Origin of Adeno-Associated Virus DNA Replication Is a Target of Carcinogen-Inducible DNA Amplification

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DNA amplification of the helper-dependent parvovirus AAV (adeno-associated virus) can be induced by a variety of genotoxic agents in the absence of coinfecting helper virus. Here we investigated whether the origin of AAV type 2 DNA replication cloned into a plasmid is sufficient to promote replication activity in cells treated by the carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). A pUC19-based plasmid, designated pA2Y1, which contains the left terminal repeat sequences (TRs) representing the AAV origin of replication and the p5 and p19 promoter but lacks any functional parvoviral genes is shown to confer replication activity and to allow selective DNA amplification in carcinogen-treated cells. Following transfection of plasmid pA2Y1 or plasmid pUC19 as a control, density labeling by a bromodeoxyuridine and *Dpn*I resistance assay suggested a semi-conservative mode of replication of the AAV origin-containing plasmid. Furthermore, the amount of *Dpn*I-resistant full-length pA2Y1 DNA molecules was increased by MNNG treatment of cells in a dose-dependent manner. In addition, DNA synthesis of plasmid pA2Y1 was studied *in vitro*. Extracts derived from MNNG-treated CHO-9 and L1210 cells displayed greater synthesis of *Dpn*I-resistant full-length pA2Y1 molecules than did nontreated controls. Experiments with specific enzyme inhibitors suggested that the reaction is largely dependent on DNA polymerase α , DNA primase, and DNA topoisomerase I. Furthermore, restriction endonuclease mapping analysis of the *in vitro* reaction products revealed the occurrence of specific initiation at the AAV origin of DNA replication. Though elongation was not very extensive, extracts from carcinogen-treated cells markedly amplified the AAV origin region. Our results, including electron microscopic examination, suggest that the AAV origin/terminal repeat structure is recognized by the cellular DNA replicative machinery induced or modulated by carcinogen treatment in the absence of parvoviral gene products.

Adeno-associated viruses (AAVs) or dependoviruses are members of the parvovirus family, a group characterized by their linear, single-stranded DNA genome with palindromic inverted terminal repeats (TRs). In contrast to autonomous parvoviruses which can replicate independently in proliferating cells, AAV normally requires coinfection with either adenovirus or a member of the herpesvirus family for its productive replication (7). Recent studies, however, demonstrated that the helper dependency of AAV is not absolute. Some cell lines can be rendered permissive for AAV in the absence of helper virus by treatment with genotoxic agents (52, 65–67).

Most intriguingly, members of both groups interfere with oncogenesis irrespective of the mode of tumor induction (for a review, see reference 47). Parvoviruses have been shown to inhibit spontaneous tumor formation and tumors induced by various oncogenic viruses as well as by chemical carcinogens in rodents (47). In addition, seroepidemiological studies revealed that high antibody titers against the human AAV types 2, 3, and 5 (AAV-2, AAV-3, and AAV-5) are associated with a reduced incidence of certain human cancers (47).

The mechanisms by which AAV in particular exerts its oncosuppressive effect are not yet understood. However, two elements essential to AAV replication appear to be also involved in oncosuppression. The first is the *rep* gene within the large open reading frame (ORF) on the left-hand side of

the genome which codes for a family of multifunctional nonstructural AAV proteins. The mRNAs coding for the two large *rep* proteins, *rep*78 and the spliced *rep*68, start at the p5 promoter, and those coding for *rep*52 and the spliced *rep*40 start at the p19 promoter. Lesions within the *rep* ORF inhibit or abolish AAV DNA synthesis (7). The *rep*78 and *rep*68 proteins specifically bind to the AAV origin/TRs, provided that the structure is in the hairpin or covalently closed form (2, 23). More recently, evidence was provided demonstrating that *rep*68 is an ATP-dependent site-specific endonuclease with DNA helicase activity, thus playing a crucial role in the resolution of the covalently joined terminal ends in lytic replication of AAV (24, 58). In addition to being required for AAV DNA replication, the *rep* proteins are involved in AAV gene regulation. Depending on the presence or absence of adenovirus helper functions, the *rep* proteins pleiotropically regulate *in trans* AAV transcription in both negative and positive ways (7, 30, 60). Under nonpermissive conditions, the *rep* proteins appear to inhibit AAV DNA synthesis and gene expression starting from the two leftward promoters, p5 and p19, whereas infection with helper adenovirus relieves this repression (4, 6). Similarly, experiments involving AAV-simian virus 40 (SV40) hybrid genomes have shown that the *rep* proteins inhibit SV40 T-antigen-driven replication if the AAV TR target sequences are present *in cis* (28). Moreover, the *rep* ORF product(s) could be shown to inhibit gene expression starting from a number of heterologous promoters (29) and to suppress transformation of the mouse cell line C127 by bovine papillomavirus (20). More recently, the AAV *rep* gene was identified as being responsible for the

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complete inhibition of herpes simplex virus-induced SV40 DNA amplification (18).

The second element essential for AAV replication is represented by the 145-bp inverted TRs which are the origins for AAV DNA synthesis. The first 125 bases of the TR consist of a complex palindromic sequence (42) that forms a T-shaped hairpin structure, thus functioning as a primer for DNA synthesis at the 3' terminus (17, 42, 57). Nevertheless, the TRs have multiple functions. In addition to being origins, they are required for integration and excision of AAV proviruses and packaging of viral DNA (15, 48). Remarkably, AAV DNA from replication-defective particles containing solely the TRs has been shown to efficiently inhibit adenovirus oncogenicity in hamsters (11). Thus, the TRs may function as potential target sites for viral or cellular *trans*-acting factors.

