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Replication of Adeno-Associated Virus in Cells Irradiated with UV Light at 254 nm

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Irradiation of simian virus 40 (*ori* mutant)-transformed Chinese hamster embryo cells (OD4 line) with UV light induced a cellular capacity which supported a full cycle of helper-independent adeno-associated virus replication. Monochromatic UV light at 254 nm was about 1,000-fold more effective than UV light at 313 nm, indicating that cellular nucleic acid is the primary chromophore in the UV-induced process leading to permissiveness for adeno-associated virus replication. The UV irradiation and the infection could be separated for up to 12 h without substantial loss of permissiveness. During this time interval, the induction process was partly sensitive to cycloheximide, suggesting a requirement for de novo protein synthesis.

The parvoviruses make up a large family (*Parvoviridae*) of single-stranded DNA viruses which display a spectrum of special replication requirements (for reviews, see references 4, 5, 7, 11, and 53). Members of the autonomous group replicate independently provided the host cell expresses certain undefined functions which are cell cycle dependent and which are associated, in some cases, with the transformed phenotype or state of cellular differentiation (8, 11, 53). In contrast, the replication of the human adeno-associated viruses (AAV) in cell culture generally requires the presence of a coinfecting adenovirus or herpesvirus helper (4, 7). The taxonomic distinction (52) between the autonomous parvoviruses and helper-dependent AAV is not, however, clear-cut. Early findings showed that although the autonomous H-1 parvovirus replicates independently in some cells, full replication in cultures of presenescent human embryonic lung cells required coinfection with adenovirus type 12 (26). Furthermore, it has recently become clear that the requirement of AAV for a helper virus is by no means absolute; AAV will replicate independently in some established cell lines if the cells are treated with chemical carcinogens, UV irradiation, cycloheximide, hydroxyurea, and other inhibitors of cell cycle progression (15, 47, 48, 63, 64). The nature of the parvovirus replication requirements provided by the helper virus or satisfied by the phenotype of the host cell (or by an induced modification of that phenotype) has remained elusive. We are interested in studying the nature of such helper functions as part of the more general problem of understanding how cellular permissiveness for viral infection may be regulated.

We previously showed that simian virus 40 (SV40)-transformed cells of Chinese hamster (OD4 line) or human (NBE line) origin will support a complete cycle of independent AAV replication if the cells are treated with 1 mM hydroxyurea (an inhibitor of ribonucleotide reductase) for 36 to 40 h or with 5 mM α-difluoromethylornithine (an inhibitor of ornithine decarboxylase) for 96 h before infection (63). In the absence of the pretreatment, the incoming AAV particle was uncoated, but the conversion of the single-stranded parental virus DNA to duplex replicative forms and amplification of the replicative intermediates were not detectable. Hence, the pretreatment induced the expression of cellular functions required for both the initiation and completion of AAV replication. The mechanism of the induction is not understood. Prolonged treatment with hydroxyurea or α-difluoromethylornithine arrests Chinese hamster OD4 cells at the G1-S boundary of the cell cycle; upon removal of the cell cycle block at the time of AAV infection, the cells moved into the S phase and remained in the S or G2 phase for the entire period of AAV replication (63). Cell synchronization by itself, however, is not a sufficient condition for the induction of AAV replication since synchronized populations derived by mitotic detachment (a procedure which avoids the use of all drugs) supported AAV DNA synthesis at a level which was about 100-fold lower than that induced by the drug pretreatments (63).

A common feature of the treatments inducing AAV replication is that they are DNA-damaging agents which perturb cell cycle progression, mainly by prolongation of the S phase (46, 63). DNA-damaging agents also induce a transient overexpression of certain cellular genes (17, 20), and we have suggested that it is the overexpression of one of these genes which facilitates AAV replication (63). The connection between damage to cellular DNA and the induced permissiveness for AAV is, however, circumstantial. In the present study, we explored the use of UV irradiation of the cells (prior to infection) as an effective inducing treatment for full AAV replication. By determining the level of induction as a function of fluence and wavelength, we present evidence that cellular nucleic acids, rather than proteins or membrane components, are the primary targets (chromophores) whose perturbation leads to the acquisition of a cellular capacity to support AAV replication. In addition, we show that this UV-induced process requires de novo protein synthesis.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The Chinese hamster embryo line OD4, transformed by SV40 *ori* mutant DNA (25), was provided by S. Lavi. OD4 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. HeLa cells (American Type Culture Collection, Rockville, Md.) were cultured in Dulbecco modified Eagle medium with 10% calf serum.

