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DNA Amplification of Adeno-associated Virus as a Response to Cellular Genotoxic Stress[1]

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ABSTRACT

We studied DNA amplification of helper virus-dependent parvoviruses [adeno-associated virus (AAV)] following genotoxic treatment of a number of mammalian cell lines from different species including primary, immortalized, and tumorigenic cells. All cell lines, either infected with AAV or transfected with parvoviral DNA, readily amplified AAV DNA in the absence of helper virus following treatment of cells with a wide variety of genotoxic agents like chemical carcinogens, UV, heat shock, and metabolic inhibitors of DNA replication or protein synthesis. In addition, we show that in the SV40-transformed Chinese hamster cell lines CO60 and CO631 carcinogen-induced AAV DNA amplification may result in a complete AAV replication cycle giving rise to infectious AAV progeny. Our results demonstrate that AAV DNA amplification induced by genotoxic agents is completely independent of the presence of viral helper functions. Because its induction is not restricted to a specific cell type or to a malignant phenotype, AAV DNA amplification may represent a marker for cellular genotoxic stress response.

INTRODUCTION

The SOS-like genotoxic stress response of mammalian cells (1) appears to involve a complex pattern of inducible events, one of which seems to be selective DNA amplification (2). SDA[3] also represents an important adaptive mechanism in the development of drug resistance (2–4). While evidence is accumulating suggesting a role for SDA in the processes of tumor initiation (5) and tumor progression (6), the cellular mechanisms underlying SDA are poorly understood thus far.

The observation that helper virus-dependent parvoviruses (AAV) (7, 8), in addition to autonomous parvoviruses, inhibit oncogenesis (9–17) stimulated us to analyze their effect on carcinogen-inducible SDA.

In experiments using the SV40-transformed Chinese hamster cell lines CO60 and CO631 as well as the SV40-transformed human kidney cell line NB-E as model systems for induction of SDA, we have shown previously that infection of these cells with AAV types 2 or 5 (AAV-2, AAV-5) effectively inhibits carcinogen-induced amplification of SV40 DNA sequences (18, 19), pointing to a possible mechanism by which AAV may exert its effect on inhibition of oncogenesis. In addition, AAV-mediated inhibition of SV40 DNA amplification is accompanied by helper virus-independent amplification of AAV DNA, expression of structural antigens of AAV (18, 19), and selective killing of carcinogen-treated host cells (20), suggesting a helper effect of carcinogen treatment for AAV replication. Recently, Yakobson *et al.* (21) provided direct evidence for the occurrence of helper virus-independent production of infectious AAV particles in SV40 *ori*$^-$ mutant DNA-transformed Chinese hamster cells (OD4) which had been pretreated with hydroxyurea.

Since carcinogen-inducible AAV DNA amplification was initially observed in SV40-transformed cell lines containing a functional origin of SV40 replication and an active SV40 large T-antigen both of which are required for SV40 DNA amplification (5, 22), we investigated whether AAV DNA amplification and production of infectious AAV progeny is inducible by carcinogen treatment in the absence of both SV40 functions and helper virus. Here we report that AAV DNA introduced by virion infection or transfection of cloned replication-competent AAV DNA is readily amplified in the absence of helper virus in several SV40-free cell lines, including human diploid skin fibroblasts, following treatment of cells with a wide spectrum of genotoxic agents. We show that this effect is not restricted to one specific AAV type. However, although AAV DNA amplification was inducible by genotoxic treatment in all cell lines tested, only the SV40-transformed cells (CO60 and CO631) supported the production of infectious AAV progeny in the absence of helper virus. Our data suggest that carcinogen-inducible AAV DNA amplification represents a novel marker for detecting cellular genotoxic stress responses inasmuch as it is not linked to a specific cell type or to a malignant phenotype.

MATERIALS AND METHODS

Cells. Monolayer cultures of CHO-K1, a spontaneously transformed Chinese hamster ovary cell line (23), and XP29mal, a spontaneously transformed fibroblast line derived from an xeroderma pigmentosum patient (24), both kindly provided by Dr. H-W. Thielmann, were grown in Ham's F-12 medium (Biochrom, Berlin, Federal Republic of Germany).

The SV40-transformed Chinese hamster cell lines CO60 and CO631 (25), kindly provided by Dr. S. Lavi, BALB/c 3T3, a BALB/c mouse fibroblast line (26), F_1BGH_12C, a rat pituitary cell line (27), kindly provided by Dr. D. Biswas, HeLa and KB, human cervical carcinoma cell lines, and E6, human diploid skin fibroblasts, were maintained in minimal essential medium (Biochrom).

L1210, a methylcholanthrene-induced leukemia cell line (28), was grown as suspension culture in RPMI 1640 (Biochrom).

All media were supplemented with L-glutamine (1 mM), antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml) and 10% heat-inactivated (30 min, 56°C) fetal calf serum (Biochrom). Routine screening of cells for *Mycoplasma* contamination was negative.

Viruses. The source and propagation of AAV-5 in HeLa or KB cells was reported previously (29). AAV-2 was propagated similarly, except that Ad2 served as helper virus. In some experiments herpes simplex virus type 1 (HSV-1), strain 17 syn$^+$, kindly provided by Dr. B. Matz, was used as helper virus for AAV.

