Effect of UV-irradiation on DNA replication of the parvovirus minute-virus-of-mice in mouse fibroblasts

Jean Rommelaere^{1,2} and David C. Ward¹

¹Dep. Human Genetics and Molecular Biophysics and Biochemistry, Yale Univ. School of Medicine, 333 Cedar St., New Haven, CT 06510, USA, and ²Lab. Radiobiology and Biophysics, Dep. Molecular Biology, Univ. Libre de Bruxelles, 67 rue des Chevaux, 1640 Rhode St. Genèse, Brussels, Belgium

Received 8 January 1982; Revised and Accepted 5 April 1982

Abstract

The effect of UV-irradiation on the conversion of the single-stranded DNA of the parvovirus Minute-Virus-of-Mice (MVM) to duplex Replicative Forms (RF) was studied after infection of mouse A9 fibroblasts. UV-irradiation of the virus prior to infection of unirradiated cells resulted in a dose-dependent, single-hit, inhibition of RF formation. Restriction fragment analysis indicated that this inhibition could be ascribed to the introduction of absolute blocks which prevent elongation of the newly synthesized complementary strand. Cell exposure to UV-light prior to infection with UV-irradiated MVM enhanced the fraction of input viral DNA which was converted to RF. This enhancement required *de novo* protein synthesis during the interval between cell irradiation and virus infection. These results suggest that DNA replication constitutes a target in the viral life cycle that leads to the UV-enhanced Reactivation of virus survival, however they do not permit us to identify the step of RF formation which is enhanced in UV-pretreated cells.

Introduction

UV-irradiation of eukaryotic cells causes periods during which the rate of DNA synthesis is reduced. UV-induced DNA damage prevents replicon initiation (1). Moreover, pyrimidine dimers, the predominant UV-induced DNA lesions, appear to pose obstacles to the replication machinery and to inhibit the elongation of growing DNA strands (2). *In vitro* experiments have shown that mammalian DNA polymerase alpha is blocked at dimers during replication on a UV-irradiated single-stranded DNA template (3). *In vivo*, dimers are responsible for the formation of discontinuities in newly synthesized DNA (4-6). The *in vivo* structure of blocked DNA replication intermediates is poorly understood in mammalian cells. In particular, it is disputed whether replication halts (7,8) or whether it resumes beyond dimers to leave gaps in the daughter strands synthesized from lesion-containing templates (9-12). The complexity of the cellular DNA replication process (13-14) makes it difficult to resolve this controversy unambiguously by direct analysis of cell DNA.

Another intriguing problem is how mammalian cells recover their ability to synthesize DNA both of a normal size (5,15) and at a normal rate (7,16) late after irradiation. This recovery does not parallel the excision of lesions (17-19) and cannot be ascribed to the reiterative replication of lesion-free DNA (20). The progression of postirradiation DNA replication does not seem to involve large single-strand exchanges between sister molecules (21-23) nor strand displacement at the replication fork (24).

These uncertainties prompted us to further investigate the effect of UV light on DNA replication using autonomous parvoviruses as probes and exposing the virus to UV light extracellularly prior to infection of untreated or UV-irradiated cells. Autonomous parvoviruses contain a single-stranded (SS) DNA genome which constitutes a single replicon that is converted into duplex Replicative Forms (RF) in infected cells (25). The primary event in the DNA replication cycle is in the continuous and unidirectional elongation of the 3' end palindrome of the viral genome which serves as a natural primer and a unique origin (26). It appeared to us that this event might be especially suitable to monitor the effect of UV-light on DNA replication on a leading strand. Previous studies using the autonomous parvovirus Minute-Virus-of-Mice (MVM) in A9 mouse fibroblasts showed that cell UV-irradiation prior to infection with UV-irradiated virus enhanced virus survival (27), a phenomenon called UV-Enhanced Reactivation (UV-ER) by Lytle (28). UV-ER in prokaryotes has been claimed to be related to an enhanced capacity for copying damaged DNA (29). In mammalian cells, the primary target of UV-ER is not known. In that general context, the present study was undertaken to compare untreated and UV-pre-irradiated A9 cells for their ability to convert SS-MVM DNA to duplex RF.

Materials and Methods

Virus and Cell

The plaque-purified strain T of MVM (30) was grown in the A9 variant of mouse L cells (31). Cells were maintained and virus was produced and purified according to Tattersall et al. (32). ³²P-labelled MVM virions were prepared from suspension A9 cultures as described by Ward and Dadachanji (25) except that medium contained 2% of the normal phosphate concentration; viral DNA had an initial specific activity of about 1.3×10^6 cpm/ug. Monolayers of A9 cells were accumulated in G₀ by isoleucine deprivation as described previously (27). After release from isoleucine

starvation, synchronized cells entered into S after about 15 hours.

UV-irradiation

Virus suspensions and cell monolayers were UV-irradiated as described (27).

