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Efficient Retroviral Infection of Mammalian Cells Is Blocked by Inhibition of Poly(ADP-Ribose) Polymerase Activity

JOOP A. GÄKEN,[1] MANOOCHEHR TAVASSOLI,[2] SHU-UIN GAN,[1] SADEQ VALLIAN,[1] IAN GIDDINGS,[1] DAVID C. DARLING,[1] JOANNA GALEA-LAURI,[1] MARK G. THOMAS,[1] HUSNA ABEDI,[1] VALÉRIE SCHREIBER,[3] JOSIANE MÉNISSIER-DE MURCIA,[3] MARY K. L. COLLINS,[4] SYDNEY SHALL,[2]* AND FARZIN FARZANEH[1]

Department of Molecular Medicine, The Rayne Institute, King's College School of Medicine & Dentistry, London SE5 9NU,[1] Cell and Molecular Biology Laboratory, University of Sussex, Brighton BN1 9QG,[2] and Institute of Cancer Research, London SW3 6JB,[4] United Kingdom, and Ecole Supérieure de Biotechnologie de Strasbourg, UPR 9003 du Centre National de la Recherche Scientifique, Unité de Cancérogenèse et Mutagenèse Moléculaire et Structurale, F-67400 Illkirch-Graffenstaden, France[3]

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Integration of proviral DNA into the host cell genome is a characteristic feature of the retroviral life cycle. This process involves coordinate DNA strand break formation and rejoining reactions. The full details of the integration process are not yet fully understood. However, the endonuclease and DNA strand-joining activities of the virus-encoded integrase protein (IN) are thought to act in concert with other, as-yet-unidentified, endogenous nuclear components which are involved in the DNA repair process. The nuclear enzyme poly(ADP-ribose) polymerase (PARP), which is dependent on DNA strand breaks for its activity, is involved in the efficient repair of DNA strand breaks, and maintenance of genomic integrity, in nucleated eukaryotic cells. In the present work, we examine the possible involvement of PARP in the retroviral life cycle and demonstrate that inhibition of PARP activity, by any one of three independent mechanisms, blocks the infection of mammalian cells by recombinant retroviral vectors. This requirement for PARP activity appears to be restricted to processes involved in the integration of provirus into the host cell DNA. PARP inhibition does not affect viral entry into the host cell, reverse transcription of the viral RNA genome, postintegration synthesis of viral gene products, synthesis of the viral RNA genome, or the generation of infective virions. Therefore, efficient retroviral infection of mammalian cells is blocked by inhibition of PARP activity.

Early events in retroviral infection of mammalian cells involve interaction between the viral envelope and specific host cell receptors, entry and uncoating of the enveloped virion, reverse transcription of the viral RNA genome into double-stranded proviral DNA, and the integration of a proportion of the proviral DNA molecules into the host cell chromosomal DNA (18, 55). Integration of provirus into the host cell genome involves the coordinated formation and rejoining of DNA strand breaks in the target sequence (see references 16 and 49 for reviews). This process is dependent on the virus-encoded protein IN, which has both endonuclease and DNA strand-joining activities (10). IN introduces a staggered break in the target DNA and joins the 3′ ends of the provirus DNA (from which IN has removed 2 bases) to the 5′ staggered ends of the target sequence (40). Integration is then completed by repair of the gapped intermediates. This step generates short direct repeats that flank the integrated provirus. The filling in and ligation of these gaps in the target DNA are dependent on as-yet-unidentified host cell nuclear enzymes. Therefore, although IN is both necessary and sufficient for provirus integration in model systems (7, 26), in the absence of the cell-encoded factors, this process is relatively inefficient (16, 26) and reversible (12, 57). Therefore, the efficient infection of mammalian cells requires host cell nuclear factors which are involved in DNA repair and strand ligation (9, 12).

The nuclear enzyme poly(ADP-ribose) polymerase (PARP) (EC 2.4.2.30) is a zinc finger nuclear protein which can bind both single- and double-stranded DNA breaks (17, 24). This enzyme, which is dependent on the presence of DNA strand breaks for its activity (4), participates in a range of cellular processes which involve DNA strand break formation and rejoining (see references 2, 11, 30, and 47 for reviews).

PARP is a ubiquitous enzyme in nucleated eukaryotic cells (45) and catalyzes the attachment of the ADP-ribose moiety of its specific substrate, NAD, to suitable protein acceptors, primarily the enzyme itself (1). PARP also catalyzes further condensation of additional ADP-ribose residues to generate linear and branched ADP-ribose homopolymers. Structural analysis of the 113-kDa enzyme has identified the presence of a DNA binding domain (DBD) with two zinc fingers, an automodification domain, and an NAD binding region (Fig. 1A) (25). PARP activity is stimulated by a variety of DNA-damaging agents, including ionizing radiation (50), monofunctional alkylating agents (13), and oxygen radicals (8, 21, 35). Using a cell-free DNA repair system, Satoh and Lindahl (41) have provided direct evidence for the involvement of this enzyme in DNA repair, whereby binding of PARP to DNA strand breaks, through its N-terminal DBD, results in the stimulation of its DNA-dependent catalytic activity and auto-ADP-ribosylation of PARP. The automodified enzyme then releases DNA, providing access for other components of the DNA repair system (41). Inhibition of PARP activity prevents its automodification and thus impedes the release of PARP from DNA, restricting the access of DNA repair enzymes to the breaks and blocking efficient ligation of DNA strand breaks. The likely consequence of PARP activity appears to be prevention of accidental homologous recombination promoted by the presence of free DNA strand breaks (42). This is consistent with the increased detection of homologous recombination (58), sister

* Corresponding author.

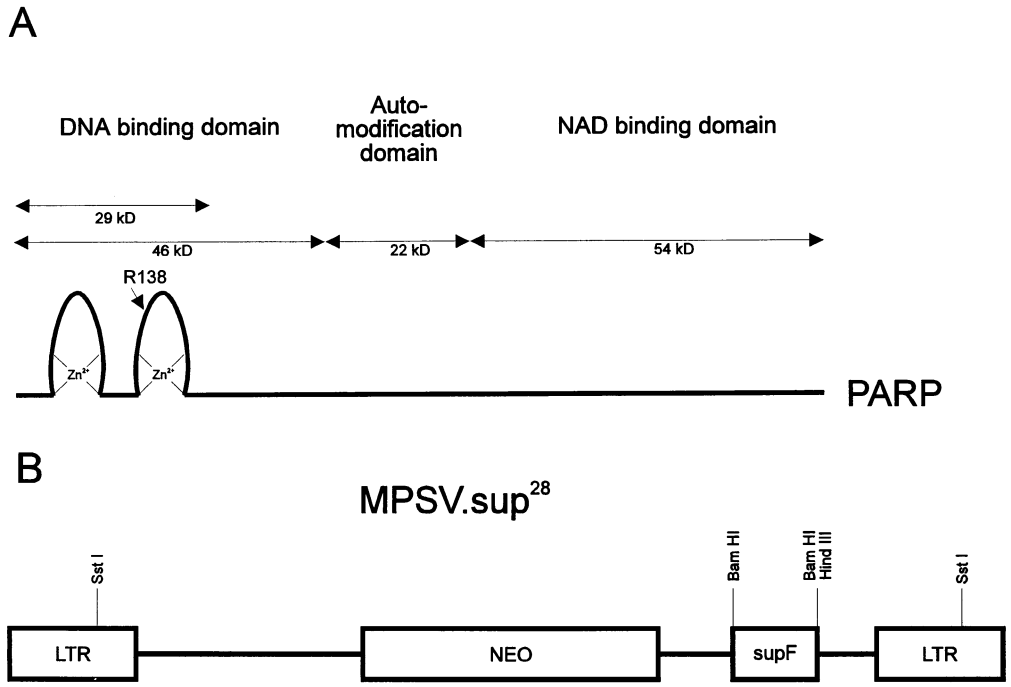

FIG. 1. PARP. (A) Schematic representation of PARP depicting its functional domains, including the presence of a DBD containing two zinc fingers, the automodification region, and the NAD binding domain (11, 25). The arrow indicates the position of arginine 138 in the second zinc finger. (B) Schematic representation of the retroviral vector MPSV.sup[28].