Molecularly cloned AAV type 2 stocks were raised, purified, and shown to be free of contaminating adenovirus type 2 (Ad2) virus as described previously (63). Ad2 (Amer-

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ican Type Culture Collection) was plaque purified twice to remove contaminating AAV, and stocks were raised, purified, titrated, and stored as described previously (63). The pAV2 (24) and pSM620 (44) plasmid constructs containing the complete AAV genome were provided by B. J. Carter and K. I. Berns, respectively.

UV irradiation of cells. Subconfluent OD4 cultures seeded 20 to 24 h previously at 2×10^5 cells per 5-cm-diameter dish were washed with phosphate-buffered saline (PBS) and irradiated (in the presence of 1 ml of PBS, with the dish covers removed) with a Philips TUV 15-W germicidal lamp at an incident fluence rate of 1.5 J/m^2/s. The fluence rate was measured with a Blak-Ray J-225 ultraviolet meter (Ultra-Violet Products, Inc., San Gabriel, Calif.).

For exposure to monochromatic sources of UV light at the Argonne National Laboratory, OD4 cells were grown on acid-cleaned glass cover slips (1 by 5 cm) placed in 5-cm-diameter culture dishes. The cover slips were rinsed in PBS and then inserted, at right angles to the beam, into a quartz cuvette with a 2-mm light path (filled with PBS) which was accurately positioned in front of the exit slit of the monochromatic light source as described elsewhere (35). After irradiation, the cover slips were returned to culture dishes containing fresh medium. The source of UV light at 254 nm was a low-pressure mercury Penray lamp. The source of light at 313 and 405 nm was a 2,500-W high-pressure Hg-Xe lamp in a Kratos-Schoeffel housing coupled to a 500-nm Bausch & Lomb grating monochromator with predispersion optics and appropriate filtration for elimination of scattered light, as described by Peak and Peak (35). The fluence rate at 254 nm was measured with a calibrated GE model UV 480 germicidal meter and that at 313 and 405 nm was measured with a calibrated YSI-Kettering model 65 A radiometer (35).

Infection and transfection of irradiated cells. At the indicated time intervals after UV irradiation, cells were infected with purified AAV at the input multiplicities noted in the figures and tables. Virus was absorbed for 90 min. The 1-ml inoculum was then removed, and the infected cells were washed three times and refed with medium. DNA transfection with plasmid-cloned AAV DNA was performed with DEAE-dextran (30) as described previously (63).

AAV DNA synthesis and proportion of cells synthesizing AAV DNA. At the indicated times, the infected cells were suspended in trypsin-EDTA, combined with floating cells centrifuged out of the medium, and counted. Triplicate samples of 50,000 cells each (for determination of AAV DNA synthesis) and 1,000 cells each (for determination of the number of cells synthesizing AAV DNA) were diluted in PBS and trapped on 25-mm-diameter nitrocellulose filters (BA 85; 0.45-μm pores; Schleicher & Schuell, Inc., Keene, N.H.) by filtration under suction. The filters, cell side up, were placed for 1 min on top of Whatman 3MM paper saturated with 0.5 N NaOH containing 1.5 M NaCl. The filters were blotted dry, and the alkali denaturation step, with intermittent drying, was repeated twice. The filters were then neutralized by three 1-min contacts with Whatman 3MM paper saturated with 1 M Tris hydrochloride (pH 7.0) plus 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked at 80°C for 2 h under vacuum. ^{32}P-labeled AAV DNA probes (1×10^8 to 3×10^8 cpm/μg of DNA) were prepared by nick translation (38) of pSM620 construct DNA. The filters were prehybridized at 68°C for 4 h in 5× Denhardt buffer (12) supplemented with 6× SSC and sonicated denatured salmon sperm DNA at 100 μg/ml; the filters were then hybridized with 300,000 cpm of AAV [^{32}P]DNA per ml in a fresh sample of the prehybridization buffer (2 ml for each filter) for 18 h at 68°C. At the end of the hybridization reaction, the filters were washed twice at room temperature with a solution containing 2× SSC and 0.1% sodium dodecyl sulfate (15 min per wash on a rotator) and twice at 68°C in 0.1× SSC with 0.1% sodium dodecyl sulfate (30 min per wash). To determine the amount of AAV DNA synthesized by 50,000 cells, the appropriate filters after hybridization were counted for ^{32}P radioactivity in a scintillation counter. To determine the proportion of cells synthesizing AAV DNA (63), the appropriate filters after hybridization were autoradiographed (24 to 48 h at −70°C in the presence of an intensifying screen). In each case, the background level of radioactivity resulting from probe hybridization to the input (nonreplicated) AAV DNA was determined in control experiments with identically infected unirradiated cells and/or with irradiated and infected cells sampled at time zero (immediately after absorption of the parental virus). These background values are given in the figures and tables.