The notion that various genotoxic agents not only induce selective DNA amplification (SDA) of cellular sequences (for reviews, see references 1, 51, and 55) or persisting viral sequences of known DNA tumor viruses (19, 31, 36, 52, 53) but also induce amplification of AAV DNA suggests that these processes may be triggered by the same inducible cellular *trans*-acting factors recognizing regulatory *cis* elements within the respective origins of replication. Cell fusion experiments provide further evidence for the role of inducible cellular *trans*-acting factors in SDA (32, 40, 44, 62). Furthermore, the amplification of both integrated viral sequences (e.g., SV40) and parvoviral DNA is sensitive to aphidicolin, thus indicating involvement of the cellular DNA polymerase α or δ in SDA (19).

The complex palindromic sequences present within the AAV origin have the potential of intrastrand base pairing, which in a negatively supercoiled molecule may lead to the formation of cruciform structures (39, 45). Palindromic sequences are also present in cellular DNA segments enriched for origins of replication (14) and have also been found in association with amplified genes (13). These observations supported the hypothesis that inverted repeat sequences may represent potential attachment sites for initiator proteins (69). We therefore decided to analyze whether the AAV origin of DNA replication/TRs cloned into a plasmid represents a specific target for cellular factors involved in the initiation of DNA replication and thus may allow SDA under conditions of cellular genotoxic stress. We report here that plasmid pA2Y1, which contains the AAV-2 origin of DNA replication as well as the two promoters p5 and p19 but lacks any functional parvoviral genes, is able to confer replication activity in carcinogen-treated cells or extracts thereof. Furthermore, we show that extracts derived from carcinogen-treated cells markedly amplify the AAV origin region in comparison with nontreated controls. The data suggest that the AAV origin/TR structure represents a specific *cis* target element which is recognized by the cellular DNA replicative machinery induced or modulated by carcinogen treatment in the complete absence of parvoviral gene products.

MATERIALS AND METHODS

Cells. Monolayer cultures of Chinese hamster ovary cells, line CHO-9, were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco modified minimal essential medium. L1210, a methylcholanthrene-induced murine leukemia cell line (67), was grown as suspension culture in RPMI 1640. All media were supplemented with 10% heat-inactivated (30 min, 56°C) fetal calf serum, L-glutamine (1 mM), and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μ g/ml). Rou-

tine screening of cells for *Mycoplasma* contamination was negative.

Plasmids. Plasmid pA2Y1 (Fig. 1), harboring the AAV-2 left TRs and the promoters p5 and p19, was generated by inserting the 1.045-kbp *Bgl*II-*Bam*HI fragment of pAV2 (35) into the *Bam*HI site of pUC19 (Boehringer GmbH, Mannheim, Federal Republic of Germany).

Chemicals and antibody. *N*-methyl-*N'*-nitro-*N*-nitroso guanidine (MNNG) was purchased from Serva, Heidelberg, Federal Republic of Germany. Bromodeoxyuridine (BrdUdR), aphidicolin, α -amanitin, coumermycin A₁, 1- β -D-arabinofuranosylcytosine-5'-triphosphate (AraCTP), spermidine, and ddTTP were obtained from Sigma, Munich, Federal Republic of Germany. *p*-*n*-Butylphenyl-dGTP, a specific DNA polymerase α inhibitor (25), was a gift from G. Wright, and monoclonal antibody SJK132-20 to DNA polymerase α (59) was purchased from the American Type Culture Collection.

DNA transfection and density labeling. DNA was introduced into CHO-9 cells by the calcium phosphate coprecipitation technique as previously described (67). After 6 h, cells were exposed to a dimethyl sulfoxide (DMSO) shock (10%, vol/vol) for 30 min and incubated in fresh medium for 12 to 16 h before MNNG treatment. Cultures were further incubated for up to 72 h; the cells were then harvested, and the DNA was extracted according to Hirt (21) and analyzed by restriction enzyme mapping.

For replication analysis by density labeling, 20 h after transfection with plasmids, the cells were exposed to BrdUrd at 12.5 μ g/ml for 24 h. Low-molecular-weight DNA was extracted by the Hirt procedure (21). After isolation, the DNA was centrifuged in an appropriate CsCl gradient (initial refractive index, 1.4150) in a Beckman TLV-100 vertical rotor for 13.5 h at 56,000 rpm (123,000 $\times$ g) at 30°C (14). Fractions were collected and diluted 1:1 with H₂O, and aliquots were analyzed in 1% agarose gels. The DNA from the gels was blotted onto GeneScreen Plus filters (DuPont, NEN Research Products) and hybridized to ³²P-labeled pA2Y1 or pUC19 DNA under conditions described previously (67). Filters were autoradiographed on Kodak XAR-5 X-ray films, using intensifying screens.

