

DNA Strand Break Rejoining Defect in *xrs-6* Is Complemented by Transfection with the Human *Ku80* Gene¹

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Abstract

The radiosensitive mutant *xrs-6*, derived from Chinese hamster ovary cell line CHO-K1, has been demonstrated to be defective in DNA double-strand break repair and also in its proficiency to undergo V(D)J recombination. Recent work has provided both genetic and biochemical evidence that the M_r 80,000 subunit of the Ku protein is able to complement the radiosensitivity and the V(D)J recombination defect in the *xrs-6* mutant. We demonstrate here that complementation of the radiosensitive phenotype in *xrs-6* cells by the introduction of Ku80 cDNA is accompanied by the concomitant restoration of DNA double-strand break rejoining proficiency to almost that of the parental CHO-K1 cells, as measured both by neutral single-cell microgel electrophoresis (Comet) technique and by pulsed-field gel electrophoresis. These results provide further biochemical evidence for the involvement of the Ku protein in the repair of DNA double-strand breaks.

Introduction

DNA dsb³ are critical lesions disrupting the integrity of the DNA duplex. Such lesions arise following ionizing radiation damage, during the repair of other DNA lesions, and as an intermediate in the recombination step of immunoglobulin gene rearrangement (1). Among the wide range of classes of damage induced by ionizing radiation, it is believed that the fate of dsb, or a subset of dsb, determines radiation-induced cytotoxicity (2).

A number of rodent mutants defective in the repair of DNA dsb have been identified and can be classified into at least three complementation groups (3). One such group includes six mutants isolated from the CHO-K1 cell line, namely *xrs* 1-6 (4, 5), and one mutant, XR-V15B, derived from V79 cells. The repair gene implicated in the determination of the phenotype of these mutants has been designated *XRCC5*.

Evidence for the localization of the human *XRCC5* gene to chromosome 2q was provided by complementation of the repair defect in *xrs-6* by transfer of human chromosome 2 using microcell-mediated chromosome transfer; this resulted in correction of both ionizing radiation sensitivity and restoration of the ability to rejoin radiation-induced DNA dsb (6). Further mapping of the gene located it to the region 2q 33-35 (7).

The recent recognition of a lack of DNA end-binding activity in *xrs* cells (8, 9) and the mapping of a M_r 80,000 subunit of a previously

identified human DNA end-binding protein, Ku, to the region 2q 33-35 (10) led to the investigation of the gene coding for Ku80 as being the candidate *XRCC5* gene. Ku binds to free double-stranded DNA ends (11) and is the DNA-binding component of DNA-dependent protein kinase (12). The ability of the Ku80 protein to correct the radiosensitivity and V(D)J recombination defect in *xrs* cells has been confirmed by a combination of genetic and biochemical approaches (13, 14). The aim of this study was to examine whether the *Ku80* gene was also able to complement the defect in the rejoining of ionizing radiation-induced DNA dsb in *xrs-6* cells.

Materials and Methods

Cell Lines. The cell lines used were the parental line, CHO-K1, its ionizing radiation-sensitive mutant *xrs-6*, and an X-ray resistant *xrs-6* Ku transfectant line designated *xrs-6/Ku80*. The derivation of these lines is described in Taccioli *et al.* (14). *xrs-6/Ku80* contained a 2200-base pair sequence containing the Ku80 open reading frame in an expression vector p220LTR under the control of the Rous sarcoma virus long terminal repeat promoter. A Ku80 transfectant that retained resistance to hygromycin but which had lost its Ku80 activity during passage (*xrs/vec*) was used as a transfection control. Confirmation that this transfectant had lost Ku80 sequences was shown by PCR using the primers described previously (14). Cell lines were maintained in a monolayer culture at 37°C in DMEM supplemented with 10% FCS, glutamine, and penicillin/streptomycin, and gassed with 5% CO₂, 3% O₂, and 92% N₂. *xrs/Ku80* and *xrs/vec* were maintained in selective conditions using 300 µg/ml hygromycin.

