[CANCER RESEARCH 61, 1585–1591, February 15, 2001]

End-joining Deficiency and Radiosensitization Induced by Gemcitabine

John W. G. van Putten,[1] Harry J. M. Groen, Kees Smid, Godefridus J. Peters, and Harm H. Kampinga

Department of Pulmonary Diseases, University Hospital, 9713 GZ Groningen [J. W. G. v. P., H. J. M. G.]; Department of Internal Oncology, University Hospital Vrije Universiteit, 1007 MB Amsterdam [K. S., G. J. P.]; and Department of Radiation and Stress Cell Biology, University of Groningen, 9713 AV Groningen, the Netherlands [H. H. K.]

ABSTRACT

The mechanism of radiosensitization by gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC) is not exactly known. We investigated the possible role of inhibition of the repair of DNA double-strand breaks by dFdC by measuring the extent of radiosensitization in different cell lines deficient and proficient in components of nonhomologous end-joining and in the parental cell lines. Different cell lines were incubated with 0.5 and 5 μM dFdC for 4 h. Cells deficient in DNA-dependent protein-kinase catalytic subunit (V3) showed sensitization similar to that of wild-type cells (AA8) and complemented cells (V3+YAC). Ku80-deficient cells (xrs5 and xrs6) showed even more radiosensitization by dFdC as compared with wild-type CHO-K1. However, Ku80-complemented cell lines (xrs5+huKu80 and xrs6+haKu80) did not show radiosensitization. The differences in dFdC-mediated radiosensitization were not attributable to different changes in deoxynucleotide triphosphate levels and cell cycle distribution. We conclude that a functional nonhomologous end-joining pathway is not required for dFdC-mediated radiosensitization.

INTRODUCTION

Gemcitabine (dFdC)[2] is a deoxycytidine analogue well known for its antitumor activity in different tumor types. It is one of the more effective drugs to sensitize cells to radiation; its radiosensitizing properties were demonstrated both *in vivo* and *in vitro* (1, 2). Yet, the exact mechanism of radiosensitization induced by dFdC is still not clear.

dFdC itself is not active; its transport into the cell is mediated by facilitated diffusion (3). Intracellularly, dFdC is phosphorylated to its active metabolites by deoxycytidine kinase to dFdCMP, dFdCDP, and dFdCTP. All three of the metabolites interfere with different steps in the processing of DNA. dFdCTP is incorporated into DNA and as such can obstruct DNA replication and repair (4). dFdCTP can also be incorporated into RNA (5) and can inhibit CTP-synthetase (6). dFdCMP can also inhibit dCMP-deaminase (7). dFdCDP is an inhibitor of ribonucleotide reductase, and its action was shown to lead to depletion of the DNA precursor pool, dNTP (2, 8, 9). These actions of dFdC will result in a self-potentiating mechanism because breakdown of dFdCMP is inhibited, whereas a reduction of dCTP will reduce feedback inhibition of deoxycytidine kinase, and inhibition of CTP-synthetase will also enhance incorporation of dFdC into RNA.

Ionizing radiation induces an array of lesions in DNA, including base damage, single-strand breaks and DSBs, and damage to the phosphodiester backbone. DNA DSBs are generally thought to be the most relevant lesion in radiation-induced killing of cells. In lower eukaryotes, such as yeast, DSBs are repaired by Rad52-dependent HR. Rad52 binds to DNA ends, thereby protecting them from exonuclease activity and activating end-to-end interactions and HR (10). In vertebrates, DSBs are primarily repaired by Ku-dependent NHEJ (11). Ku also binds to DNA ends, and NHEJ is facilitated by activating the DNA-PK (12). DNA-PK consists of three subunits: the Ku protein being a heterodimer of the M_r 70,000 Ku70, the M_r 86,000 Ku80, and the large, M_r 450,000 DNA-PKcs.

In preliminary experiments with human small cell lung carcinoma cells, we found that dFdC treatment retarded the rate and extent of repair of DNA DSBs as detected by PFGE. However, others have reported no detectable effect on DNA DSB repair by dFdC (13). To get a more clear insight into the possible role of DSB repair inhibition as a cause for dFdC radiosensitization, we measured the extent of sensitization by dFdC in cell lines defective in NHEJ. Rodent cell lines mutated in the gene *XRCC5* encoding Ku80 *(e.g., xrs*5 and 6) or in the gene *XRCC7* encoding DNA-PKcs (*e.g., V3*) have been found to be deficient in DNA DSB rejoining (14–18). Also, isogenic *xrs* cell lines complemented with either the human *Ku80* gene (*xrs5+huKu80*) or hamster *Ku80* gene (*xrs6+haKu80*) that restored their radiosensitivity and DSB repair ability were included in the study (15, 19, 20) Finally, a DNA-PKcs-deficient cell line complemented with a YAC carrying the gene for DNA-PKcs (*V3+YAC*) was included in the experiments (18, 21). Results obtained with these mutated and complemented cell lines were compared with the parental wild-type cell lines (CHO-K1 and AA8).

MATERIALS AND METHODS

Cell Culturing Procedures. The rodent cell lines were grown as monolayers at 5% CO_2 in a humidified 37°C incubator in Nunc plastic flasks (Roskilde, Denmark). The CHO cell lines CHO-K1 (wild type), xrs5 and xrs6 (both mutated in Ku80), xrs5+huKu80, and xrs6+haKu80 (xrs5 or xrs6 complemented with human or hamster Ku80, respectively) were grown on HAM's F12 medium (Life Technologies, Inc., Paisley, United Kingdom). The hamster cell lines AA8 (wild type), V3 (mutated in DNA-PKcs), and V3+YAC (V3 transfected with a YAC with the complementing human DNA-PKcs gene) were grown on HAM's F12 medium as well. All of the media were supplemented with 10% bovine calf serum (Life Technologies, Inc., Paisley, United Kingdom). Moreover, the media of xrs5+huKu80, xrs6+haKu80, and V3+YAC were supplemented with Geneticin (G418; 300 μg/ml; Life Technologies, Inc., Paisley, United Kingdom). The media of xrs5, xrs5+huKu80, xrs6, xrs6+haKu80, V3, and V3+YAC were also supplemented with glutamate and penicillin and streptomycin. Dr. Penny Jeggo (University of Sussex, Brighton, United Kingdom) kindly provided all of the cell lines.

All of our standard laboratory chemicals were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany).