chromatid exchange (31, 37), and induced DNA amplification (5, 6) as a result of PARP inhibition. Interestingly, however, PARP-deficient mouse embryos develop apparently normally, with the only reported phenotype in the absence of environmental stress being increased spontaneous development of skin disease in adult mice due to keratinocyte hyperplasia, as well as increased obesity in the aging PARP-deficient female mice (59). Furthermore, the recovery of thymocyte progenitors after whole-body gamma irradiation was found to be delayed in the PARP-deficient mice and their fibroblasts had a slower rate of proliferation in vitro. However, these PARP-negative cells were as efficient as the PARP-positive cells in unscheduled DNA synthesis after alkylating agent- or UV-induced DNA damage, although the efficiency of DNA strand break ligation in these cells was not examined (59). These observations are consistent with in vitro DNA repair studies which show that mutations in the NAD binding or catalytic domains of recombinant PARP block efficient DNA repair whereas mutant PARP with a deletion in the DBD (functionally analogous to the absence of PARP in the knockout mice) does not inhibit DNA repair (51).

Consistent with these in vivo and in vitro observations, PARP activity is not required for recovery from low levels of DNA damage but is required for efficient recovery from DNA damage at levels above a threshold, which depends on cell type and the nature and dose of the damaging agent. Such a role is also consistent with the recent demonstration of PARP as a substrate for the cysteine proteases which are activated by the induction of apoptosis (29, 36, 54). The proteolytic cleavage of PARP and separation of its DBD from its catalytic domain would result in PARP inactivation and increased sensitivity of the cell to a lower level of DNA damage and/or reduced ability to recover from otherwise-tolerable levels of environmental insult.

We have previously shown that integration of transfected DNA into the mammalian cell genome, a process involving nonhomologous DNA recombination, is blocked by competitive inhibitors of PARP activity (14, 20). This inhibition seems to be specific to the integration step and does not affect the uptake into cells, episomal maintenance, or replication of the transfected plasmid in suitable hosts. Furthermore, inhibition of PARP activity has no effect on the expression of the transduced genes either before or after integration into the host cell genome (20). Therefore, the possible involvement of PARP in the integration of provirus into the mammalian cell genome was investigated.

In this work, we examine provirus integration into the genome of host cells in which PARP activity has been inhibited by three independent procedures. Specifically, PARP activity was blocked by its competitive inhibitors, antisense-oligonucleotide-mediated inhibition of enzyme synthesis, or overexpression of transdominant negative mutants (27, 34, 43). Inhibition of PARP activity by each of these procedures impedes the successful infection of human epithelial cells (HeLa), mouse fibroblasts (NIH 3T3), or mouse lymphoblastoid cells (L1210) by recombinant retroviral vectors (pWeTAC or MPSV.sup[28]). The inhibition appears to be specific to provirus integration and does not affect viral entry, reverse transcription and synthesis of the proviral genome, postintegration expression of the viral genes, or the production of infective virions by the producer cell lines.

MATERIALS AND METHODS

Infection of NIH 3T3 and HeLa cells. Twenty-four hours prior to infection, confluent 90-mm-diameter plates of NIH 3T3 cells were trypsinized and replated at a density of 10^5 cells per 90-mm-diameter plate. At the time of infection, Polybrene (8 μg/ml) and 150 CFU of pWeTAC, encoding the *neo* gene, were added to the cells. Before addition, the viral supernatant was filtered through a 0.45-μm-pore-size filter. After 72 h, the cells were trypsinized and 1/10 aliquots were plated on 90-mm-diameter plates in the absence of the PARP inhibitors but in the presence of 1 mg of G418 per ml. The culture medium was changed after

a further 3 and 6 days. Colonies were counted 10 to 14 days after the addition of G418.

Control experiments to check for the possibility of selective killing of infected cells or of specific inhibition of the expression of the neomycin gene were carried out as follows. Previously infected and G418-selected NIH 3T3 cells (10^2) were mixed with 10^5 uninfected NIH 3T3 cells and then mock infected. The mixed cell population was then treated with the competitive inhibitors and selected for G418 resistance as described above. Infection of NIH 3T3 and HeLa cells with MPSV.sup[28] was carried out essentially as described above, at least in triplicate, except that a viral inoculum of 250 CFU (as titrated on NIH 3T3 cells) was used for the infection of approximately 10^5 NIH 3T3 or HeLa cells on 90-mm-diameter plates, in the presence of 8 μg of Polybrene per ml.

Dose dependence of the inhibition of recombinant retrovirus infection. NIH 3T3 cells at a density of 2.2×10^4 cells per 90-mm-diameter plate were exposed to the indicated concentrations of the inhibitors (see Fig. 2) for 24 h. Cells were then infected with the recombinant retrovirus, pWeTAC, in the presence of 8 μg of Polybrene per ml and the same concentration of each inhibitor. After a further 48 h, the medium was replaced with fresh medium containing 1 mg of G418 per ml but no inhibitor. Cultures were fed with fresh medium (plus 1 mg of G418 per ml) after 5 days. Five days later, cultures were fixed and stained and surviving colonies were counted.

Effect of PARP inhibitors on DNA synthesis. HeLa or NIH 3T3 cells were seeded at an initial density of 10^4 cells per cm^2 in 1 ml of complete culture medium in 24-well plates. Twenty-four hours later, 0.8 μCi of [^{3}H]thymidine (87 Ci/mmol) per ml was added to each culture together with the indicated concentration of one of the benzamide analogs. The analogs tested were 3-aminobenzamide (3 mM), 3-aminobenzoic acid (3 mM), 4-aminobenzamide (3 mM), 3-formylaminobenzamide (1 mM), 4-formylaminobenzamide (1 mM), 3-methoxybenzamide (2 mM), and 4-methoxybenzamide (2 mM). At the times indicated in the legend to Fig. 4, triplicate samples of cells were harvested, washed with phosphate-buffered saline, and precipitated with 10% (wt/vol) ice-cold trichloroacetic acid containing 2 mM tetrasodium PP_i. The precipitated material was collected by centrifugation at $12,000 \times g$ for 15 min. The pellets were washed with 70% (vol/vol) ethanol and finally air dried. Radioactivity in the precipitated material was determined by scintillation counting. Results are expressed as the percentage of radioactivity in the control samples (no inhibitors).

Analysis of provirus DNA integration. Routinely, 24 h prior to infection cells were treated with 0.8% dimethyl sulfoxide and 6 μg of Polybrene per ml. At the time of infection, viral MPSV.sup[28] supernatant (2×10^5 CFU per 90-mm-diameter plate) was added, in either the presence (+) or the absence (−) of 2 mM 3-methoxybenzamide (15 plates each). Cultures were then maintained in the continuous presence or absence of the inhibitor until harvested at various times. For Southern transfer analysis, total genomic DNA was isolated from three plates (approximately 10^7 cells for each condition) by a standard proteinase K digestion and phenol-chloroform extraction. Following ethanol precipitation and careful spooling of the high-molecular-weight genomic DNA, samples were digested with *Sst*I, which cuts in the two long terminal repeats, releasing a 4.9-kb proviral DNA fragment containing the *neo* gene. A 10-μg amount of DNA was loaded onto a 0.8% agarose gel. After electrophoresis, DNA was transferred onto GeneScreen Plus membrane (Du Pont, Stevenage, United Kingdom) and hybridized to either an oligonucleotide-labelled 1.6-kb *Xba*I-*Hin*dIII fragment of the plasmid pM5neo, which contains the *neo* gene plus 400 bp of myeloproliferative sarcoma virus (MPSV) sequences; a 762-*Pvu*II *neo* cDNA fragment; or a 1.1-kb mouse β-actin cDNA probe. After hybridization, the blots were washed three times for 5 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then twice for 30 min each time in 0.2× SSC plus 0.1% sodium dodecyl sulfate (SDS) at 65°C.