Preparation of cell extracts. Ten 150-mm plates of exponentially growing CHO-9 cells were pulse treated for 2 h with 10 or 20 μ M MNNG freshly dissolved in DMSO. Control cells were treated with DMSO alone. The final concentration of DMSO was 0.1% in all culture media used for experiments. At the end, both treated and control cultures were washed and then again supplemented with standard growth medium. L1210 cells were pulse treated (2 h) with 5 μ M MNNG at a density of 5×10^5 cells per ml. Thereafter, cells were pelleted by centrifugation at 400 $\times$ g for 10 min, washed once, and replaced in growth medium at the initial density. After an incubation period of 24 h, both CHO-9 and L1210 cells were harvested to prepare cytoplasmic and nuclear extracts. All subsequent steps were carried out at 0 to 4°C.

Monolayer cultures of CHO-9 cells were washed twice with phosphate-buffered saline and then once with TES buffer (50 mM Tris-HCl [pH 8.0]), 1 mM EDTA, 250 mM sucrose). Excess buffer was removed by aspiration, and the CHO-9 cells were scraped free with a rubber policeman, transferred to a polypropylene tube, counted, and adjusted to 5×10^7 to 1×10^8 cells per ml of TES supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μ g of aprotinin per ml, 1 μ M pepstatin A), 1 mM dithiothreitol, 1 mM ATP, and 0.3% Triton X-100 (TES*). Cells were

transferred to a Dounce homogenizer, and lysis was completed with five strokes of a tightly fitting pestle. The lysate was centrifuged at $12,000 \times g$ for 10 min at 0°C to pellet nuclei, and the supernatant was centrifuged at $100,000 \times g$ for 30 min in a Beckman TLA 100.3 rotor. This cytoplasmic fraction in TES* was finally supplemented with 20% (vol/vol) glycerol, aliquoted, and stored in liquid N_2 until use.

The $12,000 \times g$ pellet, containing the nuclei, was resuspended in TES* containing 400 mM KCl. The nuclei were lysed with 10 to 12 strokes in a Dounce homogenizer and incubated on ice for 30 min with occasional agitation. Nuclear matrix aggregates were removed by centrifugation at $50,000 \times g$ for 30 min in a Beckman TLA 100.3 rotor. The resulting supernatant was dialyzed for 16 h against TES-20% glycerol supplemented with 1 μM pepstatin and 1 mM dithiothreitol. In some experiments, prior to dialysis, nuclear lysates were passed through a DEAE-cellulose column equilibrated with 0.3 M KCl to remove endogenous DNA. Dialyzed nuclear extracts were finally clarified by centrifugation at $30,000 \times g$ for 15 min in a Beckman TLA 100.3 rotor. Extracts were aliquoted and stored in liquid N_2 until use. Usually, 10 dishes of cells yielded a cytoplasmic fraction of about 2 ml containing 6 to 10 mg of protein per ml and a nuclear extract of 1 ml containing 3 to 6 mg of protein. L1210 cells were harvested, and the cytoplasmic and nuclear extracts were prepared as described above.

In vitro DNA synthesis. The reaction mixture in a final volume of 50 μl contained 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 7 mM MgCl_2 , 1 mM ethylene-bis(oxyethylenetriole)-tetraacetic acid (EGTA), 0.5 mM dithiothreitol, 100 μM each dATP, dGTP, and dCTP, 10 μM [α - ^{32}P]dTTP or [^3H]dTTP (specific activity, 1×10^3 to 4×10^3 cpm/pmol; Radiochemical Centre, Amersham, England), 4 mM ATP, 100 μM each CTP, GTP, and UTP, 5 μg (0.2 U/ μg) of pyruvate kinase (Boehringer), 10 mM phosphoenol pyruvate, 5% (wt/vol) polyethylene glycol (PEG; 8,000 Da; Sigma), 0.3 μg of supercoiled template DNA (pA2Y1 or pUC19), and a combination of cytoplasmic (60 μg) and nuclear (30 μg) extracts. The reaction mixtures were preincubated on ice for 10 min and then incubated at 37°C for the times indicated. To measure the extent of DNA synthesis, reactions were terminated by placing the samples of 0°C and adding 10% (wt/vol) trichloroacetic acid (TCA) in 0.1 M sodium pyrophosphate. TCA-precipitable material was determined as described by Hübscher and Kornberg (22).

Product analysis. DNA products were purified as described previously (37) and dissolved in $1 \times \text{TE}$ (10 mM Tris [pH 7.5], 1 mM EDTA). Restriction enzyme digestions were carried out in 30- μl reaction mixtures under conditions specified by the manufacturer (Boehringer). Samples were electrophoresed on a 0.8 or 1% agarose gel in E buffer (40 mM Tris [pH 7.4], 5 mM sodium acetate, 1 mM EDTA) or on an 8% polyacrylamide gel (29:1, acrylamide/bisacrylamide) in TBE buffer (0.1 M Tris borate [pH 8.5], 2 mM EDTA). The gel was dried on a Bio-Rad gel dryer and autoradiographed on a Kodak XAR-5 X-ray film, using an intensifying screen. Purified DNA was prepared for electron microscopy by the cytochrome *c* droplet diffusion technique (33). Specimens were stained with uranyl acetate (10) and then rotary shadowed with Pt/Pd (80:20) at an angle of 8° . Micrographs were taken with a Zeiss EM-10 electron microscope at 40 kV. The magnification indicator was routinely controlled by the use of a grating replica. For contrast enhancement, micrographs were printed in reversed contrast.