Gemcitabine Incubation and Irradiation Treatments. Exponentially growing cells were incubated with 0.5 and 5 μM gemcitabine (dFdC; Eli Lilly, Nieuwegein, the Netherlands) for 4 h. Thereafter, cells were trypsinized, followed by neutralization of the trypsin with medium. Cell suspensions were diluted in fresh complete medium to a density of about 10^6 cells/ml. Immediately thereafter, cells were irradiated, using a ^{137}Cs gamma-ray machine (IBL 637; CIS Biointernational, Gif-sur-Yvette, France) at a dose rate of 0.9 Gy/min. The dosimetry was performed with an ionization chamber (Philips 37489/19; Eindhoven, the Netherlands) calibrated with a ^{90}Sr source (Philips2011/00).

Cell Survival. Cell survival (colony-forming ability) was determined by plating 100 μl of an appropriately diluted sample to triplicate plastic Petri

Received 1/13/00; accepted 12/7/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Pulmonary Diseases, University Hospital, Hanzeplein 1, 9713 GZ Groningen, the Netherlands. Phone: 31-50-3612357; Fax: 31-50-3619320; E-mail: j.w.g.van.putten@int.azg.nl.

[2] The abbreviations used are: dFdC, 2′,2′-difluoro-2′-deoxycytidine; dFdCMP, dFdC monophosphate; dFdCDP, dFdC diphosphate; dFdCTP, dFdC triphosphate; dNTP, deoxynucleotide triphosphate; DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end-joining; DNA-PK, DNA-dependent protein kinase complex; DNA-PKcs, DNA-PK catalytic subunit; PFGE, pulsed field gel electrophoresis; YAC, yeast artificial chromosome; LSC, liquid scintillation counting; CHO, Chinese hamster ovary.

dishes (Nunc, Roskilde, Denmark), containing 5 ml of complete growth medium. After 6–8 days of incubation, colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. Colonies containing more than 50 cells were counted. The dose-enhancement ratio ($DER_{10\%}$) was calculated by taking the following ratio:

$$\frac{\text{Radiation dose required for inducing 90\% cell kill for radiation alone}}{\text{Radiation dose required for inducing 90\% cell kill for radiation combined with dFdC}}$$

The mean $DER_{10\%}$ was determined from the $DER_{10\%}$ calculated for each individual experiment.

PFGE Assay. PFGE was used as developed by Blöcher *et al.* (22). After incubation with or without 5 μM dFdC for 4 h, cells were irradiated on ice and either processed immediately for estimating induction of DNA DSBs or incubated up to 24 h at 37°C to allow for DNA repair. For each individual sample, 2×10^7 cells were mixed with 1% low melting point agarose (Bio-Rad, Hercules, CA) at 37°C to form plugs. Next, cells were lysed by SDS-proteinase K treatment [2% sarkosyl, 0.5 mg/ml proteinase K (Sigma), and 500 mM EDTA (pH 7.6) in PBS] for 2 h at 0°C followed by 16 h at 37°C; they were then washed with PBS and treated for 1 h with RNase A (0.2 mg/ml in PBS; Sigma) at 37°C. After washing with TBE [45 mM Tris-base, 45 mM boric acid, and 2 mM EDTA (pH 8.2)], the plugs were inserted into the wells of 0.5% agarose gels (Chromosomal Grade; Bio-Rad). After clamped homogeneous electric field (CHEF) electrophoresis (at 14°C and 40 V for 25 h with switching interval of 75 min), the gel was stained with ethidium bromide. The fraction of DNA migrating from the plug into the lane (% DNA extracted) was measured using a UV-transilluminator (312 nM) and image analysis, as described previously (23).

Deoxyribonucleotide Extraction and Measurement. Levels of dATP, dTTP, dCTP, and dGTP were measured in one run using the DNA polymerase elongation assay method. dNTPs were extracted as described previously (24). Briefly, cells were suspended in 150 μl of ice-cold PBS, followed by adding 50 μl of 40% trichloro-acetic acid (10% final concentration) to precipitate proteins and nucleic acids; the cells were then chilled on ice for 20 min and centrifuged for 10 min at 10,000 $\times$ *g* at 4°C. A 2-fold excess of freshly prepared trioctylamine and 1,1,2-trichlorotrifluoroethane (1:4) was added to neutralize the supernatant. After centrifugation for 1 min at 10,000 $\times$ *g* at 4°C, the cellular extract containing dNTPs (upper aqueous layer) was carefully taken off, divided in equal parts, and stored at −20°C. Before assaying, the trichloro-acetic acid extract was dried using a Speedvac drying system at room temperature and, if necessary, stored at −20°C until analysis. The assay for dNTP was based on the original DNA polymerase assay (25), optimized by the use of 96-well plates (26) and tailor-made oligonucleotides (27, 28) and was performed as previously described for dCTP (29). Dried samples were reconstituted in assay buffer [200 mM HEPES and 20 mM $MgCl_2$ (pH 7.3)] to a final concentration equivalent to 10^7 cells/ml. Samples and standards of 0, 1, 2.5, and 5 pmol of each dNTP were added to DEAE filter plates (Millipore, Etten-Leur, the Netherlands). To all wells, demiwater was added up to 30 μl, followed by 70 μl of a reaction mix, consisting of 10 μl of {[8-^{3}H]dATP (25 μM; 1.6 Ci/mmol; 0.04 μCi/μl) for detection of dCTP, dTTP, and dGTP and 10 μl of [CH_3-^{3}H]dTTP (25 μM; 30 Ci/mmol; 0.04 μCi/μl) for dATP detection, 5 μl T-P mix [primer annealed to a tailor-made (for each dNTP) oligo template], 5 μl of Klenow DNA pol I, and 50 μl of assay buffer}. Filter plates were gently vortexed and incubated at room temperature for 2 h. Each well was washed 4 times [in 0.25 M KH_2PO_4 and 0.5 M KCl (pH 4.3)], the wet filters were punched out in LSC vials, using a Multiscreen assay system as described

