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DNA Double-Strand Break Repair Functions Defend against Parvovirus Infection

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We measured parvovirus replication and sensitivity to X-ray damage in nine CHO cell lines representing a variety of DNA repair deficiencies. We found that parvovirus replication efficiency increases with radiosensitivity. Parvovirus replication is disrupted at an early stage of infection in DNA repair-proficient cells, before conversion of the single-stranded viral DNA genome into the double-stranded replicative form. Thus, status of the DNA repair machinery inversely correlates with parvovirus replication and is proportional to the host's ability to repair X-ray-induced damage.

The majority of human cancers appear to arise from mutations caused by DNA damage (5). One significant source of mutations is error-prone repair of DNA double-strand breaks (DSB). DSB can result from normal oxidative metabolism, replication errors, and exposure to X-rays. Cells eliminate most of this damage through DNA repair pathways by homologous recombination or nonhomologous end-joining pathways. The latter are more heavily utilized in mammalian cells and more error prone. These repair pathways are essential for maintenance of the overall integrity of the DNA genome. Mammalian cells that are defective in repairing DSB are significantly more sensitive to X-rays than are their normal counterparts. A number of cell lines with defective DNA DSB repair and V(D)J recombination have been established from Chinese hamster ovary (CHO) cells (23). V(D)J recombination is necessary for normal B- and T-cell immunity, and these specialized forms of DNA rearrangements interface with the system for generic DNA break repair. Defects in this recombination pathway can result in severe combined immune deficiency and sensitivity to ionizing radiation (17).

Because parvoviruses have a linear DNA genome (3), we investigated what role DNA repair functions may play in parvovirus infection. We hypothesized that the linear viral genomes may be substrates for the DNA repair machinery. For this study, we assembled a variety of cell lines with DNA repair defects and characterized their infection with the parvovirus minute virus of mice (MVM). During the early stages of infection, the virus is translocated to the nucleus and the single-stranded DNA (ssDNA) viral genome is converted to the double-stranded (ds) replicative form prior to expression of viral genes. MVM is the parvovirus known to infect CHO cells most efficiently, although C. R. Astell (1) reported that a multiplicity of infection (MOI) of 1,200 PFU of MVM was needed to infect at least 90% of the cells.

We measured the X-ray sensitivity of nine CHO cell lines (Fig. 1A). These cell lines included mutants in several complementation groups and the cell lines from which they were directly derived. xrs5, xrs6 (X-ray repair cross-complementing group [XRCC] 5), V-3 (XRCC 7), and XR-1 (XRCC 4) cells have defects in DSB repair. The xrs5 cell line is deficient in functional Ku protein (11), also called human DNA helicase (21), and ssDNA-dependent ATPase (2, 22). Expression of the human p80 subunit of Ku (Hp80) in CHO XRCC 5 cells partially reverses the mutant phenotype (19, 20). However, the mutant phenotype is reported to be completely rescued by expression of the hamster p80 subunit (7). Ku is a DNA binding protein heterodimer with a high affinity for DNA ends, and it has been identified as the DNA-binding portion of a larger complex called DNA-dependent protein kinase (6, 12). The xrs5 revertant (xrs5-rev) cell line used in our studies was derived from xrs5 cells that spontaneously reverted to X-ray resistance. The EM9 cell line is defective in ssDNA break repair and shows no increase in X-ray sensitivity compared with the parental cell line, CHO-AA8. However, the UV light-sensitive cell line UV41 does show a slight increase in X-ray sensitivity compared with the parental cell line, CHO-AA8. The most X-ray-sensitive cell lines were XR-1, xrs5, and V-3. The CHO V-3, irs-20, and murine severe combined immunodeficiency cell lines define the XRCC 7 complementation group, which has been assigned the gene for the catalytic subunit of DNA-dependent protein kinase (23).

We infected these CHO cell lines with MVM at an MOI of 30 and assessed the proportion of infected cells by immunofluorescence assay for nonstructural protein 1 (NS1) expression at 24 h postinfection (hpi). NS1 is expressed early during parvovirus infection and is found largely in the nucleus. Less than 2% of CHO-K1 and xrs5-rev cells stained NS1 positive, while approximately 23% of xrs5 and V-3 cells were positive. Morphologically, the CHO-K1 and xrs5-rev cell lines remained virtually unchanged after infection with MVM; in contrast, xrs5 and V-3 cells exhibited a severe cytopathic effect within 72 hpi. When we infected xrs5-rev and xrs5 cells with MVM at an MOI of 5,000, the proportions of positive-staining cells increased to 39 and 89%, respectively.