FIG. 1. Restriction map of plasmid pA2Y1. Abbreviations and symbols: M, *Mae*I; B, *Bam*HI; solid bar, the AAV-2 *Bgl*II-*Bam*HI fragment (1.045 kbp) inserted into the *Bam*HI site of pUC19; open bar, pUC19 vector. Digestion of pA2Y1 with the restriction endonuclease *Mae*I generates five fragments (A to E; see also Fig. 6). Fragment E harbors the TRs representing the origin of AAV-2 DNA replication. p5 and p19 indicate the positions of the leftward promoters of AAV-2.

RESULTS

Plasmid pA2Y1 carrying the left-hand 1-kb AAV fragment with the AAV origin/TRs confers replication activity. Amplification of AAV DNA can readily be induced under conditions of genotoxic stress in a number of cell lines without necessarily leading to progeny virus production (67). To analyze whether the left-hand AAV fragment containing the AAV-2 origin/TR structure is able to promote replication activity in carcinogen-treated cells in the absence of parvoviral gene products, we inserted the 1.045-kbp *Bgl*II-*Bam*HI fragment of the AAV-2 wild-type clone pAV2 (35) into the *Bgl*II site of pUC19 to yield plasmid pA2Y1 (Fig. 1). Plasmid pA2Y1 contains the left TR sequences and the p5 and p19 promoters of AAV-2 but is lacking functional parvoviral genes. Plasmid pA2Y1 and plasmid pUC19 as a control were transfected into CHO-9 cells for transient replication assays as described in Materials and Methods. Twenty hours after transfection, DNA replication was monitored by *in vivo* labeling with BrdUrd for 24 h at 12.5 $\mu\text{g}/\text{ml}$. DNAs were extracted according to Hirt (21), and their density distribution was analyzed in CsCl gradients (Fig. 2). Figure 2A shows the distribution of pA2Y1 plasmid DNA after centrifugation in a CsCl equilibrium density gradient as monitored by DNA agarose gel electrophoresis and Southern blot hybridization. Remarkably, the plasmid was replicated, and the DNA distribution resulted in heavy-light (HL; one strand replicated) and to a lesser extent heavy-heavy (HH; both strands replicated) peaks after a 24-h BrdUrd incorporation period. The light-light (LL) DNA represents unreplicated material. These data are consistent with a semiconservative mode of DNA replication. In Fig. 2B, the DNA distribution of the control plasmid pUC19 (dimer) lacking the AAV origin is analyzed. In contrast to pA2Y1, incorporation of BrdUrd into pUC19 DNA did not result in characteristic peaks of HL or HH density. Likewise, overexposure of the pUC19 autoradiogram did not reveal any discrete peak of HL DNA (data not shown). It should be noted that BrdUrd treatment at the concentration used is genotoxic and induces selective amplification of AAV DNA, which cannot be detected under

FIG. 2. Replication analysis by density labeling with BrdUrd. AAV origin-containing plasmid pA2Y1 (A) and control plasmid pUC19 (B) were transfected to CHO-9 cells. Twenty hours later, the cells were exposed to BrdUrd (12.5 μ g/ml) for 24 h. DNA was then extracted according to Hirt (21) and centrifuged to equilibrium in CsCl (initial refractive index, 1.4150). The densities of collected fractions were determined by refractometry (graphs). Fractions were diluted 1:1 with H_2O , and aliquots were analyzed in 1% agarose gels. The DNA from the gels was blotted onto GeneScreen Plus filters (DuPont) and hybridized with the nick-translated pUC19 and pA2Y1 DNAs. Both filters were finally exposed for 1 h. The arrows indicate supercoiled (I) and relaxed (II) forms of pA2Y1. HH, HL, and LL represent the density regions expected upon BrdUrd incorporation.

physiological conditions. An additional set of experiments confirmed the occurrence of pA2Y1 DNA replication. The plasmid DNAs were tested for their sensitivity to the restriction endonuclease *DpnI* 72 h following transfection into CHO-9 cells. The template plasmids used in all experiments were isolated from *Escherichia coli* (*dam*⁺); they were fully methylated and thus sensitive to *DpnI*. DNA that is replicated after transfection into eukaryotic cells will become hemimethylated or even unmethylated and consequently *DpnI* resistant (37, 38). As shown in Fig. 3, the AAV origin-containing plasmid pA2Y1 gave rise to *DpnI*-resistant full-length molecules (forms II and III), the amount of which was increased by treatment of cells with the carcinogen MNNG in a dose-dependent manner. By contrast, accumulation of *DpnI*-resistant pUC19 molecules was not detected (Fig. 3). However, though the amount of *DpnI*-resistant pA2Y1 DNA molecules was increased by MNNG treatment of cells, the corresponding amount of *MboI*-resistant (unreplicated) molecules was not significantly diminished. This finding clearly indicated that only a small fraction (approximately 5 to 10%) of the transfected molecules became replicated, which is consistent with the data obtained by density labeling with BrdUrd (Fig. 2A). Similar results were