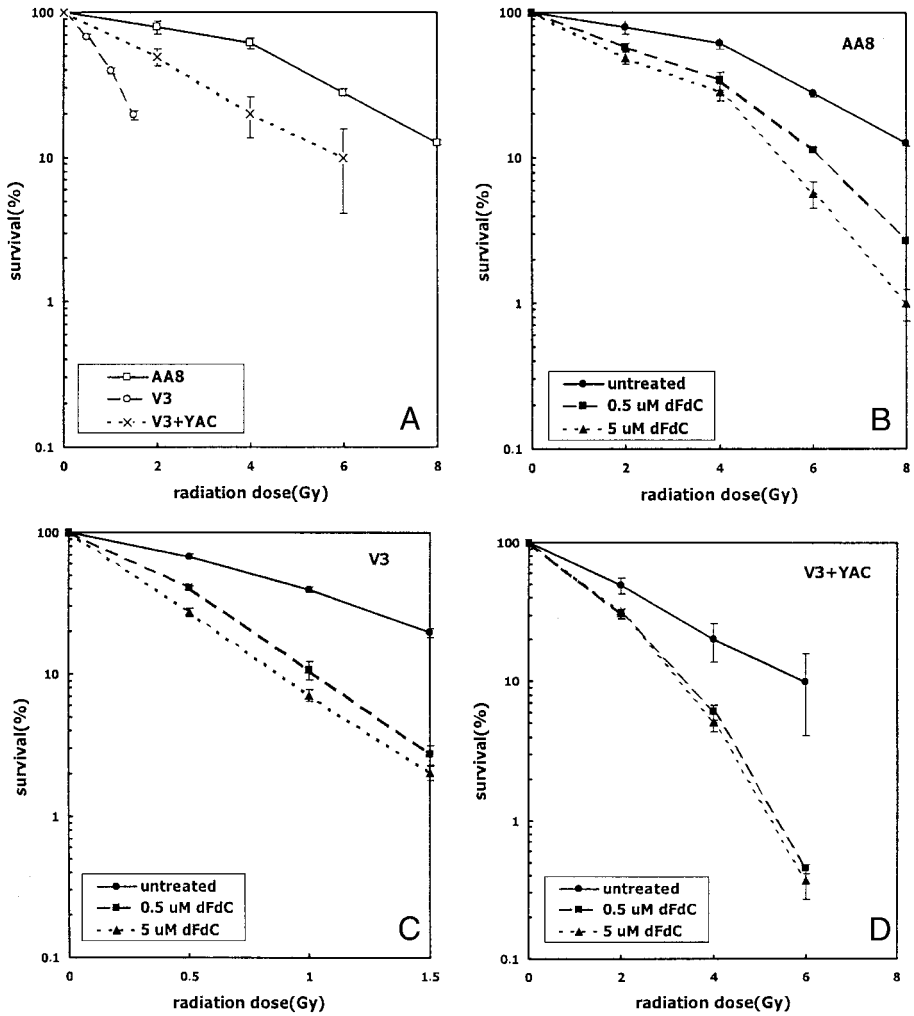

Fig. 1. Effect of dFdC on cellular radiosensitivity of DNA-PKcs-proficient and -deficient cell lines. Cells (10^6/ml) were exposed to graded doses of X-rays without (●) or with a 4-h preincubation with either 0.5 μM (■) or 5 μM (▲) dFdC. Thereafter, their clonogenic ability was determined. Survival curves are corrected for the cell-killing effect of dFdC alone. Data are the mean of at least three independent experiments; *bars*, SE, shown when they exceed the symbol. *A*, comparison of radiosensitivity of AA8 [parental (□)], V3 [DNA-PKcs-deficient (○)], and V3+YAC [V3 cells complemented with the YAC containing DNA-PKcs (×)]. *B–D*, effect of dFdC on radiosensitivity of AA8 (*B*), V3 (*C*), and V3+YAC (*D*).

previously (29), Thereafter, 500 μl of 2 M NaOH was added to each vial, followed after 3-h shaking by 4.5 ml of LSC fluid (Ultima Gold; Packard, Groningen, the Netherlands). Samples were counted in a LSC counter (1900TR; Packard). Levels were expressed as pmol/10^6 cells and, for each experiment, were calculated as a percentage of the levels measured in untreated cells.

Cell Cycle Analysis. The amount of cells in a particular cell cycle phase was measured by DNA flow cytometry (using FACSSTAR; Becton Dickenson) using the histogram analysis software ModFit (Verity Software House; Topsham, ME). Cells were diluted to a concentration of 10^6/ml, washed with PBS and 5 mM $MgCl_2$, and afterward dissolved in 80% ethanol/acetone in a 1:1 ratio. This solution was stored for at least 18 h below 5°C. Cells were stained with propidium iodide for at least 30 min at 37°C, followed by 10 μl RNase A using the manufacturer's protocol. Individual histograms of cells incubated with and without dFdC were compared.

Measurement of Ku80 Protein Expression. Cell lysates from CHO-K1, xrs6, and xrs6+haKu80 were loaded onto a denaturing polyacramide gel. After electrophoresis, the proteins were electrotransferred onto nitrocellulose (Bio-Rad) and probed for total Ku80 protein using the anti-Ku80 antibody (kindly provided by Prof. Steve Jackson, Wellcome/CRC Institute, Cambridge, United Kingdom) at a 1:2500 dilution. As a loading control, anti-α-tubulin (T5168; Sigma) was used at a 1:5000 dilution. This was followed by binding of 1:2000 diluted peroxidase-conjugated donkey-antirabbit for Ku80 (Amersham, Roosendaal, the Netherlands) or 1:5000 diluted peroxidase-conjugated sheep-antimouse for α-tubulin (Amersham) and detection of the proteins by enhanced chemiluminescence (Amersham).