Isolation of intracellular viral DNA

G₀-arrested monolayer cultures of A9 cells (about 2×10^7 cells per 150 mm dish) were UV-irradiated (0 or 4.5 J m^{-2}), further incubated in isoleucine-depleted medium for 12 hours and infected with approximately 15 pfu/cell of ^{32}P -labelled MVM. The input virus multiplicity was estimated from the specific activity of viral DNA and the particle to infectivity ratio of MVM (30) and refer to inocula prior to irradiation. The time of cell irradiation was selected to give maximal UV-ER of virus survival (27). Infected cells were incubated in complete minimum essential medium and harvested at intervals. Intracellular DNA was fractionated by a modification of the Hirt procedure (32). The DNA from the supernatant fraction was precipitated with 3 volumes of ethanol at -20°C . The DNA precipitate derived from each dish was redissolved in 0.75 ml of 10 mM Tris, 1 mM EDTA (pH 7.5) and incubated for 1 hour at 37°C with Proteinase K (50 $\mu\text{g/ml}$). The DNA solution was purified by three extractions with an equal volume of a 1:1 mixture of phenol (saturated with 10 mM Tris-HCl buffer, pH 8.0) and chloroform-isoamyl alcohol (24:1, vol:vol). After ether-extraction the aqueous phase was made 0.3 N in sodium acetate and DNA was precipitated with 3 volumes of ethanol after standing at -70°C for 2 hrs. In some experiments the Hirt pellet derived from individual cultures was extracted three times with 1.5 ml of acetone; the precipitate was dried under vacuum, suspended in 3 ml of 10 mM Tris, 10 mM EDTA (pH 7.5) and incubated with Pronase (1 mg/ml) for 2 hrs at 37°C . After repeated extraction with phenol-chloroform:isoamyl alcohol, the pellet-associated DNA was ethanol-precipitated. The overall recovery of the ^{32}P -labelled DNA was 92 and 83% for the Hirt supernatant and pellet, respectively. In reconstruction experiments, about 5% of the purified viral DNA added to uninfected cells lysates was found in the Hirt pellet, irrespectively of whether it was single-or double-stranded (data not shown).

Enzyme-assay conditions

The ethanol-precipitate of one tenth of the DNA extracted from one culture was redissolved in 50 μl (Hirt supernatant) or 250 μl (Hirt pellet) of appropriate buffers, at respective concentrations of about 0.3 and 0.05 μg radio-labelled viral DNA per ml (as calculated from Cerenkov counting of the samples and the specific radioactivity of input viral DNA).

Reaction mixtures contained (i) for digestion with single-strand-specific S1 nuclease: 0.25 M NaCl, 1 mM ZnSO₄, 0.03 M sodium acetate (pH 4.5) and 0.15-100 units of enzyme (Sigma); for digestion with Hinf I restriction endonuclease: 0.05 M NaCl, 6 mM Tris-HCl (pH 7.4), 6 mM MgCl₂, 6 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin and 10 units of enzyme (New England Biolabs); for digestion with EcoRI restriction endonuclease: 0.05 M NaCl, 0.1 M Tris-HCl (pH 7.5), 0.01 M MgCl₂ and 30 units of enzyme (Boehringer-Mannheim). Digestions were for 1 hour at 37°C. Reactions were stopped by adding EDTA (10 mM final concentration), sodium acetate (0.3 M), magnesium acetate (10 mM) and 3 volumes of ethanol.

Physical maps of the restriction fragments produced after cleavage of MVM DNA with EcoRI and Hinf I endonucleases have been reported by Chow and Ward (33) and Mc Master et al. (34), respectively.

Fractionation of viral DNA by gel electrophoresis

Samples of ethanol-precipitated DNA [$(17 \pm 10) \times 10^3$ cpm] were redissolved in 20-40 µl of appropriate buffers containing 0.4 M Urea and applied to different wells in agarose (1 or 1.4%) or polyacrylamide (4%) slab gels. Electrophoresis was carried out as described by Sharp et al. (35) and Jeppesen (36), respectively. Electrophoresis through 1% agarose, 1.4% agarose and 4% polyacrylamide was performed for 450, 750 and 1200 V.hr, respectively. In some experiments, ³²P-labelled monomer duplex DNA (about 2.5×10^3 cpm), synthesized in vitro as described by Bourguignon et al. (37), was added to the samples before electrophoresis.

Gels were dried under vacuum onto Whatman 1 paper and exposed to Kodak XR-5 X-ray film. Presensitized film (38) was used for exposure with an intensifying screen (Dupont-Cronex). Autoradiograms were quantitated by scanning with a Joyce-Loebl densitometer. Bands of ³²P-labelled DNA detected by autoradiography were excised from dried gels and counted by liquid-scintillation spectrometry in Econofluor fluid (New England Nuclear). Blanks were excised from the area ahead of the DNA bands.

Results

1. Processing of parental viral DNA in unirradiated A9 cells infected with UV-irradiated MVM

Figure 1 illustrates the types of viral DNA intermediates that have been observed in MVM infected cells and a model proposed for their mechanism of replication (25,26). In addition to unreplicated single-stranded (SS) DNA, duplex replicative forms of monomeric (RF 1) and oligomeric (RF 2) length are seen. As the result of the palindromic

Figure 1. Model for MVM DNA replication after Tattersall and Ward (26)

The parental viral (v) DNA is first converted into a monomer-length DNA duplex (RF 1) by undirectional and continuous elongation of the 3' terminus that serves as a natural primer for the synthesis of the complementary (c) strand. This DNA has the turnaround (ta) configuration, in which the v and c strands are linked covalently through the 3' hairpin duplex. RF DNA is then amplified by a process in which both monomeric (RF 1) and concatemeric (RF 2) DNA species are generated (see ref. 25 and 26). This process gives rise to dimeric RF (see Figure) and higher multimeric RF forms (not shown). Site-specific cleavage of concatemers would generate monomeric RF molecules in the extended configuration (RF 1e) containing a copy of the 3' hairpin sequence in its original orientation. The final stage of replication is postulated to involve the asymmetric strand displacement synthesis of SS progeny DNA from RF DNA.