Analysis of the effect of PARP inhibitors on virus expression and titer. PA317 packaging cells (32) producing the retroviral vector MPSV.sup[28] were plated at 5×10^5 cells per 90-mm-diameter plate in Dulbecco's modified Eagle medium containing 10% newborn calf serum. Approximately 24 h later the medium was removed and fresh medium containing the indicated (see above) concentration of each of the PARP inhibitors or analogs was added. Three days later, the titer of the virus shed into the culture supernatant was determined by infection of NIH 3T3 cells (10^4 cells per well of a 24-well plate) with serial dilutions of the medium from each of the inhibitor-treated cultures. Two days later, 1 mg of G418 per ml was added to each well, and after a further 10 days the virus titer was determined by fixation and Giemsa staining of Neo^r NIH 3T3 colonies. For virus expression studies, RNA was extracted from the producer cells by using RNAzol B according to the recommendations of the manufacturer (CINNA/BIOTECX). A 10-μg amount of total RNA was fractionated on a 1% agarose–formaldehyde gel, transferred to GeneScreen membrane, and hybridized with the *neo* cDNA described above. After autoradiography the filter was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase as a control for loading and transfer.

Detection of reverse-transcribed viral DNA. NIH 3T3 cells were infected with MPSV.sup[28] vector as described above, in either the presence or the absence of 2 mM 3-methoxybenzamide. Cells were harvested (10^7 for each condition), and the low-molecular-weight DNA was extracted by the procedure described by Hirt (22). DNA was treated with proteinase K at 400 μg/ml (overnight at 37°C), extracted with phenol-chloroform, and ethanol precipitated. Each DNA sample (from 10^7 cells) was redissolved in 40 μl of Tris-EDTA (TE) buffer. A 10-μl volume of each sample was finally digested with RNase A (20 μg/ml, 1 h), and the DNA was size fractionated on a 0.8% agarose gel. After transfer of the DNA to a GeneScreen Plus membrane, the blot was hybridized to the 762-bp *Pvu*II fragment of the *neo* gene.

Inhibition of PARP synthesis by antisense oligonucleotides. The Genetics Computer Group program FOLDRNA (60) was used to predict the secondary structure of minimal free energy for mouse PARP mRNA (23). Several oligonucleotides directed against the predicted regions with a low degree of base pairing were examined for inhibition of enzyme activity in L1210 cells. In this study, the antisense oligonucleotide used is directed against one such region of low base pairing spanning the translation initiation site. The selected oligonucleotides were as follows: antisense, 5′ **GCCTCC**gccatc**CTTCTC** (positions 95 to 78); sense, 5′ **GAGAAG**gatggc**GGAGGC** (positions 78 to 95); and scrambled, **TCCGCT**cgctca**CTCCTC**. All oligonucleotides used were partially sulfurized to increase their stability (52). The 6 nucleotides at the 5′ and 3′ ends were sulfurized (boldface), while the central 6 nucleotides (lowercase) were oxidized. Oligonucleotides were added to L1210 cell cultures (initial density, 10^5 cells per ml) at a concentration of 50 μM, and cultures were replenished every 24 h by addition of 2 volumes of fresh medium containing the appropriate oligonucleotides at the same concentration. After 72 h of oligonucleotide treatment, 2×10^5 L1210 cells were infected with the MPSV.sup[28] vector (10^5 CFU) as described above and maintained in medium containing the indicated oligonucleotides for a further 48 h. Cells were then placed in semisolid medium (0.3% agar) in the presence or absence of 1 mg of G418 per ml as the selectable marker. The G418-resistant colonies were counted 2 weeks later. At the time of infection, PARP activity was determined by measuring the level of [^{3}H]NAD^+ incorporation into acid-insoluble material as previously described (15) by using nuclei isolated according to the method of Greenberg and Ziff (19). PARP activity was also determined by activity blot studies as described by Scovassi et al. (46) and Simonin et al. (48). Briefly, nuclear proteins were extracted from the L1210 cells. These proteins were size fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The filters were then incubated in a PARP reaction mixture containing [^{32}P]NAD^+ in order to enable the auto-ADP-ribosylation of the enzyme. The intensity of PARP automodification, detected as a radioactive band at 113 kDa, is a reflection of enzyme level and/or activity in the nuclear extracts. In order to confirm equal loading and transfer of the proteins in each lane, the level of an unrelated control protein, HSP73, was measured in a duplicate filter by immunodetection. The filter was preblocked in Tris-buffered-saline (TBS) (10 mM Tris and 100 mM NaCl, pH 7.5) containing 2% bovine serum albumin, washed in TBS containing 0.1% Tween 20 (TBS-Tween), and then incubated at room temperature for 1 h in TBS containing 1 μg of mouse anti-HSP73 antibody SPA-802 (Sigma) per ml. The membrane was finally washed extensively in TBS-Tween and incubated for a further hour in the wash buffer containing polyclonal rabbit anti-mouse immunoglobulin (1/1,000) conjugated to horseradish peroxidase (Dako). Antibody binding was detected by enhanced chemiluminescence as described by the manufacturer of the kit (Amersham).

Expression of transdominant PARP DBD. Cloning in the eukaryotic episomal expression vector pECV 23 (3) of the cDNA expressing either the 46-kDa or the mutated DBD has been described previously (34). The 29-kDa version of the PARP DBD was cloned as a *Pst*I insert in the *Xho*I site of pECV 23 by using *Pst*I-*Xho*I linkers. The establishment of the cell lines has been described (43). Following hygromycin B selection, resistant clones were pooled (HpECV) or isolated individually (HR138I, H29-10, H29-11, H46-1, and H46-5). The selected clones show high-level PARP DBD expression as assayed by Western blot (immunoblot) analysis as described elsewhere (43). These cell lines were infected with an inoculum of MPSV.sup[28] retroviral vector (containing approximately 250 CFU), as described above, and the infected cells were selected in 1 mg of G418 per ml. The number of G418-resistant colonies was determined 2 weeks later.

RESULTS

Competitive inhibitors of PARP activity block retrovirus infection. The recombinant retroviral vector pWeTAC contains the neomycin phosphotransferase gene (*neo*), the expression of which confers resistance to the neomycin analog G418. Successful infection of NIH 3T3 cells by this vector was assayed by selecting the infected cells with 1 mg of G418 per ml. This long-term selection requires the stable integration and expression of the *neo* gene. The presence of a competitive inhibitor of PARP activity, 3-aminobenzamide, 3-formylaminobenzamide, or 3-methoxybenzamide (38), significantly inhibited the formation of Neo^r colonies (Fig. 2A). By contrast, the noninhibitory analogs of the inhibitors (4-aminobenzamide, 3-aminobenzoic acid, 4-formylaminobenzamide, and 4-methoxybenzamide) had little or no inhibitory effect on the formation of Neo^r colonies (Fig. 2A). In these experiments, PARP inhibitors were applied for only the first 3 days of infection. After this period, the

