Illegitimate and homologous recombination in mammalian cells: differential sensitivity to an inhibitor of poly(ADP-ribosylation)

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ABSTRACT

We determined the effect of 3-methoxybenzamide (3-MB), a competitive inhibitor of poly(ADP-ribose)polymerase (E.C. 2.4.2.30), on illegitimate and extrachromosomal homologous recombination in mouse Ltk$^-$ cells. Cells were transfected with a wild type Herpes thymidine kinase (tk) gene or with two defective tk gene sequences followed by selection for tk-positive colonies. Using a wild type tk gene, colony formation required uptake, integration, and expression of the tk gene. Using defective tk genes, colony formation had the additional requirement for homologous recombination to reconstruct a functional tk gene. The presence of non-cytotoxic levels of 3-MB during and after transfection reduced the number of colonies recovered with a wild type tk gene in a dose-dependent manner, with 2 mM 3-MB causing a 10 to 20-fold reduction. 3-MB reduced the number of colonies recovered with defective tk genes only to the same extent as in transfections with a wild type gene. Treatment with 3-methoxybenzoic acid, a non-inhibitory analog of 3-MB, did not reduce the recovery of colonies in any experiment. Similar results were obtained using linear or supercoiled molecules and when defective tk genes were transfected into cells on one or two different DNA molecules. By assaying for transient expression of the tk gene, we found that 3-MB did not inhibit uptake or expression of the tk gene. We conclude that poly(ADP-ribosylation) plays a role in random integration (illegitimate recombination) of DNA but does not play an important role in extrachromosomal homologous recombination, demonstrating that these two recombination pathways in cultured mouse fibroblasts are biochemically distinct.

INTRODUCTION

Homologous recombination can be defined as any process in which two similar DNA molecules interact and undergo an exchange of sequence information. This exchange of sequence can be reciprocal or non-reciprocal, and may occur between two chromosomal sequences, two extrachromosomal sequences, or between one chromosomal and one extrachromosomal sequence. Homologous recombination in its various forms has been implicated in many diverse cellular processes including gene expression, evolution, and carcinogenesis. In recent years, homologous recombination in mammalian cells has received increased attention because of the powerful tool that targeted recombination between a transfected DNA molecule and a chromosomal sequence promises to bring to molecular genetics. 'Gene-targeting' potentially provides a means for: (i) studying gene structure and function by targeted gene knock-out; (ii) developing animal models for disease by producing chimeric mice from embryonic stem cells modified by targeted recombination *in vitro*; 3) gene therapy to correct human genetic disorders (see 1,2 and 3 for reviews).

One difficulty with approaches involving targeted recombination is the inefficiency with which mammalian cells carry out this type of homologous recombination. In contrast to fungi and bacteria, mammalian cells efficiently integrate transfected DNA into random, nonhomologous genomic loci (reviewed in 4). It is possible that such 'illegitimate recombination' competes with targeted homologous recombination, although there is some data that suggests that this may not be the case (5,6,7).

In general, very little is known about the biochemical mechanism(s) of either homologous or illegitimate recombination in mammalian cells. Recently, studies by Farzaneh et al. (8) have indicated that inhibition of poly(ADP-ribosylation) results in the inhibition of random integration of transfected DNA molecules into the genomes of several different types of mammalian cells. The rationale for these previous studies was the knowledge that poly(ADP-ribosylation) plays a role in the repair of DNA-strand breaks (reviewed in 9) possibly by activation of DNA ligase II (10). Because random integration of transfected DNA is generally viewed as an end-joining or ligation process (4), Farzaneh et. al. predicted and then confirmed that poly(ADP-ribosylation) plays a role in stable integration of DNA. However, it was not

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determined if inhibition of poly(ADP-ribosylation) also affects homologous recombination.

To further understand the extent to which the pathways of homologous recombination and illegitimate recombination might overlap, we made an assessment of the effect of an inhibitor of poly(ADP-ribosylation) on these two pathways. In this paper, we report that treatment with 3-methoxybenzamide reduces random genomic integration (illegitimate recombination) but does not reduce either intra- or intermolecular extrachromosomal homologous recombination of DNA molecules transfected into cultured mouse fibroblasts.

MATERIALS AND METHODS

Materials

3-methoxybenzamide (3-MB) and m-anisic acid (m-AA) were purchased from Aldrich Chemical Company. Restriction endonucleases were purchased from New England Biolabs and were used as prescribed by the supplier. [Methyl-^{3}H]thymidine (46 Ci/mmole) was purchased from Amersham.

Recombination substrates

All DNA constructs used, with the exception of p1.33, are based on a vector derived from pSV2neo (11) with restriction site modifications as described previously (12). Plasmid p1.33 contains the Herpes simplex virus type 1 (HSV-1) thymidine kinase (tk) gene on a 2.5 kb Bam HI-Hind III fragment inserted into pBR322 (13).