Assay for infectious AAV. The infected or transfected cultures were lysed by six cycles of freeze-thawing. The amount of infectious AAV in the lysate was assayed on Ad2-coinfected HeLa cell monolayers by the following modification of in situ plaque hybridization (61, 62). Dishes (9-cm diameter) were seeded with 6×10^6 HeLa cells. Twenty hours later, the cells were infected with 1 ml of medium (lacking serum) containing 10^8 PFU of plaque-purified Ad2 and serial dilutions of AAV. After 90 min at 37°C for virus absorption, the inoculum was removed and 20 ml of agar overlay (Dulbecco modified Eagle medium with 0.9% agar and 2% calf serum) was added. At precisely 30 h postinfection, the agar overlay was removed and the cell monolayer was transferred physically intact to a nitrocellulose filter (61). The transfer was carried out by carefully placing a dry 8-cm-diameter nitrocellulose filter (BA 85) on top of the bared sheet of cells and firmly pressing the filter down. After being moistened with PBS (by placing a Kimwipe soaked in 5 ml of PBS in contact with the dry filter for a few minutes), the filter with the entire cell sheet stuck to its underside was peeled off and placed, cell side up, for 1 min on top of Whatman 3MM paper saturated with 0.5 M NaOH containing 1.5 M NaCl. Subsequent alkali denaturation steps, neutralization, baking, and hybridization with AAV [^{32}P]DNA probes were as above except that the volume of hybridization buffer per filter was increased from 2 to 5 ml. Foci of cells synthesizing AAV DNA were visualized by autoradiography for 48 h at −70°C in the presence of an intensifying screen. The number of discrete autoradiographic signals was directly proportional to the dilution of AAV in the inoculum, within the countable range of 5 to 150 foci per 8-cm-diameter filter (Fig. 1A to C). Pretreatment of the inoculum with AAV antiserum prevented the appearance of the foci (Fig. 1D). Addition of AAV antiserum to a liquid or agar medium (after completion of virus adsorption) did not alter the size of the autoradiographic foci, suggesting that each focus represents one infected cell actively synthesizing AAV DNA. The titer of the virus is given by the number of autoradiographic foci multiplied by the dilution factor and is expressed as AAV infectious units (IU) per milliliter or IU per 10^6 cells. Each assay included a positive calibration control (a known number of AAV IU) and a negative control (infection with Ad2 alone). Compared with the dilution endpoint assay used previously (63), we found the above titration procedure to be more sensitive and precise. In addition, by aligning the autoradiogram with the agar overlay (61), AAV virions (wild type, mutants, recombinants) released by single cells into

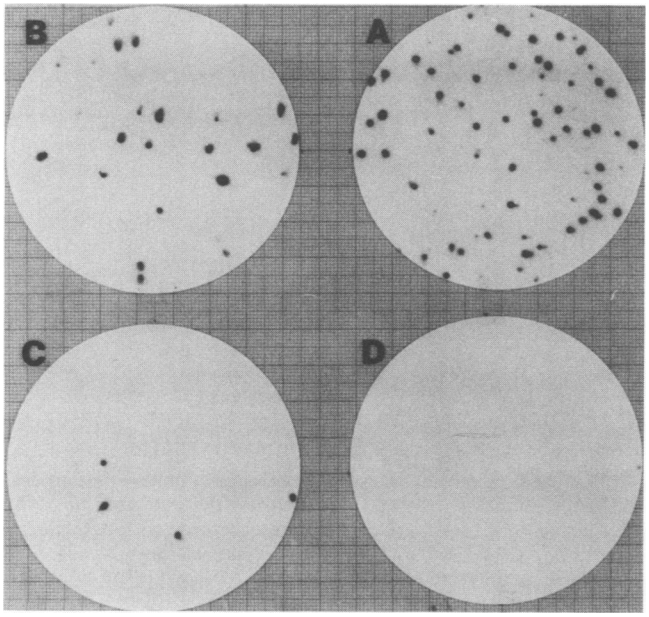

FIG. 1. In situ hybridization assay for infectious AAV. (A to C) Ad2-coinfected HeLa cell monolayers inoculated with fivefold dilutions of AAV. (D) The AAV dilution in panel A was incubated with anti-AAV capsid antiserum (63) before inoculation onto the cell monolayer. At 30 h postinoculation, the cell monolayers were transferred to nitrocellulose filters and hybridized with AAV [^{32}P]DNA as described in Materials and Methods. The photograph shows the autoradiogram of the hybridized filters. Each autoradiographic spot represents one cell actively synthesizing AAV DNA (see text).

the agar can be recovered (L. Tenenbaum and E. Winocour, unpublished data).