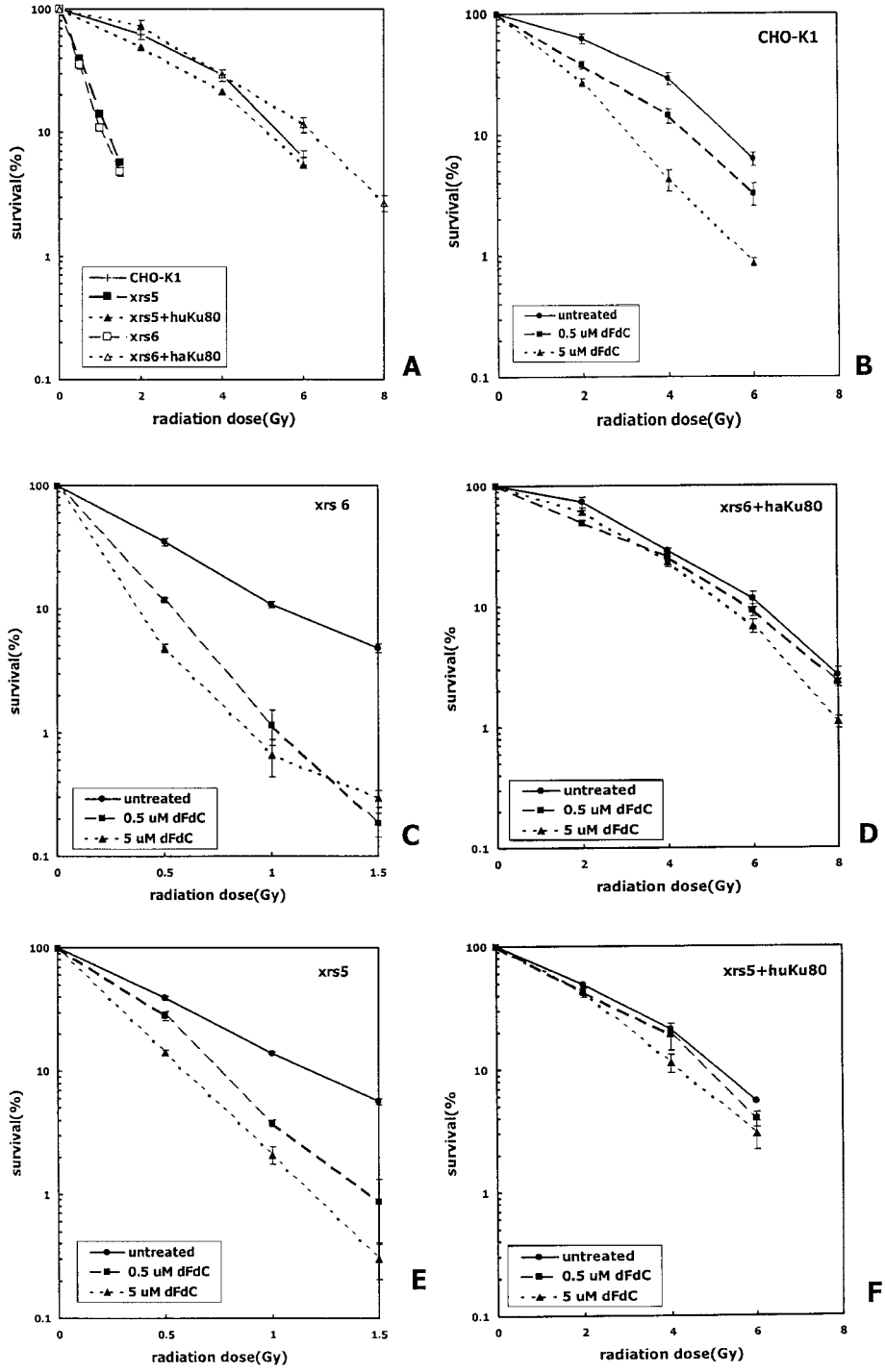

Fig. 2. Effect of dFdC on cellular radiosensitivity of Ku80-proficient and -deficient cell lines. Cells (10^6/ml) were exposed to graded doses of X-rays without (●) or with a 4-h preincubation with either 0.5 μM (■) or 5 μM (▲) dFdC. Thereafter, their clonogenic ability was determined. Survival curves are corrected for the cell-killing effect of dFdC alone. Data are the mean of at least three independent experiments; *bars*, SE, shown when they exceed the symbol. *A*, comparison of radiosensitivity of CHO-K1 [parental (+)], xrs5 [Ku80-deficient (■)] and xrs6 [Ku80-deficient (□)], and xrs5+huKu80 [xrs5 cells complemented with human Ku80 (▲)], and xrs6+haKu80 [xrs6 cells complemented with hamster Ku80 (△)]. *B–F*, effect of dFdC on radiosensitivity of CHO-K1 (*B*), xrs6 (*C*), xrs6+haKu80 (*D*), xrs5 (*E*), and xrs5+huKu80 (*F*).

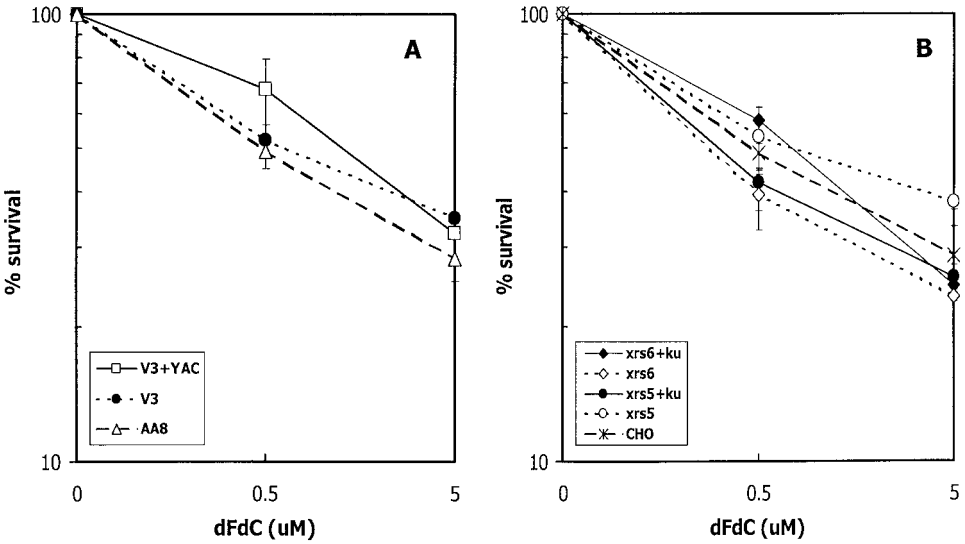

Fig. 3. Relationship between DNA-PKcs and Ku80 status and cytotoxicity of dFdC alone. Plotted are the cytotoxic effect before radiation (*% survival*) and the concentration of dFdC. Cells (10^6/ml) were exposed for 4 h to 0.5 or 5 μM dFdC. Their clonogenic ability was determined for the different cell lines used: *A*, AA8 (△), V3 (●), and V3 +YAC (□); and *B*, CHO-K1 (×), xrs5 (○), xrs5+Ku80 (●), xrs6 (◇), xrs6+Ku80 (◆). Data are the mean of at least three individual experiments; *bars*, SE when they exceed the symbol.

Statistical Analysis. Data of cell survival were analyzed using ANOVA in a repeated measurement design with dose dFdC as factor. A $P < 0.05$ was considered significant. Differences between $DER_{10\%}$ and changes in dNTP pools between the different cell lines were compared with Levene's *t* test for independent samples assuming equality of variances.