The mutant no. 8 HSV-1 tk gene (14) contains an 8 bp Xho I linker inserted at nucleotide 1215 of the HSV-1 tk gene [numbering according to Wagner et al (15)]. Plasmid pTK1−8 contains the mutant no. 8 tk gene on a 2.5 kb Bam HI fragment [previously reported (14) erroneously as a 2.4 kb fragment] inserted into the unique Bam HI site of the pSV2neo-derived vector.

Plasmid pAL2 contains the 1.2 kb Hinc II-Sma I fragment of the wild type HSV-1 tk gene inserted into the unique Hind III site of the pSV2neo vector, using Hind III linkers. This fragment contains most of the coding region but lacks the tk gene promoter and polyadenylylation signals (15). Plasmid pAL5 is identical to pAL2 except that in addition to the tk insert at the Hind III site it contains the mutant no. 8 tk gene on a 2.5 kb fragment inserted into the unique Bam HI site. Plasmids pAL2 and pAL5 have been described previously (14). Plasmid pTK12 contains the HSV-1 tk gene on a 2.5 kb Bam HI fragment inserted into the Bam HI site of the pSV2neo vector and the defective 800 bp Eco RV-Stu I fragment of the HSV-2 tk gene (16) inserted into the Hind III site of the vector.

Cell culture and transfections

The mouse Ltk$^-$ fibroblast cell line was grown in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum, MEM non-essential amino acids (GIBCO), and 50 ug of gentamicin sulfate per ml. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Stock solutions (2M) of 3-MB and m-AA were prepared in DMSO (Aldrich or Sigma) and stored at −20°C for up to one day. Cell culture media containing 3-MB or m-AA were prepared by the addition of the appropriate amount of stock solution.

Cells were transfected by the calcium phosphate coprecipitation method as described previously (14). Briefly, cells were plated at a density of 5×10^5 cells per 75 cm^2 flask on day 1. On day 2, the DNA/calcium phosphate coprecipitate was prepared and applied to the cells. Three hours prior to applying the precipitate, cells were re-fed with medium containing either no drug or containing 3-MB or m-AA at the indicated concentration; the drug was present in the medium throughout the subsequent selection period. This drug treatment protocol is taken from previous studies (8) that have shown such a regimen to have the greatest effect on illegitimate recombination. Salmon sperm DNA was used in all transfections as carrier to bring the total amount of DNA to 20 ug per 75 cm^2 flask. Cells were treated with the precipitate for 5 hours after which the precipitate was removed by washing with phosphate buffered saline (PBS). The cells were then re-fed with medium containing the appropriate drug. On day 3, 20 hours after removal of the precipitate, the cells were fed with medium containing hypoxanthine/aminopterin/thymidine (HAT) (17), supplemented with the appropriate drug, to select for tk$^+$ clones. HAT-resistant (HAT^r) colonies were counted 14 days later. Transfection of mouse L cells with salmon sperm DNA alone gives rise to fewer than 1 HAT^r colony per 10^7 cells (our unpublished observations).

DNA Isolation and Southern Blot Analysis

High molecular weight genomic DNA was isolated from mouse Ltk$^-$ cells as previously described (12). DNA samples were digested with restriction endonucleases and analyzed by Southern blots by hybridization to a probe homologous to the HSV-1 tk gene as previously described (12).

Assay for transient expression of the HSV-1 tk gene in mouse Ltk$^-$ cells

Cells were transfected by the calcium phosphate coprecipitate method as described above, with the following modifications. Cells were plated into 35mm dishes at a density of 1.5×10^5 cells per dish. Three hours before transfection, cells were refed with medium containing either no drug or 2 mM 3-MB. Salmon sperm DNA was used in transfections to bring the total amount of DNA to 2.5 ug per dish. Twenty hours after removal of the precipitate, cells were refed with 1.5 ml of medium containing either no drug or 2 mM 3-MB and supplemented with [methyl-^{3}H]thymidine (46 Ci/mmole) at a concentration of 20 uCi per ml. The uptake of tritium into the nuclei of cells was assayed by a procedure essentially described previously (18). Twenty-four hours after the addition of [methyl-^{3}H]thymidine to the medium, the cells were washed once with PBS and then fixed by washing twice with methanol. After air-drying, the dishes were coated with autoradiography emulsion (Kodak type NTB2). The dishes were placed in a light-tight container and the emulsion was exposed at 4°C for 48 hours. The emulsion was developed for 2.5 min. at 20°C with Kodak Dektol developer diluted 1:1 with water. The developer was stopped by washing the dishes with distilled water and the autoradiographs were fixed for 5 min in Kodak Fixer. Cells were viewed with a phase-contrast microscope (Nikon Diaphot) at ×100 magnification. Cells were scored positive for tk if they contained heavily localized grains over their nuclei.

RESULTS

Effect of 3-MB and m-AA on the viability of mouse Ltk$^-$ cells

We were interested in determining if inhibitors of poly(ADP-ribose)polymerase (E.C. 2.4.2.30) can influence homologous as

well as illegitimate recombination in mammalian cells. In this work, we made use of 3-MB, a competitive inhibitor of poly(ADP-ribose)polymerase (19). In some experiments, we also used m-AA (3-methoxybenzoic acid), a non-inhibitory analog of 3-MB (19), as a control. If any 3-MB were to break down either inside or outside of a cell, the most likely initial hydrolysis product would be m-AA.