DNA Plasmids. The plasmids pAV1 and pAV2 (30) comprising the complete genome of AAV-2 wild type were generous gifts of Dr. C. Laughlin. A rat albumin complementary DNA cloned into pSP62 was kindly provided by Dr. G. Scherer.

Chemicals or Physical Treatment. MNNG and CH were purchased from Serva, Heidelberg, Federal Republic of Germany. 4-Nitro-quinoline *N*-oxide, APH, HU, and MTX were purchased from Sigma, Munich, Federal Republic of Germany.

N-AAAF was kindly provided by Dr. H-W. Thielmann, and 7,12-

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[3] The abbreviations used are: SDA, selective DNA amplification; AAV, adeno-associated virus; Ad2, Ad12, adenoviruses 2 and 12; HSV, herpes simplex virus; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; CH, cycloheximide; APH, aphidicolin; HU, hydroxyurea; MTX, methotrexate; N-AAAF, *N*-acetoxy-2-acetylaminofluorene; 7,12-DMBA, 7,12-dimethylbenz(a)anthracene; 7CM-12MBA, 7-chloromethyl-12-methylbenz(a)anthracene; 3Mo-7CM-12-MBA, 3-methoxy-7-chloromethyl-12-methylbenz(a)anthracene; m-AMSA, 4'-(9-acridinylamino) methanesulfon-*m*-anisidide; DMSO, dimethyl sulfoxide; RF, replicative form.

DMBA, 7CM-12MBA, and 3Mo-7CM-12-MBA were generous gifts of Dr. H. Friesel and Dr. E. Hecker. m-AMSA was a kind gift of Warner-Lambert Co., Ann Arbor, MI.

All chemicals were freshly dissolved in DMSO, except HU, MTX, and CH which were prepared in phosphate-buffered saline. The final concentration of DMSO was 0.1% in all culture media used for experiments.

Irradiation with UV (254 nm, fluence rate 1 J/m/s) was performed as described (24). Heat shock was delivered by incubating cells growing in plastic flasks in a water bath adjusted to 44°C for periods of time as indicated.

Infection Protocol. Infection was performed by inoculating cells at a multiplicity of infection of 1–10 infectious units/cell (as defined below) with AAV-2 or AAV-5 (preincubated at 60°C for 30 min in order to inactivate contaminating adenovirus) for 1 h at 37°C. Unadsorbed virus was removed, and cells were washed twice with medium and then subjected to chemical or physical treatment at concentrations or conditions as indicated. In experiments with APH, HU, or MTX, cells were incubated for 24 h in the presence of the drug prior to AAV-infection. After removal of drug-containing medium, cells were washed twice with medium and infected with AAV as described above. Unadsorbed virus was removed, and after two additional washings cells were refed with fresh medium. HSV-1 infection was performed as described (19).

DNA Transfection Protocol. For DNA transfection experiments cells were seeded at a density of 2×10^5/100-mm dish and grown for 12 h prior to transfection with AAV-2 DNA (10 μg DNA/dish, either supercoiled pAV1 or pAV2 or linearized by *BglII* digestion) by the calcium phosphate coprecipitation method (31). Six h later, cells were exposed to a DMSO shock (10%) for 30 min and incubated in fresh medium for further 12 h before MNNG treatment or infection with helper virus (adenovirus or HSV). Cultures were further incubated for up to 72 h until extraction of total cellular DNA as described (32).

For assay of infectious progeny, cells were lysed by three rounds of freezing and thawing after various periods of time (0, 12, 24, 48, and 72 h) following carcinogen treatment, and infectious AAV progeny was titrated as described below.

Analysis of AAV DNA Amplification. AAV DNA amplification was measured either by *in situ* hybridization of cells ("dispersed cell assay") as described by Winocour and Keshet (33) or by the quantitative DNA slot blot technique by hybridization with ^{32}P-labeled AAV DNA and a rat albumin probe as internal control. This procedure has been described recently in detail by Bürkle *et al.* (34). AAV-infected (or AAV DNA-transfected) cells with and without carcinogen treatment were compared. The ratio of the values from carcinogen-treated and AAV-infected (or AAV DNA-transfected) cultures to those of controls solely infected with AAV (or transfected with AAV DNA) gives the relative factor of AAV DNA amplification for the respective treatment of the cells.

In other experiments AAV DNA amplification was studied by Southern blot analysis (35) of restriction enzyme-cleaved DNA. High molecular weight DNA was extracted according to standard procedures (32). Gels were blotted onto Gene screen plus filters (DuPont, NEN Research Products, Boston, MA) and hybridized with ^{32}P-labeled AAV-DNA as described (36).

