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Gene Recombination in X-Ray-Sensitive Hamster Cells

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Recombination was measured in Chinese hamster ovary (CHO-K1) cells and in the X-ray-sensitive mutants *xrs*1 and *xrs*7, which show a defect in DNA double-strand break repair. To assay recombination, pairs of derivatives of the plasmid pSV2*gpt* were constructed with nonoverlapping deletions in the *gpt* gene region and cotransferred into the different cell types. Recombination efficiencies, measured as the transformation frequency with a pair of deletion plasmids relative to that with the complete pSV2*gpt* plasmid, were about 6% in both CHO-K1 and the *xrs* mutants for plasmids linearized at a site outside the *gpt* gene. However, these efficiencies were substantially enhanced by the introduction of a double-strand break into the homologous region of the *gpt* gene in one of a pair of deletion plasmids before cotransfer. This enhancement was apparently only about half as great for the *xrs* cells as for CHO-K1, but variation in the data was considerable. A much larger difference between CHO-K1 and the *xrs* mutants was found when the DNA concentration dependence of transformation was explored. While the transformation frequency of CHO-K1 increased linearly with DNA concentration, no such increase occurred with the *xrs* mutants irrespective of whether complete plasmids or pairs of deletion plasmids were transferred. The fraction of cells taking up DNA, assayed autoradiographically, was similar in all cell types. Therefore we suggest that while homologous recombination of plasmid molecules may not be substantially reduced in the *xrs* mutants, processes involved in the stable integration of plasmid DNA into genomic DNA are significantly impaired.

In microbes, a genetic link has been established between ionizing-radiation resistance and recombination proficiency; prominent examples of the genes involved are found in the RecF pathway of *Escherichia coli* (41) and in the *RAD52* group of *Saccharomyces cerevisiae* (14). Additionally, mutation of such genes often affects the ability to repair DNA double-strand breaks. Thus, it has been hypothesised that double-strand breaks induced by ionizing radiation are rejoined by a process of recombination between homologous chromosomal regions (24).

To investigate the relationship between ionizing-radiation resistance and recombination in mammalian cells, we have examined the recombination proficiencies of wild-type and X-ray-sensitive (*xrs*) hamster cells. The *xrs* mutants were isolated from a Chinese hamster ovary (CHO) cell line by Jeggo and Kemp (10). These mutants lack the ability to recover from potentially lethal radiation damage (38) and show reduced repair of DNA double-strand breaks (11, 43). The two mutants selected for this study, *xrs*1 and *xrs*7, belong to the same complementation group (9) and differ only slightly in their responses to ionizing radiations (10, 38), but show substantially different responses to other genotoxic agents (10).

The study of recombination in mammalian cells has been facilitated in the last few years by the use of virus- and plasmid-based gene transfer methods. It has been shown that mammalian cells have the enzymatic processes necessary for both homologous and nonhomologous recombination of introduced DNA (6, 12, 23, 27, 31, 32, 34, 39, 40, 44). We have attempted to apply these methods quantitatively by carefully controlling DNA transfer conditions to determine plasmid recombination efficiencies in the parent and mutant CHO cells. Additionally we have followed up the observation, made initially for plasmid-chromosome recombination in *S. cerevisiae* (21), that a double-strand break in regions of homology greatly stimulates recombination. While our experiments were in progress on plasmid-plasmid recombination in CHO cells, it was reported that double-strand breaks are recombinogenic for plasmids introduced into mouse and human cells (13, 17). Our results support this finding but suggest that the defective repair of double-strand breaks in the *xrs* mutants may not result from a reduction in homologous recombination proficiency.

(Preliminary results of this study have been published [J. Thacker, A. Stretch, A. Hamilton, and N. Jones, Br. J. Cancer **51**:609, 1985].)