Because of a lack of detailed reports in the literature on the cytotoxic effects of 3-MB or m-AA on mouse Ltk$^-$ cells, we first determined the effects of these compounds on cell viability. Dose response curves were generated by measuring the effects of these compounds on the plating efficiency of mouse Ltk$^-$ cells (Fig. 1). Both 3-MB and m-AA decreased the plating efficiency of the mouse Ltk$^-$ cell line in a dose-dependent manner. A 50% reduction in plating required a dose of approximately 2.3 mM 3-MB or greater than 4.0 mM m-AA (Fig. 1).

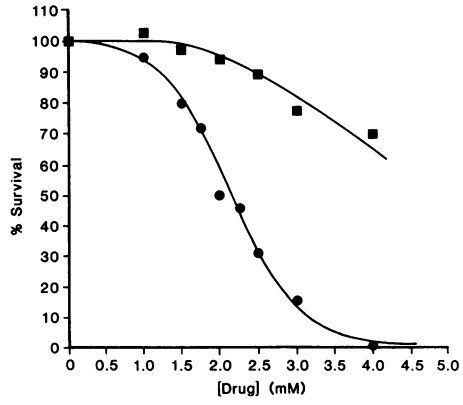

Figure 1. Survival of mouse Ltk$^-$ cells plated in the presence of 3-MB or m-AA. Mouse L cells, 100 cells per 25 cm^2 flask, were seeded in medium supplemented with either no drug or the indicated concentration of 3-MB (●) or m-AA (■). At least three flasks were used for each drug concentration. Colonies were counted after 11 days of incubation at 37°C. '% Survival' (relative plating efficiency) was calculated as the average number of colonies per flask at a given drug concentration divided by the average number of colonies obtained in the absence of drug (times 100). In the absence of drug (100% survival), the average number of colonies was 71.

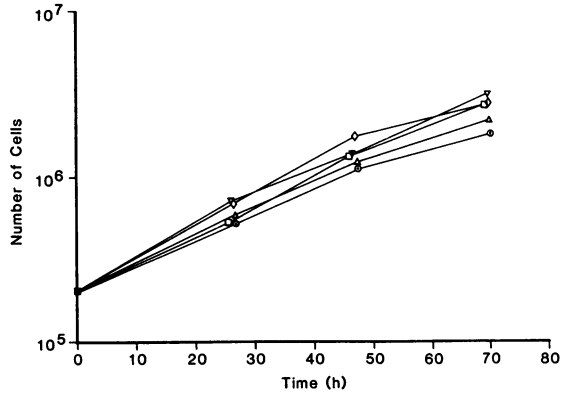

Figure 2. Growth rate of mouse Ltk$^-$ cells transfected in the presence of 3-MB or m-AA. Cells (1.4×10^5) were transfected with 7.5 ug carrier DNA plus 15 ng of p1.33 per 60 mm dish in the absence of drug or in the presence of the indicated amount of 3-MB or m-AA. Cells were maintained in medium with appropriate level of drug for the duration of the experiment. No HAT selection was applied. The time of refeeding of cells prior to applying the calcium phosphate/DNA coprecipitate was set as t=0. At various times, cells were harvested by trypsinization from two dishes for each case and counted. Cells transfected in the absence of drug (□); in the presence of 1 mM (△) or 2 mM (⊙) 3-MB; in the presence of 1 mM (▽) or 2 mM (◇) m-AA.

We were interested in measuring the effects of 3-MB and m-AA on recombination processes involving DNA molecules transfected into mouse L cells. It was conceivable that the cells might internalize 3-MB or m-AA during transfection and thus become hypersensitive to these compounds. The effect of 3-MB and m-AA on the growth of cells following transfection in the presence of these compounds was therefore determined. As shown in Fig. 2, the growth rate for mouse L cells varied very little under all conditions tested. We estimated the doubling time to be 16.5 hours in the absence of drug, 15.5 hours in the presence of 1 mM or 2 mM m-AA, and 18 hours in the presence of 1 mM or 2 mM 3-MB. By 70 hours post-transfection, all dishes had approached confluence and the growth curves could not be continued. Since the transfection experiments described below did not involve actual plating of cells in the presence of 3-MB or m-AA, a level of 2 mM or lower of these compounds was acceptable for use [and within the range used in previously reported studies (8)].

