains, at 0.5 p.f.u./cell, there was a delay of about 8 h before an equivalent concentration of virus mRNA was detected in cells infected with virus irradiated for 1116 ergs/mm² compared with unirradiated controls (Fig. 1a).

Chick embryo cells were infected at 0.5 p.f.u./cell with unirradiated virus or virus irradiated with 1116 ergs/mm². No uninfected cell contained more than 8 grains (mean = 4) and 10 grains/cell was therefore taken as the lower limit for the detection of virus mRNA. At the time of maximum graining, 28 h p.i. for unirradiated virus and 32 h p.i. for irradiated virus, the average number of grains/positive cell were 23 and 29 with maximum grains/cell 55 and 58 respectively. Judged by a number of cells with more than 10 grains there was a delay of about 16 h before mRNA produced from irradiated virus reached the levels of unirradiated controls (Fig. 1b). Photoreactivation from 2 to 3 h p.i. shortened this delay considerably.

Photoreactivation did not affect the final percentage of cells found to contain mRNA. RNase treatment [at 37 °C for 30 min with 50 µg/ml pancreatic RNase (Worthington), previously boiled for 3 min at pH 5 to destroy DNase activity] reduced the percentage of cells infected with unirradiated virus containing mRNA from 42 to 6% at 24 h p.i.

The distribution of grains/cell in the photoreactivation experiment (Fig. 2) also demonstrates the delay in the production of mRNA with u.v.-irradiated herpes virus. For example, at 24 h p.i. with irradiated virus the pattern is similar to that at 12 h p.i. with irradiated virus which was photoreactivated.
Fig. 2. Development of herpes-specific mRNA in chick-embryo cells infected with herpes virus: (a) unirradiated, (b) irradiated and (c) irradiated and photoreactivated from 2 to 3 h p.i (see legend for Fig. 1b).

**Delay in translation**

When cells were infected at 1 p.f.u./cell with unirradiated virus or virus irradiated with 1116 ergs/mm², a delay was found before the levels of thymidine kinase from irradiated samples became the equivalent of those in unirradiated samples (Fig. 3). For BHK cells this delay was 8 to 10 h and for chick cells the delay was 12 to 14 h. Photoreactivation 2 to 3 h after infection of chick cells reduced the delay found with irradiated virus by about 6 h but did not affect the unirradiated controls. The delays found in the production of Band II antigen, DNA polymerase, DNase and infectivity were the same as for thymidine kinase in BHK cells. With chick cells, levels of DNase were too low for detection but the other functions again showed the same delay as thymidine kinase; photoreactivation reduced these delays by approx. 6 h.
Fig. 3. Thymidine kinase activity in cell cultures at various times after infection with u.v.-irradiated (1116 ergs/mm²) and unirradiated virus. (a) BHK cells. •—•, Unirradiated virus; ■—■, irradiated virus. (b) Chick-embryo cells. Unirradiated virus: •—•, kept in dark; ○—○, exposed to light. Irradiated virus: ■—■, kept in dark; □—□, exposed to light. Exposure to light was from 2 to 3 h p.i.

Fig. 4. U.v. survival curve of virus-specified functions at 12 h p.i. •—•, Infectivity; △—△, Band II antigen; ▲—▲, thymidine kinase; □—□, alkaline DNase; ○—○, DNA polymerase. Bars represent the range of values.
In the course of the work it was necessary to examine the u.v. sensitivity of the ability to induce several virus proteins. Virus, u.v.-irradiated with different doses, was inoculated on to BHK cells at 0.5 to 1 p.f.u./cell and after 12 h the cells were assayed for thymidine kinase, DNA polymerase, DNase and Band II antigen. The results (Fig. 4) indicate that the survival curves cluster into two groups. The group less sensitive to u.v. light consists of thymidine kinase, alkaline DNase and DNA polymerase with D37 (the 37% survival dose for one-hit curves) of 300 to 400 ergs/mm². Band II antigen and infectivity are more sensitive to u.v. light with D37 about 100 ergs/mm². The levels of virus functions induced by the irradiated virus exceed those of unirradiated virus diluted to a multiplicity of infection equivalent to that recovered from u.v.-irradiated samples. Therefore the induced abilities cannot be accounted for by virus escaping irradiation damage.