MATERIALS AND METHODS

Cell culture. The CHO-K1 Chinese hamster cell line and its *xrs*1 and *xrs*7 X-ray-sensitive derivatives were kindly supplied by P. A. Jeggo (National Institute for Medical Research, London). Cells were removed from a stock held at −70°C and grown as monolayers for 2 to 3 days prior to each experiment in α-complete medium, as described previously (38).

Construction of deletion plasmids. The plasmid pSV2*gpt*, kindly supplied by P. Berg (Stanford University), was maintained in *E. coli* HB101, and plasmid DNA was prepared by the method of Clewell and Helinski (5) to give preparations containing predominantly supercoiled molecules. Deletion plasmids, as shown in Fig. 1, were constructed by cutting with the relevant restriction endonucleases and conversion of cohesive ends to blunt ends (19) before religation at a DNA concentration of 20 μg/ml. *E. coli* HB101 was transformed with the ligation mix by the method of Kushner (15). Minipreparations of plasmid DNA from selected bacterial clones were made (4), and restriction endonuclease digests were examined on agarose gels to select the correct deletion plasmids.

DNA transfer. Prior to transfer, plasmid DNA was cut with the appropriate restriction endonuclease, checked for complete cutting on a 0.8% agarose minigel, and phenol extracted. After ethanol precipitation and dissolving in TE

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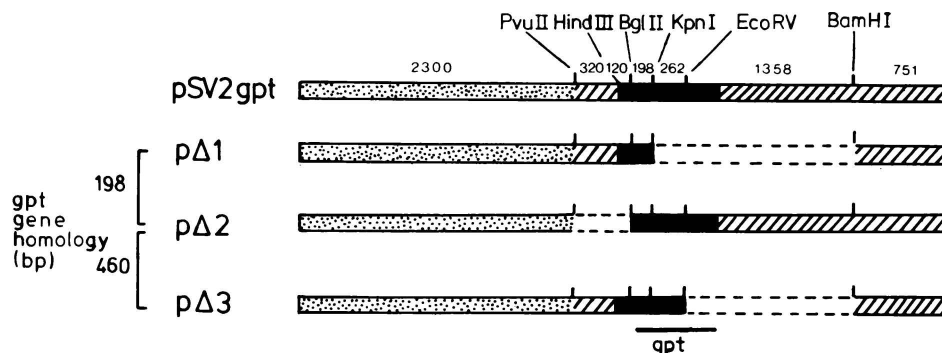

FIG. 1. Structure of pSV2*gpt* and derived deletion plasmids, all shown linearized at the *Eco*RI site. Dotted area, pBR322 plasmid sequence; hatched area, SV40 sequence; solid area, *E. coli gpt* gene and flanking sequence. Dashed lines indicate deleted sequence; lengths are shown in base pairs (bp). The position of the *gpt* gene is indicated (bar), and to the left, the length of the homologous region within *gpt* is given for the two pairs of deletion plasmids.

buffer (10 mM Tris, pH 7.6, 1 mM EDTA), the DNA was filtered on a Centricon column (Amicon) and washed several times with TE buffer before the concentration was checked against standards on a minigel as before. Monolayers of cells seeded the previous day at 4×10^5 per 25-cm^2 flask (CHO) or 5×10^5 per flask (*xrs* mutants) were exposed to a calcium phosphate-DNA precipitate by the method of Frost and Williams (7). No carrier DNA was included. To prepare the DNA precipitate, plasmid DNA was diluted into IHS buffer (137 mM NaCl, 21 mM HEPES [*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid], 6 mM glucose, 5 mM KCl, 0.7 mM $Na_2HPO_4 \cdot 2H_2O$, adjusted to pH 7.14 with NaOH) to a concentration of 20 μg/ml for pSV2*gpt* and single deletion plasmid transfers or 40 μg/ml when deletion plasmids were cotransferred (i.e., 20 μg of each per ml) and 1/16 volume of 2 M $CaCl_2$ was added. This mixture was left at room temperature for 20 min with occasional agitation for the precipitate to form. The medium was removed from the flasks, and 0.5 ml of DNA precipitate was added to each before incubation for 20 min at 37°C on a rocking platform. α-Complete medium was then added, and the cells were incubated for a further 6 h at 37°C. After this time, the medium was removed and 15% glycerol in IHS buffer (1 ml per flask) was added for 3 min at 37°C. The glycerol was replaced by fresh medium, and unless otherwise stated, the cells were incubated for a further 2 days before respreading into selective medium.

