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Cancer Res 1989;49:6044-6051. Published online November 1, 1989.

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[CANCER RESEARCH 49, 6044–6051, November 1, 1989]

Increase in Nitrosourea Resistance in Mammalian Cells by Retrovirally Mediated Gene Transfer of Bacterial O^6-Alkylguanine–DNA Alkyltransferase[1]

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ABSTRACT

Maloney murine leukemia virus-based, replication-defective retroviral vectors containing the neomycin resistance gene (*neo*) were developed to transfer the *Escherichia coli ada* gene coding for O^6-alkylguanine–DNA alkyltransferase, into mammalian cells. To optimize gene transfer and expression, the following promoters were linked to *ada*: the Maloney murine leukemia virus promoter within the long-terminal repeat, the Rous sarcoma virus promoter, the thymidine kinase promoter, or the human phosphoglycerate kinase promoter. Sequences were transfected into the helper virus-free retroviral packaging ψ-2 cell line. Recombinant retroviruses were tested in CCL-1 cells, which, like most murine tissues, have low levels of alkyltransferase and are sensitive to 1,3-bis(2-chloroethyl)nitrosourea (BCNU), and in NIH-3T3 cells, which are BCNU resistant and have high levels of alkyltransferase. Lines infected with each of the four retroviruses were selected for *neo* expression and found to have intact proviral integration and *ada* gene expression. Alkyltransferase activity was greatest with retrovirus containing the Rous sarcoma virus-*ada* gene; infected NIH-3T3 cells had up to 2300 units of alkyltransferase/mg of protein compared with 151 units/mg of protein in control cells, and infected CCL-1 cells had up to 1231 units/mg of protein compared with 33 units/mg of protein in control cells. CCL-1 cells expressing *ada* were more resistant to BCNU cytotoxicity than were controls. However, NIH-3T3 cells expressing *ada* were only slightly more resistant to BCNU than controls, possibly because most of the *ada* protein was cytoplasmic rather than nuclear as suggested by immunohistochemical stain. These studies establish a series of retroviruses containing the bacterial *ada* gene, which efficiently infect mammalian cells. *ada* expression increases nitrosourea resistance in cells with low mammalian alkyltransferase activity.

INTRODUCTION

The DNA repair protein O^6-alkylguanine–DNA alkyltransferase (alkyltransferase) is thought to diminish the cytotoxic and mutagenic effects of DNA-damaging agents (1, 2) and thus to protect cells from chemotherapeutic environmental agents (1, 3, 4) and carcinogen-induced malignant transformation (5). Because many normal cells and tissues contain low alkyltransferase activity, we developed a series of retroviral vectors to transfer the alkyltransferase gene into normal untransformed cells. By using these retroviral vectors, we could determine whether increased alkyltransferase activity increased resistance to DNA-damaging agents in these cells and ultimately whether or not normal animal tissues could be protected from the cytotoxic and carcinogenic effects of *N*-nitroso compounds by increased alkyltransferase activity.

We utilized the bacterial alkyltransferase coded for by the *Escherichia coli ada* gene. This protein, like its mammalian counterpart, repairs O^6-alkylguanine–DNA adducts formed by methylating agents, the chemotherapeutic nitrosoureas, and other *N*-nitroso alkylating agents (6–8). When these adducts are left unrepaired, cytotoxic DNA-DNA cross-links and guanine:cytosine to adenine:thymine point mutations occur (9, 10). Cells with high levels of this protein are protected from the cytotoxic effects of nitrosoureas and have low levels of mutation and sister chromatid exchanges following drug exposure (1, 3, 9). In contrast, cells with low levels of alkyltransferase are sensitive to the cytotoxic and mutagenic effects of these agents (1, 3, 11, 12). In addition, murine and human tissues such as bronchial epithelium, brain, thymus, and bone marrow which experience malignant transformation following exposure to different *N*-nitroso compounds all have low levels of alkyltransferase activity (13–15).

One way to test the hypothesis that alkyltransferase protects tissues from this transformation is to increase the alkyltransferase activity in normal cells. Previous studies have transfected the bacterial gene *ada* into mammalian cells and have documented the functional activity of this bacterial protein in these cells (16–18). Cells expressing high levels of the bacterial alkyltransferase have increased resistance to *N*-nitroso compounds and decreased frequency of sister chromatid exchanges following exposure to nitrosoureas (16–18). These studies have been limited to cell lines which contain very low or unmeasurable levels of alkyltransferase activity, raising the question of whether or not the bacterial alkyltransferase will be protective in cells which already have moderate to high levels of mammalian alkyltransferase activity, such as may occur when introducing *ada* into normal cells and tissues. Furthermore, one of the drawbacks of the transfection method is that stable gene transfer is achieved in only a limited number of cell lines and is difficult in tissue explants. In addition, transfection results in multiple gene copies per cell, making it impossible to assess the physiological effect of expression from a single gene copy which is desirable for *in vivo* studies.

With these issues in mind, we turned to retroviral vectors to transfer a single copy of the *ada* gene and to test its expression in cells with low as well as high levels of mammalian alkyltransferase activity. Retroviral vectors have the advantage of being able to efficiently and stably introduce a single gene copy and have been used to study the multidrug resistance gene as well as the expression of many other genes in normal cells and in tissues *in vivo* (19–21). We studied 4 different promoters linked to the *ada* gene (the MoMLV[3] LTR, the RSV LTR, the TK promoter from herpes simplex virus, and the human PGK promoter). The MoMLV LTR, TK, and PGK promoters have been shown to be expressed from within proviral sequences following retroviral infection (22–25), and the RSV, TK, and PGK promoters are preferentially expressed in hematopoietic

Received 4/14/89; revised 8/1/89; accepted 8/7/89.

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[1] Supported in part by USPHS Grants ESCA-00134, CA-45609, CA-08644, and P30CA-43703 from the NIH.

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[3] The abbreviations used are: MoMLV, Maloney murine leukemia virus; BCNU, 1,3-bis(2-chloroethyl)nitrosourea; CNU, chloroethylnitrosourea; LTR, long-terminal repeat; RSV, Rous sarcoma virus; TK, thymidine kinase; PGK, phosphoglycerate kinase; CS, calf serum; FCS, fetal calf serum; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CFU, colony-forming unit.