Effect of 3-MB and m-AA on transfection of mouse Ltk$^-$ cells

We next determined the effect of 3-MB and m-AA on transfection of mouse Ltk$^-$ cells. This cell line was not used in earlier studies (8) that demonstrated that 3-MB reduced random integration of transfected DNA into the genomes of several mammalian cell lines. Additionally, we made determinations

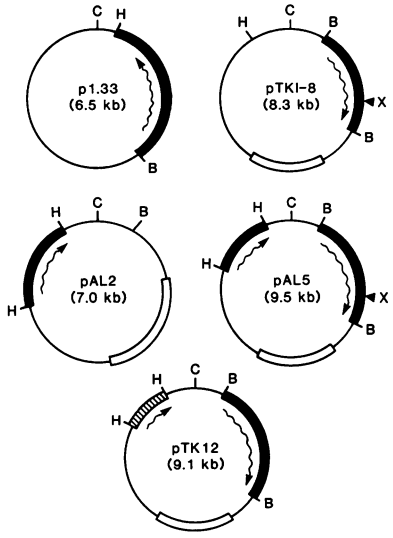

Figure 3. DNA substrates. Plasmids p1.33 and pTK12 both contain the wild type tk gene from HSV-1. Plasmids pTK1−8 and pAL5 contain a Xho I linker insertion (X) mutant tk gene cloned into the Bam HI site (B) of the vector while pAL5 and pAL2 each contain a defective internal fragment of the tk gene inserted at the Hind III site (H). The unique Cla I site (C) in each plasmid is also shown. Vector sequences are shown as a thin line. HSV-1 sequences are depicted as ■ and the direction of transcription of tk sequences is illustrated by wavy arrows. The neo gene of pSV2neo is shown as □. The defective fragment of the HSV-2 tk gene (▥) contained in pTK12 is not relevant to these studies. See Materials and Methods for construction details.

using both circular and linear DNA substrates (the previous studies used only circular substrates).

The DNA substrate used in these experiments, p1.33, is shown in Fig. 3. This plasmid contains a wild type tk gene from HSV-1. Plasmid p1.33 was transfected into mouse L cells in the absence of any drug or in the presence of 2 mM 3-MB or m-AA. The DNA was introduced either as a supercoiled molecule or after linearization with Cla I. Cells were fed with HAT medium to select for transformants that were stably tk-positive. The formation of colonies in HAT medium required the uptake, integration, and expression of the HSV-1 tk gene.

Table 1 shows the results of two representative experiments. At a level of 2 mM, 3-MB reduced the recovery of HAT^r colonies to a similar extent after transfection of the mouse cells

Table 1. Effect of 2 mM 3-MB and m-AA on Stable Transfection of Mouse Ltk$^-$ Cells

	DNA[a]	Drug	# Colonies[b]	Colony Recovery % no drug[c]	Colony Recovery % m-AA[d]
Expt. 1	p1.33, circular	None	472	100	–
		m-AA	743	157	100
		3-MB	53	11	7
Expt. 2	p1.33, linear	None	243	100	–
		m-AA	313	129	100
		3-MB	12	5	4

[a] 50 ng of plasmid DNA (plus carrier) were used per flask. Linear p1.33 was produced by cleavage with Cla I.
[b] Average of five flasks for each case.
[c] The number of colonies recovered for each case divided by the number of colonies obtained in the absence of drug, multiplied by 100.
[d] The number of colonies recovered in the presence of drug divided by the number of colonies obtained in the presence of an equal concentration of m-AA, multiplied by 100.

Table 2. Effect of 3-MB and m-AA on Intramolecular Extrachromosomal Homologous Recombination in Mouse Ltk$^-$ Cells

	DNA[a]	Drug	Conc.[b] (mM)	# Colonies[c]	Colony Recovery[d] % no drug	Colony Recovery[d] % m-AA
Expt. 1 (circular)	pAL5	None		16	100	–
	"	m-AA	1	23	143	100
	"	3-MB	1	4	25	18
	p1.33	None		271	100	–
	"	m-AA	1	293	108	100
	"	3-MB	1	45	17	15
Expt. 2 (circular)	pAL5	None		27	100	–
	"	m-AA	2	54	203	100
	"	3-MB	2	3	11	6
	p1.33	None		243	100	–
	"	m-AA	2	287	118	100
	"	3-MB	2	14	6	5
Expt. 3 (linear)	pAL5	None		134	100	–
	"	m-AA	1	86	64	100
	"	3-MB	1	34	25	40
	p1.33	None		118	100	–
	"	m-AA	1	120	102	100
	"	3-MB	1	27	23	23
Expt. 4 (linear)	pAL5	None		119	100	–
	"	m-AA	2	119	100	100
	"	3-MB	2	7	6	6
	p1.33	None		243	100	–
	"	m-AA	2	313	129	100
	"	3-MB	2	12	5	4

[a] 50 ng of p1.33 or 3 ug of pAL5 (plus carrier) were used per flask. Linear p1.33 and pAL5 were produced by cleavage with Cla I and XhoI, respectively.
[b] Concentration of m-AA or 3-MB.
[c] Average of five flasks for each case.
[d] As defined for Table 1.

with either supercoiled or linear p1.33. For supercoiled p1.33, the reduction was about ten-fold compared to the recovery of colonies in the absence of drug (Table 1). For linear p1.33, the corresponding reduction in colonies was about twenty-fold (Table 1). Based on previous work (8), we inferred that 3-MB inhibited the integration of the transfected DNA into the mouse L cell genome. In contrast, m-AA caused no reduction of HAT^r colonies for either supercoiled or linear p1.33 (Table 1). In some experiments the presence of 2 mM m-AA may have slightly increased the number of colonies recovered after transfection with supercoiled or linear p1.33. For this reason, the recovery of colonies in the presence of 3-MB was also normalized to the number of colonies obtained in the presence of m-AA (Table 1, far-right column). When normalized in this manner, 3-MB reduced colony recovery similarly for both linear and circular molecules of p1.33.