Determination of AAV Infectivity. AAV infectivity was determined by a "dilution end point dot blot hybridization method" as follows. Infected or transfected cells were lysed by three rounds of freezing and thawing, and the cleared supernatants of lysates were incubated at 60°C for 30 min to inactivate contaminating adenovirus. Aliquots (50 μl) of a 1:4 dilution series of the cell-free supernatants were inoculated onto adenovirus-infected HeLa cells grown on 96-well microtitration plates. Infected cultures were allowed to progress to complete adenovirus-induced cytopathic effect (7 days) and then were lysed by three rounds of freezing and thawing. Aliquots containing lysates of approximately 2×10^4 cells were trapped onto a nitrocellulose filter (BA85; Schleicher & Schüll, Dassel, Federal Republic of Germany) by using a 96-well dot blot apparatus (BRL, Neu-Isenburg, Federal Republic of Germany). The DNA of the spotted lysates was denatured by placing the filters for 10 min onto Whatman No. 3MM paper saturated with 0.5 M NaOH/1.5 M NaCl. Filters were dried, neutralized by placing them for 10 min onto Whatman No. 3MM paper saturated with 1 M Tris-HCl (pH 7.0)/20× standard saline-citrate (1× standard saline-citrate is 150 mM NaCl/15 mM sodium citrate), baked for 1 h at 80°C, and hybridized with nick-translated (37) ^{32}P-AAV-DNA as described (36). One infectious unit of AAV was defined as the highest dilution that resulted in a specific AAV hybridization signal.

In order to exclude the presence of any residual infectious helper virus (Ad2 or Ad12) following heat inactivation (60°C, 30 min), undiluted AAV stocks (preincubated at 60°C for 30 min) were inoculated at a high multiplicity of infection (approximately 5×10^2 infectious units/cell) onto HeLa cells. Even after prolonged time of incubation (14 days) adenovirus-induced cytopathic effect was not observed nor was adenovirus DNA detectable by dot blot hybridization of lysed cells using ^{32}P-labeled adenovirus DNA as probe (data not shown).

RESULTS

Amplification of AAV DNA in CHO-K1 Cells Treated with Genotoxic Agents. AAV grows on many cell lines in the presence of different viral helpers (38–42). Helper virus-independent amplification of AAV DNA and production of infectious AAV progeny has been reported only in SV40-transformed cell lines following carcinogen treatment or cell synchronization (18, 19, 21), although SV40 is not able to support AAV replication in cell lines permissive for SV40. We wanted to elucidate whether amplification of AAV DNA and production of infectious AAV progeny is inducible by treatment of cells with genotoxic agents independent of the presence of SV40 functions or coinfection with a helper virus.

In initial experiments we studied whether the spontaneously transformed Chinese hamster ovary cell line CHO-K1 was able to amplify AAV DNA following genotoxic treatment. This cell line was confirmed to be free of SV40 DNA sequences by Southern blot hybridization with ^{32}P-labeled SV40 DNA as probe (data not shown). Furthermore, the AAV virus inocula were verified to be free of infectious helper virus (Ad2 or Ad12) as described in "Materials and Methods."

As shown by the dispersed cell assay, treatment of AAV-2-infected CHO-K1 cells with chemical carcinogens (MNNG, N-AAAF, 4-nitroquinoline *N*-oxide, 3Mo-7CM-12MBA), UV, heat shock (44°C), and inhibitors of DNA synthesis (APH, HU, m-AMSA) or of protein synthesis (CH), respectively, induces amplification of AAV DNA, in most cases in a dose-dependent manner (Fig. 1). AAV DNA amplification is inducible whether chemical or physical treatment is applied prior to or after infection with AAV. In the case of treatment with metabolic inhibitors such as APH (an inhibitor of DNA polymerase α or δ), HU (an inhibitor of ribonucleotide reductase), MTX (an inhibitor of dihydrofolate reductase), or CH (an inhibitor of protein synthesis), the highest amplitudes of AAV DNA amplification were achieved only after drug removal as expected. Permanent presence of inhibitory doses of these drugs inhibit both cellular DNA replication and AAV DNA amplification. MNNG appears to be one of the strongest inducers of AAV DNA amplification in relation to its cytotoxicity (measured by trypan blue exclusion method). In the following experiments we therefore used MNNG as inducer.

MNNG strongly induces AAV DNA amplification in AAV-2-infected CHO-K1 cells in a dose-dependent manner reaching an up to 420-fold amplification at 40 μM MNNG as determined by quantitative DNA slot blot hybridization (Table 1). Similar results were obtained in parallel experiments using AAV-5 (see Figs. 2 and 3).