— parental DNA; newly synthesized DNA

structures at both ends of the viral genome, the 3'- and 5'-termini of all RF molecules can be found in extended (e) or turnaround (ta) configurations, a reflection of whether the nucleotide sequence within the palindrome has been replicated or not, respectively. The gel fractionation systems used in the present study will resolve the two configurations of terminal restriction fragments up to 2 Kb in size; however, because of the small size of the palindromes, full length RF molecules containing different terminal configurations cannot be separated.

To study the effect of UV-irradiation on the replication of parental viral DNA, untreated cells were infected with 32 P-labelled, UV-irradiated

MVM and harvested at intervals after infection. Intracellular-viral-DNA replicative intermediates were isolated by the Hirt procedure and fractionated by gel electrophoresis. It is apparent from Figure 2 that the total radioactivity recovered in the Hirt supernatant fraction was not affected significantly by virus UV-irradiation nor by postinfection incubation time within the interval studied. Consequently, differences in the fraction of the radioactivity of the Hirt supernatant present as RF molecules, are unlikely to result from viral DNA degradation or changes in the distribution of viral DNA between the Hirt supernatant and pellet fractions. The percentage of total Hirt supernatant activity in duplex DNA will thus be considered to be proportional to the level of SS - RF conversion and to constitute a direct measurement thereof.

Figure 3 illustrates the gel electrophoretic pattern of viral DNA intermediates, either undigested (panel a) or treated with Hinf I restriction endonuclease prior to analysis (panel b). The samples shown on each panel were countmatched before loading the gel. Therefore, each gel slot provides a direct comparison of the amount of each DNA species in the Hirt supernatant migrating at a given position under the different experimental conditions. Two dose-dependent effects of virus UV-irradiation are apparent from panel a: (i) the decrease in the fraction

Figure 2. Recovery of input radioactivity in the Hirt-supernatant

Twelve hours after irradiation with UV-light (0 or 4.5 Jm^{-2}), C_{60} -arrested cell cells were infected with ^{32}P -labelled intact or UV-irradiated (50 Jm^{-2}) MVM, incubated in complete medium, and harvested at intervals. The radioactivity of the inoculum was the same for all batches of cells. Viral DNA was extracted (32) and the total ^{32}P -activity in the Hirt supernatant fraction was measured by liquid scintillation spectrometry. Average values from two independent experiments (standard deviation less than 30%). Data were pooled to calculate the regression line. Δ , unirradiated virus in unirradiated cells; $\circ$, UV-irradiated virus in unirradiated cells; $\bullet$, UV-irradiated virus in UV-irradiated cells.

Figure 3. Processing of parental viral DNA in unirradiated A9 cells infected with UV-irradiated MVM

G₀-arrested cells were infected with UV-irradiated ³²P-labelled MVM and incubated for 40 hr in complete medium. After harvesting, the intracellular viral DNA was extracted and the Hirt supernatant fraction analyzed by gel electrophoresis. Each panel is the autoradiograph of a dried gel. Numbers on top of lanes are UV-doses (J m⁻²) given to virus. Samples loaded on each gel were count-matched.

Panel a: Fractionation of viral DNA on a 1.4% agarose gel. SS: single-stranded DNA; RF 1: monomer-length DNA duplex; RF 2: dimeric DNA duplex.

Panel b: Fractionation of viral DNA on a 4% polyacrylamide gel after digestion with Hinf I restriction endonuclease. The restriction fragments G and F have map coordinates between 0-4 and 53-58, respectively. Gta is the turn-around form of the G fragment in which the 3' terminus of the parental DNA strand remains in the foldback configuration.

of DNA that migrates as RF molecules and (ii) the smear of the band of SS DNA towards lower molecular weights. We conclude from Figure 2 and 3a that virus exposure to the range of UV-doses used inhibits the conversion of viral SS DNA to RF forms and causes a limited degradation of SS DNA into acid-precipitable products.

The conversion of viral DNA to duplex molecules confers to it a sensitivity to restriction endonucleases. This property was used to investigate the effect of UV-irradiation on the synthesis of specific segments of viral DNA, in particular the F and Gta restriction fragments generated by Hinf I endonuclease and located in the middle and at the 3' end of the genome, respectively. As shown in Figure 3 (panel b) virus UV-irradiation also inhibited the formation of these fragments, but to a lesser extent than for full RF molecules.

Figure 4 shows that virus UV-irradiation lowered the level of RF accumulation in the Hirt supernatant but did not alter significantly its time course. The relative amount of duplex DNA increased with time and reached a plateau around 40 hr post-infection. However the plateau level obtained decreased as the UV dose given to virus increased. Virus irradiation therefore caused a permanent inhibition of RF formation but no significant delay in the rate at which residual DNA was converted to RF.

The fraction of the radioactivity of the Hirt supernatant present in RF molecules or their restriction fragments at 40 hr post-infection was taken as a measurement of the level of conversion achieved in infected cells and used to quantitate the dose response of viral DNA synthesis, as shown in Figure 5. It is apparent from panel a that RF formation drops exponentially with the dose administered to the virus. The inhibition curve is consistent with a linear UV-induction of replication blocks randomly distributed among the pool of viral DNA molecules. The frequency

Figure 4: Kinetics of conversion of parental viral DNA to duplex replicative forms in unirradiated A9 cells infected with UV-irradiated MVM

G_0 -arrested cultures were infected with UV-irradiated ^{32}P -labelled MVM and incubated for different times in complete medium. Dose to virus was 0 ($\bullet$), 25 ($\circ$) or 50 (Δ) J m^{-2} . Viral DNA was extracted and the fraction of the label of the Hirt supernatant migrating as duplex replicative forms (RF) was determined by electrophoresis through 1.4% agarose gels. RF formation is shown relative to that given by unirradiated virus at 40 hr post-infection. The 1.0 value corresponds to 14% of the total label in the Hirt supernatant. Average values and standard deviations from 2 independent experiments.