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cells (23–27), a potential target for *ada* gene expression *in vivo*. By constructing a series of retroviral vectors, we determined: (*a*) which construct produced the greatest level of bacterial alkyltransferase activity and whether or not this varied between cell lines; (*b*) certain factors which influenced the ability of the bacterial alkyltransferase to function *in situ* and to protect cells from *N*-nitroso compounds; and (*c*) which of the four retroviral constructs could be used to achieve the highest level of bacterial alkyltransferase in normal mammalian cells.

MATERIALS AND METHODS

Chemicals and Reagents. DNA-modifying enzymes and deoxynucleotides were purchased from Boehringer-Mannheim and were used according to instructions of the manufacturer. Nitrocellulose was purchased from Schleicher and Schuell. Gene Screen Plus and [α-^{32}P]-dCTP (800 Ci/mmol) were purchased from New England Nuclear Corp. Formamide (Boehringer-Mannheim) was deionized before use with Ag-50-XA8 resin (Bio-Rad). Dextran sulfate was obtained from Pharmacia. Guanidium thiocyanate was obtained from Fluka A.G. Tissue culture reagents were obtained from Hazelton Laboratories, and fetal bovine serum was obtained from Hyclone Laboratories. G418 was obtained from Gibco. BCNU and CNU were obtained from the Drug Synthesis Branch, Drug Therapeutics Branch, National Cancer Institute. O^6-Methylguanine was synthesized using the method of Demple *et al.* (28) by Dr. Paul Howard, Department of Environmental Health Sciences, Case Western Reserve University School of Medicine. Rabbit antiserum to the M_r 19,000 fragment of the bacterial alkyltransferase protein used for immunocytochemical studies was obtained from Applied Genetics, Inc. (29). Normal swine serum and swine anti-rabbit IgG were obtained from Dako. All other chemicals were obtained from Sigma.

Plasmids. pSV2*ada-alkB* (16), which codes for the bacterial *ada-alkB* operon originally isolated by Lemotte and Walker (30), was kindly provided by Dr. Leona Samson (Harvard School of Public Health). The retroviral vector pLJ (also called DOL-) (31) was a generous gift from Dr. Richard C. Mulligan (Whitehead Institute, Massachusetts Institute of Technology). The retroviral vector pTKneoPGKpr was kindly provided by Dr. D. A. Williams and Dr. S. Orkin (Harvard Medical School). pTk109 was a gift of Dr. S. McKnight (University of Washington) (32). pRSVCat (27) was obtained from Dr. Mark Tykocinski, Department of Pathology, Case Western Reserve University.

Construction of Retroviral Vector Plasmids. Three of 4 recombinant retroviral vector plasmids were derived from pLJ. pLJ contains the SV-40 early promoter ligated to the neomycin resistance gene, *neo* [which confers resistance to the neomycin analogue, G418 (33)], inserted between the MoMLV LTRs, forming a replication-incompetent virus. For two of the pLJ-based retrovirus vectors, the internal SV-40 promoter was removed, creating pLJ(-SV40) (34).

The second parent retroviral vector used was pTKneoPGKpr. It contains the 109-base pair TK promoter derived from herpes simplex virus linked to the *neo* gene with a transcriptional orientation opposite to the 5′ MoMLV LTR. The 0.5-kilobase promoter of the human PGK gene is located 3′ to the TK-*neo* chimeric gene and is in the same transcriptional orientation as the 5′ LTR (see Fig. 1). The parent plasmids also contain the ψ region necessary for retroviral packaging, but lack intact *env*, *pol*, and *gag* genes necessary for wild-type virus production (35). Transfection of these defective retroviral sequences into ψ-2 cells (35) allows release of only replication-defective retrovirus particles containing the recombinant sequences.

General Methods. Purification of plasmid DNA, enzyme treatment, ligation reactions, and transformations were performed as previously described (36, 37). Construction of the retroviruses used (see Fig. 1) proceeded as follows.

pLJB*ada*. The 1320-base pair *Hin*dIII-*Sma*I *ada* fragment which codes only for the *E. coli* alkyltransferase protein was isolated from pSV2*ada-alkB* (16). The *ada* fragment was then ligated into the unique *Bam*HI site of pLJ after blunt ending both fragments. Two mRNA transcripts are expected from this provirus: a 5.7-kilobase mRNA which initiates in the 5′ LTR; and a 3.5-kilobase mRNA which initiates in the internal SV-40 promoter. Both transcripts terminate in the 3′ LTR.

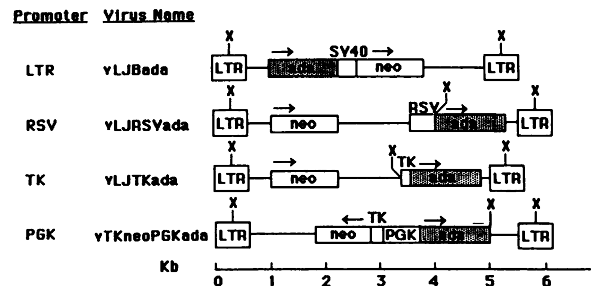

Fig. 1. Defective retroviral constructs containing *ada*. The construction of each of the 4 retroviral constructs is described completely in "Materials and Methods." *Promoter* identifies exogenous promoters expected to generate *ada*-containing transcripts; *virus name*, the name of the defective retrovirus; *X*, *Xba*I, the pertinent restriction enzyme recognition sites used to identify the intact integration of the provirus. *Arrows* indicate the direction of mRNA transcripts. *kb*, kilobases.

pLJTK*ada*. To construct TK*ada*, the 164-base pair *Bam*HI-*Bgl*II fragment, isolated from pTK109 and containing 109 base pairs of the TK promoter (31), was ligated to the 1320-base pair *Hin*dIII-*Sma*I *ada* fragment in the polylinker region of the Cla12N plasmid (34). The TK*ada* chimeric gene was then inserted into pLJ(-SV40) at the unique *Cla*I site in the same orientation as the 5′ LTR. The two expected transcripts are: a 5.8-kilobase mRNA, initiating in the 5′ LTR; and a 2.3-kilobase mRNA, initiating in the TK promoter. Both terminate in the 3′ LTR.