**DISCUSSION**

Previously published evidence showed that after u.v.-irradiation with 1116 ergs/mm² the replication of herpes virus in BHK cells was delayed by about 14 h, judged by plaque formation. This could not be explained by delayed adsorption to or penetration into cells (Ross et al. 1972). Despite the work of Miyamoto & Morgan (1971) our present experiments show that the delay in replication observed after u.v. irradiation with this dose cannot be due to impaired uncoating either.

Our previous work showed that the delay in replication was matched by the time of appearance of virus antigens. It is not surprising therefore that the appearance of specific virus enzymes is also delayed. We previously found that, though cooperative reactivation of infectivity occurs, this has no influence on the delay, judged by size of plaques; it is therefore to be expected that the prime cause of the delay precedes DNA replication. This not only accords with the finding that the induction of enzymes involved in DNA replication is delayed but also with the present finding that overall virus transcription is delayed and that this can be partly reversed by photoreactivation. Similar results have been reported for u.v.-irradiated bacteriophage and bacteria (Lewin, 1974) and SV40 (Carp et al. 1966).

The experiments with BHK cells indicate that a u.v. dose of 1116 ergs/mm² caused a delay of 8 h in overall transcription (Fig. 1) and probably a similar period for the induction of enzymes (Fig. 3). As expected this is less than the delay in plaque formation found by Ross et al. (1972). In chick cells longer delays were observed (Fig. 1) but since the activities had not reached their peak by the end of the experiment the length of the delay was uncertain. No attempt was made to determine the length of the delay in more prolonged experiments.

It is difficult to correlate the delays of transcription and translation because in the former, though we detect virus specific sequences, they may be all gene products whereas the enzymes and Band II antigen represent single or limited gene products.

We conclude with some confidence that the prime cause of the u.v.-induced delay is that transcription is prevented by cyclobutane-dimers of adjacent pyrimidine residues until such time as these lesions can be repaired.

During the course of these experiments we found that the u.v. sensitivity of the ability to induce thymidine kinase was only about four times less than that of infectivity (the results of Ogino & Rapp (1972) appear to show approx. a sixfold difference). We also find similar sensitivities with respect to DNase and DNA polymerase. This finding is surprising in view of the different target sizes of the functions; infectivity might be expected to require many (say 100) functional genes whereas any of the enzymes might require one only and thus show relative u.v. resistance. Virus-induced enzymes of bacteriophage T2 are 20 more times resistant than infectivity (Dirksen et al. 1960). Probably the survival of infectivity (judged by
plaque formation in BHK cells) is falsely high owing to the ability of these cells to repair the irradiation damage during the long period of the assay. Thus we estimate a D37 of about 94 ergs/mm² for infectivity assayed in BHK cells, but Lytle et al. (1972) estimated 35 ergs/mm² for virus assayed in xeroderma pigmentosum cells which are deficient in the ability to excise dimers. Similarly we find a D37 of 30 ergs/mm² for virus titrated in mouse brain (R. P. Eglin & P. Wildy, unpublished data). It is likely that, in the absence of repair mechanisms, infectivity would be at least ten times as sensitive to u.v. light as, for example, thymidine kinase.

It is interesting to consider the u.v. sensitivity of these functions in relation to what is known of transcription and the protein regulation scheme put forward by Honess & Roizman (1975). Three groups of proteins are recognized which appear sequentially and which seem to interact. Thus the formation of γ proteins is dependent on the presence of β proteins and the formation of β proteins on the presence of α proteins. Hence the sensitivity of the ability to produce these would be expected to be as follows: α < β < γ < infectivity. Though our results must not be overinterpreted, they are consistent with this pattern. For the probable β proteins (DNA polymerase, alkaline DNase and thymidine kinase) the D37 is about 350 ergs/mm², for γ proteins (Band II antigen) it is about 110 ergs/mm² and for infectivity less.

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