Effect of 3-MB and m-AA on intramolecular, extrachromosomal homologous recombination

In order to determine the effect of 3-MB on homologous recombination, we transfected cultured mouse Ltk$^-$ cells with either p1.33, which contains a wild type HSV-1 tk gene, or with pAL5, which contains two defective HSV-1 tk sequences (Fig. 3). Transfections were carried out in the presence or absence of 3-MB or m-AA. Plasmid DNA was introduced as supercoiled molecules or after linearization by digestion with Cla I (in the case of p1.33) or Xho I (in the case of pAL5). Following transfections, cells were fed with HAT medium to select for tk-positive clones.

In the transfections with p1.33, colony formation required DNA uptake, integration and expression of the tk gene, as in the above experiments. In the case of transfections with pAL5, colony formation had the additional requirement for reconstruction of a functional tk gene by homologous recombination prior to integration. The number of colonies recovered after transfection of cells with pAL5 was previously shown to be linearly related to the amount of transfected DNA, in the range of DNA concentrations used in these studies (14). Therefore, each colony functionally represents a single recombination event and the colony count is thus a valid measure of extrachromosomal recombination rates. Furthermore, the *linear* relationship between colonies recovered and amount of DNA used implies that homologous recombination among the tk sequences of pAL5 occurs primarily intramolecularly. Additionally, because one of the defective tk gene sequences on pAL5 has 5′ as well as 3′ deletions (Fig. 3), only gene conversion events or double reciprocal exchanges between the defective tk sequences are recoverable; any single crossover event would yield a tk gene with a 5′ or 3′ deletion.

By comparing the effect of 3-MB on the recovery of colonies after transfection with either p1.33 or pAL5, we made an assessment of whether 3-MB inhibited homologous recombination. If 3-MB inhibited homologous recombination *in addition to* random integration, then 3-MB should have a more pronounced effect on the recovery of colonies following transfection with pAL5 compared to transfection with p1.33. If 3-MB inhibited random integration only, then 3-MB would reduce the number of colonies to the same extent in either case. As shown in Table 2, the presence of 3-MB at 1 mM or 2 mM reduced the number of HAT^r colonies recovered after transfection with either supercoiled (experiments 1 and 2) or linearized (experiments 3 and 4) pAL5. Compared to controls using no drug, colony recovery was reduced approximately four-fold by 1 mM 3-MB and ten to twenty-fold by 2 mM 3-MB. The reduction in colonies was no greater than the reduction observed in parallel transfections in which the L cells were transfected with the wild type tk gene on p1.33 (Table 2). 3-MB therefore did not appear to inhibit intramolecular homologous recombination.

Southern analysis of recombinant HSV-1 tk genes produced by extrachromosomal recombination

To ascertain that colonies recovered after transfection with pAL5 arose from homologous recombination between the defective HSV-1 tk genes and did not represent rare aminopterin-resistant L cell variants, DNA was isolated from several such HAT^r clones and these samples were digested with Bam HI or with Bam HI plus Xho I. The DNA samples were analyzed by Southern analysis using a probe specific for HSV-1 tk sequences. A representative Southern blot is shown in Fig. 4. Although the hybridization pattern was sometimes complex, each of the fourteen clones examined (seven of which are shown in Fig. 4) displayed a 2.5 kb Bam HI fragment that was resistant to cleavage with Xho I. This DNA band is consistent with an HSV-1 tk gene that had undergone a correction of the original Xho I linker insertion mutation via gene conversion or double reciprocal exchange. Such a band was always detected in HAT^r clones arising from cells treated with no drug, 3-MB, or m-AA, regardless of the physical form of the transfected DNA (circular or linear). In some clones, we detected a 1.5 kb and a 1.0 kb band in the Bam HI plus Xho I digestion (see Fig. 4 lanes f and l) that were not present in the Bam HI digestions (Fig. 4 lanes e and k). These bands were probably due to cleavage of an uncorrected copy of the tk gene at the site of the Xho I linker insertion mutation. Most samples examined displayed additional hybridizing bands which represent additional rearrangements that are not readily interpretable.