RESULTS

UV-irradiated cells support a complete cycle of helper-independent AAV replication. The OD4 line of Chinese hamster embryonic cells was established by transformation with a replication-deficient *ori* mutant of SV40 DNA (25). As shown previously (63), AAV does not replicate in untreated OD4 cells. To investigate the replication of AAV in UV-irradiated OD4 cells, we exposed subconfluent cultures to a germicidal UV lamp and infected them with purified AAV (Table 1). UV irradiation induced a cellular capacity which supported the synthesis of AAV DNA and infectious viral progeny in a fluence-dependent manner. The correspondence between the levels of induced AAV DNA synthesis and the production of viral progeny was satisfactory up to fluences of 18 J/m^2 (we assume that at higher fluences, the selective lysis of irradiated cells replicating AAV led to the apparent decrease in viral DNA synthesis, as measured by the AAV DNA content of 50,000 intact cells). At a fluence of 15 J/m^2, approximately 10% of the irradiated cells supported AAV DNA synthesis (see Fig. 2, below). The efficiency of induction by UV light was very strongly influenced by cell density at the time of irradiation. Optimum conditions were found to be a seeding level of 2×10^5 cells per 5-cm-diameter dish and irradiation at 20 to 24 h after seeding (average cell density of 2×10^4 cells per cm^2 at the time of irradiation).

Transfection of UV-irradiated OD4 cells with plasmid-cloned AAV DNA also gave rise to infectious virus progeny

TABLE 1. Full AAV replication in OD4 cells irradiated with UV light: fluence dependency[a]

J/m^2	AAV DNA replication (^{32}P cpm/50,000 cells)	AAV progeny (IU/10^6 cells)
0	318 (± 21)	1.5×10^4[b]
3	313 (± 38)	1.5×10^4
6	1,261 (± 166)	8.0×10^5
9	6,606 (± 149)	4.1×10^6
12	13,941 (± 3,922)	7.2×10^7
15	25,977 (± 3,596)	8.8×10^7
18	34,182 (± 4,888)	1.4×10^8
21	29,141 (± 6,042)	1.6×10^8
24	17,456 (± 1,805)	2.1×10^8

[a] OD4 cells at a density of 5×10^5 cells per 5-cm-diameter dish were exposed to a UV germicidal lamp at the above incident fluences and then infected with AAV (0.3 IU per cell). AAV DNA synthesis at 24 h postinfection and infectious AAV progeny at 72 h postinfection were measured as described in Materials and Methods. The values shown for AAV DNA synthesis are the means (± standard deviation) of triplicate filters.

[b] Surviving fraction of the input virus.

(Table 2). In this case, the virus yields are comparatively low (cf. Tables 1 and 2) because only a minor proportion of the cells can be successfully transfected with DNA. Hence, the probability that a successfully induced cell will be successfully transfected is correspondingly reduced. The virus harvested from the transfected UV-irradiated cells behaved like wild-type AAV in subsequent infections; that is, it replicated in UV-irradiated cells but not in unirradiated cells (data not shown).

The above experiments demonstrate that UV light induces cellular functions which facilitate a complete cycle of independent AAV replication. In unirradiated cells, AAV replication requires coinfection with a helper virus such as Ad2. The data in Table 3 compare the growth of AAV in UV-irradiated OD4 cells with the AAV replication which is mediated by Ad2 coinfection of unirradiated OD4 cells. The AAV yields generated by Ad2 coinfection of OD4 cells were 50-fold higher, and in Ad2-coinfected HeLa cells (an optimum host cell system for helper virus-mediated AAV replication), the yields were 300-fold higher than those obtained from irradiated OD4 cells in the absence of helper virus. Thus, although helper-independent growth of AAV in UV-irradiated OD4 cells is substantial (1,000-fold increases over the input virus) and generates yields of progeny virus comparable to those of the minute virus of mice autonomous parvovirus in nonrestrictive host cells (11, 39), the Ad2-mediated helper activity is remarkably more efficient.