pLJRSV*ada*. A chimeric gene containing the RSV promoter linked to *ada* was constructed with the 621-base pair *Nde*I-*Hin*dIII fragment of the 3′ LTR of RSV (27, 38) ligated to the 1320-base pair *ada* fragment in the Cla12N polylinker. The isolated chimeric gene was then ligated into pLJ(-SV40) at the *Cla*I site. The two transcripts expected are: a 6.1-kilobase mRNA, initiating from the 5′ LTR; and a 2.1-kilobase mRNA initiating in the RSV promoter. Both terminate in the 3′ LTR.

pTKneoPGK*ada*. The 1320-base pair *ada* fragment was inserted using *Bam*HI linkers into the unique *Bam*HI site located 3′ to the human PGK (23, 39) promoter in pTKneoPGKpr. The three transcripts expected are: a 2.9-kilobase mRNA coding for *neo* initiating in the TK promoter and terminating in the 5′ LTR; a 6.1-kilobase mRNA initiating in the 5′ LTR; and a 2.5-kilobase mRNA coding for *ada* initiating in the PGK promoter, both of which terminate in the 3′ LTR.

Cell Culture and DNA Transfection. The murine fibroblast cell line NIH-3T3 was maintained in 50% Ham's F-12 medium/50% Dulbecco's modified Eagle's medium supplemented with 7% FCS and 3% CS at 37°C in an atmosphere of 5% CO_2/95% air. The CCL-1 murine fibroblast cell line was maintained in α-modified Eagle's medium, supplemented with 7% FCS and 3% CS at 37°C. The retroviral replication-deficient packaging ψ-2 cell line was maintained in 50% Ham's F-12 medium/50% Dulbecco's modified Eagle's medium supplemented with 10% CS at 37°C, in 5% CO_2/95% air. Transfection of DNA into the ψ-2 cell line was performed by the calcium phosphate method (35–37) using 20 μg of retroviral vector DNA per 2×10^5 ψ-2 cells. Colonies containing the integrated recombinant DNA were selected in medium containing 1 mg/ml of G418 and were maintained in 0.2 mg/ml. Transfected cells were maintained both as mass cultures and as clonally derived cell lines after selection.

Harvest of Retroviral Particles and Infection of NIH-3T3 and CCL-1 Cells. Retrovirus was harvested from confluent plates of transfected ψ-2 cells (34, 35), concentrated 20-fold with a YM-100 Amicon filter (40), and sterilized by passage through a 0.45-μm Nalgene filter. Clonally isolated ψ-2 transfectants were screened for virus production by the ability of culture supernatant to transmit G418 resistance to NIH-3T3 cells. Virus infection was quantitated by culturing 5×10^5 NIH-3T3 or CCL-1 cells in the presence of limiting dilutions of ψ-2 supernatant in a 100-mm^2 dish, at 37°C with 8 μg/ml of polybrene. After 6 h, 10 ml of culture medium were added, the cells were incubated for an additional 48 h, and then they were split 1:3 into medium containing 1 mg/ml of G418. Alternatively, cells received 0.06 mM BCNU. Colonies were

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counted and isolated 10 days after infection.

Probes. All DNA probes were labeled with [^{32}P]dCTP using the Pharmacia Random Primer kit. The specific activity of the labeled DNA was approximately 4×10^5 cpm/ng of DNA. The following probes were used: *ada* DNA, 1320-base pair *Hin*dIII-*Sma*I fragment from pSV2 *ada-alkB* as described above; *neo* DNA, 1.3-base pair *Bgl*II-*Sma*I fragment from pLJ isolated as previously described (34); and *actin* cDNA, the 1.35-base pair murine α-actin cDNA isolated as previously described (41).

Isolation and Analysis of Cellular DNA and RNA. Cellular genomic DNA was prepared as previously described (36). Total cellular RNA was prepared by standard procedures using 4 M guanidium thiocyanate and purification on CsCl cushions by ultracentrifugation (36, 42).

DNA Analysis. Twenty μg of DNA from various cell lines were digested with a 3-fold excess of *Xba*I overnight at 37°C. The digested DNA was separated by electrophoresis on a 1% agarose gel and transferred to nitrocellulose as described (37). Hybridization was at 42°C for 48 h. Filters were washed 3 times in 0.1× standard saline citrate/0.1% SDS at 55°C, air dried, and exposed to Kodak XAR film at −70°C. Dot blot analysis was performed on genomic DNA samples for more accurate assessment of gene copy number.

RNA Analysis. Twenty μg of total cellular RNA from various cell lines were separated by formaldehyde agarose gel electrophoresis and hybridized to the *ada* and *neo* probes sequentially using the RNA transfer techniques previously described (36, 37).

Determination of Alkyltransferase Activity. Cells grown to late log phase in 100-mm Petri dishes were trypsinized and washed 3 times with cold PBS-1 mM EDTA by centrifugation at $400 \times g$ for 10 min. The cell pellets were resuspended in 1 ml of cell extract buffer (70 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/0.1 mM EDTA/5% glycerol/1 mM dithiothreitol, pH 7.8) and processed as previously described (43). The activity of alkyltransferase in each cell extract was measured as removal of the [^{3}H]methyl adduct from O^6-[^{3}H]methylguanine in [^{3}H]methyl DNA alkylated with *N*-[^{3}H]methylnitrosourea as previously described (13, 43). The reaction mixture consisted of 250 μg of cellular protein, 4 μg of [^{3}H]methyl DNA, 1 mM dithiothreitol, and 25 μM spermidine in a total volume of 0.3 ml of cell extract buffer. An alkyltransferase unit of activity is defined as the removal of one fmol of O^6-methylguanine from the substrate DNA and is reported as units/mg of cellular protein (13, 43).