FIG. 3. Replication analysis by restriction endonucleases *DpnI* and *MboI*. CHO-9 cells were transfected with pUC19 (A) or pA2Y1 (B) form I DNA as described in Materials and Methods. The following day, cells were pulse treated for 2 h with either MNNG at 2 μ M (lanes 3, 4, 9, and 10) and 5 μ M (lanes 5, 6, 11, and 12) or the solvent DMSO as a control (lanes 1, 2, 7, and 8). The concentration of DMSO was 0.1% in all culture media during treatment. After washing and replacement of medium, cells were incubated for 72 h until DNA extraction according to Hirt (21). Aliquots (10 μ g) of the DNAs were subjected to restriction endonuclease digestion with either *DpnI* (lanes 1, 3, 5, 7, 9, and 11) or *MboI* (lanes 2, 4, 6, 8, 10, and 12), electrophoresed on a 1% agarose gel, transferred to GeneScreen Plus filter (DuPont), and then hybridized with nick-translated pUC19 or pA2Y1 DNA. The arrows in panel B indicate the positions of forms I, II, and III of pA2Y1 DNA. In addition, *DpnI*-resistant full-length pA2Y1 molecules (form II) are indicated by arrows in the lanes 9 and 11.

obtained with L1210 cells (data not shown). Taken together, these observations suggested to us that the left-hand 1-kb AAV-2 fragment containing the origin is functionally active in circular plasmids and may serve as a specific target site for carcinogen-inducible cellular factors involved in SDA, obviously in the complete absence of parvoviral gene products.

In vitro DNA replication of pA2Y1 with extracts from MNNG-treated and nontreated cells. To analyze the molecular mechanisms of DNA replication, cell-free systems have proven to be powerful, as demonstrated in prokaryotes (27) and eukaryotes (56). The two viral systems of adenovirus DNA and SV40 DNA (for a review, see reference 8) have been most instructive so far. Replication of SV40, however, depends on a permissive intracellular milieu and the presence of sufficient amounts of SV40 T antigen (37), which mediates specific initiation of SV40 DNA replication and DNA helicase activity (54). In this study, we attempted to establish a cell-free DNA replication system, using the AAV origin-containing plasmid pA2Y1 to analyze carcinogen-inducible SDA in the absence of viral factors. The system consisted of a combination of cytoplasmic and nuclear extract, the four deoxyribonucleoside triphosphates, the four ribonucleoside triphosphates, an ATP-regenerating system, $MgCl_2$, polyethylene glycol (PEG 8000), and form I pA2Y1 DNA. The important point is that this system is lacking helper virus functions and AAV-encoded proteins; thus, it depends completely on cellular replicative functions.

Twenty-four hours after pulse treatment (2 h) of CHO-9 or

FIG. 4. DNA synthesis in vitro on pA2Y1 by extracts from L1210 cells. (A) Time course of in vitro DNA synthesis on form I pA2Y1 by combined crude cytoplasmic extracts (CE) and nuclear extracts (NE) derived from MNNG-treated or DMSO-treated (control) L1210 cells. Reaction conditions were as described in Materials and Methods. At indicated times, aliquots were assayed for TCA-insoluble radioactivity. (B) Corresponding autoradiogram after agarose gel electrophoresis (0.8%) of the reaction products (n.c., nicked circular form II DNA; lin., form III molecules). Arrowheads represent positions of λ HindIII size markers: from top to bottom, 23.130, 9.416, 6.557, 4.361, 2.322, and 2.027 kbp. The thin arrow represents putative replicative intermediates.

L1210 cells with the carcinogen MNNG, crude cytoplasmic and nuclear extracts were prepared, and combinations of cytoplasmic and nuclear extracts were incubated at 37°C in the standard in vitro reaction mixture containing [α -³²P]dTTP or [α -³²P]dCTP as described in Materials and Methods. As shown in Fig. 4A, in vitro DNA synthesis by extracts from MNNG-treated cells (L1210) was 2 to 10 times higher than synthesis by control extracts. The ³²P-labeled products migrated as nicked circular and linear molecules (Fig. 4B). However, the overall increase of precursor incorporation into double-stranded form I pA2Y1 DNA by extracts from carcinogen-treated cells also correlated with an increased background smear resulting from label incorporated into degraded forms of the plasmid, thus indicating that MNNG treatment caused an apparent increase in nuclease and repair activity. Similar results were obtained with CHO-9 cell extracts (data not shown). To elucidate whether this in vitro system allows synthesis of full-length molecules, the DNA was tested for its sensitivity to *DpnI* in relation to the incubation time (Fig. 5). As presented in Fig. 5, *DpnI*-resistant full-length molecules which migrated as relaxed circular plasmids appeared at about 30 min after onset of the reaction. Consistent with the increased rate of incorporation of dTMP into TCA-precipitable material by extracts from MNNG-treated cells, the amount of newly synthesized *DpnI*-resistant full-length DNA was enhanced as well. Similar to in vitro SV40 DNA synthesis by extracts from permissive cells in the presence of T antigen, we observed a lag period of 5 to 10 min in both types of extracts. Although our system is lacking extensive elongation, the data show that a small fraction (5 to 10%) of the input pA2Y1 DNA is replicated by the crude cell extracts in the absence of parvoviral gene products, thus confirming our in vivo results. Moreover, enhanced DNA synthesis by extracts from MNNG-treated cells appeared to mimic the in vivo situation.

FIG. 5. *DpnI* endonuclease restriction analysis of in vitro DNA synthesis on pA2Y1 by CHO-9 cell extracts. In vitro replication of pA2Y1 DNA was performed with combinations of cytoplasmic extracts (CE) and nuclear extracts (NE) from nontreated control (mock) or MNNG-treated (20 μ M) CHO-9 cells as described in Materials and Methods. Reactions were terminated at the times indicated, DNA products were isolated, and aliquots were treated with restriction endonuclease *DpnI* (+*DpnI*) or the isochizomer *Sau3A* (+*Sau3A*), and electrophoresed in a 0.8% agarose gel. For comparison, an aliquot of the products from 60-min reactions was electrophoresed without restriction endonuclease treatment. The gel was dried and then subjected to autoradiography. The bars above the lanes indicate reactions carried out by extracts from MNNG-treated cells. Arrows mark the positions of relaxed circular (r.c.) pA2Y1 DNA.