We measured MVM replication efficiency in the nine CHO

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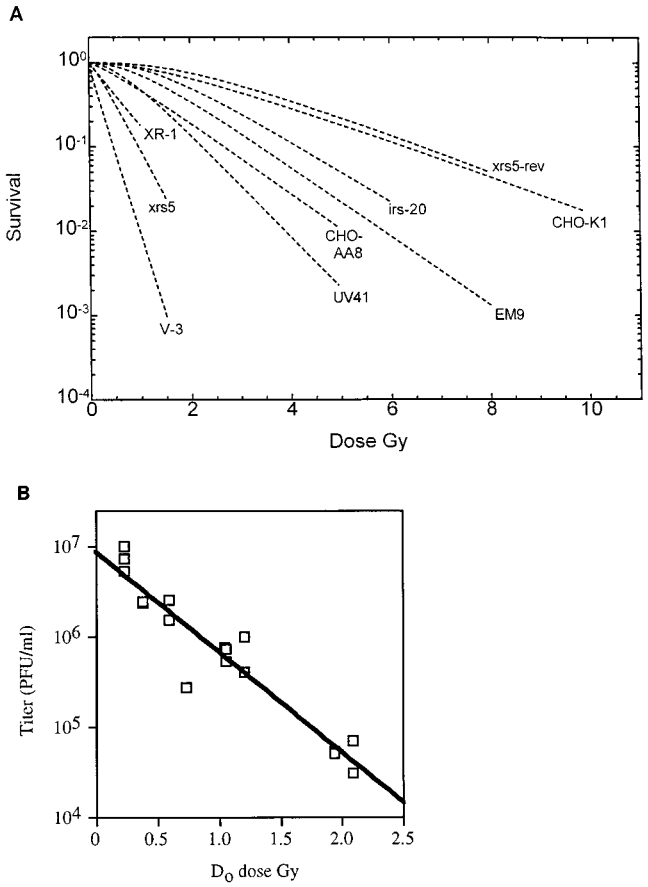

FIG. 1. Analysis of radiosensitivity and parvovirus replication. (A) Cell survival after exposure to X-rays as previously described (18). For clarity, the datum points have been omitted. (B) Correlation between radiosensitivity and parvovirus replication efficiency. The scatter plot was generated with the data in Table 1 (data for xrs5Hp80 are not included). The line represents a best-fit exponential equation ($y = 8506049.189 \times 10^{-0.012x}$).

cell lines (Fig. 1A). The virus titers and D_0 values of X-ray survival curves for each cell line were determined (Table 1). The D_0 value is the dose increment that reduces survival on the exponential portion of a log survival-dose curve to 37% of the previous value. XR-1, xrs5, and V-3 are the only cell lines that were considerably more radiosensitive than the parental cell lines.

The scatter plot in Fig. 1B shows the correlation between radiosensitivity and culture yields of MVM. In general, as radiosensitivity increases so does virus replication efficiency. The excision repair mutant UV41 was an exception and appeared to have reduced virus yields compared with the parental cell line, and we did not characterize UV41 cells further. It should be noted that xrs5-rev cells have the same radiosensitivity as CHO-K1 cells and similar MVM replication efficiency (data in Table 1). Thus, xrs5 cells that spontaneously revert to the radioresistant phenotype also revert to less suitability as a host for parvovirus replication.

We attempted to replicate the marker rescue of xrs5 cells by transforming them with Hp80 cDNA as previously reported (20), but despite expression of Hp80, no change in phenotype occurred. Stably transformed cells were selected for G418 resistance in McCoy's 5a modified medium with 10% serum and 20% xrs5-conditioned medium. We found that xrs5 cells had a lower plating efficiency than xrs5-rev cells in standard medium. Addition of conditioned medium enhanced xrs5 plating efficiency and increased the G418-resistant colony survival rate by a factor of 10 (>50 colonies 2.5×10^5 transfected cells^{-1} μg of plasmid DNA^{-1}). However, xrs5-rev cells still had a higher transformation efficiency than xrs5 cells in conditioned medium. We pooled more than 50 colonies of xrs5 cells transformed with Hp80 cDNA (xrs5Hp80) and tested for Ku expression. Functional Ku binding activity was detected via electrophoretic mobility shift assay. The Ku DNA binding activity was identified as human by using polyclonal rabbit antibodies raised against the human DNA helicase II p90 subunit (21) by a supershift of the electrophoretic mobility shift assay band (data not shown). This antibody did not cross-react with CHO Ku (data not shown). The Ku DNA binding activity of xrs5Hp80 was 58% of that of xrs5-rev cells as measured by electrophoretic mobility shift assay (data not shown). Similar levels of expression have been reported for XRCC 5 cells rescued with Hp80 cDNA (19, 20).