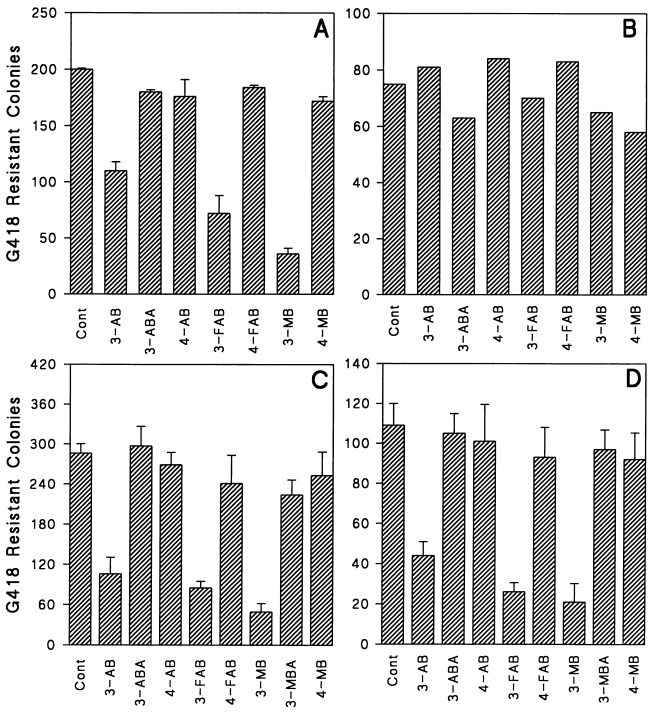

FIG. 2. Inhibition of infection by recombinant retroviral vectors due to the inhibition of PARP activity, as assessed by the formation of G418-resistant colonies. NIH 3T3 (A, B, and C) or HeLa (D) cells were infected with the same titer of pWeTAC (A and B) or MPSV.sup[28] (C and D) in the absence (Cont) or presence of the PARP inhibitors 3 mM 3-aminobenzamide (3-AB), 1 mM 3-formylaminobenzamide (3-FAB), and 2 mM 3-methoxybenzamide (3-MB) or their noninhibitory analogs 3 mM 3-aminobenzoic acid (3-ABA), 3 mM 4-aminobenzamide (4-AB), 1 mM 4-formylaminobenzamide (4-FAB), 2 mM 3-methoxybenzoic acid (3-MBA) and 2 mM 4-methoxybenzamide (4-MB). The medium was replaced after 3 days with fresh medium containing G418 at 1 mg/ml. G418-resistant colonies were counted after a further 10 to 14 days following fixation and staining. For the data shown in panel B, the protocol was the same as that for panel A except that previously infected and selected NIH 3T3 cells were mixed with uninfected NIH 3T3 cells, mock infected, and then reselected for G418 resistance. Results are the means and standard deviations (error bars) for three experiments.

medium was changed and G418 was added to allow selection of Neo^r cells. Therefore, PARP inhibition and G418 selection were temporally separated to avoid the possible cytotoxicity due to long-term exposure of cells to combinations of G418 and enzyme inhibitors. Furthermore, the inhibition of colony formation by the inhibitors was not due either to nonspecific cytotoxicity or to inhibition of expression of neomycin resistance. This was demonstrated by the absence of an effect on the formation of Neo^r colonies by cells which had been previously infected and selected for Neo^r (in the absence of the enzyme inhibitors) and then mixed with uninfected NIH 3T3 cells, mock infected in the presence of the enzyme inhibitors, and once again selected for Neo^r (Fig. 2B). It can therefore be concluded that PARP inhibitors do not block the expression of the virus-encoded *neo* gene and do not selectively kill the infected cells or exhibit nonspecific cytotoxicity at the concentrations used.

Inhibition of PARP activity also blocks the infection of NIH 3T3 or HeLa cells by another recombinant vector, MPSV.sup[28] (28, 53). This vector, which is derived from the murine MPSV, also carries the *neo* gene (Fig. 1B). Inhibition of viral infection was assayed both by counting of Neo^r colonies and by Southern hybridization analysis of high-molecular-weight DNA. The results show that the number of Neo^r colonies obtained after infection of either NIH 3T3 or HeLa cells is markedly reduced by the addition of PARP inhibitors but, again, not by the noninhibitory analogs (Fig. 2C and D). The observed inhibition of retroviral infection by PARP inhibitors is dose dependent, and the rank order of their effectiveness in blocking the enzyme activity correlates with their potency in inhibition of retroviral infection (Fig. 2 and 3).

Inhibition of infection is not due to the inhibition of host cell DNA synthesis. Transduction of cells by retroviruses is most efficient when the host cells are cycling during infection (56) because of a requirement for host cell DNA synthesis (33) and access to the nucleus during the nuclear membrane breakdown in mitosis (39). In order to ascertain whether the observed inhibition of retrovirus infection was possibly due to the nonspecific inhibition of cell cycling, the effect of the inhibitors on host cell DNA synthesis was examined. Analysis of thymidine incorporation in the presence of PARP inhibitors or their noninhibitory analogs demonstrates the absence of an inhibitor-specific reduction in the level of DNA synthesis (Fig. 4). In HeLa cells none of the three inhibitors or their analogs, tested at concentrations equal to those used in the infection assays, could significantly inhibit DNA synthesis (Fig. 4A). A slight inhibition of DNA synthesis was observed to occur in NIH 3T3 cells (Fig. 4B), but this was not specific to PARP inhibitors. There was therefore no correlation between the effect of these compounds on DNA synthesis and their inhibition of retrovirus infection (Fig. 2). Therefore, the inhibition of retroviral infection by PARP inhibitors cannot be due to the inhibition of genomic DNA synthesis.

PARP inhibitors block integration of provirus into the host cell DNA. Analysis of the high-molecular-weight genomic DNA isolated from mixed populations of NIH 3T3 cells infected in either the presence or the absence of 2 mM 3-methoxybenzamide demonstrates that inhibition of PARP activity blocks integration of provirus into the host cell genome. This is shown by the presence of a 4.9-kb proviral DNA fragment, which is released by *Sst*I digestion, only in cells infected in the absence of 3-methoxybenzamide (Fig. 5A). The inhibition of proviral DNA integration is clearly visible at 24 and 48 h after infection. A number of other bands, generated by hybridization of the probe to the mouse endogenous retroviral sequences,

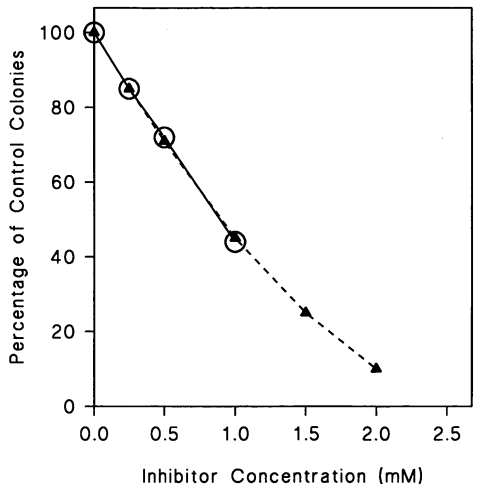

FIG. 3. Dose-dependent inhibition of recombinant retroviral infection by PARP inhibitors. NIH 3T3 cells were infected with pWeTAC in the presence of the indicated concentrations of either 3-methoxybenzamide (▲) or 3-formylaminobenzamide (○) as described in the legend to Fig. 2.