Determination of Molecular Weight of the Alkyltransferase. Both the mammalian and bacterial alkyltransferase proteins function by covalently transferring alkyl groups from DNA to the active site in the protein, thereby repairing the DNA adduct and inactivating the alkyltransferase. The molecular weight of the alkyltransferase was determined by incubating cell extract containing 1 mg of protein in excess (25 μg) ^{3}H-methylated DNA for 45 min at 37°C, followed by precipitation in 9% trichloroacetic acid and boiling in 2% SDS/15% glycerin/0.5 M Tris (pH 8.8)/1% β-mercaptoethanol for 15 min followed by separation on an 11% SDS-PAGE as previously described (44). The species of origin can be inferred from the molecular weight of the protein, as the expected molecular weight of the mammalian enzyme is 26,000, whereas the expected molecular weight of the *E. coli* enzyme is 39,000 with active M_r 21,000 and 19,000 cleavage products (45, 46).

BCNU and CNU Cytotoxicity Studies. The effects of BCNU and CNU on clonal survival in CCL-1 and NIH-3T3 cells were determined. Exponentially growing cells were diluted 10-fold into fresh culture medium in either the absence or presence of 0.5 mM O^6-methylguanine. Twenty-four h later, the exponentially growing cells were treated with trypsin, and 1000 cells were plated on each 100-mm tissue culture plate in serum-free medium. Four h after attachment, 0 to 80 μM BCNU or 0 to 160 μM CNU was added to the medium for 2 h. The cells were then washed in drug-free medium containing serum and maintained an additional 7 days in the case of NIH-3T3 or 10 days for CCL-1 at 37°C. Colonies containing greater than 50 cells were identified following methylene blue staining. The cloning efficiency of these cell lines was approximately 65% for NIH-3T3 and 35% for CCL-1.

Immunocytochemical Localization of the Alkyltransferase. Cells were grown to late log phase on single chamber Lab Tek glass slides, rinsed with PBS, and air dried. The cells were fixed in 50% methanol/50% acetone for 2 min and nonspecific antibody binding blocked with a 1:50 dilution of swine serum in 0.01 M Tris buffer (pH 7.4) at 22°C in a humidified chamber. Rabbit anti-bacterial alkyltransferase polyclonal IgG antibody was added for 30 min as described (29). After the slides were rinsed in Tris buffer, a 1:12 dilution of swine anti-rabbit IgG antibody was added for 20 min at 22°C, and the slides were rinsed in Tris buffer and incubated in rabbit horseradish peroxidase/antiperoxidase complexes in Tris buffer for 20 min and with 3-amino-9-ethylcarbazole for 15 min to produce a red color product.

RESULTS

Establishment of Retrovirus Producer ψ-2 Clones. The recombinant retroviral vectors containing the viruses shown in Fig. 1 were transfected into ψ-2 packaging cells, and producer clones were selected by exposure to G418. Approximately 20 clones from each transfection were screened for alkyltransferase activity and virus production. The virus produced from the ψ-2 cells transfected with pLJB*ada*, pLJTK*ada*, pLJRSV*ada*, and pTKneoPGK*ada* was designated vLJB*ada*, vLJTK*ada*, vLJRSV*ada*, and vTKneoPGK*ada*, respectively. The maximum virus titers from the ψ-2 clones transfected with each construct were as follows: vLJB*ada*, 6×10^4 CFU/ml; vLJTK*ada*, 1.2×10^5 CFU/ml; vLJRSV*ada*, 6×10^4 CFU/ml; and vTKneo-PGK*ada*, 5×10^4 CFU/ml. Virus-producing ψ-2 clones had increased alkyltransferase activity which was of bacterial origin by SDS-PAGE (data not shown). However, alkyltransferase activity in ψ-2 lines did not correlate with viral titer. Viral titers were stable over time except when ψ-2 cells were transfected with pTKneoPGK*ada*, where titers decreased 10- to 20-fold over 2 mo. No wild-type virus was produced, as determined by the inability of medium from infected cells to transmit G418 resistance to NIH-3T3 cells.

To demonstrate the efficacy of retroviral mediated gene transfer of *ada* into untransformed mammalian cells, two murine cell lines were infected with each retroviral construct. The cell lines NIH-3T3 and CCL-1 were chosen because of their different levels of endogenous mammalian alkyltransferase activity: 151 ± 31 units/mg of protein in NIH-3T3 cells *versus* 33 ± 11 units/mg of protein in CCL-1 cells. NIH-3T3 cells selected using either G418 or BCNU yielded similar numbers of infected clones containing the integrated provirus. The infection efficacy of CCL-1 cells was approximately 60% that of NIH-3T3 cells. Following infection with each retroviral construct, 20 G418-resistant colonies were screened for alkyltransferase activity.

Alkyltransferase Activity in Infected NIH-3T3 and CCL-1 Cells. The range in alkyltransferase activity seen in the infected clones is shown in Fig. 2. Virus vLJRSV*ada* generated the highest levels of alkyltransferase activity in both NIH-3T3 and CCL-1 cells. High alkyltransferase activity was also seen in some clones infected with vTKneoPGK*ada* and vLJTK*ada* in CCL-1 cells and with vLJB*ada* in NIH-3T3 cells. Following clonal isolation, alkyltransferase activity was stable in culture for greater than 4 mo with the exception of clones containing the vTKneoPGK*ada* provirus, where activity increased up to 3-fold. The activity achieved in NIH-3T3 clones infected with vLJRSV*ada* is among the highest reported for alkyltransferase activity in mammalian cells (13, 17, 47). Cell lines with high alkyltransferase were selected for further analysis.