Effect of 3-MB on intermolecular, extrachromosomal homologous recombination

Since 3-MB appeared capable of inhibiting illegitimate recombination between a transfected DNA molecule and the mouse genome, an *intermolecular* process, we wanted to determine if 3-MB could inhibit an homologous recombination event that required interactions between two different DNA molecules. Mouse Ltk$^-$ cells were cotransfected with pAL2 (supercoiled) and pTK1−8 (linearized with XhoI) (Fig. 3) in the presence or absence of 3-MB, and HAT^r cells were selected. In this case, colony formation required intermolecular homologous recombination to reconstruct a functional tk gene. (Transfection of cells with pTK1−8 alone produces no HAT^r colonies (14)). As shown in Table 3, 3-MB reduced the number of HAT^r colonies arising after cotransfection of cells with pAL2 and pTK1−8. Colonies were reduced about 3-fold and ten-fold by 1 mM and 2 mM 3-MB, respectively. The reduction in the recovery of colonies was equal to that observed in parallel experiments in which the cells were transfected with a wild type tk gene (pTK12, Table 3). There was no indication that 3-MB could inhibit intermolecular homologous recombination.

In these experiments and the ones described below, pTK12 (Fig. 3) rather than p1.33 was used as a source of the wild type tk gene. Plasmid pTK12 is derived from the pSV2neo vector that the recombination substrates are based on, whereas p1.33 is derived from pBR322. Transfection with pTK12 exhibited similar sensitivity to 3-MB as transfections with p1.33 (compare Table 2, expts. 1 and 2, and Table 3).

Effect of 3-MB on transient expression of the HSV-1 tk gene

In each of the above experiments, scoring for homologous recombination required stable integration (illegitimate recombination) of a reconstructed functional tk gene into the L cell genome in order to produce a HAT^r colony. It is conceivable that the mechanism of integration following extrachromosomal homologous recombination might be different from that of integration in the absence of recombination. Because of the possibility of such complications, we assayed for transient expression of the HSV-1 tk gene. We used this assay to monitor extrachromosomal homologous recombination independent of integration. Cells were transfected either with pTK12 or pAL5 (both linearized) in the presence or absence of 3-MB. One day after transfection, cells were fed with ^{3}H-thymidine. Only tk-positive cells could phosphorylate and then incorporate this labeled nucleotide into their genomes. Tk-positive cells were identified by autoradiography as those cells displaying heavily localized grains over their nuclei. A representative autoradiogram of cells transfected with pTK12 is shown in Fig. 5. In this assay, DNA uptake and expression of the tk gene was required for a positive cell to arise. When pAL5 was used, DNA uptake, homologous recombination, and expression of the reconstructed tk gene was required for the production of a positive cell.

As shown in Table 4, 2 mM 3-MB produced little or no effect on the recovery of tk-positive cells after transfection with either pTK12 or pAL5, as measured by the transient expression assay. These results implied that 3-MB did not inhibit uptake, expression, or extrachromosomal homologous recombination of transfected DNA molecules in mouse Ltk$^-$ cells. We concluded that 3-MB exerts its influence on transfection by inhibiting random integration of DNA into the mammalian genome.

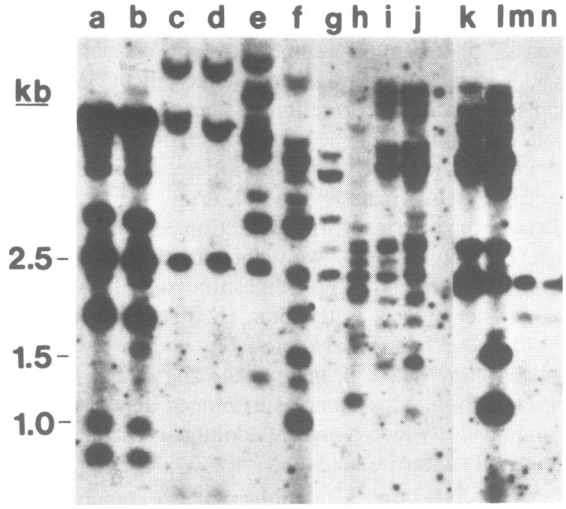

Figure 4. Representative Southern analysis of HAT-resistant colonies arising after transfection with pAL5. DNA was isolated from cells transfected with: circular pAL5 in the presence of 2 mM 3-MB (lanes a & b), 1 mM 3-MB (e & f), or no drug (k & l); linear pAL5 in the presence of 2 mM 3-MB (c & d), 1 mM m-AA (g & h), 1 mM 3-MB (i & j) or no drug (m & n). Pairwise lanes (e.g., lanes a & b) contain a single DNA sample digested with Bam HI alone or Bam HI plus Xho I, respectively. Samples were hybridized with a probe specific for HSV-1 tk sequences. Seven out of fourteen samples analyzed by Southern blotting are shown. Each sample displays a 2.5 kb Bam HI fragment that was resistant to cleavage by Xho I, consistent with correction of the Xho I linker insertion via homologous recombination. See text for details.

DISCUSSION

The results presented in this paper demonstrate that, at non-cytotoxic levels, the poly(ADP-ribosylation) inhibitor 3-MB inhibits random integration of DNA into the mammalian genome but does not affect extrachromosomal homologous recombination. Farzaneh et. al. had previously demonstrated (8) that poly(ADP-ribosylation) plays a role in random integration of transfected circular DNA molecules into the genomes of several mammalian cell lines. We have extended those studies by showing that 3-MB exerts similar effects on stable transformation of mouse Ltk$^-$ cells by either circular or linear DNA molecules.