Figure 5: Dose response for the conversion of parental viral DNA to duplex forms in unirradiated A9 cells infected with UV-irradiated MVM

G₀-arrested cultures were infected with UV-irradiated ³²P-labelled MVM and incubated for 40 hr in complete medium. Extracted intra-cellular viral DNA from the Hirt supernatant was analyzed.

Panel a: The fraction of labelled viral DNA converted to duplex replicative forms (RF) was determined by electrophoresis through 1.4% agarose gels and is shown relative to that given by unirradiated virus. Virus survival measured independently with unlabelled virus was an inverse exponential function of the UV-dose (27), and is indicated for reference on the top x-axis. The line drawn is the theoretical curve expected if every pyrimidine dimer [as given by Proctor et al, (39) for equivalent survivals of the closely related parvovirus KRV] constituted an absolute block to DNA conversion.

Panel b: Viral DNA was digested with Hinf I restriction endonuclease and analyzed by electrophoresis through 4% polyacrylamide gels. The formation of the F and Gta restriction fragments and of full RF is shown for virus irradiated with 10 (●) or 25 (○) J m⁻² UV-light, relative to unirradiated virus. Residual synthesis is plotted as a function of the distance between the origin of replication (3' hairpin terminus of the genome) and the 5' end of the fragments as derived from the Hinf I restriction map of MVM DNA (34). Lines are the theoretical curves expected from elongation blocks at randomly located pyrimidine dimers, for levels of residual RF synthesis corresponding to 10 (—) or 25 (---) J m⁻² UV-light to virus. Average values and standard deviations from 2 independent experiments.

of these blocks is similar to the reported frequency of UV-induced pyrimidine dimers (39), suggesting that these might be the predominant lesions causing an irreversible arrest of parvoviral DNA conversion. In panel b, the inhibition of the formation of RF molecules is compared to that of the F and Gta Hinf I restriction fragments. The residual synthesis of these three DNA species is plotted as a function of the distance between their respective 5' end and the origin of replication. This distance is equivalent to the length of single-stranded DNA whose conversion is required for the formation of each of these duplex DNAs. It can be seen that the level of synthesis is inversely related to the distance from the replication origin: the formation of the Gta fragment, which comprises the

origin, is barely affected, whereas the synthesis of the F middle fragment is inhibited significantly but to a lesser extent than that of full RF. The shape of the curves is consistent with the blockage of replication at randomly located sites along the viral genome. Most replication blocks in DNA of UV-irradiated virus thus apparently occur at the level of complementary-strand elongation.

2. Effect of UV-irradiation of the cells on their ability to convert parental parvoviral DNA to duplex replicative forms

Figure 6 illustrates electrophoretic profiles of viral DNA extracted from cells which had been infected with ^{32}P -labelled and UV-irradiated virus 12 hours after the culture itself was exposed (or not) to a sublethal UV dose. It is apparent that cell preirradiation increased the fraction of the Hirt supernatant-DNA migrating at higher molecular weight positions than single-stranded DNA, in particular in the RF1 and RF2 sites. This effect was irrespective of the intensity of the RF1 and RF2 bands one relative to the other.

Figure 6: Effect of cell UV-irradiation on the processing of parental viral DNA after infection with UV-irradiated MVM

Twelve hours after irradiation with UV-light (0 or 4.5 J m^{-2}), Go-arrested cultures were infected with ^{32}P -labelled, UV-irradiated (50 J m^{-2}) MVM and incubated for 32-42 hours in complete medium. Viral DNA was extracted and the Hirt supernatant was analyzed by electrophoresis through 1.4% (panel a) or 1% (panel b) agarose gels. Panels (a) and (b) are autoradiograms of dried gels derived from two independent experiments. Samples loaded on each gel were count-matched. Single-strand regions were overexposed and have been cut off. (-) and (+) on top of lanes denote that cells were exposed to 0 or 4.5 J m^{-2} UV-light, respectively. Symbols are as in Figure 1. The distribution of radioactivity between the RF 1 and RF 2 bands varies with time post infection and might reflect the precursor-product relationship between the different RF forms.