RESULTS

Cell Survival. V3 cells lack functional DNA-PKcs and hence are deficient in NHEJ, which leads to their high radiosensitivity. The YAC containing the DNA-PKcs gene partly restores radioresistance to the level of the wild-type AA8 (Fig. 1*A*). AA8, V3, and V3+YAC were equally sensitized when pretreated with 0.5 μM dFdC for 4 h. This indicates that the status of DNA-PKcs is not relevant for dFdC-induced radiosensitization. The extent of radiosensitization did not increase or only slightly increased when using 5 μM dFdC (Fig. 1, *B–D*). Next, we used xrs5 and xrs6 cells without functional Ku80, which also leads to a defect in NHEJ and high radiosensitivity (Fig. 2*A*; Ref. 14, 16). Both concentrations of dFdC caused substantial radiosensitization in the xrs5 and xrs6 cells despite their lack of functional Ku80 (Fig. 2, *C* and *E*). The extent of radiosensitization seemed even larger than in the parental CHO cell line (Fig. 2*B*). Interestingly, radiosensitization by dFdC was significantly less in the xrs5 and xrs6 cells complemented with the human or hamster Ku80, respectively ($P < 0.001$; Fig. 2, *D* and *F*), which suggested at least some effect of Ku80 expression on dFdC-mediated radiosensitization. The status of DNA-PKcs or Ku80 did not significantly affect the cytotoxic effects of dFdC (Fig. 3). These data demonstrate that the absence of functional Ku80 or DNA-PKcs does not impair dFdC-mediated radiosensitization. Hence, NHEJ is not a prerequisite for the dFdC-mediated enhancement of cellular radiosensitivity. However, cells with different Ku80 expression showed a different extent of radiosensitization, as expressed by $DER_{10\%}$ at given dFdC concentrations (Fig. 4). In fact, cells without Ku80 (xrs5 and xrs6) showed higher dFdC-mediated radiosensitization than cells expressing Ku80 (CHO-K1 or xrs5+huKu80 and xrs6+haKu80).

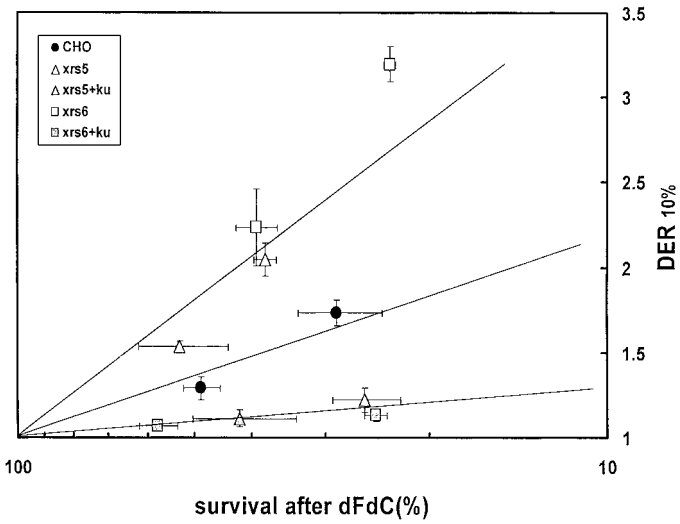

Fig. 4. Relationship between cytotoxicity and the radiosensitizing effect of dFdC. Plotted are the cytotoxic effect before radiation [*survival after dFdC (%)*] and the radiosensitizing effect of dFdC ($DER_{10\%}$) for the different cell lines used [CHO-K1 (●), xrs5 (△), xrs5+Ku80 (▲), xrs6 (□), and xrs6+Ku80 (▨)]. For each cell line, two points are shown: the one with the higher survival percentage has been exposed to 0.5 μM dFdC and the one with the lower to 5 μM dFdC for 4 h. Data are the mean of at least three individual experiments; *bars*, SE when they exceed the symbol. *Lines*, drawn to compare cell lines with different Ku80 status and extent of radiosensitization. $DER_{10\%}$ was calculated by taking the ratio:

$$\frac{\text{Radiation dose required for inducing 90\% cell kill for radiation}}{\text{Radiation dose required for inducing 90\% cell kill for radiation combined with dFdC}}$$

PFGE Assay. To test whether dFdC enhances radiosensitivity by interfering with the processing of radiation-induced DNA DSBs, we performed PFGE in the parental CHO-K1 cells. Incubation with dFdC alone did not cause DSBs (data not shown). Preincubation with dFdC caused no changes in the induction of radiation-induced DSBs (Fig. 5*A*). Also, kinetics of DSB repair after 30 Gy of radiation was unchanged by prior incubation with dFdC (Fig. 5*B*). The finding that dFdC had no detectable effect on the repair of the majority of radiation-induced DSBs further supports our survival data that dFdC does not interfere with the NHEJ pathway that is responsible for processing most DSBs. An enormous increase in the number of DSBs was found at 24 h after exposure to dFdC. This effect was attributable to the large amount of apoptotic cells induced by the cytotoxicity of dFdC, which limits the interpretation of the number of residual DSBs at 24 h (data not shown).

Deoxynucleotide Pools. It has been shown that dFdC induces changes in dNTP pools (2). Especially, a decrease in dATP should be responsible for the dFdC-induced radiosensitization (30). To investigate why Ku-complemented xrs cells did not show dFdC-mediated radiosensitization, we measured dNTP pools both in CHO-K1 and