The observation that poly(ADP-ribosylation) appears to play a role in random integration has led us to consider the significance of this finding. The effect of 3-MB on random integration can perhaps be best understood in terms of the important role that DNA ends play in integration. It has been shown that transfected linear molecules preferentially insert into a chromosome using the ends of the DNA molecule (reviewed in 4). It has also been reported (20) that, when fewer than 50 molecules of DNA are microinjected into the nuclei of cultured mammalian cells, linear molecules transfect mammalian cells with a greater efficiency than do circular molecules. It might therefore be inferred that a linear DNA molecule is the preferred topological form of DNA for stable integration and that a supercoiled molecule must first become nicked or linearized before it may integrate. Our finding that 3-MB inhibits transfection of mammalian cells by either linear or supercoiled molecules to similar extents suggests that the

Table 3. Effect of 3-MB on Intermolecular Extrachromosomal Homologous Recombination in Mouse Ltk$^-$ Cells

	DNA[a]	drug	# Colonies[b]	Colony Recovery (% no drug)
Expt. 1	pAL2 + pTK1−8	None	52	100
	" "	3-MB, 1mM	18	35
	pTK12	None	387	100
	"	3-MB, 1mM	136	35
Expt. 2	pAL2 + pTK1−8	None	29	100
	" "	3-MB, 2mM	3	10
	pTK12	None	222	100
	"	3-MB, 2mM	23	10

[a] 5 ug each of pAL2 and pTK1−8 or 50 ng of pTK12 (plus carrier) were used per flask. pTK1−8 was linearized by digestion with Xho I prior to transfection.
[b] Average of five flasks for each case.

pathways of integration of these two DNA forms overlap biochemically. This observation is consistent with the notion that integration of DNA proceeds by the obligate formation of free DNA ends (either *in vivo* or *in vitro*) and is an end-joining process. Our data imply that it is not the formation but rather the repair of DNA breaks that normally is rate-limiting for stable integration since 3-MB inhibits break repair. Others have shown (21,22) that transfected DNA suffers much damage, including double-strand breaks. Therefore, free DNA-strand ends probably become available rapidly after transfection with supercoiled substrates. Perhaps only when a small amount of circular DNA is used in transfection can the production of a broken molecule become rate-limiting.

It is possible that *induction* of poly(ADP-ribose)polymerase activity during transfection plays an important role in stable integration. It has been reported that poly(ADP-ribose)polymerase is tightly associated with the chromatin of higher eucaryotes and is preferentially localized to linker DNA (9,23), in domains containing significantly elevated amounts of single-strand DNA breaks compared to bulk chromatin (24). Poly(ADP-ribose)polymerase is known to be inducible by free ends of double-stranded DNA fragments (9,25) and has been referred to as a 'nuclear alarm signal' for the repair of DNA strand discontinuities (26). When transfected DNA enters the nucleus of a mammalian cell, the ends of the exogenous DNA molecules have the potential to activate poly(ADP-ribose)polymerase which in turn may induce a high level of break repair/end-joining. One consequence could be the fortuitous integration via end-joining of the transfected molecule into the genome at the site of a genomic break, perhaps in proximity to the location of the polymerase.

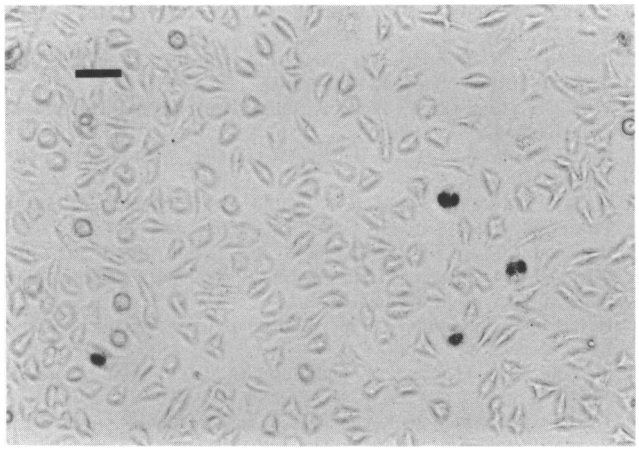

Figure 5. Autoradiogram of cells assayed for transient expression of HSV-1 tk. Cells were transfected with pTK12 and then assayed for uptake of ^{3}H-thymidine, as described in Materials and Methods. A representative picture of an autoradiogram is shown. Cells exibiting heavily-labelled nuclei were counted as positive. Radiolabelled mitotic figures, two of which are shown in this autoradiogram, were counted as one positive transformant. The field shown thus contains four positives. Bar = 50 microns.

Alternatively, it has been reported that activation of poly(ADP-ribose)polymerase can result in the poly(ADP-ribosylation) of histone H1 (see 9). This modification can result in the loss of H1 from polynucleosomes which brings about a localized relaxation of chromatin structure (9). Such relaxed domains may serve as preferential targets for strand breakage and in turn serve as sites for integration of transfected DNA via end-joining. Domains of relaxed chromatin might also be targets for additional rearrangements, as integration of DNA is often accompanied by rearrangements at the integration site (4).