This increase in the proportion of RF DNA is unlikely to result merely from an enhancement of the degradation of SS DNA or of its entrapment in the Hirt pellet since the total radioactivity in the Hirt supernatant was not affected by cell preirradiation (Figure 2). Thus the absolute amount of RF DNA appears to be higher in the Hirt supernatant extracted from cells preexposed to UV light. Treatment of the cells might stimulate their ability to convert parental DNA to duplex forms. Alternatively, cell irradiation might release into the Hirt supernatant a fraction of RF DNA which would normally be retained in the pellet fraction. The latter possibility is unlikely since (i) no significant loss of purified duplex MVM DNA into the Hirt pellet was found (data not shown) and (ii) the Hirt pellet extracted from infected-unirradiated-cells was not richer in MVM duplex DNA than that prepared from UV-damaged cells. An analysis of the DNA in the Hirt pellet is illustrated in Figure 7. The majority of this DNA stuck on the top of the gel and was sensitive to digestion by S1 nuclease under conditions that leave duplex DNA intact. After extensive deproteinization this DNA enters into the gel and forms a major band at the position of MVM SS-DNA (data not shown). Altogether these data suggest that most of the radioactivity in the pellet fraction resides in viral SS DNA that is associated with proteins, probably nucleocapsids. However, an overexposure of the gels reveals that a small proportion of the pellet radioactivity was S1-resistant; as shown in Figure 7, this duplex DNA gave rise to the characteristic MVM DNA fragments after digestion with EcoRI restriction endonuclease. Densitometric tracing of these autoradiograms indicate that the amount of RF in both pellet fractions was about 4 times lower than in the corresponding supernatant. In addition, the intensity of the bands of viral duplex DNA obtained from UV-irradiated cells was equal to, or greater than, that obtained from control cells. These data rule out the possibility that a selective release of RF molecules from the Hirt pellet is responsible for the enrichment of duplex DNA in the supernatant fraction from cells preexposed to U.V. We conclude that the enhanced proportion of RF molecules in the Hirt supernatant is indicative of a higher degree of DNA conversion in pretreated cells.

The enhanced capacity of UV-irradiated cells to replicate viral DNA was characterized by the following results. a Increased DNA conversion did not merely result from an earlier onset of viral DNA synthesis. As shown in Figure 8, RF formation occurred at a higher level but with a similar time-course in both treated and control cells.

Figure 7: Analysis of the DNA in Hirt pellets

Twelve hours after UV-irradiation (0 or 4.5 J m^{-2}), Go_2 -arrested cells were infected with ^{32}P -labelled UV-irradiated MVM (50 J m^{-2}) and incubated for 42 hrs in complete medium. After extraction, intra-cellular DNA from the Hirt pellet fractions was incubated with or without S1 nuclease (10 units) and EcoRI restriction endonuclease. Countmatched samples were then electrophoresed in 1.4% agarose gels for 450 V. hr. Exogeneous RF DNA added to the samples was not degraded by the S1 treatment under these conditions (not shown). The autoradiogram of a dried gel is shown. (-) and (+) denote that cells were irradiated with 0 or 4.5 J m^{-2} UV-light, respectively. The position of the A, B and C fragments generated by EcoRI digestion of duplex viral DNA is indicated. The B and C terminal fragments appear as doublets corresponding to the extended and foldback configurations.

b The factor of enhancement varied from one experiment to the other.

Values of 1.0 (no effect) to over 2.0 were found, as illustrated by the scattering of the points in Figure 8. Errors inherent to the procedures can only account in part for these fluctuations since the standard deviation in individual experiments was about 20%. A variable enhancement factor was also reported for the survival of virus infectivity in pretreated cells (40). Such fluctuations might reflect the dependence of induced processes on poorly controlled parameters of cell physiology.

c Viral DNA conversion was stimulated by cell pretreatment for both intact

Figure 8: Effect of cell irradiation on the time-course of the conversion of parental viral DNA to duplex replicative forms (RF) following infection with UV-irradiated MVM

Twelve hours after irradiation with UV light (0 or 4.5 J m^{-2}), Go_2 arrested cells were infected with ^{32}P -labelled-UV-irradiated (50 J m^{-2}) MVM, incubated in complete medium, and harvested at intervals. Viral DNA extracted from the Hirt supernatant was analyzed by electrophoresis through agarose gels and the fraction of ^{32}P -label migrating as duplex DNA was measured. RF formation in UV-irradiated (---) and control (—) cells is shown relative to the plateau level of RF in unirradiated cells. Values obtained for irradiated cells in independent experiments (o, ●, Δ, ▲) are plotted to illustrate the scattering of the results. For the sake of clarity, the control data are illustrated as a single control curve, although the stimulation factor in each experiment was calculated relative to an internal unirradiated cell control. Actual data for control cells were similar to those of Figure 4.

and UV-irradiated virus, but to a greater extent for the latter. In the experiments shown in Table 1 (lines 1-4), the enhancement for unirradiated virus (around 1.25) was 16% of that for UV-damaged virus.

d The enhancement of DNA conversion was dependent on active protein synthesis during the interval between cell irradiation and virus infection. Indeed, cycloheximide, a potent protein synthesis inhibitor, abolished the stimulation, if present during that interval (Table 1, lines 5-7 and Figure 9). Cycloheximide was used at concentrations which did not affect detectably RF formation in unirradiated cells; moreover such a cycloheximide treatment had little effect on the titer of intact virus and the survival of UV-damaged virus in unirradiated cells (27). The drug thus appeared to inhibit selectively the UV-induced enhancement of viral DNA conversion.

Discussion

1. Parvoviruses as probes of replication on a UV-irradiated leading strand

The conversion of parvoviral SS DNA to duplex RF involves the synthesis

Table 1. Effect of cell UV-irradiation on the conversion of MVM SS-DNA to duplex RF

UV Dose to MVM ($J\ m^{-2}$)	Cycloheximide Concentration ($\mu g/ml$)	Time postinfection (hours)	Relative RF* Formation
1. 0	0	32	1.2 ± 0.1
2. 0	0	42	1.3 ± 0.3
3. 50	0	32	2.6 ± 0.3
4. 50	0	42	2.5 ± 0.6
5. 50	0	48	1.7 ± 0.2
6. 50	10	48	1.2 ± 0.1
7. 50	15	48	0.9 ± 0.1

Cells arrested in G_0 by isoleucine starvation were UV-irradiated (0 or $4.5\ J\ m^{-2}$), incubated for 12 hrs in isoleucine-depleted medium (with or without cycloheximide), rinsed and infected with ^{32}P -labelled MVM exposed or not to UV-light. Extracted viral DNA from the Hirt supernatant was analyzed by agarose gel electrophoresis and the fraction of labelled parental DNA migrating as duplex RF was determined.