In another set of experiments using *in situ* hybridization of

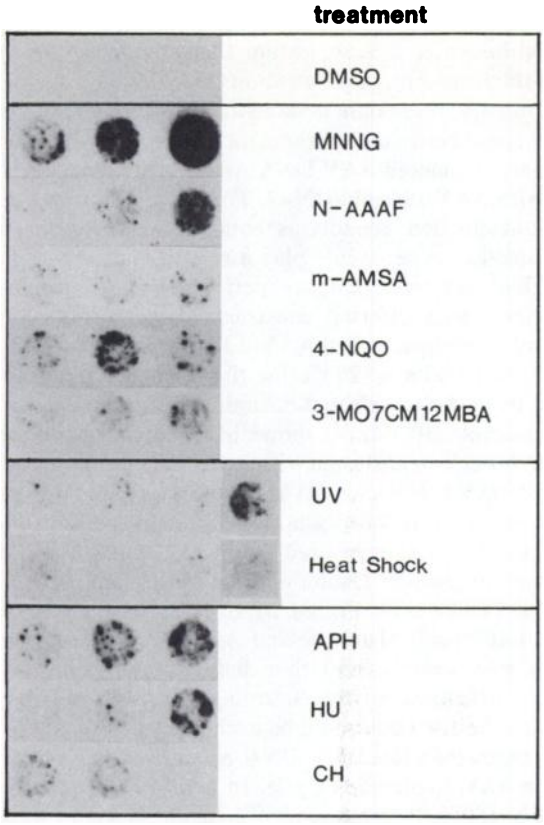

Fig. 1. AAV-2 DNA amplification in CHO-K1 cells treated with various genotoxic agents. Cells were infected with AAV-2 (multiplicity of infection, 1) and 12 h later treated with either solvent (DMSO) or increasing doses of the chemical carcinogen at the following concentrations (from *left* to *right*): MNNG, 15, 30, 60 (μM); N-AAAF, 2, 4, 8 (μM); m-AMSA, 0.1, 0.2, 0.4 (μM); 4-nitroquinoline *N*-oxide (*4-NQO*), 0.5, 1, 2 (μM); 3Mo-7CM-12MBA, 2.5, 5, 10 (μM); CH, 1, 5, 10 (μg/ml). UV irradiation was performed 18 h postinfection at 1, 3, 6, and 12 J/m^2 (fluence rate, 1 J/m^2/s). Heat shock (44°C) was applied 10 h following AAV infection for 2.5, 5, 10, and 20 min. APH and HU treatment, respectively, was performed transiently for 24 h prior to infection with AAV-2 as described in "Materials and Methods." The doses of APH were 5, 10, and 20 μM and those of HU were 0.5, 1, and 2 mM from *left* to *right*. After 3 days of incubation adherent and floating cells were harvested and analyzed by the dispersed cell assay with 5×10^4 cells/filter. Filters were hybridized with ^{32}P-labeled pAV1 and autoradiographed as described in "Materials and Methods."

cells (dispersed cell assay), we determined the time course and the proportion of CHO-K1 cells synthesizing AAV-5 DNA following infection and MNNG treatment (Fig. 2). By applying a low number of cells (10^3 or 10^4) onto nitrocellulose filters and hybridizing these with a ^{32}P-labeled AAV-5 DNA probe, detection and quantitation of individual cells synthesizing parvoviral DNA was possible (data not shown). As shown in Fig. 2, AAV DNA synthesis is already traceable in approximately 0.05% of the total cell population 12 h postinfection. Thereafter the proportion of cells synthesizing AAV-5 DNA continuously increases reaching a maximum of approximately 1% between 72 and 120 h postinfection. Similar experiments with CO60 cells revealed that the proportion of cells synthesizing AAV DNA reaches a maximum of approximately 2% (data not shown). The time course of AAV DNA amplification follows the kinetics of carcinogen-induced SV40 DNA amplification in CO60 or CO631 cells (25, 43).

Southern blot analysis of DNA obtained from MNNG-treated and AAV-5-infected CHO-K1 cells revealed that carcinogen treatment generates similar replicative intermediates as formed in the presence of coinfecting HSV-1 as helper (Fig. 3). While in all cells infected with AAV-5 a band corresponds to the position of the monomer replicative form (RFI), the dimer replicative form (RFII) is detectable only in AAV-infected cells

Table 1 *Mammalian cell lines tested for MNNG-induced AAV DNA amplification*

Cells were infected with either AAV-2 or AAV-5 as described, treated with MNNG at the concentrations indicated, and incubated for 72 h until extraction of total cellular DNA. AAV DNA amplification factors were determined by the quantitative DNA slot blot hybridization described in "Materials and Methods." By definition, the ratio of the values from carcinogen-treated and AAV-infected cultures to those of controls solely infected with AAV gives the factor of AAV DNA amplification.

Cell type	MNNG (μM)	AAV DNA amplification factor
CHO-K1[a]	0	1
	5	6.0
	10	15.5
	20	132.2
	40	419.0
L1210[a]	0	1
	5	2.3
	10	3.0
	20	117.0
	40	368.0
BALB/c 3T3	0	1
	20	31.0
	40	750.0
F_1BGH_12C[a]	0	1
	25	51.9
HeLa	0	1
	30	32.0
XP29mal	0	1
	30	71.1
Human diploid fibroblasts (E6)		1
	0	1.7
	1	2.8
	15	3.7
	20	3.9
	40	29.6
	60	131.4

[a] Factors of AAV DNA amplification are mean values from two independent cultures treated in parallel.

coinfected with HSV-1 or treated with MNNG (Fig. 3, lanes of *Hind*III-digested DNAs). Consistently, *Eco*RI digestion of RFII AAV DNA creates a 5.3-kilobase pair fragment corresponding to tandem head to head dimer of the 2.65-kilobase pair fragment. The 2.45-kilobase pair *Eco*RI fragment which is also detectable only in cells synthesizing AAV DNA probably results from first-strand DNA synthesis ending at the 5′-primed hairpin structure thus creating double-stranded monomers with an incomplete terminus. Alternatively, the 2.45-kilobase pair fragment might be generated by sequence-specific endonuclease cleavage of the RFI template near the terminal palindromic sequences as discussed by Samulski *et al.* (44). This suggests that carcinogen-inducible AAV DNA amplification resembles the regular AAV DNA replication cycle induced by helper viruses.