TABLE 2. UV fluence response for the production of infectious AAV progeny in cells transfected with cloned AAV DNA[a]

J/m^2	IU/10^6 cells
0	<10[b]
3	<10[b]
6	5×10^4
9	5×10^4
12	1×10^5
15	2×10^5
18	9×10^4
21	1×10^5
24	8×10^4

[a] OD4 cells irradiated as described in Table 1, footnote *a*, were transfected with 1 μg of pAV2 DNA per ml (after *Bgl*II digestion which excises AAV DNA from the plasmid [24]) in Tris-buffered medium supplemented with 500 μg of DEAE-dextran per ml. At 72 h after DNA transfection, infectious AAV in the culture lysates was assayed as described in Materials and Methods.

[b] No IU of AAV were detected in a 1:10 dilution of the cell culture lysates.

TABLE 3. AAV replication in UV-irradiated cells compared with AAV replication mediated by Ad2 coinfection of unirradiated cells[a]

Cells	Pretreatment	Virus	h p.i.	IU/10^6 cells
OD4	UV	AAV	1	1.3×10^5
			12	1.1×10^7
			48	2.5×10^8
			72	2.0×10^8
OD4	None	AAV + Ad2	1	1.5×10^5
			72	1.0×10^{10}
HeLa	None	AAV + Ad2	1	1.4×10^5
			72	6.0×10^{10}

[a] UV-irradiated (15 J/m^2) or unirradiated cells were infected with AAV alone (0.5 IU per cell) or with AAV (1.0 IU per cell) plus Ad2 (200 IU per cell), respectively. At the indicated times postinfection (p.i.), the titers of infectious AAV in the lysates were determined as described in Materials and Methods.

Wavelength dependence of UV light-induced replication of AAV DNA. An important advantage of UV light is that the relationship between wavelength and induction efficiency provides information on the primary cellular target (chromophore) whose perturbation leads to the induced process (9, 42). Although the treatments used to induce AAV replication are also known to damage cellular DNA, proof of a connection between DNA damage and the capacity to support AAV replication was lacking. We therefore investigated the wavelength dependence of the induced synthesis of AAV DNA by exposing OD4 cells to the fluences of monochromatic UV light described in Table 4. Under the conditions noted in Table 4, maximum AAV DNA synthesis occurred at a fluence of 25 J/m^2 with light at 254 nm. With increasing wavelength, the efficiency of the induced process was drastically reduced. Thus, despite the use of energy levels at least 1,000-fold higher, the induction at 313 nm was less than that at 254 nm. When cells were irradiated with monochromatic UV light at 405 nm, no induction of AAV DNA synthesis could be detected, although very high levels of energy were employed. However, irradiation with 405 nm light appeared to be deleterious to the cells since a subsequent irradiation at 254 nm (405 + 254 in Table 4) failed to induce AAV DNA replication, in contrast to cells irradiated solely at 254 nm. Consequently, the lack of induction at 405 nm must be viewed with caution. Nevertheless, the data in Table 4 clearly demonstrate that irradiation with UV light at 254 nm is far superior to irradiation with UV light at 313 nm in the induction of the cellular capacity to replicate AAV DNA. Since the absorption maximum for nucleic acids occurs at 260 nm, the data indicate that cellular DNA (or RNA) is the primary target whose perturbation leads to cellular permissiveness for AAV replication. Indeed, when the efficiencies of induction at 254 and 313 nm were plotted as an action spectrum, the data points fitted closely the DNA spectrum derived by Setlow (51) (data not shown).

TABLE 4. AAV DNA synthesis in cells exposed to different wavelengths of UV light[a]

Wavelength (nm)	Fluence (J/m^2)[b]	AAV DNA replication (^{32}P cpm/50,000 cells)
254	0	1,211 (± 75)
	5	1,903 (± 111)
	15	8,301 (± 1,299)
	25	36,650 (± 1,659)
	35	19,756 (± 1,431)
254	0	1,074 (± 39)
	10	5,107 (± 2,122)
	25	22,346 (± 4,421)
313	7,687	2,571 (± 211)
	18,450	4,055 (± 841)
	30,750	2,874 (± 68)
	46,125	5,276 (± 1,282)
405	4,579,200	647 (± 63)
405 + 254[c]	4,579,239	1,057 (± 86)

[a] OD4 cells were exposed to the indicated wavelengths and fluences of monochromatic UV light (at the Argonne National Laboratory) and infected with AAV (3 IU per cell). AAV DNA replication was measured at 22 h postinfection (mean values of two to three replicate filters [± standard deviation]).