*RF formation in UV-irradiated cells is shown relative to that in similarly treated but unirradiated cells. Average values and standard deviations from 2 independent experiments. Under the conditions used cycloheximide did not affect detectably RF formation in unirradiated cells.

Figure 9: Effect of cycloheximide on the processing of parental viral DNA in control and UV-irradiated A9 cells infected with UV-irradiated MVM.

A9 cells arrested in G_0 by isoleucine starvation were UV-irradiated (0 or $4.5\ J\ m^{-2}$), incubated for 12 hrs in isoleucine-depleted medium in the presence of cycloheximide (CH) (0, 10 or $15\ \mu g/ml$), rinsed extensively and infected with ^{32}P -labelled UV-irradiated ($50\ J\ m^{-2}$) MVM. After further incubation in complete medium for 48 hrs, cells were harvested and intracellular viral DNA was extracted. The Hirt supernatant was analyzed by electrophoresis through 1% agarose gels. The autoradiogram of a dried gel is shown. (-) and (+) on top of lanes indicate control and UV-irradiated cells, respectively. Symbols are as in Figure 1.

of a complementary strand by continuous elongation of the 3' palindromic end of the genome (26), thereby providing unique probes for analyzing DNA synthesis on a leading strand. Moreover the conversion step of parvoviral DNA replication is very likely to rely entirely on host cell functions (41) and may thus be considered as a monitor of cellular replication activities.

The data presented in this paper indicate that UV-irradiation of the parvovirus MVM inhibited the SS to RF conversion. Neither the site and the frequency of the initiation events nor the temporal sequence of synthesis seem to be altered by virus exposure to UV-light. The time-course of the residual RF formation in cells infected with UV-irradiated MVM was the same as for intact virus, arguing against a general slowing of DNA synthesis. In contrast, it appears that the inhibition of MVM DNA conversion can be quantitatively accounted for by a blockage in the elongation of the complementary strand. The sites of blockage are distributed along the genome and occur at a frequency similar to that reported by Proctor et al.(39) for intrastrand pyrimidine dimers, the major photoproduct induced by UV-irradiation of both SS and duplex DNA. Pyrimidine dimers are likely to present an obstacle to DNA replication by preventing normal base pairing to occur opposite the dimer site. The quantification of the recovery of input radioactivity does not support the interpretation of the results in terms of a preferential loss of SS DNA or duplex DNA after infection with intact or UV-irradiated virus, respectively. However, since only parental strands were labelled, it cannot be ruled out that conversion is unimpeded opposite dimers but that the complementary strand is subsequently degraded from the damaged site towards the 5' end of the genome. This possibility is, however, very unlikely from the weight of evidence suggesting that elongation of the newly synthesized strand is blocked at many sites of UV damage in the template strand (reviewed in Ref. 2).

Convincing evidence either confirming or disproving the occurrence of reinitiation of DNA synthesis downstream from the dimers, i.e. gap formation opposite the dimers, is lacking in mammalian cells. Studies of SV40 virus DNA replication indicated that some dimers block progression of the replication fork and led to the hypothesis that these are the dimers residing in the leading strand (42). DNA damage in the leading strand would arrest the elongation of the complementary strand which would not resume beyond the lesions, thus blocking the ongoing replication fork. In contrast, the discontinuous mode of replication on the lagging strand would

allow the reinitiation of DNA synthesis downstream from the lesions in that strand. The evidence presented in this paper supports such a model by arguing against gap formation during replication on a UV-irradiated leading strand. Molecules of UV-damaged parvoviral DNA which were blocked in the formation of a small restriction fragment comprising the replication origin did not synthesize a fragment located downstream in the middle of the genome. In contrast, only a small fraction of the molecules blocked in the formation of the middle fragment were affected in the synthesis of the origin fragment. These results suggest that the elongation of the complementary strand stopped irreversibly in the vicinity of the lesions in the viral strand, although the formation of gaps large enough to overlap both fragments (about 2500 nucleotides) cannot be ruled out. Extrapolation of the results obtained with SV40 or parvovirus to the cells should take into account that these viruses comprise an unique replicon, whereas the cell genome is made up with multiple replicons each containing its own origin of replication. Consequently, the possibility should be considered that in mammalian cells, DNA synthesis downstream from a lesion in a leading strand of a given replicon might occur through the extension of the replication of an adjacent replicon beyond its normal termination point (13).