Retroviral Integration into Genomic DNA after Infection of NIH-3T3 and CCL-1 Cells. In order to confirm *ada* gene incorporation into the infected cell lines, genomic DNA was digested with *Xba*I and analyzed by Southern blotting, utilizing sequentially the *ada* and *neo* probes to document that the provirus was intact in each case (Fig. 3). Most NIH-3T3 and

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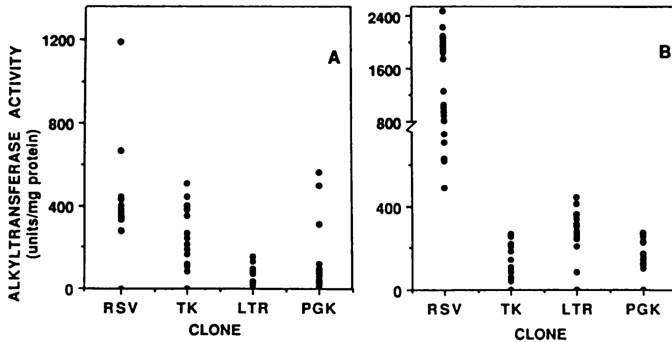

Fig. 2. Alkyltransferase activity in retrovirally infected cell lines. Individual CCL-1 (*A*) and NIH-3T3 (*B*) clones were assayed for alkyltransferase activity as described in "Materials and Methods." *Values* shown represent the mean of 2 to 6 determinations per clone. The clone is identified by the exogenous promoter controlling *ada* expression in the retrovirus used: *RSV*, vLJRSV*ada*; *TK*, vLJTK*ada*; and *PGK*, TKneoPGK*ada*; or the *LTR* in the case of vLJB*ada*.

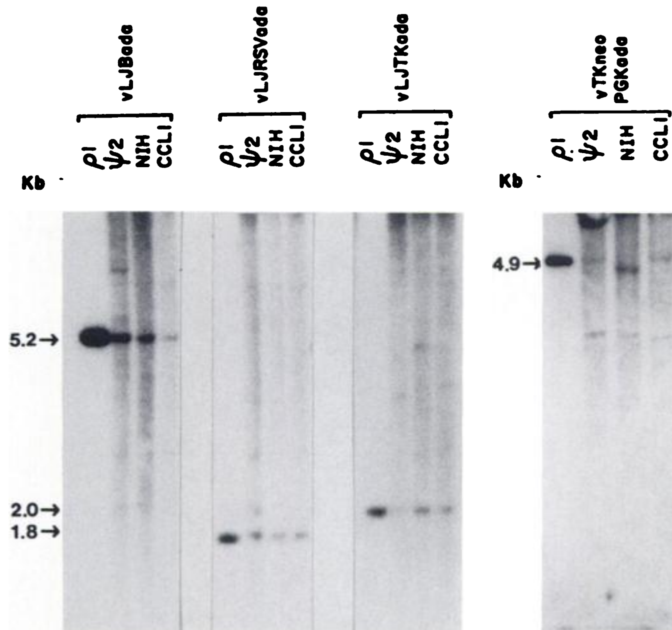

Fig. 3. Proviral integration into ψ-2, NIH-3T3, and CCL-1 cells. Plasmids containing each of the proviral constructs were separately transfected into ψ-2 cells, and culture supernatant containing retroviral particles was used to infect both NIH-3T3 and CCL-1 cells. Following selection for approximately 4 wk in 0.5 mg/ml of G418, resultant colonies of NIH-3T3 and CCL-1 cells were isolated and expanded. Genomic DNA was extracted, digested with the restriction enzyme *Xba*I, separated by agarose gel electrophoresis, transferred to a nylon membrane, and analyzed for proviral sequences by hybridization to 1.3-kilobase *ada* probe. Complementary sequences were identified using the 1.3-kilobase *neo* probe on the same membrane (data not shown). *pl*, plasmid DNA containing the retrovirus; *ψ2*, ψ-2 cells transfected with the appropriate plasmid; *NIH* or *CCL1*, NIH-3T3 or CCL-1 cells infected with the designated virus. The expected fragments hybridizing with *ada* are: vLJBada, 5.2 kilobases; vLJRSVada, 1.8 kilobases; vLJTKada, 2.0 kilobases; and vTKneoPGKada, 4.9 kilobases.

CCL-1 clones with increased alkyltransferase activity contained only the expected proviral restriction fragment, although some virus-producing ψ-2 clones had additional bands consistent with either internal deletions, insertions, or point mutations at the *Xba*I site within the provirus. Dot blot analysis of each retrovirally infected NIH-3T3 or CCL-1 clone indicated the presence of 0.5 to 1 gene copy per cell (data not shown).

Analysis of mRNA Transcripts in Retrovirally Infected NIH-3T3 and CCL-1 Cells. *ada* expression in individual cloned cell lines infected with each retroviral construct was analyzed by Northern blot (Fig. 4). *ada* transcripts were expressed in each cell line with the appropriately sized mRNA transcripts generated from both the internal promoter, as well as from the 5′

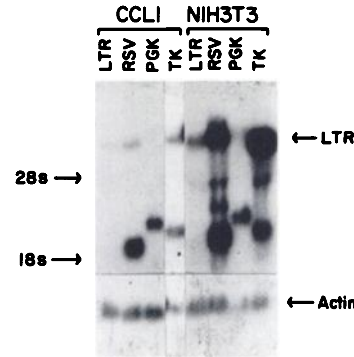

Fig. 4. Expression of *ada* in retrovirally infected cell lines. Total cellular RNA was prepared from representative clones of each cell line infected with each of the 4 retroviruses followed by formaldehyde-agarose electrophoresis, transfer to a nylon membrane, and hybridization with *ada* probe. The same membrane was then stripped and rehybridized with *actin* probe to control for variation in RNA loading. *LTR* on the *right* indicates the expected size of the full-length genomic transcripts initiating in the 5′ LTR. The transcript lengths predicted to hybridize with *ada* from each provirus are as follows: vLJB*ada* (*LTR*), 5.7 kilobases; vLJRSVada (*RSV*), 6.1 and 2.1 kilobases; TKneoPGKada (*PGK*), 6.1 and 2.5 kilobases; and vLJTK*ada* (*TK*), 5.8 and 2.3 kilobases.

LTR. There was variation in the relative intensity of the signals from the LTR and internal promoters when the same retroviral construct was used to infect the two cells lines. Specifically, the amount of RNA containing *ada* was greater in NIH-3T3 cell clones than CCL-1 clones for each virus measured, an observation that was consistently seen with multiple RNA analyses. *ada* mRNA initiating in the 5′ LTR was greatest in cells infected with vLJRSV*ada* and vLJTK*ada*, less with vLJB*ada*, and lowest with vTKneoPGK*ada*. In NIH-3T3 clones, relative promoter strength was RSV > PGK = TK; and in CCL-1 clones, the order was RSV > PGK > TK. In CCL-1 clones, the *ada* mRNA initiating from the internal promoters was greater than that initiating from the 5′ LTR. It is interesting to note the lack of correlation between *ada* mRNA levels and alkyltransferase activity in the NIH-3T3 cell line infected with VLJTK*ada*, whereas there is a reasonably good correlation between *ada* mRNA and alkyltransferase in the other infected clones.