Carcinogen-induced AAV DNA Amplification in Cell Lines of Other Origin. In order to see whether carcinogen-inducible amplification of AAV DNA is restricted to CHO-K1 cells, we studied a number of cell lines from different species by quantitative DNA slot blot hybridization.

In all cell lines tested [CHO-K1, Chinese hamster; L1210, BALB/c/3T3, murine; F_1BGH_12C, rat; HeLa, XP29mal, human diploid skin fibroblasts (E6)], MNNG treatment induced AAV DNA amplification in a dose-dependent manner (Table 1).

Carcinogen-inducible AAV DNA amplification was also observed in human diploid skin fibroblasts (E6) (see Table 1). Increasing doses of MNNG given to AAV-infected human

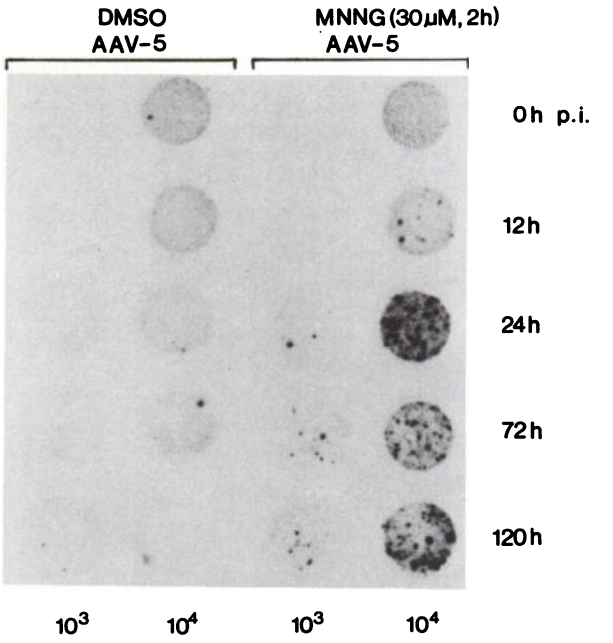

Fig. 2. Determination of the time course and the proportion of MNNG-treated CHO-K1 cells synthesizing AAV DNA (dispersed cell assay). Cells were treated with MNNG (30 μM, 2 h) or solvent (DMSO), and after extensive washing were infected with AAV-5 (1 infectious unit/cell) as described. At the times indicated in the figure, 10^3 and 10^4 cells/filter were analyzed by the dispersed cell assay using ^{32}P-labeled AAV-5 DNA as probe, as described in "Materials and Methods." *p.i.*, postinfection.

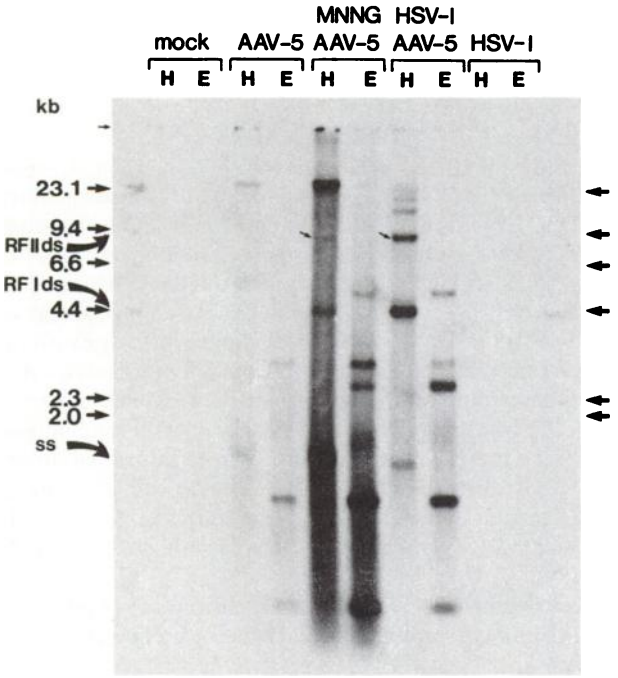

Fig. 3. Southern blot analysis of AAV-5 DNA amplification in MNNG-treated and HSV-1-coinfected CHO-K1 cells. AAV-5-infected CHO-K1 cells (10 infectious units/cell) were either coinfected with HSV-1 as helper virus or treated with MNNG (30 μM, 2 h). After 3 days cells were harvested and total cellular DNAs (10 μg) were digested with either *Hind*III (*H*, non-cut enzyme for AAV-5 DNA) or *Eco*RI (*E*, triple-cut enzyme (see Ref. 29), generating 2.65-, 1.10-, 0.47-, and 0.34-kilobase pair fragments the smallest of which is not visible here) and electrophoresed in a 1% agarose gel (30 V, 18 h). Southern blots were hybridized with a ^{32}P-labeled AAV-5 DNA probe and autoradiographed. *Large arrows*, different replicative forms of parvoviral DNA (*ss*, single-stranded DNA; RFI, double-stranded monomer; RFII, double-stranded dimer). *Small arrows* in *lanes* of *Hind*III-digested DNAs from MNNG-treated and HSV-1-coinfected cultures, RFII.

fibroblasts resulted in a significant amplification of parvoviral DNA, although to a lesser extent than found in transformed cells at the respective concentration of MNNG.