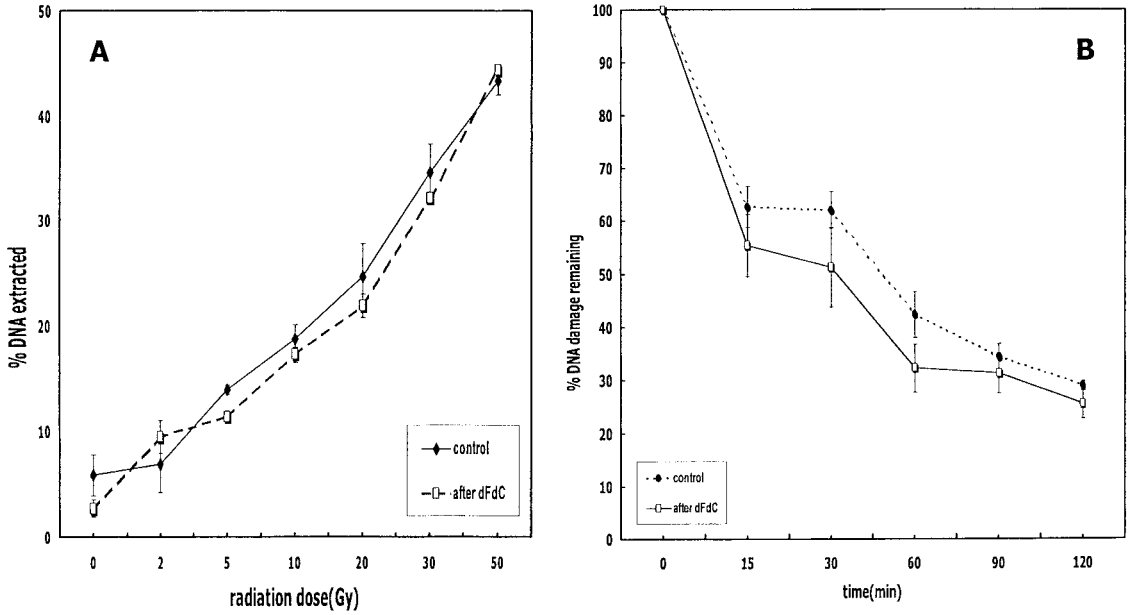

Fig. 5. Effects on induction and repair of radiation-induced DNA DSBs without and with dFdC. *A*, CHO cells were exposed to graded doses of X-rays without (◆) or with a 4-h preincubation with 5 μm dFdC (□). Induction of DNA DSBs is represented as percentage of extracted DNA in the lane by PFGE as was described in "Materials and Methods." *B*, repair of DNA DSBs in CHO cells represented as percentage of the total amount of extracted DNA in the lane by PFGE during different time intervals in 2 h after radiation with 30 Gy without (●) or after incubation with 5 μM dFdC (□) for 4 h prior to irradiation. Data are the mean of at least three individual experiments; *bars*, SE when they exceed the symbol.

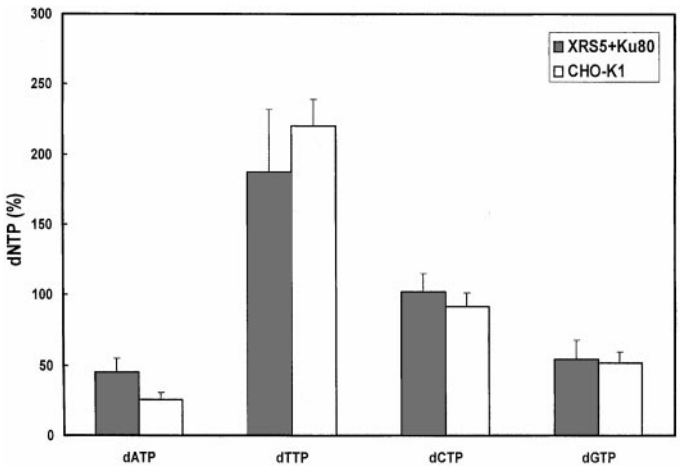

Fig. 6. Effect of dFdC on deoxynucleotide pools. Two different cell lines (CHO-K1 and xrs5+huKu80) were exposed to 5 μM dFdC for 4 h; dATP, dTTP, dCTP, and dGTP were measured. For each experiment, dNTP levels were expressed as percentage of the level measured in untreated cells. Data are the mean of at least three separate experiments; *bars*, SE.

xrs5+huKu80 cells. Consistent with previous data in different cell lines, we confirmed a decrease in dATP and dGTP levels by incubation with dFdC. The levels of dCTP did not change significantly; levels of dTTP increased after dFdC treatment (Fig. 6). Radiation itself induced no changes in dNTP pools. No significant differences in the dFdC-induced effects on the dNTP pools could be detected between CHO-K1 and xrs5+huKu80 cells (dATP $P = 0.11$; dTTP $P = 0.21$; Fig. 6). Although a decrease in dATP level may be a prerequisite for radiosensitization induced by dFdC, other (downstream) events need to be invoked to explain the presence (CHO-K1) or absence (xrs5+huKu80) of a radiosensitizing effect of dFdC.

Cell Cycle. Cellular radiosensitivity varies over the cell cycle. Prolonged dFdC incubation is known to alter cell cycle distribution (2). Because dFdC-mediated radiosensitization is highest in S phase and low in G_1 (31), the absence (xrs5+Ku80) or presence (CHO-K1) of enhanced radiosensitivity of dFdC-pretreated cells might be attributable to differences in cell cycle redistribution. Using flow cytometric analysis, we found that both of the cell lines showed rather similar cell cycle distribution prior to dFdC exposure. The short, 4-h incubation with dFdC prior to irradiating cells did not induce significant alterations in the cell cycle distribution of either CHO-K1 or xrs5+huKu80 ($P = 0.24$; data not shown). Therefore, the presence or absence of the radiosensitizing effect of dFdC is not caused by changes in cell cycle (re)distribution.

Relationship between Different Ku80 Status and the Extent of dFdC-mediated Radiosensitization. xrs5 cells and xrs6 cells that are complemented with human or hamster Ku80, respectively, have

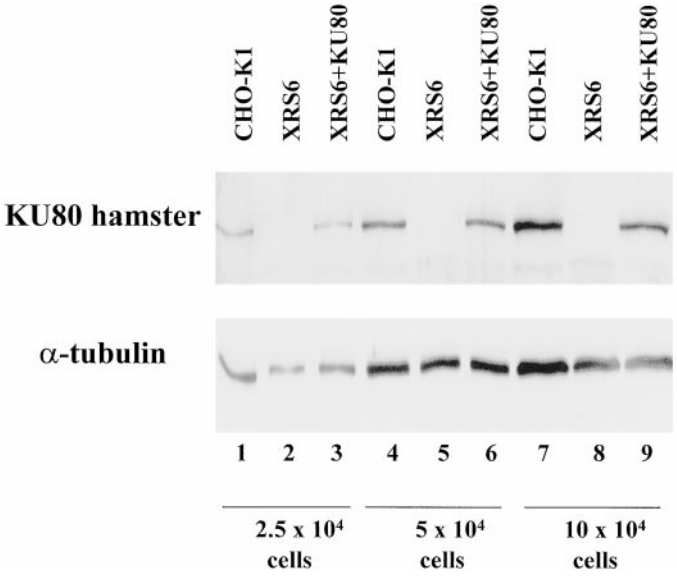

Fig. 7. Expression levels of Ku80 in parental CHO-K1, xrs6, and xrs6 cells complemented with haKu80. Cell lysates from CHO-K1, xrs6, and xrs6+haKu80 cells, at three different cell concentrations were processed on SDS-polyacrylamide gels and, after transfer to nitrocellulose, probed for ha-Ku80 expression and α-tubulin (as a loading control). A typical experiment is shown.