Selection of *gpt*-transformed cells. Cells expressing *gpt* activity (guanine-xanthine phosphoribosyltransferase enzyme) were selected by respreading at 2×10^5 cells per 9-cm dish in XHATM medium (α-complete medium supplemented with xanthine [10 μg/ml], hypoxanthine [6.8 μg/ml], azaserine [3.5 μg/ml], thymidine [1.8 μg/ml], and mycophenolic acid [10 μg/ml]), with 5 to 20 dishes per treatment. Cells from the same cultures were also diluted and respread at 200 per 9-cm dish in nonselective medium to assess viability (cloning efficiency). Cells were incubated for 8 to 11 days (viability) or 11 to 14 days (transformation), depending on the cell line, to achieve comparable colony sizes. Colonies were counted with a binocular microscope, with a 50-cell size as the criterion for acceptance. In these experiments, average cell viabilities were found to vary as follows: CHO, 98.8%; *xrs*1, 61.7%; and *xrs*7, 69.8%. It was important to measure cell viability in DNA transfer experiments since at its extremes for any one cell type the range of viability was threefold.

DNA uptake. *Eco*RI-cut pSV2*gpt* was radioactively labeled by nick translation (26) with [^{3}H]dTTP (~100 Ci/mmol; Amersham International). DNA transfer was carried out as described above, except that unlabeled thymidine was added to the growth medium (final concentration, 2.5×10^{-8} M) in considerable excess of that potentially available from the labeled nucleotide. After exposure to the plasmid, cells were treated as recommended by Loyter et al. (18) to remove exogenous DNA, including EDTA treatment followed by incubation in DNase I (60 μg/ml) and micrococcal nuclease (60 U/ml).

The cells were then prepared for autoradiography by treatment with hypotonic solution (0.56% KCl for 6 min) and fixative (3:1, methanol-acetic acid) before spreading onto glass slides. Photographic emulsion (Ilford K5) was applied, and grains were counted 7 to 9 days later. The labeled fraction (see Results) is given as the fraction of the cell population having more than 10 grains per cell from a count of 200 cells per treatment.

Isolation of independent transformants. Cells were seeded into multiwell dishes at 2×10^5 per well and incubated for 24 h. DNA transfer with pΔ2 plus pΔ3 was carried out as described above, and the contents of each well were separately subjected to selection for *gpt* proficiency. A single transformant was selected from the colonies arising from each well.

Molecular analysis of transformant DNA. Methods of DNA isolation, gel electrophoresis, Southern blotting, and probe hybridization were as described previously (36). The probe was prepared as an *Hin*dIII-*Bam*HI fragment of plasmid pL10, kindly supplied by P. Berg (Stanford University). pL10 has the bacterial *gpt* gene inserted between the *Hin*dIII and *Bam*HI sites of pBR322.

RESULTS

Experiments with deletion plasmids 1 and 2. pΔ1 and pΔ2 were constructed with convenient endonuclease cut sites in pSV2*gpt* and have a 198-base region of homology (Fig. 1). Plasmids were linearized with *Eco*RI (as shown in Fig. 1) and precipitated onto cells at 10 μg per flask (or at 10 μg of each per flask for mixtures of the two deletion plasmids) to keep the concentration of complete *gpt* gene sequences constant. CHO cells exposed to precipitates of each deletion plasmid separately gave no measurable transformation to *gpt* proficiency (frequency per viable cell, $<2.2 \times 10^{-7}$). Similar experiments with pΔ1 and pΔ2 mixed together immediately before precipitating onto cells yielded *gpt* transformants at