In summary, these data demonstrate that AAV DNA amplification is not restricted to a specific cell type.

Carcinogen-induced AAV DNA Amplification in Cells Transfected with AAV Plasmid DNAs. To exclude that involvement of heat-inactivated adenovirus contaminants present in the AAV infection experiments play a role in carcinogen-induced AAV DNA amplification, we performed DNA transfection experiments with plasmid constructs containing replication-competent double-stranded AAV-2 DNA (pAV1 or pAV2). In Ad2-infected HeLa or 293 cells, the AAV genome is readily rescued from the transfected plasmids resulting in a complete infectious cycle (30, 44). As shown in Fig. 4, MNNG treatment of CHO-K1 cells transfected with either supercoiled circular or *Bgl*II-cut pAV1 DNA (*Bgl*II releases intact double-stranded AAV-2 DNA from the plasmid) resulted in amplification of transfected DNA as measured by quantitative DNA slot blot hybridization. Similar results were obtained with pAV1 DNA-transfected HeLa cells (Fig. 5). MNNG treatment led to significant amplification of transfected AAV-2 DNA, although expectedly to a lesser extent than induced by the helper virus Ad12. As evident from the controls, reactivation of the heat-inactivated helper virus could be excluded (see Fig. 5).

Carcinogen-inducible AAV DNA Amplification Results in an Abortive AAV Replication Cycle. In additional experiments it was analyzed whether carcinogen-inducible AAV DNA amplification is accompanied by the production of infectious viral progeny in SV40-free and SV40-transformed cell lines. As shown in Fig. 6, HSV-1 infection or treatment with 7,12-DMBA (0.1 μg/ml) induced AAV-5 DNA amplification in

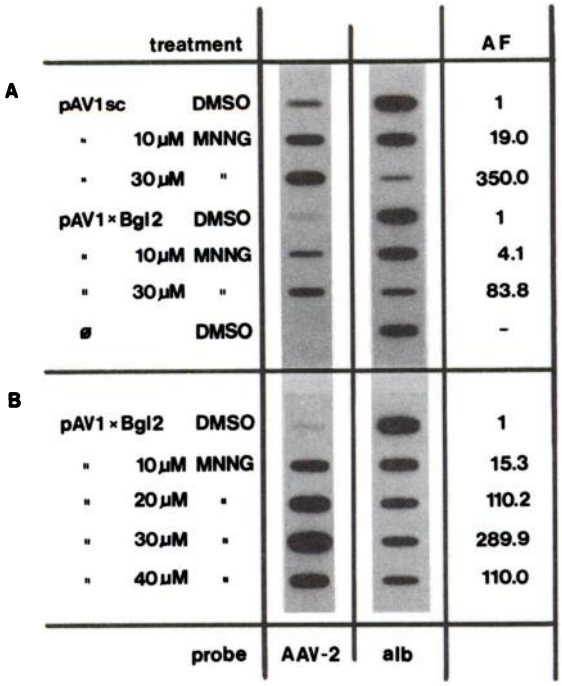

Fig. 4. MNNG-induced amplification of transfected pAV1 DNA in CHO-K1 cells. CHO-K1 cells were transfected with pAV1 DNA and treated with MNNG (2 h pulse) as described in "Materials and Methods." Cells were allowed to grow for a further 3 days until analysis by quantitative slot blot hybridization with ^{32}P-labeled AAV-2 DNA (pAV1) or ^{32}P-labeled rat albumin (*alb*) DNA as probes. *AF*, "amplification factor," determined by correcting for the internal control hybridization (albumin) as described in "Materials and Methods." *pAV1sc*, supercoiled circular pAV1 DNA; *pAV1×Bgl2*, pAV1 DNA cut with *Bgl*II. *A* and *B* represent independent experiments.

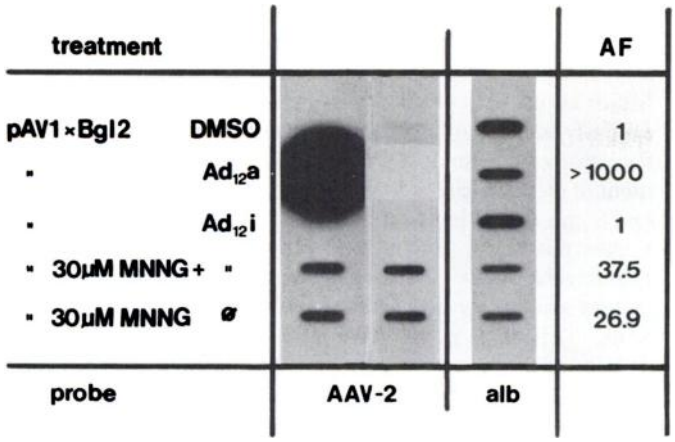

Fig. 5. Amplification of transfected AAV-2 DNA in HeLa cells. HeLa cells were transfected with *Bgl*II-cut pAV1 DNA (*pAV1×Bgl2*) as described in "Materials and Methods." Six h later cells were shock treated with DMSO (10%, 30 min), infected with either heat-inactivated (Ad_{12i}) or noninactivated adenovirus type 12 (Ad_{12a}) as indicated, and treated with MNNG (30 μM, 2 h) or solvent (DMSO) as indicated. Cells were incubated for a further 72 h and analyzed for AAV DNA amplification as described in Fig. 6. The slot for active adenovirus type 12 (Ad_{12a}) (*left column*) providing a strong helper effect for AAV DNA amplification was cut out on a second exposure to make signals in the neighboring slots visible (*middle column*). *AF*, amplification factor; *alb*, albumin.