[b] After application of a quantum correction (33, 36).

[c] Immediately after irradiation at 405 nm, the cells were exposed to 25 J/m^2 of light at 254 nm.

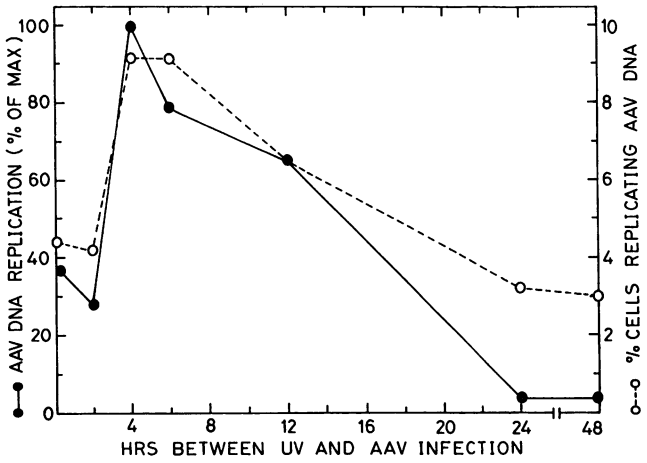

FIG. 2. Dependency of AAV DNA replication on time interval between UV irradiation and infection. At the indicated time after UV irradiation (15 J/m^2), OD4 cells were infected with AAV at 3 IU per cell. At 24 h later, AAV DNA synthesis was measured as described in Materials and Methods. The 100% value for AAV DNA replication was 71,005 (± 1,995) cpm of AAV [^{32}P]DNA hybridized per 50,000 cells. The value for the control nonirradiated cells was 122 (± 9) cpm of AAV [^{32}P]DNA hybridized per 50,000 cells. At each time point, the percentage of cells replicating AAV DNA was measured as described in Materials and Methods.

Time course of UV-induced process. To test the sensitivity of the UV light-induced process to inhibitors of protein synthesis (see below), it was important to determine the stability of that process in terms of the time interval between irradiation and infection. An optimum level of AAV DNA synthesis was obtained when the cells were infected 4 h after irradiation (Fig. 2) (this level, however, was only twofold higher than the level obtained by infecting cells immediately after irradiation). Increasing the time interval between irradiation and infection to 12 h resulted in a modest drop in induction efficiency (about 60% of the maximum level), but time intervals longer than 12 h resulted in an increasingly rapid loss of the induced capacity to replicate AAV DNA. At present, the main conclusion that we wish to draw from Fig. 2 is that the time interval between irradiation and infection can be increased to 12 h without a drastic reduction in the detectable level of induced AAV DNA synthesis. In a reversal of the protocol described in the legend to Fig. 2, cells were also infected prior to UV irradiation. At time

intervals up to 4 h postinfection, UV irradiation induced AAV DNA synthesis with efficiencies comparable to those shown in Fig. 2. However, the induction efficiency decreased when cells were irradiated at times beyond 4 h postinfection (data not shown).

Does the UV light-induced process require de novo protein synthesis? UV irradiation modulates the expression of several cellular genes (reviewed in reference 17). At least two types of mechanisms appear to be involved, depending on the requirement for de novo protein synthesis. The enhanced expressions of the mouse p53 gene (29), the human collagenase gene (1), and the human metallothionein IIA gene (3) after UV irradiation were found to be relatively insensitive to the inhibitor of protein synthesis cycloheximide, observations which have led to the suggestion that DNA-damaging agents increase the activity of preexisting proteins by inducing posttranslational modifications (2). In support of this view, the modulation of gene expression by the phorbol esters seems to arise, ultimately, from the posttranslational modification of DNA-binding proteins (2, 27). On the other hand, the UV light-induced activation of chromosomally integrated viral replicons has been reported to be sensitive to cycloheximide (34, 40). It was therefore important to determine the cycloheximide sensitivity of the UV light-induced process which renders OD4 cells permissive for AAV replication.