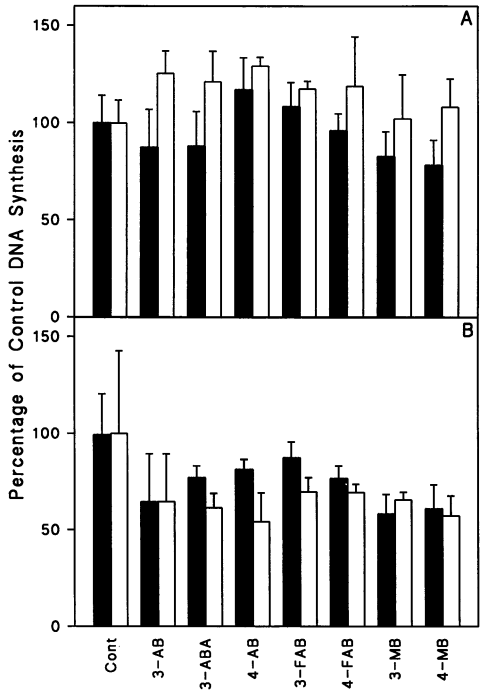

FIG. 4. Effect of PARP inhibitors on cellular DNA synthesis. HeLa (A) and NIH 3T3 (B) cells were cultured in the presence of [^{3}H]thymidine and either no inhibitor (Cont) or 3 mM 3-aminobenzamide (3-AB), 3 mM 3-aminobenzoic acid (3-ABA), or 3 mM 4-aminobenzamide (4-AB); 1 mM 3-formylaminobenzamide (3-FAB) or 1 mM 4-formylaminobenzamide (4-FAB); or 2 mM 3-methoxybenzamide (3-MB) or 2 mM 4-methoxybenzamide (4-MB). The amount of incorporated radioactive thymidine in triplicate samples was determined at 8 h (solid bars) and 24 h (open bars). Error bars indicate standard deviations.

are also visible and serve as internal controls for digestion, loading, transfer, and hybridization (Fig. 5A). This was further confirmed by rehybridization of this blot with a 1.1-kb murine β-actin-specific cDNA (Fig. 5B). In a repeat study, using a *neo*-specific probe which identifies only the 4.9-kb MPSV.sup[28] band and not the endogenous provirus sequences, the presence of integrated provirus in the control cells and its reduced level in the PARP-inhibited cultures are evident as early as 6 h postinfection (Fig. 5C). In order to confirm that the detected provirus sequences were the product of genomic integrations and not unintegrated provirus DNA, the same genomic DNA samples as used for Fig. 5C were digested with *Bam*HI, which cuts within the provirus (Fig. 1B), and hybridized with the *neo* probe. Discrete bands were not detectable in the *Bam*HI-digested DNA samples isolated from mixed populations of infected and selected NIH 3T3 cells, confirming the absence of unintegrated proviral DNA (data not shown). Therefore, the *neo*-hybridizing bands detected in the *Sst*I-digested samples were in fact products of provirus DNA integrations in the high-molecular-weight genomic DNA.

In contrast to their inhibition of provirus integration, PARP inhibitors had no detectable effect on virus-host cell receptor interaction, host cell entry, uncoating of the viral capsid, or reverse transcription and synthesis of the provirus DNA. This was demonstrated by the detection of similar levels of provirus DNA in the low-molecular-weight DNA fractions isolated by Hirt extraction (22) from the NIH 3T3 cells infected both in the presence and in the absence of 2 mM 3-methoxybenzamide (data not shown). The effect of the inhibitors on the viral reverse transcriptase was assayed by direct analysis of the enzyme activity present in cell extracts from pWeTAC producer cells incubated in the presence of PARP inhibitors. There was no detectable effect on the reverse transcriptase activity (data not shown). Therefore, inhibition of host cell infection was not due to inhibition of reverse transcriptase or reduced synthesis of reverse-transcribed provirus DNA.

PARP inhibitors do not block provirus transcription or generation of infective virions. In order to study the possible involvement of PARP in the postintegration transcription of the proviral genome, the effects of enzyme inhibitors on the expression of the virally encoded *neo* gene and production of virus by MPSV.sup[28] producer cells were examined. Producer cells were incubated in the absence or presence of either PARP inhibitors or their noninhibitory analogs for 3 days. Northern (RNA) blot analysis of total RNA extracted from these cells showed comparable levels of *neo* gene expression (Fig. 6). Furthermore, there was little or no difference in the titers of the virus produced by these cells (Table 1). In addition, the blocking of PARP activity had no effect on formation of Neo^r colonies by cells which had already been retrovirally infected and selected (Fig. 2B). Therefore, PARP activity was required neither for the postintegration expression of virally encoded genes nor for the transcription, packaging, and release of infective retrovirus.

In order to confirm that the results obtained with the com-

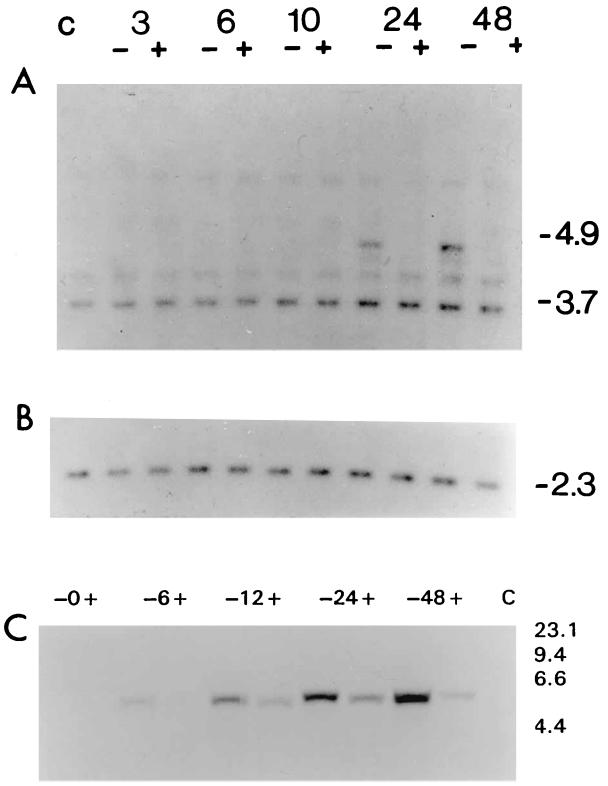

FIG. 5. Inhibition of MPSV.sup[28] provirus integration in NIH 3T3 cells. (A) Southern transfer analysis of genomic DNA isolated at the indicated times (hours) from a mixed population of NIH 3T3 cells infected with the MPSV.sup[28] retroviral vector in either the absence (−) or the presence (+) of 2 mM 3-methoxybenzamide. The isolated DNA was digested with *Sst*I, size fractionated on a 0.8% agarose gel, transferred to a GeneScreen Plus membrane, and hybridized to a 1.6-kb *Xba*I-*Hin*dIII fragment of the vector containing 1.2 kb of the *neo* cDNA and 400 bp of flanking (*gag* gene) virus sequences. Lane c, control uninfected cells. (B) The filter used for panel A was stripped and reprobed with a 1.1-kb mouse β-actin cDNA. (C) In a parallel study, *Sst*I-digested DNA samples were probed with a 762-bp *Pvu*II *neo* cDNA fragment. Lane c, control uninfected cells. Numbers on the right are molecular sizes (in kilobases).

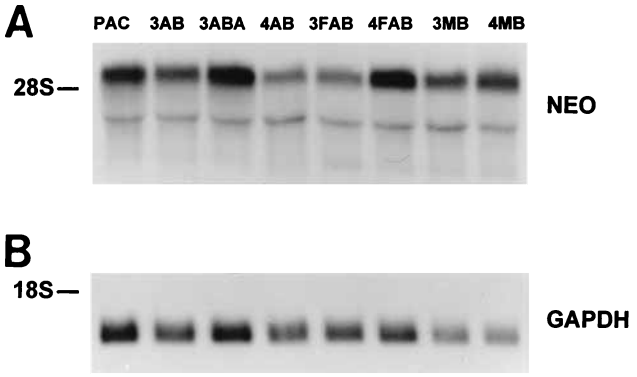

FIG. 6. Inhibition of PARP activity does not block viral gene expression. Shown is Northern blot analysis of total RNA isolated from PA317 cells producing MPSV.sup[28] incubated in the absence or presence of the indicated PARP inhibitors or their analogs. The concentrations of the compounds used and abbreviations are as given in the legend to Fig. 4. The filter was first hybridized to a *neo* probe (A) and then stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (B).

petitive inhibitors were not due to nonspecific effects of these compounds, two alternative means of inhibiting PARP activity were investigated.