Cellular Sensitivity to Ionizing Radiation. Cellular sensitivity to ionizing radiation was assessed in CHO-K1, *xrs-6*, *xrs/Ku80*, and *xrs/vec* by measuring clonogenic cell survival following acute exposure at high dose rate. Briefly, exponential phase cells were plated at a range of cell densities, and 4 h later were irradiated at 37°C using a 33TBq ⁶⁰Co source at a dose-rate of 1-2 Gy/min over an acute dose range of 1-8 Gy. Cultures were returned to incubate at 37°C for a period of 6-9 days. Cultures were fixed and stained with Giemsa; then colonies of greater than 50 cells were scored. The plating efficiencies (*PE*) were calculated as the colony number/cells plated and the surviving fraction (*SF*) is given by $PE_{treated}/PE_{control}$. Parameters of cellular radiosensitivity were obtained by fitting the surviving fraction data as a function of dose using nonlinear regression to the linear-quadratic model of radiation action ($\ln SF = -\alpha d - \beta d^2$), where α and β are constants and d = dose).

Rejoining of DNA dsb Using SCGE. Initial DNA damage induction and the kinetics of repair were evaluated in all four cell lines by a neutral SCGE or "comet" assay, modified from Olive *et al.* (15). Briefly, cells were irradiated on ice over the dose range 10-100 Gy, then cells were harvested immediately (no repair) or incubated at 37°C for periods ranging from 15-360 min. Monolayers were harvested by trypsinization (0.05% trypsin-0.02% EDTA), and 5000 cells were embedded in 1% low gelling temperature agarose (Sigma Type VII) at a final concentration of 0.7% agarose and pipetted onto fully frosted, glass microscope slides (Dakin). Following the gelling of agarose on ice, cell lysis was undertaken using 0.5% SDS-30 mM EDTA and 0.25 mg/ml proteinase K (pH 8.0)(Boehringer) at 4°C for 1 h, followed by incubation in a

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³ The abbreviations used are: dsb, double-strand break(s); SCGE, single-cell microgel electrophoresis; PGFE, pulsed-field gel electrophoresis.

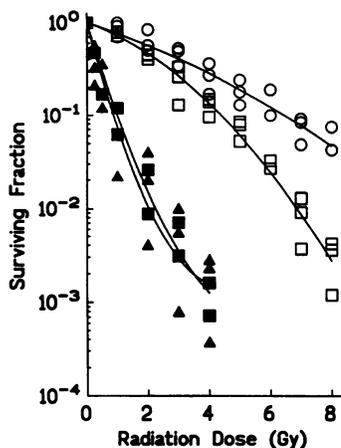


Fig. 1. Cellular radiosensitivity of wild-type CHO-K1 (○), *xrs-6* (▲), *xrs/Ku* (□), and *xrs/vec* (■). Each data point represents the mean value for an individual experiment.

waterbath at 37°C overnight. Slides were rinsed in 0.5X Tris-borate-EDTA before electrophoresis at 1 V/cm for 25 min in 0.5 TBE at 10°C. Slides were stained with ethidium bromide (20 ng/ml) and visualized by epifluorescence using a Zeiss Axioskop microscope. Images were digitalized, and DNA damage was expressed as a comet moment (16), defined as:

$$\text{Comet moment} = \frac{\sum_{0 \rightarrow n} \{(\text{Amount of DNA at distance } x) \times (\text{Distance migrated})\}}{\text{Total DNA}}$$

where x = distance from center of mass of head and n = no. of estimations at regular intervals from the proximal end of the head to the distal end of the tail. Approximately 50 comets/data point were acquired, and the results presented are the pooled data from a minimum of three independent experiments on each cell line.