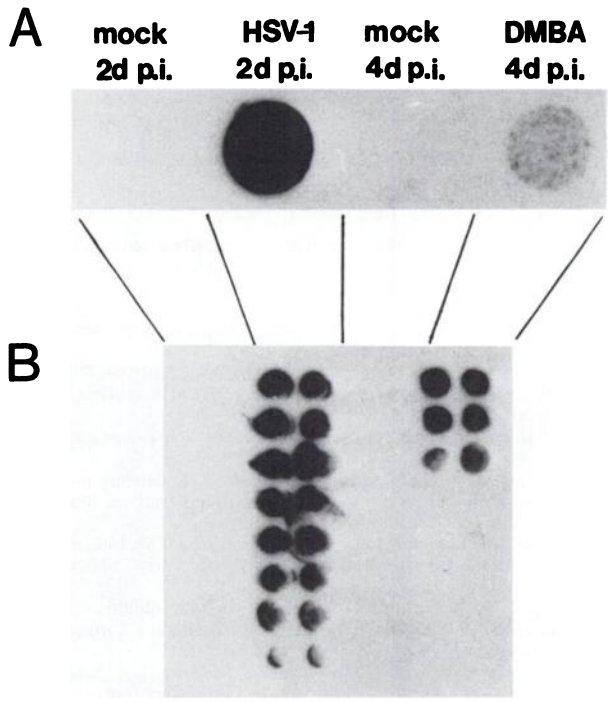

Fig. 6. DNA amplification of AAV-5 and rescue of infectious particles in CO631 cells after carcinogen treatment or superinfection with the helper virus HSV-1. CO631 cells were infected with AAV-5 and propagated for 10 passages without further treatment. Thereafter, cells were either infected with HSV-1 as described (19) or treated with 7,12-DMBA at 0.1 μg/ml. After 2 days (HSV-1) or 4 days (DMBA) cells were harvested and analyzed. *A*, analysis of AAV-5 DNA amplification. Five × 10^5 cells per filter were analyzed by the dispersed cell assay using ^{32}P-labeled AAV-5 DNA as probe. *B*, determination of AAV-5 infectivity. Cells from parallel cultures were lysed by 3 rounds of freezing and thawing and the cleared supernatants were tested for AAV infectivity by the dilution end point dot blot technique described in "Materials and Methods." AAV-5 titers were 10^5 infectious units/ml for HSV-1 coinfection and 10^3 infectious units/ml for DMBA treatment. *p.i.*, postinfection.

CO631 cells which had been infected with AAV-5 10 passages before treatment (Fig. 6*A*, dispersed cell assay). Cell-free supernatants from lysed cultures were found to contain infectious AAV particles only after infection with helper virus or treatment with carcinogen as shown by dilution end point dot blot hybridization (Fig. 6*B*). No infectious virus particles were present in untreated control cells after 10 passages following AAV infection (see Fig. 6*B*, mock treatment). The titers of the rescued AAV-5 were 10^5 and 10^3 infectious units/ml for HSV-1 infection and DMBA treatment, respectively. Also in CO60 cells transfected with *Bgl*II-digested pAV1 DNA and treated with MNNG (30 μM, 2 h), carcinogen-induced AAV DNA amplification was accompanied by the production of infectious AAV progeny reaching approximately 10^2 infectious units/ml at 72 h posttransfection (data not shown).

In contrast to the results obtained with CO631 and CO60 cells, we were unable to rescue infectious AAV by carcinogen treatment from the SV40-free cell lines CHO-K1 or HeLa although both cell lines readily amplified AAV DNA following carcinogen treatment.

DISCUSSION

Although cellular functions expressed upon genotoxic stress in mammalian systems are poorly understood and not characterized at the molecular level, a number of inducible phenomena indicate the existence of a pleiotropic SOS-like genotoxic stress response in mammalian cells (1). Its nature appears to be analogous to the well-characterized SOS response in prokaryotic organisms (45) in that it involves inducible error-prone DNA repair and DNA synthesis, leading to enhanced mutagenesis (45), and the occurrence of selective DNA amplification (SDA), resulting in chromosomal instability (46–49). Both processes seem to represent an adaptive response of individual target cells which may facilitate cell survival under altered microenvironmental conditions, as evidenced by the acquisition of drug resistance (2–4).

Viral probes have proved to be valuable tools to analyze mammalian SOS functions (50). Using animal viruses (HSV, SV40, adenovirus and autonomous parvoviruses) which grow lytically in their respective host cells it has been shown that pretreatment of these cells with a variety of chemical or physical genotoxic agents, heat shock, as well as inhibitors of DNA replication or protein synthesis induces phenomena termed "enhanced virus reactivation," "enhanced virus mutagenesis," or "enhanced virus induction" (1, 50). It is well established that genotoxic agents readily mediate SDA of chromosomally integrated viral DNA sequences in certain cell lines transformed by SV40 (5, 19, 22, 25, 43), mouse polyoma (51, 52), and the B-lymphotropic papovavirus (53). Genotoxic treatment also induces or enhances amplification of cellular DNA sequences (43, 54–56).