Similar experiments with pUC19 as control did not reveal any *DpnI*-resistant full-length molecules (data not shown).

To visualize the replicative intermediates from the in vitro reaction, purified DNA samples were examined by electron microscopy. Figure 6 shows some examples of pA2Y1 replicating intermediates from the reaction carried out by using extracts from MNNG-treated cells. Approximately 99% of the input DNA molecules are converted into relaxed circular form. A significant fraction (10 to 20%) of these molecules is intertwined, suggesting involvement of DNA topoisomerase I or II. Remarkably, a small fraction (about 0.1%) of the in vitro products displayed replication eyes at different stages of elongation (Fig. 6c to g). This finding clearly demonstrated the occurrence of Cairns-like replicative intermediates. By contrast, we could not detect any replicating intermediates with pUC19 DNA (data not shown).

To measure the contribution of the various replicative enzymes on pA2Y1 DNA synthesis in vitro, experiments with specific inhibitors of DNA replication enzymes were performed. The data summarized in Table 1 indicate that approximately 60% of synthesis catalyzed by extracts from carcinogen-treated cells was carried out by DNA polymerase α , as concluded from its inhibition by aphidicolin and *p-n*-butylphenyl-dGTP, as well as by a neutralizing monoclonal antibody directed against DNA polymerase α . Furthermore, the reaction is highly sensitive to AraCTP, which inhibits DNA polymerase α and primase activity by chain termination (27), and to coumermycin A₁, which specifically inhibits eukaryotic DNA topoisomerase I (27). However, as expected, a significant proportion of the reaction carried out by extracts from MNNG-treated cells reflected repair synthesis as well. ddTTP can selectively inhibit DNA polymerases β and γ at appropriate concentrations without inhibiting DNA polymerases α and δ (12, 21a). ddTTP/dTTP ratios that preferentially inhibit DNA polymerases β and γ could be shown to inhibit precursor incorporation into pA2Y1 DNA by about 40%. α -Amanitin concentrations sufficient to completely inhibit RNA polymerases II and III had no effect on pA2Y1 DNA synthesis. This finding suggests that transcription does not play a role in this system.

Initiation of AAV-2 DNA replication is origin specific and enhanced by extracts from MNNG-treated cells. To determine whether the enhanced repair synthesis by extracts from carcinogen-treated cells was superimposing SDA, we tested whether specific initiation of DNA replication at the AAV origin occurred that was greater than for control extracts. Newly synthesized DNA labeled for different incubation times (10 to 70 min) was extracted and digested with *MaeI*,

FIG. 6. Electron microscopy of spread preparations of pA2Y1 DNA after incubation in extracts from MNNG-treated cells. In vitro replication of pA2Y1 DNA was performed with combinations of cytoplasmic and nuclear extracts from MNNG-treated (20 μ M) CHO-9 cells as described in Materials and Methods. Reactions were terminated after 30 min of incubation, DNA products were isolated, and aliquots were spread for electron microscopy as described in Materials and Methods. Approximately 99% of the input DNA molecules (a) are converted into relaxed circles (b), often revealing intertwined configurations (b). Replication eyes in monomeric (c and e to g) and trimeric (d) DNA molecules at different stages of elongation are visible. Quantitation of the relaxed molecules gave a fraction of 10 to 20% of intertwined configurations and a small fraction (about 0.1%) of molecules displaying replication eyes. Bars: (a) 250 nm; (b, c and e to g) 200 nm; (d) 500 nm.

TABLE 1. Effects of inhibitors on in vitro pA2Y1 DNA synthesis by extracts from MNNG-treated L1210 cells^a

Conditions	Concn	DNA synthesis (%)
Complete system (CE/NE) without inhibitor		100
Aphidicolin	10 μ M	52
	100 μ M	41
<i>p</i> - <i>n</i> -Butylphenyl-dGTP	10 μ M	53
	100 μ M	38
Anti-polymerase α anti-body (SJK132-20)	20 μ g/ml	49
AraCTP	1 μ M	47
	10 μ M	33
	100 μ M	30
Coumermycin A ₁	1 μ g/ml	41
	10 μ g/ml	33
	100 μ g/ml	25
α -Amanitin	20 μ g/ml	100
	200 μ g/ml	88
Spermidine	1 mM	37
ddTTP/dTTP	2:1	68
	20:1	58
	40:1	53

^a DNA synthesis activity was measured for 60 min in the standard reaction assay as described in Materials and Methods; 100% DNA synthesis corresponded to 7.2 pmol of deoxynucleoside monophosphate incorporated per h per 300 ng of pA2Y1 DNA. The mean values of duplicates are listed. CE, cytoplasmic extract; NE, nuclear extract.