Antisense-oligonucleotide-mediated inhibition of PARP synthesis blocks retrovirus infection. An important factor in reducing the level of specific gene products by antisense oligonucleotides directed against their mRNA sequences is the stability of the protein product. Because of the long intracellular half-life of PARP, the inhibition of its synthesis in cells with long cell division cycles is unlikely to result in a large decrease in its intracellular level. However, in mouse lymphoblastoid L1210 cells, which have a relatively short cell cycle time, approximately 12 h, the complete inhibition of PARP synthesis could result in the reduction of the enzyme level by one-half with every population doubling. L1210 cells were therefore chosen for these studies and incubated for 72 h in the presence of an antisense oligonucleotide directed against the PARP mRNA translation initiation site. The level of active enzyme in the nuclear extract of these cells was measured by determining the enzyme activity either in isolated nuclei (Table 2) or by activity gel assays (Fig. 7) and was more than 10-fold lower than the level detected in untreated control cells. In cells treated with the corresponding sense oligonucleotide or a random oligonucleotide with the same content of each of the four nucleotides there was no decrease in the enzyme level. The cells treated with oligonucleotides were infected with the MPSV.sup[28] vector, and the number of infected colonies was determined by soft-agar cloning in the presence of 1 mg of G418 per ml. In the cells treated with antisense oligonucleotides there was a fourfold reduction in the level of G418-resistant colonies relative to the level with either the control cultures or those treated with either the sense or the scrambled oligonucleotides (Table 2). Therefore, antisense-oligonucleotide-mediated inhibition of PARP synthesis reduces the number of retrovirus-infected L1210 cells.

TABLE 1. Effect of PARP inhibitors on retrovirus production by MPSV.sup[28] producer cells

Inhibitor	Concn (mM)	Virus titer (10^5) (mean ± SD)
None (control)		1.6 ± 0.9
3-Aminobenzamide	3	1.4 ± 0.8
3-Aminobenzoic acid	3	0.9 ± 1.4
4-Aminobenzamide	3	1.7 ± 1.9
3-Formylaminobenzamide	1	0.9 ± 1.7
4-Formylaminobenzamide	1	1.1 ± 1.2
3-Methoxybenzamide	2	1.2 ± 1.2
4-Methoxybenzamide	2	1.1 ± 0.9

TABLE 2. Effect of antisense oligonucleotide treatment on PARP activity and retroviral infection of L1210 cells

Oligonucleotide	PARP activity		Retroviral infection	
	pmol/min/10^6 cells (mean ± SD)	% Inhibition	No. of G418^r colonies (mean ± SD)	% Inhibition
None	848 ± 44	0	120 ± 37	0
Antisense	220 ± 32	74	29 ± 19	76
Sense	851 ± 29	0	106 ± 24	12
Scrambled	790 ± 39	7	122 ± 31	0

Inhibition of PARP by expression of its truncated DBD blocks retrovirus infection. Previous studies have demonstrated that in cells transfected with expression vectors encoding the PARP DBD of 46 kDa there is transdominant inhibition of endogenous enzyme activity due to an apparent competition for DNA strand breaks which are necessary for the activation of the enzyme (27, 34, 43). We have shown that overproduction of the DBD restricted to 29 kDa results also in a dominant negative inhibition of PARP (unpublished results). Both the 29- and 46-kDa fragments contain the nuclear localization domain but lack the NAD binding and automodification regions (44). Mutations affecting the zinc finger in the DBD abolish the DNA binding property of these fragments and therefore their transdominant inhibition of PARP activity (34). HeLa cells stably transfected with either the vector alone or vectors expressing different DBD constructs were infected with the MPSV.sup[28] vector (Fig. 8). There was clear inhibition of infection in cells expressing either the 29- or 46-kDa DBD. In contrast, in cells transfected with the control vector

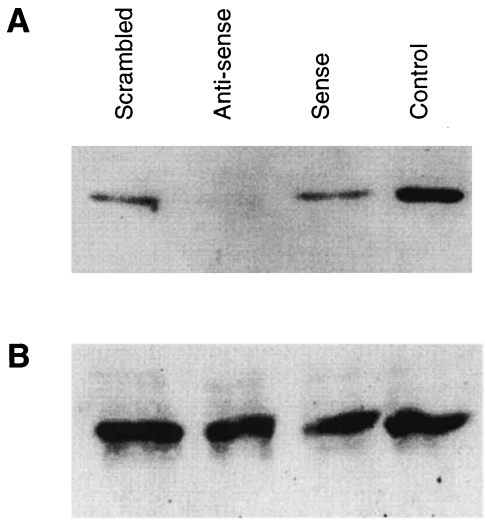

FIG. 7. Antisense oligonucleotide treatment inhibits PARP expression. L1210 cells were incubated in the presence of the indicated oligonucleotides (50 μM) for 72 h. Every 24 h, the cells were diluted threefold and fresh oligonucleotides were added. (A) The level of PARP in nuclear extracts was determined by activity blot analysis. (B) Western blot analysis of a duplicate filter probed with anti-HSP73 antibody.

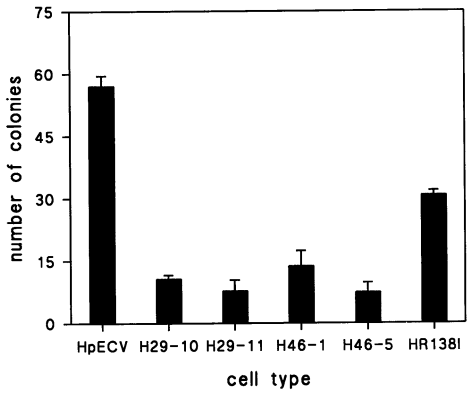

FIG. 8. Expression of transdominant PARP DBD inhibits retroviral infection in HeLa cells. HeLa cells were infected with the MPSV.sup[28] retroviral vector and selected in the presence of 1 mg of G418 per ml for 2 weeks, and resistant colonies were counted. The HeLa cell lines were previously transfected and selected for the constitutive expression of either the vector (HpECV), the 29- or 46-kDa PARP DBD (clones H29-10 and H29-11 and clones H46-1 and H46-5, respectively), or a mutant 46-kDa domain with an arginine-to-isoleucine substitution at amino acid position 138 (HR138I). Error bars indicate standard deviations.

alone there was no inhibition of infection. Mutation of the arginine at position 138 to isoleucine, which reduces the DNA binding activity (34), resulted in a reduced inhibitory effect, decreasing the number of retrovirus-infected cells by only 50%. This result is consistent with those previously described (43) indicating that in the HR138I line, PARP is partially inhibited although the R138I mutant has no affinity for DNA in vitro. Therefore, the dominant negative effect may be due to mechanisms other than competition between DBD and endogenous PARP for free DNA strand breaks.

DISCUSSION

The data presented here demonstrate that competitive inhibitors of PARP block the efficient infection of mammalian cells by retroviral vectors. This inhibition appears to be mediated through a process, or processes, which intervenes between proviral DNA synthesis and stable integration of provirus into the host cell genome. The enzyme inhibitors do not seem to affect the necessary interactions between the virus envelope and host cell receptors, virus entry into the cell, uncoating of the viral RNA genome, or its reverse transcription and synthesis of the proviral DNA. Furthermore, the inhibitors do not prevent genomic DNA synthesis in HeLa cells. The slight inhibition of DNA synthesis in NIH 3T3 cells was detectable in the presence of both PARP inhibitors and the noninhibitory analogs. Therefore, there is no correlation between the effect of the inhibitors on DNA synthesis and their inhibition of retroviral infection. The inhibitors appear not to affect the postintegration expression of the virally encoded genes, transcription of the viral genome, or the packaging and release of infective virions by the producer cells.