Determination of *E. coli* Origin of Alkyltransferase in Retrovirally Infected Cell Lines by SDS-PAGE. To confirm that the increase in alkyltransferase was due to *ada* gene expression, SDS-PAGE of the cell extracts was performed after reacting the extracts with [^{3}H]methylnitrosourea to label the alkyltransferase (Fig. 5). Each infected clone of NIH-3T3 and CCL-1 had evidence of the bacterial M_r 39,000 protein. Occasionally the active M_r 19,000 and 21,000 proteolytic breakdown products of the bacterial protein were observed. In retrovirally infected NIH-3T3 clones, the intensity of the endogenous mammalian protein band (M_r 26,000) is similar to the NIH-3T3 parent cell line. This mammalian band is faintly seen in CCL-1 cells and clones.

Nitrosourea Resistance in Cell Lines Expressing the *ada* Gene. Virally infected CCL-1 clones were more resistant to BCNU (Fig. 6) and CNU (Fig. 7) than was the parent CCL-1 cell line. The relative drug resistance corresponded to the level of alkyltransferase activity, with the CCL-1 clone carrying vLJRSV*ada* (CCL1-RSV) being the most resistant. The nitrosourea resistance could be reversed by the specific alkyltransferase inhibitor, O^6-methylguanine, in CCL1-RSV cells (Fig. 7). In contrast, clones derived from the NIH-3T3 parent cell line, which is normally much more resistant to BCNU than the CCL-1 parent cell line, were only marginally more resistant to CNU- or BCNU-induced cytotoxicity despite the fact that some virally

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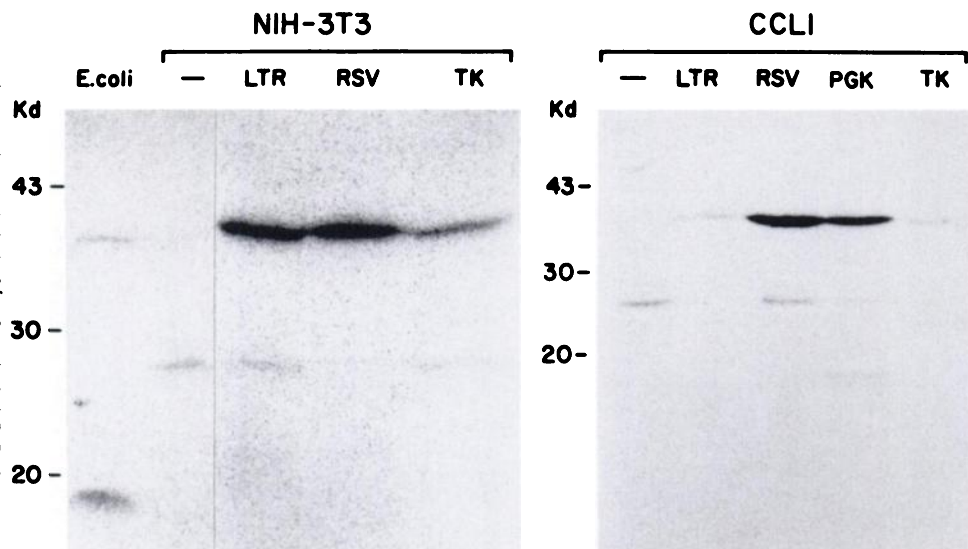

Fig. 5. *E. coli* origin of alkyltransferase activity in selected clones. Cell extracts from representative clonally derived cells from each retrovirally infected cell line were incubated with [^{3}H]methyl DNA containing O^6-[^{3}H] methylguanine. To detect transfer of the [^{3}H] methyl group to the alkyltransferase, these extracts were separated by SDS-PAGE, and the radioactive bands were identified by autoradiography. The same amount of cell extract was applied to each lane; however, the exposure of the gel containing NIT-3T3 cell extracts was for 3 wk and that of the gel containing CCL-1 extracts for 5 wk, increasing the relative intensity of the bands in the latter gel. The expected bands are: intact *E. coli* alkyltransferase at M_r 39,000 with proteolytic M_r 21,000 and 19,000 active fragments and mammalian M_r 26,000 alkyltransferase. Abbreviations are the same as in Fig. 2.

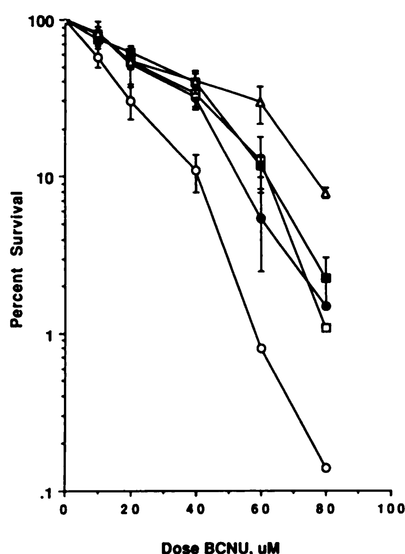

Fig. 6. BCNU cytotoxicity in CCL-1 clones containing *ada*. CCL-1 cells or individual retrovirally infected CCL-1 clones were exposed to increasing concentrations of BCNU in serum-free medium for 2 h and cultured in fresh medium for 10 days, after which colonies containing greater than 50 cells were counted. The individual cell lines and selected single clones of retrovirally infected cell lines used in these experiments and their mean alkyltransferase activity are as follows: ○, CCL-1, 35 units/mg of protein; △, CCL1-RSV (infected with vLJRSV*ada*), 1219 units/mg of protein; ■, CCL1-PGK (infected with vTKneoPGK*ada*), 564 units/mg of protein; ●, CCL1-TK (infected with vLJTK*ada*), 223 units/mg of protein; and □, CCL1-LTR (infected with vLJB*ada*), 138 units/mg of protein. *Points*, mean of 5 separate experiments; *bars*, SE.