To investigate the cycloheximide sensitivity of the UV-induced process, it was essential to distinguish the requirement for protein synthesis during the interval between UV irradiation and virus infection from that requirement during virus replication. UV-induced AAV DNA synthesis can be readily detected even when the time interval between UV irradiation and infection is extended to 12 h (Fig. 2). As measured by the incorporation of radioactive methionine into an acid-precipitable fraction, protein synthesis was totally inhibited in OD4 cells treated with 100 μg of cycloheximide per ml (in the medium) for 1 h, and protein synthesis was restored to 70% of the control value within 2 h of drug removal (data not shown). Although low concentrations (1 μg/ml) of cycloheximide induce the cellular capacity to replicate AAV (15), the high concentrations of cycloheximide (100 μg/ml) used to inhibit protein synthesis do not detectably induce this capacity (B. Yacobson and E. Winocour, unpublished data). Accordingly, the conditions set for the cycloheximide sensitivity tests described in Table 5 were a 4-h (experiment 1A) and 12-h (experiment 2A) exposure to 100 μg of cycloheximide per ml between UV irradiation and AAV infection. In each case, the drug was removed at the time of infection. To evaluate the recovery of the cells from the cycloheximide treatments (and to determine whether such cells can support virus replication), similarly treated cultures of unirradiated cells were infected with AAV and the Ad2 helper virus (experiments 1B and 2B, Table 5). When the UV-irradiated cells were exposed to the inhibitor for 4 h, their ability to replicate AAV DNA was reduced by about 30% (experiment 1A); in contrast, the same cycloheximide pretreatment did not significantly influence AAV DNA replication mediated by Ad2 coinfection in unirradiated cells (experiment 1B). A stronger inhibition of AAV DNA synthesis was observed in irradiated cells treated with cycloheximide for 12 h (92 and 57% inhibition); however, this increased level of inhibition was offset by the finding that a 12-h exposure to the drug inhibited the capacity of unirradiated cells to support the Ad2 helper-mediated replication of AAV DNA by 22%. Nevertheless, the data in Table 5 indicate that the UV light-induced permissiveness for AAV replication is partly sensitive to cycloheximide, suggesting at least a partial dependence on de novo protein synthesis. Conceivably, the UV light-induced process may be totally dependent on de novo protein synthesis. The observed lack of 100% inhibition could result from partly restored synthesis of UV-induced permissiveness factors after drug removal at the time of AAV infection. It was not possible to confirm this possibility experimentally since extension of the cycloheximide treatment beyond the time of infection interfered (as expected) with normal viral DNA synthesis.

TABLE 5. Cycloheximide sensitivity of the UV-induced permissiveness for AAV DNA replication

Expt[a]	Virus	Cycloheximide	h p.i.	AAV DNA synthesis (^{32}P cpm/50,000 cells)[b]	% Inhibition
1A	AAV	−	10	12,464 (± 637)	
		+	10	8,440 (± 427)	32
		−	18	56,342 (± 5,184)	
		+	18	40,129 (± 1,612)	29
		−	24	104,674 (± 498)	
		+	24	68,853 (± 3,466)	34
1B	AAV + Ad2	−	24	32,601 (± 3,324)	
		+	24	32,390 (± 36)	0.6
2A	AAV	−	10	15,009 (± 1,059)	
		+	10	1,174 (± 89)	92
		−	20	42,615 (± 2,991)	
		+	20	18,182 (± 18)	57
2B	AAV + Ad2	−	24	27,392 (± 4,033)	
		+	24	21,389 (± 2,122)	22

[a] UV-irradiated (15 J/m^2) OD4 cells were maintained in normal medium (−) or medium containing 100 μg of cycloheximide per ml (+) for 4 h (experiment 1A) or 12 h (experiment 2A); thereafter, the cells were washed, infected with AAV (1 IU per cell), incubated in normal medium, and analyzed for AAV DNA synthesis at the indicated times (hours postinfection [p.i.]). To monitor the recovery of the cells from the cycloheximide treatment (see text), we maintained unirradiated OD4 cells for 4 h (experiment 1B) or 12 h (experiment 2B) in medium containing or lacking cycloheximide; thereafter, they were coinfected with AAV (5 to 10 IU per cell) and Ad2 (10 PFU per cell) and analyzed for AAV DNA synthesis.

[b] Mean ± standard deviation.