Analysis of PARP enzyme activity in cell-free systems and direct measurement of the enzyme product, poly(ADP-ribose), in intact cells have demonstrated the dose-dependent inhibition of this reaction by the competitive inhibitor 3-aminobenzamide and a number of its related derivatives. However, the acid analogs or 4-substituted derivatives of these compounds are much less inhibitory (the K_i values for the latter compounds are about 100-fold higher than the K_i values for the inhibitors). This dose dependency and the rank order of inhibition are reflected in the inhibition of recombinant retroviral infection, which suggests that an ADP-ribosylation reaction is required at some stage of the viral infection. Rankin et al. (38) have shown that the competitive inhibitors of PARP, in the concentration range used in this study, allow the selective inhibition of this enzyme in intact C3H10T1/2 cells, with no or little effect on the activity of the mono(ADP-ribosyl) transferase or NAD^+ glycohydrolases. Therefore, the studies with PARP competitive inhibitors suggest its involvement in the efficient infection of mammalian cells by retroviral vectors. This suggestion is strengthened by the inhibition of retroviral infection in mouse L1210 cells, in which antisense-oligonucleotide treatment has inhibited PARP expression as determined both by direct enzyme assays with isolated nuclei and in activity blots measuring the level of active enzyme present in nuclear extracts. In this case, the enzyme itself is diminished, rather than being inhibited, suggesting that the enzyme is actively needed for efficient retroviral infection. Furthermore, inhibition of enzyme activity in HeLa cells expressing the PARP DBD inhibits retroviral infection. However, expression of a mutated DBD fragment (HR138I) which has lost its ability to bind DNA shows reduced inhibition of retroviral infection, thus demonstrating the specificity of the requirement for PARP activity in the efficient retroviral infection of mammalian cells. Previous studies have shown that expression of the PARP DBD has no effect on either the normal rate of DNA synthesis or the proliferation of these transduced HeLa cells (43). Therefore, the inhibition of retroviral infection in these cells must be due to the inhibition of enzyme activity and not the result of possible alterations in the rate of DNA synthesis or cell proliferation.

Several studies with purified and recombinant IN protein have demonstrated the presence of both endonuclease and DNA strand-joining activities in this protein (7, 10, 26). These studies show that IN is both necessary and sufficient for the integration of retrovirus, or retrovirus-like DNA molecules, into target DNA. However, the efficiency of IN-mediated provirus integration is greatly enhanced by the presence of cellular components (16, 49). The IN protein itself, or other nuclear factors, may be the subject of regulation by such cellular factors. The increased efficiency of provirus integration in the presence of cellular components appears to be due to a need for nuclear factors involved in DNA strand repair and ligation (9). On the basis of the present data, the nuclear enzyme PARP appears to be a strong candidate for such a factor required for efficient retroviral infection of mammalian cells at one or more steps between reverse transcription and stable integration of the provirus into the host cell genome. This is consistent with the known physical properties of PARP, namely, its binding to DNA strand breaks followed by the activation of its catalytic property, resulting in its auto-ADP-ribosylation and release from DNA strand breaks (see reference 30). This is also consistent with the demonstrated biological involvement of PARP in a range of eukaryotic cellular processes which involve the formation and removal of DNA strand breaks.

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J.A.G. and M.T. contributed equally to this study.