full complementation of cellular radiosensitivity compared with xrs5 and xrs6. Xrs6+haKu80 has been reported to express even higher levels of Ku80 protein than the parental CHO-K1 (14). These higher Ku80 levels could suggest the possibility of an inverse relationship between the level of Ku80 expression and the extent of dFdC-induced radiosensitization (as might be suggested from Fig 4). To investigate this, Ku80 protein levels were analyzed in CHO-K1, xrs6, and xrs6+haKu80. Using serial dilutions and α-tubulin as a loading control, we confirmed the absence of Ku expression in xrs6 cells. However, in our hands, Ku80 expression levels were similar in wild-type and complemented cells (Fig 7). Thus, an increase in Ku80 expression levels does not seem to explain the observed differences in radiosensitization by dFdC. Direct comparison of levels of Ku80 expression is not possible in the xrs5 and xrs5+huKu80 panel because of differences in cross-reactivity of the antibody to human Ku80 as present in the xrs5+huKu80 cells.

DISCUSSION

We have shown that the radiosensitizing effect of dFdC is also observed in cells lacking either functional DNA-PKcs or Ku80. This suggests that a functional NHEJ pathway, which is required for the repair of the majority of radiation-induced DNA DSBs in mammals, is not a prerequisite for radiosensitization by dFdC. Consistently, we found no gross effects of dFdC on the rejoining of DNA DSBs in PFGE analyses. Hence, NHEJ and its individual components are not essential for radiosensitization by dFdC.

A remarkable finding was that the Ku80-complemented cells were significantly less sensitized to radiation by dFdC compared with parental cells, and Ku80-deficient cells even showed the highest levels of radiosensitization. Although this demonstrates that Ku80 is not the target for dFdC-mediated radiosensitization, it does suggest that Ku80 somehow interferes with the radiosensitizing action of dFdC. This interference is not attributable to different effects of dFdC on dNTP pools or cell cycle (re)distribution because these changed to the same extent in Ku80-complemented cells as in parental CHO-K1 cells. Although dFdC effects on cell cycle redistribution and dNTP pools may contribute to, or even be necessary for, dFdC-mediated radiosensitization (13), we showed that they do not ultimately determine whether or not dFdC treatment is going to result in enhanced cellular radiosensitivity.

So, what could possibly be the critical action of dFdC leading to radiosensitization, and why is it strongly reduced in Ku80-complemented cells? If the observed decline in dATP is crucial for the radiosensitizing effect as has been suggested (2, 30), it is rather unlikely that this would affect a short-patch repair like NHEJ or base excision repair, both crucial for the repair of DNA damage induced by ionizing irradiation. Rather, effects on long-patch repair pathways such as nucleotide excision repair or HR would be expected. Indeed, recent data suggest that dFdC interferes with nucleotide excision repair (32). However, because nucleotide excision repair plays no role in the repair of ionizing radiation-induced DNA damage, the most likely pathway remaining as a target for dFdC-mediated radiosensitization is HR. Although, in mammalian cells, HR is thought to be less dominant than NHEJ in the repair of DSBs, defects in HR do enhance cellular radiosensitivity (33). It is currently thought that in mammalian cells HR is especially operational in (late) S and G_2 phases of the cell cycle (10). Interestingly, the extent of dFdC-induced radiosensitization was also shown to be higher for cells in late S phase and G_2 phase than for the same cells in G_1 phase (31). Xrs5 and xrs6 are proficient in HR and still show radiosensitization after induction with dFdC. They have also shown to have near-to-normal S-phase radiosensitivity (34, 35). Taken together, these observations suggest that HR is involved in radiation sensitivity and in radiosensitization by dFdC. Along with this assumption, one may postulate why dFdC-mediated radiosensitization is higher in cells without Ku80 and lower in cells with Ku80. In the nucleus, Ku80 (NHEJ) competes with Rad52 (HR) for the DNA DSB ends (10). In the absence of Ku80, a higher fraction of DNA DSBs may be processed by HR rather than by NHEJ. By assuming that HR is a target for dFdC, the proportion of DSBs processed by either NHEJ or HR determines the radiosensitizing efficacy of dFdC. This will result in higher levels of dFdC-mediated radiosensitization in Ku80-deficient cells. Inversely, higher levels of Ku80 could reduce the fraction of DNA DSBs being processed by HR, resulting in less dFdC-mediated radiosensitization. Although, we did not find higher expression levels of Ku80 in the Ku80-complemented xrs cells that showed the lowest level of radiosensitization, one could still assume that in the Ku80-complemented cells, the functional level of end-binding activity of Ku80 is higher.