and the distribution of radioactivity on each fragment was examined following native polyacrylamide gel electrophoresis and autoradiography (Fig. 7). Digestion of plasmid pA2Y1 with *Mae*I yielded five fragments (A to E), the smallest of which represented the AAV-2 TRs (Fig. 1). As shown in Fig. 7B (5-h exposure), discrete fragments of expected length were found to be labeled by extracts from both controls and MNNG-treated cells. Fragment E, however, comprising the AAV origin/TRs, was overexposed after 5 h. Interestingly, short-time (1-h) exposure of the gel (Fig. 7A) revealed that the AAV TRs (fragment E) was labeled first, as shown by the 10- and 20-min incubations. Most remarkably, extracts from MNNG-treated cells continued to initiate at the AAV-2 origin of replication even after later times of incubation. This was not detected in the reaction carried out by extracts from solvent (DMSO)-treated control cells. Labeling of the neighboring fragments A and B, however, appeared to be more extensive by the extracts from control cells (Fig. 7). These observations demonstrate that the AAV-2 origin/TR structure is the preferred site of initiation of DNA synthesis in this cell-free system. Furthermore, extracts from treated cells have an enhanced capacity for specific initiation at the AAV origin, thus leading to local amplification. Densitometric analysis of the radioactivity in each DNA fragment relative to the number of base pairs per restriction fragment further confirmed the observed high specificity of initiation at the AAV

FIG. 7. Specific initiation of DNA replication at the AAV origin and selective amplification in vitro. In vitro replication of pA2Y1 was performed with combinations of cytoplasmic and nuclear extracts from DMSO-treated control or MNNG-treated L1210 cells as described in Materials and Methods. Reactions were terminated at the times indicated; DNA products were isolated, treated with restriction endonuclease *Mae*I, and electrophoresed in a 5% acrylamide gel in 0.1 M Tris borate (pH 8.5) containing 2 mM EDTA. The gel was dried and then subjected to autoradiography. A to E, fragments generated by digestion with *Mae*I (see Fig. 1): A, 2127 bp; B, 877 bp; C, 335 bp; D, 253 bp; E, 144 bp. Fragment E contains the left AAV-2 TRs representing the origin. (A) 1-h exposure (optimized for fragment E); (B) 5-h exposure (optimized for the bands of fragments A, B, C, and D). Note that band E is overexposed after 5 h.

origin (data not shown). The most distant fragments (C and D) from the AAV origin region were only weakly labeled in both extracts, implying that elongation in this system was not extensive. Parallel experiments with pUC19 as a control did not reveal any site-specific initiation rather than low-level random labeling of the corresponding fragments proportional to their size (data not shown). Taken together, the results suggest that the AAV origin/TRs represents a functional *cis* element which is recognized by the cellular DNA replication machinery induced or modulated by carcinogen treatment.

DISCUSSION

Recent studies have demonstrated that genotoxic treatment of cells induces a permissive milieu for AAV propagation in some continuous lines of animal cells (52, 65–67). Without such treatment or coinfection by a helper virus, AAV is unable to replicate in cell culture. The mechanisms leading to AAV DNA amplification and progeny virus production in the absence of helper virus functions are not yet understood. However, indirect evidence suggests that induction or modulation of regulatory cellular factors involved in cellular genotoxic stress responses appears to be important (50). In addition, substantial evidence has accumulated indicating that AAV transcription and DNA replication are strongly autoregulated in both negative and positive ways by the products of the AAV *rep* ORF, depending on the presence of viral helper functions (7, 30, 60). Under permissive conditions, *rep* ORF products are required for AAV gene expression and DNA replication. Under nonpermissive conditions, however, limited expression of the *rep* ORF appears to inhibit both AAV gene expression and DNA synthesis (4, 6).

In this study, we investigated the role of the AAV origin/TRs as a *cis* target element in carcinogen-inducible amplification of AAV DNA in the absence of AAV *rep* ORF products. In initial experiments, we monitored replication of the AAV origin-containing plasmid pA2Y1 (Fig. 1) following transfection into CHO-9 cells by density labeling with BrdUrd and subsequent analysis of the DNA in CsCl gradients. Interestingly, the AAV origin-containing plasmid was found to peak at the characteristic HL and to a lower extent at the HH position, thus indicating semiconservative DNA replication. By contrast, control plasmids (pUC19) faintly smeared through the CsCl gradient, indicating statistical incorporation of BrdUrd (Fig. 2). Consistently, we found an accumulation of *DpnI*-resistant full-length pA2Y1 molecules after treatment of cells with the carcinogen MNNG in a dose-dependent manner. Although only a small fraction of pA2Y1 DNA molecules (approximately 5 to 10%) appeared to be replicated, these observations demonstrated that the left-hand 1-kb AAV fragment containing the origin/TR structure is able to promote replication activity in cell culture in the absence of parvoviral gene products. Apparently, the AAV origin/TR structure or adjacent sequences are recognized by the cellular DNA replication machinery induced or modulated by carcinogen treatment. Elucidation of the molecular basis of alterations of the DNA synthesis machinery may help us to understand the cellular control mechanisms which normally prevent reinitiation in a single cell cycle (16). A number of reports involving cell fusion experiments have shown that *trans*-acting cellular factors controlling selective amplification of viral sequences such as SV40 or polyomavirus DNA are activated in genotoxically treated cells (32, 40, 41, 44, 62).