Rejoining of DNA dsb Using Pulsed-field Gel Electrophoresis. For pulsed-field gel electrophoresis, cells were labeled with $2[^{14}\text{C}]$ thymidine (specific activity, 1.90 GBq/mmol; 51.4 mCi/mmol) at 1 mCi/ml for 48 h and chased with nonradioactive medium for 18 h. After irradiation, cells were harvested at various time points from 0 to 4 h following a single dose of 20 Gy or at 4 h following a series of dose points from 0 to 70 Gy. Further repair was prevented by the addition of ice-cold phosphate buffered saline (PBS-A) and by keeping the flasks on ice.

After suspension in PBS-A, the cells were centrifuged at 4°C at 1000 rpm for 8 min. The cell pellets were then resuspended in 0.8% low-melting-point agarose (LMP agarose; Sigma Chemical Co.) in PBS-A at 37°C at a concentration of 4×10^6 cells/ml. The suspension was pipetted into plug moulds (250 μ l Bio-Rad) which had been stored in 0.1 M HCl to inhibit exogenous nuclease activity. These were placed at 4°C until the agarose set. The plugs were removed and suspended in ice-cold lysis buffer containing 1 mg/ml proteinase-K (Boehringer-Mannheim) in 2% lauryl-sarcosine (Sigma)/0.5 M EDTA at pH 7.6. Plugs in lysis solution were held on ice for 1 h and then incubated at 37°C for 24 h (17). Plugs were then stored at 4°C and were assessed by pulsed-field gel electrophoresis within 1 week of irradiation.

For electrophoresis, cell plugs were sectioned into samples of approximately 25 μ l and loaded into wells of a 0.8% agarose gel and run in a Bio-Rad CHEF-DR11 PFGE system. Electrophoresis conditions were: 0.5X TBE buffer, switching time 60 min, 45 V for 96 h. Buffer temperature was maintained at 14°C by circulating through a cooling bath. Yeast chromosome molecular weight markers from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were run to standardize DNA migration measures. Following electrophoresis, the gels were stained in 0.5 μ l/ml ethidium bromide for at least 1 h and then destained in distilled water for 2–4 h. Gels were photographed under UV illumination (Chromato-vue Transilluminator, TM-15;UVP, Inc., Cambridge, MA), using Polaroid “55” film.

Following photography under UV light, each lane of the gel was cut into 5-mm sections. Gel pieces were heated slowly until melted in 1 ml of 1 M HCl and neutralized with 1 ml of 1 M NaOH; the liquified samples were then mixed

with scintillation fluid (UniScint). Isotope activity (disintegrations/min, dpm) was determined on a 2000 CA Tricarb liquid scintillation analyzer (Packard).

The number of dsb induced in DNA of irradiated cells is related to the fraction of DNA that is able to migrate out of the well under PGFE. The fraction extracted (*FE*) is the ratio of isotope counts detected in the sample lane to the total counts.

$$FE = \frac{\text{dpm lane}}{\text{dpm lane} + \text{dpm well}}$$

For dsb rejoining experiments, the results were expressed at each time point as the percentage of the damage initially induced.

Results

Complementation of Cellular Radiosensitivity. Fig. 1 shows the comparative radiosensitivity of the four cell lines. The *xrs-6* mutant was hypersensitive to ionizing radiation by comparison with the parental line CHO-K1 as shown by surviving fractions at 2 Gy of 0.01 and 0.63, respectively. Transfection of the *xrs* cells with the human *Ku80* gene greatly increased its radioresistance but not completely to the level of the parental cell line. *xrs-6/vec*, in which the transfected *Ku* cDNA sequences had been lost, exhibited identical sensitivity to the *xrs-6* mutant.

Induction and Repair Kinetics of DNA Damage. Fig. 2 shows that initial levels of DNA damage were indistinguishable in all three cell lines examined by SCGE. Fig. 3a shows dsb rejoining data measured using the SCGE assay. For CHO-K1, disappearance of DNA damage was rapid over the first 60 min. The levels of dsb were indistinguishable from background in CHO-K1 beyond 240 min. By contrast, *xrs-6* showed final levels of residual dsb in the region of 60% of initial damage. *xrs-6/Ku* showed similar kinetics of rejoining to CHO-K1, although the amount of residual damage after 180 min was significantly greater for the *xrs/Ku* cell line.