We infected xrs5Hp80 cells with MVM at an MOI of 30 and stained for NS1 via immunofluorescence assay. Approximately 24% of the cells stained positive, which is about the same percentage found in xrs5 cells. xrs5Hp80 also developed a severe cytopathic effect at 72 hpi. Furthermore, we tested for X-ray sensitivity and virus replication efficiency. We found no decrease in either radiosensitivity or MVM replication efficiency (Table 1). Our inability to reverse the phenotype with xrs5Hp80 could be due to the differences in the selection processes used to isolate the transformed xrs5 cells. However, recent evidence suggests that full reversion of the X-ray-sensitive phenotype is dependent on high levels of expression of the hamster p80 gene (7).

Next, we examined the step in parvovirus replication that is inhibited in repair-proficient CHO cells. We tested for differences in the relative number of MVM receptors expressed in CHO cells (Fig. 2) with purified radioactive MVM. No significant difference was seen between CHO-K1 and xrs5-rev cells; however, xrs5 cells had fewer receptors. Thus, receptor levels do not explain the lower infection efficiency found in CHO-K1 and xrs5-rev cells.

After attachment, the viral genome must translocate to the nucleus to replicate. We tested for viral localization with purified MVM with radiolabeled DNA (Table 2). All of the CHO cells tested had a significant amount of radioactive viral DNA in their nuclei by 8 hpi. Therefore, the virus attaches to the cell and translocates to the nucleus with closely similar efficiencies for the various cell lines tested.

TABLE 1. Analysis of radiation survival curves and parvovirus replication in CHO cell lines[a]

Cell line	Titer(s) (PFU)[b]	D_0 (Gy)[c]
CHO-K1	6.9×10^4, 3.0×10^4	2.09
xrs5-rev	5.2×10^4, 5.0×10^4	1.93
irs-20	1.0×10^6, 4.0×10^5	1.20
EM9	5.3×10^5, 7.3×10^5	1.05
CHO-AA8	7.6×10^5	1.04
UV41	2.7×10^5	0.72
XR-1	1.5×10^6, 2.5×10^6	0.58
xrs5	2.3×10^6, 2.4×10^6	0.37
xrs5Hp80	7.3×10^6	0.28
V-3	1.0×10^7, 5.3×10^6, 7.2×10^6	0.22

[a] Cells were infected with a common stock of the parvovirus MVM at an MOI of 15 and harvested at 72 hpi.

[b] PFU per 2.0×10^5 cells as determined by plaque assays in the NBE or A9 cell line. Each titer was determined from a separate sample.

[c] D_0 values were determined from the radiation survival curves in Fig. 1A.

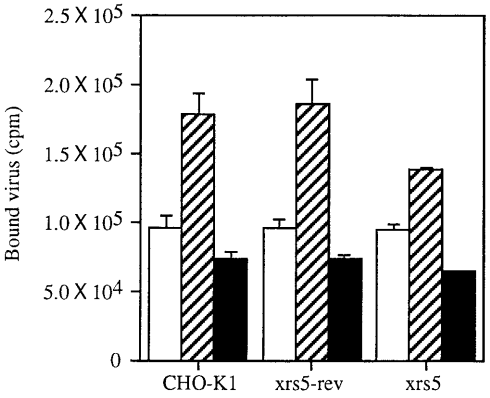

FIG. 2. Comparison of the relative number of parvovirus receptors on CHO cells. Cells were infected with purified ^{32}P-labeled MVM at 3.8×10^6 cpm (open histogram) or 7.6×10^6 cpm (striped) or with unlabeled MVM followed by 3.8×10^6 cpm of labeled MVM (filled). Cells were then collected, and radioactivity was counted. Error bars equal 2 standard deviations ($n = 3$).