To facilitate analysis of the cellular *trans*-acting factors involved in SDA, DNA synthesis of pA2Y1 was tested in vitro. Accordingly, combinations of crude cytosolic and nuclear extracts from MNNG-treated and nontreated CHO-9 and L1210 cells, respectively, were prepared and tested for their ability to support pA2Y1 DNA synthesis. As expected, extracts from carcinogen-treated cells displayed an overall increase of DNA synthesis in comparison with control extracts (Fig. 4). Though the reaction also involved repair-like synthesis, a small fraction (5 to 10%) of the input pA2Y1 DNA was converted to *DpnI*-resistant full-length molecules (Fig. 5). Remarkably, the amount of *DpnI*-resistant pA2Y1 plasmids was enhanced in reactions with extracts from treated cells (Fig. 5), similar to the situation in cell culture. By contrast, control plasmid pUC19 lacking the AAV origin did not replicate either in vivo (Fig. 2 and 3) or in vitro. Hence, the AAV origin/TR structure inserted into a plasmid appears to represent a functional target which is recognized by cellular DNA replication factors induced or modulated by carcinogen treatment.

To characterize the reaction catalyzed by extracts from MNNG-treated cells, specific enzyme inhibitors as well as specific antibody to DNA polymerase α were used (Table 1). These experiments demonstrated that approximately 60% of the reaction involves DNA polymerase α . Moreover, inhibition of the reaction by AraCTP and coumermycin A₁ indicates the requirement for DNA primase and DNA topoisomerase I, respectively. However, a significant part (approximately 40%) of the reaction also includes DNA repair synthesis, as evident from its inhibition by ddTTP/dTTP ratios known to selectively inhibit DNA polymerases β and γ . This is not unexpected since DNA-damaging agents induce repair synthesis. Therefore, it was important to determine whether repair synthesis was superimposed by specific initiation of DNA replication at the AAV origin. Our results indicated that the AAV-2 origin region was indeed the preferred site of initiation of pA2Y1 DNA replication in this cell-free system and thus facilitated SDA in treated cells or crude extracts thereof. Obviously, the barrier preventing unrestricted initiation of DNA replication is removed by carcinogen treatment of cells. Similarly, restriction of SV40 DNA replication in nonpermissive host cells can partially be overcome by carcinogen treatment of cells, as has been recently shown in an in vitro system for SV40 DNA replication (5).

The high specificity of initiation within the AAV origin might be alternatively explained by the finding of Gottlieb and Muzyczka (15), who demonstrated a cellular endonuclease activity capable of excising AAV DNA from recombinant plasmids at the G+C-rich sites of the TRs (15). The observed increase in labeling of fragment E should then result from end labeling after cleavage by this endogenous nuclease. In this case, however, one would expect that both ends of the linearized molecules are equally labeled and that exclusively form III DNA molecules are accumulated with increased incubation time. Our in vitro as well as in vivo results argue against this possibility. Moreover, electron microscopic visualization of the in vitro products revealed a high percentage of relaxed circular DNA molecules with a significant fraction of intertwined molecules and, most remarkably, a small fraction of Cairns-like replication intermediates (Fig. 6).

It is not known how selectivity for initiation at the AAV origin/TRs is accomplished in the absence of parvovirus gene products. Experiments to identify carcinogen-induced cellular proteins that specifically interact with the AAV

origin/TRs or elements of the p5 promoter may clarify some aspects of the mechanism of SDA. It is interesting to note that the AAV-2 DNA sequences upstream of the p5 promoter share some homologies with known regulatory elements of cellular or viral origins of DNA replication. The nature of the cellular factors involved and their function is currently under investigation.

Previous studies demonstrating that aphidicolin is a potent inhibitor of carcinogen-inducible SDA (19) pointed to an important role of DNA polymerase α or δ in this process. In addition, the appearance of a modified form of DNA polymerase α has been reported after treatment of HeLa cells with cycloheximide (46), a compound which effectively induces SDA. Our results suggested that DNA polymerase α plays an important role in the *in vitro* reaction. Early replication in SV40 also appears to be carried out only by the DNA polymerase α -primase complex (61). Subsequent elongation would then need both DNA polymerases α and δ (61, 64). In addition, several studies on SV40 DNA replication *in vitro* have shown that during the presynthesis stage a rate-limiting unwinding reaction occurs which involves cellular proteins such as replication factor A (also called replication protein A or single-stranded-DNA-binding protein; see reference 56 for details) and replication protein C (63). It is possible that alterations or modifications in the activities of these proteins occurred in the carcinogen-treated cells, thus facilitating SDA. Fractionation of the crude extracts from carcinogen-treated and control cells may reveal some structural and functional alterations of the replicative activities.

SDA of certain cellular genes has been shown to play an important role in tumor development and in the emergence of drug resistance (1, 51, 55). Its common inducibility by genotoxic treatments is considered to represent part of an adaptive pleiotropic SOS-like stress response (50) which appears to involve the induction of a number of *trans*-acting regulatory factors (26). Hence, competition of cellular and AAV target sequences for such inducible factors may also contribute to the known inhibitory effects of AAV on oncogenesis and tumor growth (3, 47) or on the development of drug resistance (68). In addition, the type of DNA replication observed in our experiments may be related to that seen during AAV proviral integration (9, 34, 43, 49). In those cases, a limited amplification of AAV DNA also appears to occur under nonpermissive conditions and in the absence of *rep* gene products. Further analysis of the cellular factors interacting with the AAV origin/TRs or adjacent regulatory sequences of the p5 promoter may also provide a better understanding of the events leading to helpervirus-independent replication of AAV.

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