The time course for rejoining of dsb in CHO-K1 and *xrs-6* cells was very similar using PFGE to that which we and others have reported previously for CHO-K1 and *xrs-5* cells (Fig. 3b; Ref. 18). The rejoining of dsb was rapid over the first hour and then entered a slow phase of repair. The repair kinetics are not defined well enough in this study to determine whether the rates of repair, in contrast to the final unrejoined levels, differ between the two cell lines. Rejoining appears to have been essentially complete after 3–4 h, and the final level of dsb is much higher in *xrs-6* than in CHO-K1. This is confirmed by the results of a dose-response study in each of the three cell lines measuring unrejoined breaks present 4 h after irradiation (Fig. 4). Consistent with the SCGE data, Figs. 3 and 4 demonstrate that the rejoining defect in *xrs-6* is largely complemented by the human *Ku80*

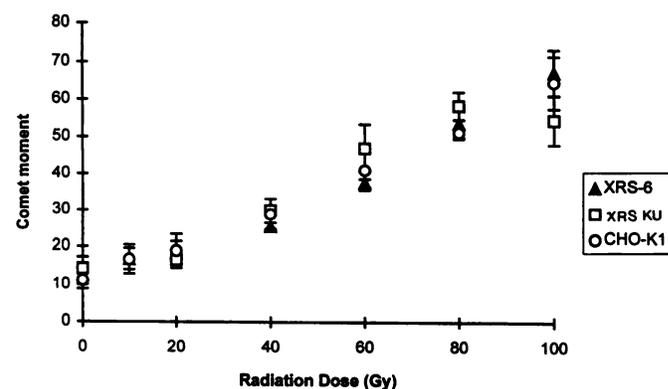


Fig. 2. Induction of DNA damage in CHO-K1 (○), *xrs-6* (▲), and *xrs/Ku* (□) measured by neutral SCGE. Data points are mean values and SEM for a minimum of three individual experiments on each cell line. Bars, SE.

gene, although again it is seen that the level of residual damage is probably higher in *xrs*/*Ku80* than in CHO-K1.

Discussion

In this study, we have demonstrated that the human *Ku80* gene can largely complement the DNA dsb rejoining defect in the *xrs-6* radiosensitive mutant of CHO-K1 cells using two independent measures of DNA strand breakage. This complementation is reflected in the increase in clonogenic cell survival in the complemented cells. Thus, we have added support to the data of Taccioli *et al.* (13) which characterized the *Ku80* gene as being the *XRCC5* gene on the basis of the reversal of the survival and V(D)J rejoining defects in *xrs* cells.

The *Ku80* protein is a subunit of the Ku protein, a DNA end-binding protein that recognizes DNA ends in a manner which is relatively independent of the structure of the end (9). Its mechanism of action in dsb rejoining is not yet known, but two possibilities have been proposed (14). The physical attachment of the Ku protein to the DNA end may protect that end from degradation by exonucleases in the cell or may act as a "helper molecule" for the repair enzymes by guiding them to the damage or holding the ends of a dsb together. In addition, the Ku protein forms a complex with another very large protein (*M*, 350,000) to form a DNA-dependent kinase (DNA-PK). *In vitro* DNA-PK can phosphorylate and, therefore, potentially control the regulation of several proteins including p53 (19). However, it is not known to date whether p53 is phosphorylated *in vivo* by DNA-PK. Thus, although there is little evidence for inducible dsb repair systems in mammalian cells, the activation of critical genes may be required for repair to take place. Resolution of this mechanism awaits the further characterization of the Ku activity.