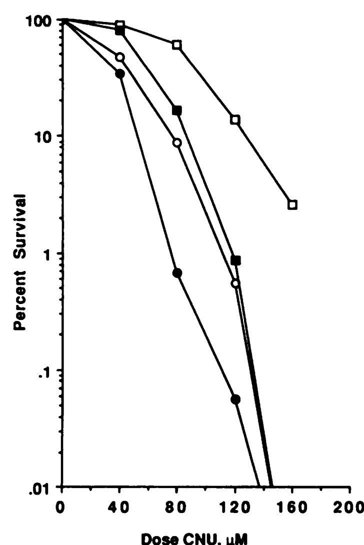

Fig. 7. Effect of O^6-methylguanine on CNU cytotoxicity. CCL-1 or CCL1-RSV cells were incubated with or without 0.5 mM O^6-methylguanine for 18 h, trypsinized, and replated at 1000 cells/dish and then exposed to increasing concentrations of CNU for 2 h. Cells were then cultured for 10 days in fresh medium and colonies counted. Similar results were obtained with the other retrovirally infected cell lines. Clonal survival was not affected by exposure to O^6-methylguanine. □, CCL1-RSV; ■, CCL1-RSV plus O^6-methylguanine; ○, CCL-1; ●, CCL-1 plus O^6-methylguanine.

infected NIH-3T3 clones contained alkyltransferase activity 2 to 15 times the level found in the parent cell line. In these clones, the 50% inhibitory concentrations in clonogenic assays were 32 µM BCNU and 112 µM CNU for NIH-3T3 cells infected with vLJRSV*ada* (NIH-3T3-RSV) and 28 µM BCNU and 86 µM CNU for NIH-3T3 cells. Even though increased alkyltransferase activity did not increase BCNU resistance in the NIH-3T3 clones, O^6-methylguanine did deplete the alkyltransferase and sensitize these cells to nitrosoureas (data not shown), indicating that drug resistance was at least partly dependent on alkyltransferase activity.

Intracellular Localization of Bacterial *ada* Protein. A possible explanation for the lack of increased BCNU resistance in NIH-3T3 clones expressing high levels of *ada* is that the bacterial alkyltransferase is not efficiently transported into the nucleus and, thus, cannot repair DNA damage. This possibility is not examined by measuring total cellular alkyltransferase but can be addressed by using an immunohistochemical technique to assess the intracellular localization of the bacterial *ada* protein. Fig. 8 shows predominantly diffuse cytoplasmic staining of the bacterial alkyltransferase in NIH-3T3 clones expressing *ada* from vLJRSV*ada*. Nuclear staining was seen in a minority of cells, usually only in cell clusters. NIH-3T3 clones infected with the three other retroviruses also had a similar diffuse cytoplasmic staining pattern with irregular or weak nuclear staining. CCL-1 clones expressing *ada*, on the other hand (Fig. 8*d*), had relatively increased staining of the nucleus relative to the cytoplasm, but even these cell lines failed to show predominant nuclear localization of the protein.

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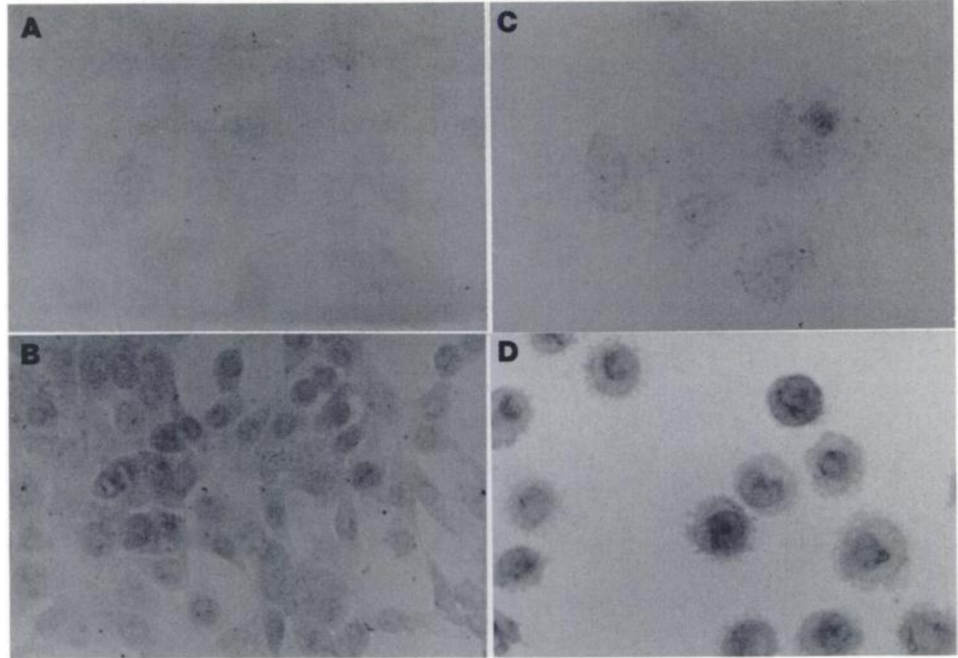

Fig. 8. Intracellular location of bacterial alkyltransferase. Cells were grown to late log phase on glass slides, incubated with rabbit antibacterial alkyltransferase polyclonal antibodies, and identified by secondary reaction with swine anti-rabbit antibody conjugated to immunoperoxidase. Multiple preparations showed a similar pattern of diffuse cytoplasmic staining with irregular and inconsistent nuclear staining, particularly in NIH-3T3-RSV. Similar exposure settings were used in *A*, *B*, and *C*. Photograph *D* was overexposed so that the staining cells can be better seen. Irregular but weak background staining was noted in CCL-1 cells. *A*, NIH-3T3; *B*, NIH-3T3RSV; *C*, CCL-1; and *D*, CCL1-RSV. Similar patterns were observed with cell lines infected with each of the other three retroviruses.