Whatever the precise mechanism may be, our work and previous work (6) suggest that poly(ADP-ribose)polymerase plays an important role in random integration. It is noteworthy that poly(ADP-ribosylation) activity has not been found in bacteria (9) and its existence in fungi remains controversial (9,27). Bacteria and fungi are considerably less efficient than mammalian cells at random integration and DNA end-joining (4). On the other hand, bacteria and fungi are more proficient at promoting targeted recombination than are mammalian cells.

In contrast to the process of illegitmate recombination, we have shown that both intra- and intermolecular extrachromosomal homologous recombination are not sensitive to inhibition of poly(ADP-ribosylation). This suggests that, unlike illegitimate integration, homologous recombination is not equivalent to a DNA end-joining or ligation process. [It is precisely this concept that was exploited in a positive-negative selection scheme for enriching for targeted homologous recombination in mammalian cells (28).] Farzaneh et. al.(8) suggested that the process of concatenation that often occurs among transfected DNA molecules would be sensitive to inhibitors of poly(ADP-ribosylation). In light of the observation of Folger et. al. (20) that concatenation is primarily due to homologous recombination rather than end-to-end ligation, we suggest that this process would not be sensitive to inhibitors of poly(ADP-ribosylation).

Our findings suggest that homologous recombination mechanisms can occur in the absence of DNA break-repair. In fact, cells that are deficient in DNA repair often exhibit elevated levels of genetic rearrangements (reviewed in 29). As one example, cells isolated from patients with Bloom's Syndrome have been shown to have altered DNA ligase I activity, are deficient in DNA break repair, and exhibit elevated levels of sister chromatid exchanges (29). (It should be mentioned that it is not presently clear if sister chromatid exchange is a process distinct from general homologous recombination.) Additionally, it has been demonstrated many times that double-strand breaks promote

Table 4. Effect of 2 mM 3-MB on Transient TK Gene Expression

DNA[a]	2 mM 3-MB	# Fields Counted[b]	# Positive Cells	Positives/Field
pAL5	–	445	515	1.2
"	+	188	255	1.4
pTK12	–	116	733	6.3
"	+	92	438	4.8

[a] pAL5 was cleaved with Xho I and pTK12 with Cla I prior to transfection. 1.5 ug of pAL5 or 50 ng of pTK12 (plus carrier) were used per 35 mm dish.
[b] Fields were viewed under phase contrast at 100-fold magnification. There were approximately 500 cells per field.

extrachromosomal recombination in mammalian cells (reviewed in 1 and 3). The current view is that a double-strand break in a sequence can enhance recombination with an homologous sequence by initiating a double-strand gap repair mechanism as originally described for yeast (30).

We have observed previously (14) as well as in this study (Table 2) that cleavage of pAL5 with Xho I, at the site of the insertion mutation, increased recombination 5–10 fold, presumably by stimulating double-strand gap repair. Under these circumstances, the presence of 3-MB still did not affect the rate of homologous recombination. If the sealing of DNA breaks does not play an important role in homologous recombination while DNA breaks themselves may serve as initiation sites for homologous recombination, then it might be inferred that the increased lifetime of DNA breaks brought about by a reduced level of DNA break repair would increase recombination rates. That such enhancement was not observed by treatment with 3-MB in these studies might reflect an abundance of un-repaired DNA breaks in transfected molecules; i.e., break availability might not have been rate-limiting in the absence of 3-MB. Perhaps treatment with 3-MB can increase levels of homologous recombination within mammalian *chromosomes*, where DNA breaks are presumably not normally readily available. Experiments addressing this issue are currently in progress. It was reported that inhibition of poly(ADP-ribosylation) might increase levels of chromosomal homologous recombination in Drosophila (26). Additionally, it has been shown that treatment of Chinese hamster ovary cells with 3-MB brings about an elevated level of sister chromatid exchange (29,31).

It is formally possible that a secondary effect of 3-MB unrelated to inhibition of poly(ADP-ribosylation) might influence random integration. The fact that m-AA did not inhibit random integration (Tables 1 and 2) makes it more likely that the effects observed with 3-MB are due to inhibition of poly(ADP-ribose)polymerase rather than some other metabolic effect of 3-MB since the most likely metabolic fate of 3-MB would be deamidation to m-AA. Regardless, our studies have demonstrated that the pathways of random integration and homologous recombination are biochemically distinct. Previous work by others (32) has shown that extrachromosomal homologous recombination of transfected linear molecules peaks in early to mid-S phase in Rat-2 cells, whereas random integration of transfected linear molecules shows no cell-cycle dependence. These observations, like ours, indicated that the cellular mechanisms for random integration and homologous recombination do not completely overlap. Our studies have demonstrated the feasibility of selectively inhibiting random integration while leaving intra- and intermolecular homologous recombination mechanisms apparently unaffected. Such selective inhibition of random integration might be useful in approaches toward improving the efficiency of gene targeting in mammalian cells.

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