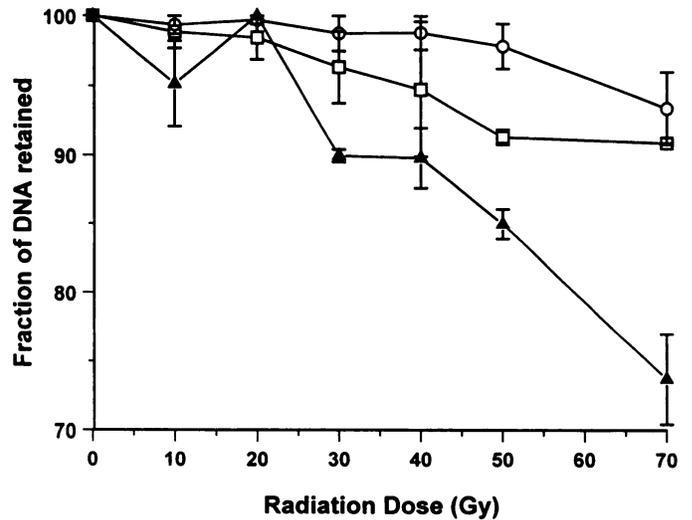


Fig. 4. Comparative levels of DNA dsb rejoining at 4 h, measured by PFGE, and expressed as the percentage DNA retained in well as a function of initial radiation dose. CHO-K1 (○), *xrs-6* (▲), *xrs*/*Ku* (□), and *xrs*/*vec* (■).

The complementation of the *xrs* phenotype by the human *Ku80* was not complete either in terms of survival or the final extent of dsb rejoining. It is not clear why this might be. The rodent and human *Ku80* are not completely homologous, as demonstrated by the ability to develop species-specific probes and antibodies (20). Thus, the human protein may not have full function in the hamster cells. In addition, the level of expression or the constitutive expression of the transfected gene may not be optimal.

A further aspect of this work is the comparison between the neutral SCGE assay and PFGE. PFGE has been well characterized for its ability to measure DNA dsb, but the neutral pH version of the SCGE assay as used here has only recently been developed for the rapid quantification of dsb. The similarity of the results obtained by the two methods in this study add further support to the view that this form of the SCGE assay has a useful place in the study of DNA dsb induced by ionizing radiation (15).

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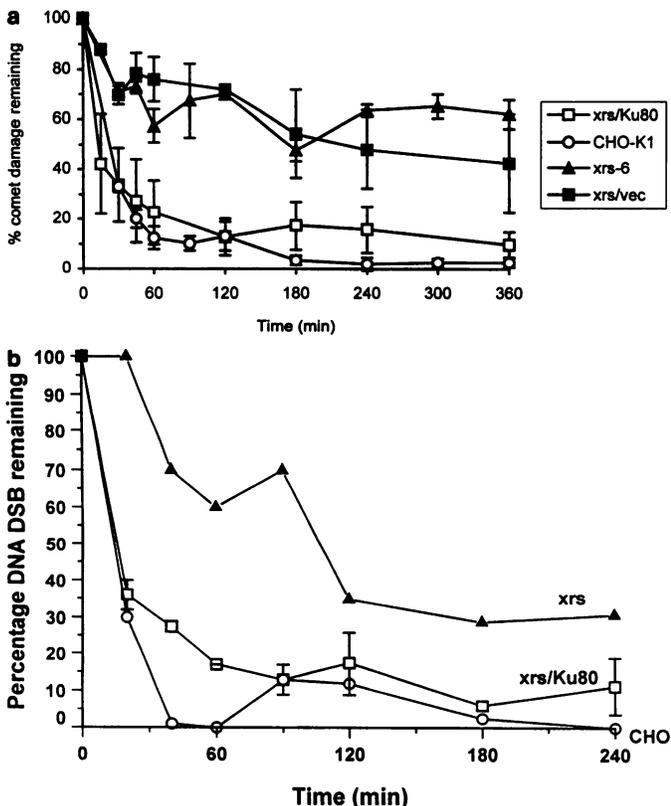


Fig. 3. a, repair kinetics of DNA damage measured by SCGE assay. b, repair kinetics of DNA damage measured by PFGE assay. CHO-K1 (○), *xrs-6* (▲), *xrs*/*Ku* (□), and *xrs*/*vec* (■).

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