A critical step in early parvovirus replication is the conversion of its ssDNA genome to the ds replicative form. Once the genome is converted to the replicative form, transcription can be initiated and viral proteins can be expressed. If early events in infection prior to or including replicative-form synthesis are restricted in repair-proficient cells, then transfection with a parvovirus infectious clone can bypass the restriction. We tested parvovirus infection efficiency in the xrs5 and xrs5-rev cell lines after transfection with pGLu883, the infectious plasmid clone of LuIII (4). LuIII was chosen because LuIII virions infect CHO cells very inefficiently and this would minimize the effects of secondary infections. Cultures were evaluated for transfection efficiency, virus yield, numbers of cells expressing NS1, and infected-cell yield by infectious-center assay at 40 h posttransfection. No differences were detected between the two cell lines that would reflect the 10-fold or greater differences seen with virus infection (Table 3). The similar virus and infected-cell yields indicate that once the viral ssDNA genome is converted to the ds replicative form, virus replication proceeds normally through DNA replication, packaging, and release of new infectious virus independently of the DNA repair status of the cells.

Collectively, our data suggest that disruption of a component of a common DSB repair pathway in CHO cells allows the viral ssDNA genome to be converted to the ds form more effectively. Autonomous parvovirus vectors have exhibited enhanced gene expression in cells pretreated with UV irradiation (16a). However, it is unknown if UV pretreatment has any effect on autonomous parvovirus replication.

TABLE 2. Nuclear localization of parvovirus in infected CHO cells[a]

Cell line	Activity (cpm)[b]
CHO-K1	2,609
xrs5-rev	2,277
irs-20	2,762
EM9	2,410
XR-1	1,790
xrs5	2,039

[a] Cells were infected with purified, ^{32}P-labeled MVM. Nuclei and cytoplasmic fractions were isolated, and radioactivity was counted at 8 hpi.

[b] Radioactivity in labeled virus coincided with viral DNA as determined by gel electrophoresis. Fifty percent of cell-bound radioactivity was chased to the nuclei.

TABLE 3. Virus replication in CHO cells transfected by the lipofectin method with an infectious parvovirus plasmid[a]

Cell line	No. of infectious centers[b]	% Staining[c]	Titer (PFU, 10^3)[d]
xrs5-rev	210	0.78	8.5
xrs5	240	1.9	5.0

[a] Cells were cotransfected with β-galactosidase expression plasmid pCMVβ (in which β-galactosidase expression is under the control of the cytomegalovirus promoter) and LuIII infectious parvovirus plasmid pGLu883 (4). Data were normalized to the proportions of cells expressing β-galactosidase. The transfection efficiencies measured by β-galactosidase staining were approximately 1.5-fold higher for xrs5-rev cells. Similar results were obtained in two additional experiments.

[b] Number of infectious centers per 10^4 transfected cells determined via the indicator cell line NBE.

[c] Percentage of cell nuclei staining positive for NS1 in an immunofluorescence assay.

[d] Number of PFU released by three cycles of freeze-thawing per 10^4 transfected cells determined via plaque assay (16) with NBE cells.

We have identified a correlation between X-ray sensitivity and parvovirus replication efficiency in CHO cells. The DNA DSB repair machinery appears to inhibit replication at an early stage, prior to synthesis of NS1 and after viral uptake and internalization, probably before the conversion of ssDNA to dsDNA. This restriction point can be overcome by increasing the MOI, thereby saturating and overwhelming the host factors which interfere with successful ssDNA-to-dsDNA conversion. We can speculate that Ku DNA-dependent protein kinase and other repair functions in that pathway carry out an inactivating reaction with virion DNA. Therefore, viral DNA may mimic a particular class of DNA DSB for which repair is defective in these mutant cell lines (13). We can also predict that MVM replication would be enhanced in severe combined immunodeficiency mice independently of any immune defect. This viral system should provide a defined substrate that is valuable for the identification of specific DNA repair proteins, as well as their importance in the repair of radiation-damaged DNA. Adeno-associated virus is a parvovirus being developed as a vector for gene therapy for a variety of applications (10, 14, 15). A major hurdle to overcome is increasing efficiency of gene transfer by stimulating the virus to convert its ssDNA genome to the ds replicative form necessary for gene expression and integration into the host cell's genome. Fisher et al. (9) and Ferrari et al. (8) have identified adenovirus E4 gene expression and UV and X-ray treatments as factors that significantly increase adeno-associated virus ssDNA-to-dsDNA genome conversion. Together, these results could lead to strategies for enhancing the ssDNA-to-dsDNA conversion of adeno-associated virus without introduction of viral genes from adenovirus, thereby increasing adeno-associated virus's effectiveness as a gene therapy vector.

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