The inhibition of the conversion of UV-irradiated parvovirus DNA was permanent within the 48 hours interval studied. As the burst of infectious progeny virus was produced within 36-48 hours (27) RF DNA synthesis is unlikely to resume at later times. Altogether, the results suggest that the RF formed within a physiologically relevant interval derive mostly from unhit, lesion-free, parental DNA. This contrasts with the ability of mammalian cells to eventually overcome the replication blocks induced in cellular or SV40 DNA, i.e. to elongate shorter daughter strands by a process often referred to as Post-Replication Repair (reviewed in Ref. 2). On the other hand, the irreversibility of the inhibition of parvoviral DNA replication in untreated cells is reminiscent of that observed for UV-irradiated ϕ X174 bacteriophage (29). Single-stranded DNA viruses, such as MVM and ϕ X174, do not allow recombination with an homologous sister molecule to contribute to the replication of damaged DNA since no complementary strand exists in virus-infected cells prior to replication. This property might be related to the persistency of the inhibition of parvovirus DNA replication, although large sister strand exchanges do not appear to play a major role in the recovery of UV-irradiated duplex DNA

synthesis in animal cells (2). Moreover, it should be noted that the replication of both UV-damaged ØX174 (29) and MVM DNA was studied, in particular, after infection of untreated cells, whereas cultures were exposed directly to UV-light in the case of cellular and SV40 DNA analysis. As discussed below, the ability of irradiated cells to replicate damaged DNA might not be the same as that of normal cells. Consequently, the possibility should be considered that the cell treatment might also contribute to the different persistency of the replication blocks observed for SS and DS DNA.

2. Regulation of the cell capacity to convert MVM parental DNA to RF DNA

When mouse cells were exposed to sublethal UV doses and incubated to allow expression of any inducible mechanism prior to MVM infection, an absolute increase of the fraction of parental DNA that became double-stranded was observed. We found no argument to support the explanation of this result in terms of a preferential loss or degradation of duplex DNA from untreated cells, or of single-stranded DNA from UV-irradiated cells. The data rather suggest that irradiated cells displayed an enhanced capacity to convert SS parental DNA to RF. The time course of RF formation was not altered by cell pre-irradiation, indicating that (a) factor(s) involved in normal conversion or in its enhancement is (are) only available transiently.

Our data do not yet allow us to identify the process whose activation in irradiated cells increases the level of RF formation. Possible mechanisms include (i) replication past pyrimidine dimers by modification of either the replication machinery or the structure of the damaged site; (ii) reinitiation of DNA synthesis downstream from the lesion in parental DNA; (iii) initiation of the conversion of new molecules and (iv) correction of the defectiveness responsible for the generation of many deleted genomes during replication of normal viral DNA. Since RF molecules were identified by their electrophoretic mobility in neutral gels, small gaps in the complementary strand would not be detected. The data, therefore, do not discriminate between possibilities (i) and (ii), i.e. continuous or discontinuous modes of enhanced RF synthesis. For practical reasons, the experiments reported in this paper were performed under conditions in which cells were infected with more than one viral plaque-forming-unit per cell. Moreover, the efficiency of conversion of SS DNA to RF was low since less than 15% of the radioactivity of the Hirt

supernatant prepared from cells infected with unirradiated MVM migrated as RF. Recombination between sister molecules cannot participate in the conversion of SS parvoviral DNA. Yet, interactions between homologous viral genomes involving the large pool of non- or partially replicated molecules might account for the increased fraction of RF molecules. It should be noted, however, that the infectivity and the survival of MVM were found to be independent of the multiplicity of infection in both control and UV-irradiated cells (27). Mechanisms (iii) and (iv) are conceivable, knowing that under normal conditions the majority of input parvoviral DNA does not initiate replication. Defective genomes are also generated rapidly in MVM infected cells (43). The greater stimulation of RF formation found for irradiated versus intact virus does not favor possibilities (iii) and (iv); however, it does not exclude them since factor(s) unrelated to the enhancement process might limit the level of RF achieved in untreated cells infected with intact MVM. Moreover the presence of defective genomes obscures the analysis of the interband regions of the gels and precludes a direct study of the accumulation and chase of blocked intermediates.

An intriguing question is the physiological relevance of the observed increase in RF formation. The conditions of cell treatment used in the present study were selected previously as giving rise to an optimal enhancement of virus survival(27), a phenomenon denoted as UV-enhanced Reactivation (28). A parallelism can be tentatively drawn between the stimulation of RF synthesis and UV-ER. Both phenomena were abolished selectively if cycloheximide was present during the induction period. Since cells were in G_0 during that period cycloheximide might suppress UV-induced phenomena directly by inhibiting the de novo synthesis of proteins required for their expression; it cannot be ruled out, however, that the drug acted indirectly by disturbing subsequent cell DNA replication. The factor of enhancement was similar for viral titer and DNA replication and was higher for irradiated than for intact virus. It can be calculated that under the conditions defined in Table 1, the average numbers of hits overcome by the preirradiation treatment were similar and close to 0.65 per virion for both RF formation (Table 1 and Fig. 5A) and virus infectivity (27). These correlations are strengthened by our previous observations showing that (i) the step of the virus life cycle sensitive to UV-ER was subsequent to virus uptake (27) but preceded virus release and secondary infection (44), and (ii) ER of parvoviruses was

associated with viral mutagenesis (45). Altogether our data thus suggest that viral DNA replication might be a candidate target of the UV-ER process. We are presently testing the tightness of the correlation between the increases in virus survival and the extent of DNA conversion.

Acknowledgments

We thank Drs. M.B. Chow, J.J. Cornelis, D.K. Dadachanji and J.M. Vos for numerous discussions.

This work was supported by United States Public Health Service grants GM-20124 and CA-16038 to D.C.Ward and European Communities contract ENV/355/B to J. Rommelaere and M. Errera. J. Rommelaere is Chercheur qualifie du Fonds National Belge de la Recherche Scientifique.