Our finding that amplification of AAV-DNA occurs under conditions of genotoxic stress in the absence of SV40 functions and coinfecting helper virus suggests that this type of cellular response involves alterations of the replicative system, thus providing functions leading to amplification of parvoviral and possibly also cellular DNA. The following observations suggest a similarity of the mechanisms underlying carcinogen-inducible SDA of cellular or integrated viral DNA sequences and carcinogen-inducible AAV DNA amplification: (*a*) both processes are inducible by the same genotoxic treatments; (*b*) there is a similar time course for carcinogen-induced SDA and AAV DNA amplification (see Fig. 2 and Refs. 25, 43, and 56); (*c*) both processes can be inhibited by the presence of APH, indicating the involvement of cellular DNA polymerase α or δ (36); (*d*) infection of SV40-transformed cells with AAV inhibits carcinogen-induced amplification of SV40 DNA sequences and concomitantly leads to AAV DNA amplification (18, 19, 36).

We favor the hypothesis that AAV DNA amplification is

likely to be mediated by the same carcinogen-inducible cellular factors that induce SDA of cellular or integrated viral sequences. Cell fusion experiments seem to support the role of inducible cellular *trans*-acting factors in SDA. Nomura and Oishi (57) and van der Lubbe *et al.* (58) have shown that UV irradiation of African green monkey cells mediated SV40 DNA replication and virus induction in SV40-transformed Syrian hamster cells following cell fusion with the SV40-negative cells. Similarly, Lücke-Huhle and Herrlich (59) demonstrated that α-particle irradiation of Chinese hamster embryo cells induced SV40 DNA amplification in nonirradiated SV40-transformed Chinese hamster cells following cell fusion. Lambert *et al.* (60) demonstrate cell fusion-mediated transmission of carcinogen-induced cellular factors that act in *trans*-enhancing polyoma virus DNA replication in untreated polyoma virus-transformed rat cells.

Since AAV DNA amplification can also be induced in human diploid skin fibroblasts by carcinogen treatment, this phenomenon is obviously not linked to a malignant phenotype. The quantitative differences observed in primary *versus* transformed cells in AAV DNA amplification may be accounted for by the differential sensitivity towards MNNG (61), the slower proliferation rate of primary cells, as well as the fact that induction of SDA has been shown to occur more readily in cells that are already genomically unstable than in diploid cells (62). Since induction of helper virus-independent AAV DNA amplification is not linked to a specific cell type this phenomenon appears to represent a general host cell response to genotoxic stress.

In this study we further analyzed whether carcinogen-induced AAV DNA amplification is also accompanied by the production of infectious parvoviral progeny in the absence of both SV40 functions and helper virus. Our previous studies with SV40-transformed cells (18, 19) revealed that carcinogens supported the synthesis of structural antigens of AAV in the absence of helper virus, indicating that AAV replication is not necessarily dependent on coinfection with a helper virus. Additional evidence for the occurrence of helper virus-independent replication of AAV has been recently provided by Yakobson *et al.* (21). They showed that SV40 *ori*$^-$ mutant DNA-transformed Chinese hamster cells (OD4) synchronized by pretreatment with HU or reversal of polyamine depletion support the production of infectious AAV particles. Our DNA transfection and AAV rescue experiments shown here yield further evidence for helper virus-independent AAV replication upon carcinogen treatment in SV40-transformed Chinese hamster cell lines (CO60 and CO631). On the basis of these findings of helper virus-independent replication of AAV in SV40-transformed cell lines, we further tested whether SV40-free cell lines would also support the production of infectious AAV progeny after carcinogen treatment. In contrast to our results obtained with SV40-transformed cells, we were unable to detect production of infectious AAV particles in the SV40-free cell lines CHO-K1 and HeLa although they readily amplified AAV DNA following MNNG treatment. Since the proportion of CO60 cells amplifying AAV DNA is not significantly higher than in CHO-K1 cells, this differential effect on the acquisition of AAV permissiveness appears to be a function distinct from that inducing AAV DNA amplification. It is possible that SV40-encoded functions interact with carcinogen-induced cellular factors, resulting in permissiveness for production of infectious AAV particles. Further analysis using cell lines transformed by temperature-sensitive mutants of SV40 (T Ag$^-$) should help to define functions required for the production of infectious AAV particles in the absence of coinfecting helper virus.

In conclusion, our data suggest that carcinogen-inducible AAV DNA amplification might represent a valuable marker for detecting and analyzing cellular genotoxic stress responses. Major advantages of the "AAV system" are: (*a*) its high amplitude of DNA amplification following treatment with a wide spectrum of genotoxic agents in short term tests; (*b*) no requirement of selective conditions to detect AAV DNA amplification; and (*c*) no restriction to transformed or immortalized cell lines. Furthermore, the AAV system may represent a useful tool to characterize those carcinogen-inducible cellular *trans*-acting factors which appear to be directly involved in the induction of SDA. This may in addition provide some insight into the cellular processes underlying tumor initiation.

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