REFERENCES

1. Milas, L., Fujii, T., Hunter, N., Elshaikh, M., Mason, K., Plunkett, W., Ang, K. K., and Hittelman, W. Enhancement of tumor radioresponse *in vivo* by gemcitabine. Cancer Res., *59:* 107–114, 1999.
2. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2′,2′-difluoro-2′-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., *54:* 3218–3223, 1994.
3. Heinemann, V., Hertel, L. W., Grindey, G. B., and Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res., *48:* 4024–4031, 1988.
4. Schy, W. E., Hertel, L. W., Kroin, J. S., Bloom, L. B., Goodman, M. F., and Richardson, F. C. Effect of a template-located 2′,2′-difluorodeoxycytidine on the kinetics and fidelity of base insertion by Klenow (3′→5′exonuclease-) fragment. Cancer Res., *53:* 4582–4587, 1993.
5. Ruiz van Haperen, V., Veerman, G., Vermorken, J. B., and Peters, G. J. 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem. Pharmacol., *46:* 762–766, 1993.
6. Heinemann, V., Schulz, L., Issels, R. D., and Plunkett, W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin. Oncol, *22:* 11–18, 1995.
7. Heinemann, V., Xu, Y. Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res., *52:* 533–539, 1992.
8. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. 2′-Deoxy-2′-methylenecytidine and 2′-deoxy-2′,2′-difluorocytidine 5′-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. J. Med. Chem., *34:* 1879–1884, 1991.
9. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2′,2′-difluoro-2′-deoxycytidine. Int. J. Radiat. Oncol. Biol. Phys., *34:* 867–872, 1996.
10. Van Dyck, E., Stasiak, A. Z., Stasiak, A., and West, S. C. Binding of double-strand breaks in DNA by human Rad52 protein. Nature (Lond.), *398:* 728–731, 1999.
11. Kanaar, R., Hoeijmakers, J. H., and van Gent, D. C. Molecular mechanisms of DNA double strand break repair. Trends Cell Biol., *8:* 483–489, 1998.
12. O'Hara, M. D., Pollard, M. D., Wheatley, G., Regine, W. F., Mohiuddin, M., and Leeper, D. B. Thermal response and hyperthermic radiosensitization of scid mouse bone marrow CFU-C. Int. J. Radiat. Oncol. Biol. Phys., *31:* 905–910, 1995.
13. Lawrence, T. S., Eisbruch, A., and Shewach, D. S. Gemcitabine-mediated radiosensitization. Semin. Oncol., *24* (Suppl. 7)*:* S24–S28, 1997.
14. Singleton, B. K., Priestley, A., Steingrimsdottir, H., Gell, D., Blunt, T., Jackson, S. P., Lehmann, A. R., and Jeggo, P. A. Molecular and biochemical characterization of xrs mutants defective in Ku80. Mol. Cell Biol., *17:* 1264–1273, 1997.
15. Smider, V., Rathmell, W. K., Lieber, M. R., and Chu, G. Restoration of X-ray resistance and V(D)J recombination in mutant cells by Ku cDNA. Science (Washington DC), *266:* 288–291, 1994.
16. Finnie, N. J., Gottlieb, T. M., Blunt, T., Jeggo, P. A., and Jackson, S. P. DNA-dependent protein kinase activity is absent in xrs-6 cells: implications for site-specific recombination and DNA double-strand break repair. Proc. Natl. Acad. Sci. USA, *92:* 320–324, 1995.
17. Ross, G. M., Eady, J. J., Mithal, N. P., Bush, C., Steel, G. G., Jeggo, P. A., and McMillan, T. J. DNA strand break rejoining defect in *xrs*-6 is complemented by transfection with the human *Ku80* gene. Cancer Res., *55:* 1235–1238, 1995.
18. Blunt, T., Taccioli, G. E., Priestley, A., Hafezparast, M., McMillan, T., Liu, J., Cole, C. C., White, J., Alt, F. W., and Jackson, S. P. A YAC contig encompassing the *XRCC5* (*Ku80*) DNA repair gene and complementation of defective cells by YAC protoplast fusion. Genomics, *30:* 320–328, 1995.
19. Fulop, G. M., and Phillips, R. A. The scid mutation in mice causes a general defect in DNA repair. Nature (Lond.), *347:* 479–482, 1990.
20. Taccioli, G. E., Cheng, H. L., Varghese, A. J., Whitmore, G., and Alt, F. W. A DNA repair defect in Chinese hamster ovary cells affects V(D)J recombination similarly to the murine scid mutation. J. Biol. Chem., *269:* 7439–7442, 1994.

21. Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., and Jeggo, P. A. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell, *80:* 813–823, 1995.
22. Blocher, D., Einspenner, M., and Zajackowski, J. CHEF electrophoresis, a sensitive technique for the determination of DNA double-strand breaks. Int. J. Radiat. Biol., *56:* 437–448, 1989.
23. Rosemann, M., Kanon, B., Konings, A. W., and Kampinga, H. H. An image analysis technique for detection of radiation-induced DNA fragmentation after CHEF electrophoresis. Int. J. Radiat. Biol., *64:* 245–249, 1993.
24. Ruiz-van Haperen, V., Veerman, G., Boven, E., Noordhuis, P., Vermorken, J. B., and Peters, G. J. Schedule dependence of sensitivity to 2′,2′-difluorodeoxycytidine (gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. Biochem. Pharmacol., *48:* 1327–1339, 1994.
25. Hunting, D., and Henderson, J. F. Determination of deoxyribonucleoside triphosphates using DNA polymerase: a critical evaluation. Can. J Biochem., *59:* 723–727, 1981.
26. Peters, G. J., De Abreu, R. A., Oosterhof, A., and Veerkamp, J. H. Concentration of nucleotides and deoxynucleotides in peripheral and phytohemagglutinin-stimulated mammalian lymphocytes. Effects of adenosine and deoxyadenosine. Biochim. Biophys. Acta, *759:* 7–15, 1983.
27. Sherman, P. A., and Fyfe, J. A. Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. Anal. Biochem., *180:* 222–226, 1989.
28. Iwasaki, H., Huang, P., Keating, M. J., and Plunkett, W. Differential incorporation of ara-C, gemcitabine, and fludarabine into replicating and repairing DNA in proliferating human leukemia cells. Blood, *90:* 270–278, 1997.
29. van Moorsel, C. J., Pinedo, H. M., Veerman, G., Guechev, A., Smid, K., Loves, W. J., Vermorken, J. B., Postmus, P. E., and Peters, G. J. Combination chemotherapy studies with gemcitabine and etoposide in non-small cell lung and ovarian cancer cell lines. Biochem. Pharmacol., *57:* 407–415, 1999.
30. Shewach, D. S., and Lawrence, T. S. Radiosensitization of human tumor cells by gemcitabine *in vitro*. Semin. Oncol., *22:* 68–71, 1995.
31. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2′,2′-difluoro-2′-deoxycytidine) within the cell cycle *in vitro*. Int. J. Radiat. Oncol. Biol. Phys., *41:* 875–882, 1998.
32. Yang, L. Y., Li, L., Jiang, H., Shen, Y., and Plunkett, W. Expression of ERCC1 antisense RNA abrogates gemicitabine-mediated cytotoxic synergism with cisplatin in human colon tumor cells defective in mismatch repair but proficient in nucleotide excision repair. Clin. Cancer Res., *6:* 773–781, 2000.
33. Essers, J., Hendriks, R. W., Swagemakers, S. M., Troelstra, C., de Wit, J., Bootsma, D., Hoeijmakers, J. H., and Kanaar, R. Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. Cell, *89:* 195–204, 1997.
34. Whitmore, G. F., Varghese, A. J., and Gulyas, S. Cell cycle responses of two X-ray sensitive mutants defective in DNA repair. Int. J. Radiat. Biol., *56:* 657–665, 1989.
35. Mateos, S., Slijepcevic, P., MacLeod, R. A., and Bryant, P. E. DNA double-strand break rejoining in xrs5 cells is more rapid in the G_2 than in the G_1 phase of the cell cycle. Mutat. Res., *315:* 181–187, 1994.