References

1. Kaufman, W.K. and Cleaver, J.E. (1981) *J. Mol. Biol.* **149**, 171-187.
2. Hall, J.D. and Mount, D.W. (1981) in *Progress in Nucleic Acid Research and Molecular Biology*, Davidson, I.N. and Cohn, W.E., Eds., Vol. 25, pp 53-126, Academic Press, New York.
3. Moore, P.D., Bose, K.K., Rabkin, S.D. and Strauss, B.S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 110-114.
4. Lehman, A.R. (1979) *Nucleic Acid Res.* **7**, 1901-1912.
5. Cleaver, J.E., Thomas, G.H. and Park, S.D. (1979) *Biochim. Biophys. Acta* **564**, 122-131.
6. Rosenstein, B.S. and Setlow, R.R. (1980) *Biophys. J.* **31**, 196-206.
7. Edenberg, H.J. (1976) *Biophys. J.* **16**, 849-860.
8. Dahle, D., Griffiths, T.D. and Carpenter, J.G. (1980) *Photochem. Photobiol.* **32**, 157-165.
9. Lehman, A.R. (1972) *J. Mol. Biol.* **66**, 319-337.
10. Buhl, S.N., Setlow, R.B. and Regan, J.D. (1972) *Int. J. Radiat. Biol.* **22**, 417-424.
11. Doniger, J. (1978) *J. Mol. Biol.* **120**, 433-446.
12. Meneghini, R., Cordeiro-Stone, M. and Schumacher, R.I. (1981) *Biophys. J.* **33**, 81-92.
13. Park, S.D. and Cleaver, J.R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3927-3931.
14. Cordeiro-Stone, M., Schumacher, R.I. and Meneghini, R. (1979) *Biophys. J.* **27**, 287-300.
15. Buhl, S.N., Setlow, R.B. and Regan, J.D. (1973) *Biophys. J.* **13**, 1265-1275.
16. Cleaver, J.E. (1965) *Biochim. Biophys. Acta* **108**, 42-52.
17. Meyn, R.E., Kasschan, M.R. and Hewitt, R.R. (1977) *Mutation Res.* **44**, 129-138.
18. Park, S.D. and Cleaver, J.E. (1979) *Nucleic Acids Res.* **6**, 1151-1159.
19. Doniger, J. and DiPaolo, J.A. (1980) *Biophys. J.* **31**, 247-254.
20. Meyn, R.E., Hewitt, R.R., Thomson, L.F. and Humphrey, R.M. (1976) *Biophys. J.* **16**, 517-525.
21. Rommelaere, J. and Miller-Faures, A. (1975) *J. Mol. Biol.* **98**, 195-218.

22. Meneghini, R. and Hanawalt, P. (1976) *Biochim. Biophys. Acta* 425, 428-437.
23. Lehman, A.R. and Kirk-Bell, S. (1978) *Photochem. Photobiol.* 27, 297-307.
24. Tatsumi, K. and Strauss, B. (1978) *Nucleic Acids Res.* 5, 331-346.
25. Ward, D.C. and Dadachanji, D.K. (1978) in *Replication of Mammalian Parvoviruses*, Ward, D.C. and Tattersall, P., Eds., pp 297-313 Cold Spring Harbor Laboratory, New York.
26. Tattersall, P. and Ward, D.C. (1976) *Nature* 263, 106-109.
27. Rommelaere, J., Vos, J.M., Cornelis, J.J. and Ward, D.C. (1981) *Photochem. Photobiol.* 33, 845-854.
28. Lytle, C.D. (1978) *Natl. Cancer Inst. Monogr.* 50, 145-149.
29. Caillet-Fauquet, P., Defais, M. and Radman, M. (1977) *J. Mol. Biol.* 117, 95-112.
30. Tattersall, P. (1972) *J. Virol.* 10, 586-590.
31. Littlefield, J.W. (1964) *Nature* 203, 1142-1144.
32. Tattersall, P., Crawford, L.V., and Shatkin, A.J. (1973) *J. Virol.* 12, 1446-1456.
33. Chow, M.B. and Ward, D.C. (1978) in *Replication of Mammalian Parvoviruses*, Ward, D.C. and Tattersall, P., Eds., pp 205-217 Cold Spring Harbor Laboratory, New York.
34. McMaster, G.K., Beard, P., Engers, H.D. and Hirt B. (1981) *J. Virol.* 38, 317-326.
35. Sharp, P.A., Sugden, B. and Sambrook, J. (1973) *Biochemistry* 12, 3055-3063.
36. Jeppesen, P.G.N. (1974) *Analyt. Biochem.* 58, 195-207.
37. Bourguignon, G.J., Tattersall, P. and Ward, D.C. (1976) *J. Virol.* 20, 290-306.
38. Laskey, R.A. and Mills, A.D. (1975) *Eur. J. Biochem.* 56, 335-341.
39. Proctor, W.R., Cook, J.S. and Tennant, R.W. (1972) *Virology* 49, 368-378.
40. Bockstahler, L.E. and Lytle, C.D. (1977) *Photo-chem. Photobiol.* 25, 477-482.
41. Rhode, S.L. (1978) in *Replication of Mammalian Parvoviruses*, Ward, D.C. and Tattersall, P., Eds., pp 279-296, Cold Spring Harbor Laboratory, New York.
42. Sarasin, A.R. and Hanawalt, P.C. (1980) *J. Mol. Biol.* 138, 299-319.
43. Faust, E.A. and Ward, D.C. (1979) *J. Virol.* 32, 276-292.
44. Luo Zu Yu, Cornelis, J.J., Vos, J.M. and Rommelaere, J. (1981) *Int. J. Radiat Biol.* 40, in press.
45. Zao Zhong Su, Cornelis, J.J. and Rommelaere, J. (1981) *Carcinogenesis* 2, 1039-1043.