TABLE 1. Average transformation frequencies per 10^4 viable cells[a]

Cell line	Avg transformation frequency ± 1 SD (no. of expt)			
	pSV2*gpt* (*Eco*RI cut)	pΔ1 + pΔ2 (*Eco*RI cut)	pSV2*gpt* (*Kpn*I cut)	pΔ2 + pΔ3 (uncut and *Kpn*I cut)
CHO	4.53 ± 2.36 (14)	0.27 ± 0.27 (4)	0.46 (2)	8.44 ± 5.64 (8)
*xrs*1	2.58 ± 1.54 (8)	0.14 (2)	0.20 (2)	1.79 ± 1.35 (5)
*xrs*7	2.71 ± 1.33 (5)	0.15 (2)	0.22 (1)	2.71 (2)

[a] Frequencies shown with 1 standard deviation of the mean for more than two experiments (the number of independent experiments is shown in parentheses). DNA at 10 μg per flask (or 10 μg of each per flask for mixtures of deletion plasmids) was used, with a 2-day expression time before respreading into selective medium. The average number of colonies counted per experiment was as follows. pSV2*gpt* (*Eco*RI cut): CHO, 597; *xrs*1, 216; *xrs*7, 215. pΔ1 plus pΔ2 (*Eco*RI cut): CHO, 147; *xrs*1, 44; *xrs*7, 46. pSV2*gpt* (*Kpn*I cut): CHO, 92; *xrs*1, 34; *xrs*7, 33. pΔ2 plus pΔ3 (uncut and *Kpn*I cut): CHO, 1,250; *xrs*1, 335; *xrs*7, 732.

frequencies of about 3×10^{-5} per viable CHO cell, while *xrs*1 and *xrs*7 cells were transformed at about half this frequency (Table 1). These transformation frequencies are approximately 6% of the respective frequencies with which each cell type was transformed by the complete (undeleted) pSV2*gpt* plasmid. It is difficult to exploit these relatively low recombination efficiencies to analyze potential differences between cell lines. Thus, following Orr-Weaver et al. (21), we explored the use of deletion plasmids with an endonuclease-generated double-strand break in the recombination region as substrates for recombination. pΔ3 was constructed for this purpose; when paired with pΔ2, the *Kpn*I endonuclease site may be used to give a double-strand break in a 460-base homologous region (Fig. 1).

Recombination of deletion plasmids 2 and 3. pΔ3 on its own gave no measurable transformation to *gpt* proficiency (frequency per viable cell, $<2.4 \times 10^{-7}$) while mixtures of pΔ2 and pΔ3 cut with *Eco*RI prior to transfer gave a transformation frequency of 2.6×10^{-5} per viable CHO cell (very similar to pΔ1 plus pΔ2, Table 1). Cutting pSV2*gpt* with *Kpn*I reduced the transformation frequencies of CHO and the mutants about 10-fold compared with those of *Eco*RI-cut pSV2*gpt* (Table 1). However, when pΔ2 and pΔ3 were both cut with *Kpn*I prior to mixing and transfer, a transformation frequency of 4.6×10^{-4} per viable CHO cell was found. Cotransfer of one circular (pΔ2) and one *Kpn*I-cut deletion plasmid (pΔ3) gave yet higher frequencies of transformation, and this procedure was adopted for the remaining experiments (Table 1, Fig. 2 and 3). As is seen in Table 1, the average transformation frequencies were higher for CHO cells than for the *xrs* mutants, particularly with pΔ2 plus pΔ3 transfers, but the data show considerable statistical variation. These data also suggest that transformation by the complete pSV2*gpt* and by pΔ2 plus pΔ3 may occur at a similar frequency in a given cell line (the largest discrepancy in this comparison is for the CHO cell line, but these data also show the largest variation).