DISCUSSION

In these studies, a series of replication-defective retroviral vectors was constructed containing a heterologous mammalian promoter linked to the bacterial *ada* gene. Each of the 4 retroviruses successfully infected the NIH-3T3 and CCL-1 cell lines, and the *ada* gene was expressed in each instance. Important differences in *ada* gene expression were noted between retroviral constructs and between cell lines. Expression of the RSV*ada* chimeric gene was greater than the other genes and, in general, *ada* expression in NIH-3T3 was greater than in CCL-1 cells. Bacterial alkyltransferase activity in CCL-1 imparted resistance to chloroethylnitrosoureas, whereas little effect was noted in NIH-3T3 cells containing *ada*. These studies indicate that vLJRSV*ada* and vTKneoPGK*ada* may be effective retroviral vectors with which to study *ada* gene expression in normal mammalian cells and tissues *in vivo*, particularly in cells and tissues which, like CCL-1, have low levels of endogenous alkyltransferase activity.

There are a number of explanations for why different retroviral vectors had such a range in *ada* expression in the cell lines tested. (*a*) In vLJB*ada*, *ada* expression from the 5′ LTR is relatively low. In the parent pLJ vector, the internal SV-40 promoter, which controls *neo* expression, decreases expression from the 5′ LTR (34). It is possible that absence of the SV-40 promoter in vLJTK*ada* and vLJRSV*ada* was responsible for the increased transcription from the 5′ LTR in NIH-3T3 and, to a lesser extent, in CCL-1 cells. (*b*) In vTKneoPGK*ada* the transcriptional orientation of the *neo* gene may have decreased virus production and possibly *ada* expression, because cells which transcribe from the 5′ LTR might be lost during continued selection in G418, since the 5′ LTR mRNA contains antisense *neo* mRNA which would hybridize with the sense *neo* mRNA, resulting in less *neo* mRNA and lack of G418 resistance. This vector was designed such that expression from the 5′ LTR would be limited once proviral integration into the targeted cell had been achieved so as to avoid the negative interaction between the LTR enhancer elements and the internal promoters (48). We are currently evaluating whether clones infected with this virus develop deletions of the 5′ LTR during continued selection. Inactivation of the 5′ LTR could explain why viral production in the ψ-2 vTKneoPGK*ada* clones decreased over time and why small proviral deletions were noted in some clones. (*c*) Proximity between internal chimeric genes can lead to promoter competition for transcription factors (48) and could have been important in vTKneoPKG*ada*. (*d*) Finally, we are currently analyzing whether or not the vLJTK*ada* virus contains an internal mutation to explain the discordance between the level of *ada* mRNA and alkyltransferase activity in the NIH-3T3 cell line.

Whether or not our results will predict expression of *ada* when these viruses are used to infect normal cell explants or tissues *in vivo* is unknown. However, it is known that, in transgenic animals, methylation of the retroviral 5′ LTR decreases transcription, whereas tissue-specific expression of regulated internal promoters is maintained (22, 49). In bone marrow cells, the RSV, TK, and PGK promoters are expressed to a greater extent than the SV-40 or MoMLV 5′ LTR promoters (23–27, 50). Thus, vLJRSV*ada* and vTKneoPGK*ada* appear to be appropriate vectors to test whether *ada* expression can be observed in bone marrow cells following retroviral infection.

The major endpoint of these gene transfer studies was to observe the effect of *ada* expression on nitrosourea drug resistance. Two important results were obtained. (*a*) CCL-1 clones expressing *ada* became more resistant to BCNU and CNU in proportion to their level of alkyltransferase activity. The parent CCL-1 cell line has low levels of alkyltransferase activity, similar to that seen in most murine cells, and is sensitive to nitrosoureas. The increased drug resistance in CCL-1 cells expressing *ada* was similar to that reported by others using transfection techniques to introduce *ada* into V79, HeLa, Chinese hamster ovary, and WEHI-3 cell lines (16–18, 51), indicating that single gene copy can result in very high levels of gene expression and transmission of drug resistance.

On the other hand, when we infected the NIH-3T3 cell line, which is normally BCNU resistant and has moderately high alkyltransferase activity, clones expressing *ada* failed to show a significant increase in drug resistance, despite the fact that total alkyltransferase activity increased 2- to 15-fold. These results indicate that there are, in fact, limits to the ability of the

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bacterial alkyltransferase to increase drug resistance in mammalian cells. Previous reports have introduced *ada* into cells with very low levels of endogenous alkyltransferase activity (16–18, 51). Lack of an effect in cells expressing high levels of endogenous alkyltransferase may be due to one of three possible factors. (*a*) Even though some cell lines had markedly increased total cellular alkyltransferase activity, much of this activity appears to be cytoplasmic rather than being concentrated in the nucleus. It seems likely that mammalian DNA repair enzymes are localized to the nucleus because of either their capacity to bind DNA or the presence of a nuclear localization signal such as exists for the SV-40 large T-antigen (52). Thus, if the bacterial alkyltransferase is not concentrated within the nucleus, it may not efficiently repair DNA damage. (*b*) The mammalian alkyltransferase repairs longer chain O^6-methylguanine–DNA adducts faster than the *E. coli* protein (6), such that in cells with moderately high levels of mammalian alkyltransferase, the addition of the bacterial protein may add little to the level of drug resistance to chloroethylating agents. (*c*) NIH-3T3 cells may rely on other mechanisms of resistance to nitrosoureas such as glutathione-*S*-transferase and/or elevated levels of glutathione (53).

Nonetheless, most murine tissues have low levels of alkyltransferase activity, similar to that observed in CCL-1 cells, making it likely that resistance to *N*-nitroso compounds including the nitrosoureas will be increased following retrovirally mediated transfer of the *ada* gene. The most obvious choice for a retroviral vector is vLJRSV*ada*, because *ada* is expressed at high levels, and vTK*neo*PGK*ada*, because the PGK promoter is expressed in hematopoietic cells (23). Once elevated levels of alkyltransferase are achieved in different tissues *in vivo*, the role of this protein in resistance from *N*-nitroso compound-induced cytotoxicity, mutagenicity, and carcinogenicity can be tested.

ACKNOWLEDGMENTS

The authors thank Dr. R. W. Hanson and Dr. L. Samson for many helpful discussions in the design of these experiments, S. Kessen for expert technical support, C. Donovan for preparation of figures, and L. Lucas for preparation of the manuscript.

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