DISCUSSION

We reported previously that pretreatment of Chinese hamster OD4 cells with hydroxyurea or α-difluoromethylornithine induced a cellular capacity which supported a full cycle of independent AAV replication (63). In the present study, we investigated the optimum conditions for the induction of AAV replication by UV light. An important advantage of UV irradiation is that the wavelength dependence of the induced process provides information on the cellular targets involved in the acquisition of the capacity which supports AAV replication. We found that light at 254 nm was about 1,000-fold more effective than light at 313 nm in the induction of this capacity. Optimum induction thus correlates with the peak absorption wavelengths of the nucleic acids, indicating that it is this cellular component which is the primary target in the induction process. Similar action spectra have been observed in other UV-inducible processes in animal cells such as cell lethality (13, 18, 43), mutations (18), transformation (13), and the activation of chromosomally integrated papovavirus replicons (10, 40). The wavelength dependence, however, does not by itself distinguish between DNA and RNA targets. Although a major effect of light at 254 nm in animal cells is the formation of pyrimidine dimers in DNA (13, 43), other correlations between DNA

damage (or subsequent repair) and permissiveness for AAV will have to be sought before a target role for RNA can be vigorously excluded. The lack of response with light at 405 nm is difficult to interpret since subsequent irradiation of the exposed cells with light at 254 nm also failed to elicit a response (Table 4). Prior exposure of *Escherichia coli* to 335-nm, 366-nm, or broad-spectrum solar UV light inhibited subsequent induction of the SOS functions by light at 254 nm (58). Whether our observations with light at 405 nm in animal cells are analogous to the anti-SOS protective effect of near-UV light in *E. coli* (6) remains to be determined.

The induction by UV light of cellular factors which can activate the synthesis of chromosomally integrated SV40 and polyomavirus DNAs has been widely reported and well documented (14, 19, 28, 40). The *trans*-acting nature of these factors has been established by cell fusions (34, 40, 60) and by the transfer, via erythrocyte vectors, of active protein extracts from irradiated to unirradiated target cells (41). In one case, the UV-inducible *trans*-acting factor has been isolated as a 60-kilodalton protein (41). The generality of UV-inducible functions which enhance or facilitate virus replication has now been extended to parvoviruses (this report) and to the human immunodeficiency type 1 retrovirus (59). The important question of whether the UV-inducible cellular functions which enhance different types of virus infections are related must await the isolation and characterization of the mediating factors. In the present report, we did not directly address the question of whether the UV-induced function which facilitates AAV replication operates by means of a *trans*-acting diffusible factor. However, the findings that the UV-induced process is sensitive to cycloheximide (Table 5) and decays with time (Fig. 2) are consistent with this notion. The nature of the AAV permissiveness factors is currently being investigated by cell fusion studies and by the cell-to-cell transfer of active protein extracts. Because AAV replication is so dependent on cellular functions induced by UV light and other DNA-damaging agents, this parvovirus can be exploited to further our understanding of cellular permissiveness for viral infections in general.

Several types of mechanisms can explain the AAV replication requirement which is satisfied by UV irradiation of the host cell. About 50% of the AAV genome encodes a remarkable series of overlapping nonstructural proteins (32, 54, 57) which regulate DNA replication (16, 55) and gene expression (22–24, 56), as shown by transfection of plasmid constructs mutagenized in vitro. In addition to the *trans*-acting regulatory proteins, the AAV inverted terminal repeats act, in *cis*, as origins and primers of DNA replication (45, 50). Experiments with AAV-SV40 hybrids, in which the AAV p5 promoter region was replaced by an SV40 replication-regulatory segment (nucleotides 5171 to 5243-1 to 270) have shown that AAV-encoded regulatory factor(s) can downregulate the functioning of the SV40 replication origin, provided that origin region is linked to the AAV terminal repeats (21). Assuming that the target SV40 replication origin in the AAV-SV40 hybrid reflects the behavior of the AAV replication origin (21), the above observations suggest that AAV encodes a suppressor of its own replication. In UV-irradiated cells, one of the UV-induced proteins (17) may compete with the AAV suppressor by binding to critical sites on the terminal repeats. An alternative possibility is that UV irradiation induces a transcriptional *trans*-activator (31) which alters the expression patterns of the AAV regulatory products such that the positive replication determinants now predominate. UV induction of a posttranslational modifier, such as a protease, would bring about a similar consequence (49). Finally, a quite separate model might specify that AAV replication depends uniquely on a modified cellular DNA polymerase which arises in response to the cellular stress induced by UV irradiation, carcinogens, and other DNA-damaging agents. It was suggested previously (15) that AAV DNA amplification in cells exposed to low concentrations of cycloheximide was related to a cycloheximide-induced change in the subunit structure of DNA polymerase alpha (37).

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