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REFERENCES

1. **Althaus, F. R., L. Hofferer, H. E. Kleczkowska, M. Malanga, H. Naegeli, P. L. Panzeter, and C. A. Realini.** 1994. Histone shuttling by poly ADP-ribosylation. Mol. Cell. Biochem. **138:**53–59.
2. **Althaus, F. R., and C. Richter.** 1987. ADP-ribosylation of proteins. Enzymology and biological significance. Mol. Biol. Biochem. Biophys. **37:**1–237.
3. **Belt, P. B., H. Groeneveld, W. J. Teubel, P. van de Putte, and C. Backendorf.** 1989. Construction and properties of an Epstein-Barr-virus-derived cDNA expression vector for human cells. Gene **84:**407–417.
4. **Benjamin, R. C., and D. M. Gill.** 1980. Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks. J. Biol. Chem. **255:**10502–10508.
5. **Burkle, A., K. Grube, and J. H. Kupper.** 1992. Poly(ADP-ribosyl)ation: its role in inducible DNA amplification, and its correlation with the longevity of mammalian species. Exp. Clin. Immunogenet. **9:**230–240.
6. **Burkle, A., R. Heilbronn, and H. zur Hausen.** 1990. Potentiation of carcinogen-induced methotrexate resistance and dihydrofolate reductase gene amplification by inhibitors of poly(adenosine diphosphate-ribose) polymerase. Cancer Res. **50:**5756–5760.
7. **Bushman, F. D., T. Fujiwara, and R. Craigie.** 1990. Retroviral DNA integration directed by HIV integration protein *in vitro*. Science **249:**1555–1558.
8. **Cantoni, O., F. Cattabeni, V. Stocchi, R. E. Meyn, P. Cerutti, and D. Murray.** 1989. Hydrogen peroxide insult in cultured mammalian cells: relationships between DNA single-strand breakage, poly(ADP-ribose) metabolism and cell killing. Biochim. Biophys. Acta **1014:**1–7.
9. **Charneau, P., and F. Clavel.** 1991. A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract. J. Virol. **65:**2415–2421.
10. **Craigie, R., T. Fujiwara, and F. Bushman.** 1990. The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. Cell **62:**829–837.
11. **de Murcia, G., and J. Menissier de Murcia.** 1994. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem. Sci. **19:**172–176.
12. **Dotan, I., B. P. Scottoline, T. S. Heuer, and P. O. Brown.** 1995. Characterization of recombinant murine leukemia virus integrase. J. Virol. **69:**456–468.
13. **Durkacz, B. W., O. Omidiji, D. A. Gray, and S. Shall.** 1980. (ADP-ribose)n participates in DNA excision repair. Nature (London) **283:**593–596.
14. **Farzaneh, F., G. N. Panayotou, L. D. Bowler, B. D. Hardas, T. Broom, C. Walther, and S. Shall.** 1988. ADP-ribosylation is involved in the integration of foreign DNA into the mammalian cell genome. Nucleic Acids Res. **16:** 11319–11326.
15. **Farzaneh, F., R. Zalin, D. Brill, and S. Shall.** 1982. DNA strand breaks and ADP-ribosyl transferase activation during cell differentiation. Nature (London) **300:**362–366.
16. **Goff, S. P.** 1992. Genetics of retroviral integration. Annu. Rev. Genet. **26:** 527–544.
17. **Gradwohl, G., J. M. Menissier de Murcia, M. Molinete, F. Simonin, M. Koken, J. H. Hoeijmakers, and G. de Murcia.** 1990. The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA. Proc. Natl. Acad. Sci. USA **87:**2990–2994.
18. **Grandgenett, D. P., and S. R. Mumm.** 1990. Unraveling retrovirus integration. Cell **60:**3–4.
19. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311:**433–438.
20. **Hardas, B. D., M. Braz, and F. Farzaneh.** 1992. ADP-ribosylation is involved in the integration of exogenous DNA into the mammalian cell genome, but is not required for the episomal replication or expression of autonomously replicating plasmids, p. 113–117. *In* G. G. Poirier and P. Moreau (ed.), ADP-ribosylation reactions. Springer-Verlag, New York.
21. **Heller, B., Z. Q. Wang, E. F. Wagner, J. Radons, A. Burkle, K. Fehsel, V. Burkart, and H. Kolb.** 1995. Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. J. Biol. Chem. **270:**11176–11180.
22. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
23. **Huppi, K., K. Bhatia, D. Siwarski, D. Klinman, B. Cherney, and M. Smulson.** 1989. Sequence and organization of the mouse poly(ADP-ribose) polymerase gene. Nucleic Acids Res. **17:**3387–3401.
24. **Ikejima, M., S. Noguchi, R. Yamashita, T. Ogura, T. Sugimura, D. M. Gill, and M. Miwa.** 1990. The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation. Other structures recognize intact DNA. J. Biol. Chem. **265:**21907–21913.
25. **Kameshita, I., Z. Matsuda, T. Taniguchi, and Y. Shizuta.** 1984. Poly(ADP-ribose) synthetase. Separation and identification of three proteolytic fragments as the substrate-binding domain, the DNA-binding domain, and the automodification domain. J. Biol. Chem. **259:**4770–4776.
26. **Katz, R. A., G. Merkel, J. Kulkosky, J. Leis, and A. M. Skalka.** 1990. The avian retroviral IN protein is both necessary and sufficient for integrative recombination *in vitro*. Cell **63:**87–95.
27. **Kupper, J. H., M. Muller, M. K. Jacobson, J. Tatsumi-Miyajima, D. L. Coyle, E. L. Jacobson, and A. Burkle.** 1995. *trans*-dominant inhibition of poly(ADP-ribosyl)ation sensitizes cells against γ-irradiation and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine but does not limit DNA replication of a polyomavirus replicon. Mol. Cell. Biol. **15:**3154–3163.
28. **Laker, C., C. Stocking, U. Bergholz, N. Hess, J. F. De Lamarter, and W. Ostertag.** 1987. Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway. Proc. Natl. Acad. Sci. USA **84:**8458–8462.
29. **Lazebnik, Y. A., S. H. Kaufmann, S. DesNoyers, G. G. Poirier, and W. C. Earnshaw.** 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature (London) **371:**346–347.
30. **Lindahl, T., M. S. Satoh, G. G. Poirier, and A. Klungland.** 1995. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem. Sci. **20:**405–411.
31. **Lindahl-Kiessling, K., and S. Shall.** 1987. Nicotinamide deficiency and benzamide-induced sister chromatid exchanges. Carcinogenesis **8:**1185–1188.
32. **Miller, A. D., and C. Buttimore.** 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6:**2895–2902.
33. **Miller, D. G., M. A. Adam, and A. D. Miller.** 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. Cell. Biol. **10:**4239–4242.
34. **Molinete, M., W. Vermeulen, A. Burkle, J. Menissier-de Murcia, J. H. Kupper, J. H. Hoeijmakers, and G. de Murcia.** 1993. Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. EMBO J. **12:**2109–2117.
35. **Muehlematter, D., R. Larsson, and P. Cerutti.** 1988. Active oxygen induced DNA strand breakage and poly ADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells. Carcinogenesis **9:**239–245.
36. **Nicholson, D. W., A. Ambereen, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, N. A. Munday, S. M. Raju, M. E. Smulson, T. Yamin, V. L. Yu, and D. K. Miller.** 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature (London) **376:**37–43.
37. **Oikawa, A., H. Tohda, M. Kanai, M. Miwa, and T. Sugimura.** 1980. Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. Biochem. Biophys. Res. Commun. **97:**1311–1316.
38. **Rankin, P. W., E. L. Jacobson, R. C. Benjamin, J. Moss, and M. K. Jacobson.** 1989. Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. J. Biol. Chem. **264:**4312–4317.
39. **Roe, T., T. C. Reynolds, G. Yu, and P. O. Brown.** 1993. Integration of murine leukemia virus DNA depends on mitosis. EMBO J. **12:**2099–2108.
40. **Roth, M. J., P. L. Schwartzberg, and S. P. Goff.** 1989. Structure of the termini of DNA intermediates in the integration of retroviral DNA: dependence on IN function and terminal DNA sequence. Cell **58:**47–54.
41. **Satoh, M. S., and T. Lindahl.** 1992. Role of poly(ADP-ribose) formation in DNA repair. Nature (London) **356:**356–358.
42. **Satoh, M. S., G. G. Poirier, and T. Lindahl.** 1994. Dual function for poly(ADP-ribose) synthesis in response to DNA strand breakage. Biochemistry **33:**7099–7106.
43. **Schreiber, V., D. Hunting, C. Trucco, B. Gowans, D. Grunwald, G. de Murcia, and J. M. de Murcia.** 1995. A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage. Proc. Natl. Acad. Sci. USA **92:** 4753–4757.
44. **Schreiber, V., M. Molinete, H. Boeuf, G. de Murcia, and J. Menissier-de Murcia.** 1992. The human poly(ADP-ribose) polymerase nuclear localization signal is a bipartite element functionally separate from DNA binding and catalytic activity. EMBO J. **11:**3263–3269.
45. **Scovassi, A. I., R. Izzo, E. Franchi, and U. Bertazzoni.** 1986. Structural analysis of poly(ADP-ribose)polymerase in higher and lower eukaryotes. Eur. J. Biochem. **159:**77–84.
46. **Scovassi, A. I., M. Stefanini, and U. Bertazzoni.** 1984. Catalytic activities of human poly(ADP-ribose) polymerase from normal and mutagenized cells detected after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **259:**10973–10977.
47. **Shall, S.** 1988. ADP-ribosylation of proteins: a ubiquitous cellular control mechanism. Adv. Exp. Med. Biol. **231:**597–611.
48. **Simonin, F., J. Menissier-de Murcia, O. Poch, S. Muller, G. Gradwohl, M. Molinete, C. Penning, G. Keith, and G. de Murcia.** 1990. Expression and site-directed mutagenesis of the catalytic domain of human poly(ADP-ribose)polymerase in Escherichia coli. Lysine 893 is critical for activity. J. Biol. Chem. **265:**19249–19256.
49. **Skalka, A. M.** 1993. Retroviral DNA integration: lessons for transposon shuffling. Gene **135:**175–182.
50. **Skidmore, C. J., M. I. Davies, P. M. Goodwin, H. Halldorsson, P. J. Lewis, S. Shall, and A. A. Ziaée.** 1979. The involvement of poly(ADP-ribose) polymerase in the degradation of NAD caused by gamma-radiation and N-methyl-N-nitrosourea. Eur. J. Biochem. **101:**135–142.
51. **Smulson, M., N. Istock, R. Ding, and B. Cherney.** 1994. Deletion mutants of poly(ADP-ribose) polymerase support a model of cyclic association and

dissociation of enzyme from DNA ends during DNA repair. Biochemistry **33:**6186–6191.
52. **Stein, C. A., C. Subasinghe, K. Shinozuka, and J. S. Cohen.** 1988. Physicochemical properties of phosphorothioate oligodeoxynucleotides. Nucleic Acids Res. **16:**3209–3221.
53. **Stocking, C., U. Bergholz, J. Friel, K. Klingler, T. Wagener, C. Starke, T. Kitamura, A. Miyajima, and W. Ostertag.** 1993. Distinct classes of factor-independent mutants can be isolated after retroviral mutagenesis of a human myeloid stem cell line. Growth Factors **8:**197–209.
54. **Tewari, M., L. T. Quan, K. O'Rourke, S. DesNoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit.** 1995. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:**801–809.
55. **Varmus, H., and P. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
56. **Varmus, H. E., T. Padgett, S. Heasley, G. Simon, and J. M. Bishop.** 1977. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. Cell **11:**307–319.
57. **Vincent, K. A., V. Ellison, S. A. Chow, and P. O. Brown.** 1993. Characterization of human immunodeficiency virus type 1 integrase expressed in *Escherichia coli* and analysis of variants with amino-terminal mutations. J. Virol. **67:**425–437.
58. **Waldman, A. S., and B. C. Waldman.** 1991. Stimulation of intrachromosomal homologous recombination in mammalian cells by an inhibitor of poly(ADP-ribosylation). Nucleic Acids Res. **19:**5943–5947.
59. **Wang, Z. Q., B. Auer, L. Stingl, H. Berghammer, D. Haidacher, M. Schweiger, and E. F. Wagner.** 1995. Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. Genes Dev. **9:**509–520.
60. **Zuker, M.** 1989. Computer prediction of RNA structure. Methods Enzymol. **180:**262–288.