In an attempt to highlight differences between CHO and the mutant lines, the effect of DNA concentration on transformation frequency was studied. As shown in Fig. 2A for pΔ2 plus pΔ3, the averages found at 10 μg of DNA per flask reflect different responses to DNA concentration: the *xrs*1 cells showed little increase in transformation frequency with DNA concentration, while the CHO cells showed an approximately 10-fold increase over the concentration range used. Also shown in Fig. 2B is an experiment with pSV2*gpt* which confirmed that the response for the complete plasmid was similar to that for the recombined deletion plasmids (see also Table 1).

A factor which may have a differential effect on the observed transformation frequencies is the length of time after DNA transfer before cells are selected for *gpt* activity (the expression time). To check this, cells exposed to pΔ2 plus pΔ3 were respread into selective medium at 1, 2, and 3 days after transfer. An example of one experiment, in which an overall high frequency was found, is given in Fig. 3. The difference between CHO and *xrs*1 cells was maintained after different expression times, and the data again indicate the similarity of pSV2*gpt* and pΔ2 plus pΔ3 transformation frequencies. Overall, a 2-day expression time was justified since a decline in transformation frequencies with longer expression times was sometimes found.

DNA uptake. A potential additional variable in the quantitative estimation of plasmid-mediated transformation of different cell types is the fraction of cells which take up DNA. To assess uptake, pSV2*gpt* was labeled by nick translation with [^{3}H]dTTP and precipitated onto CHO and

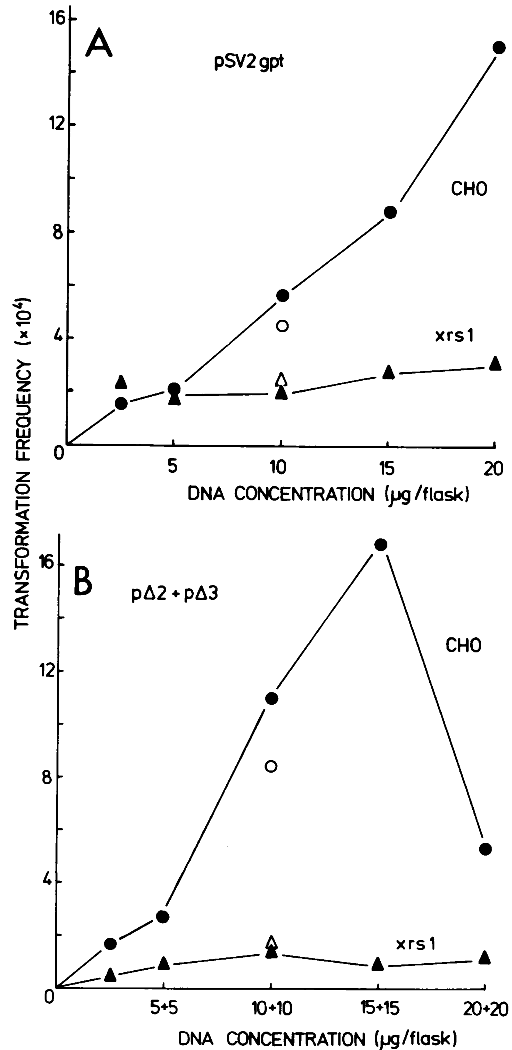

FIG. 2. Effect of concentration of DNA on the transformation frequency per 10^4 viable cells by (A) complete pSV2*gpt* or (B) uncut pΔ2 plus *Kpn*I-cut pΔ3. Solid symbols show representative data from experiments with CHO (circles) or *xrs*1 (triangles); open symbols show average data for one concentration from Table 1.

*xrs*1 cells as in the experiments described above. After a 6-h exposure (without glycerol treatment) or a 24-h exposure (with glycerol treatment at 6 h), exogenous DNA was enzymatically removed before preparation for autoradiography (see Materials and Methods). CHO cells showed a labeled fraction of 27% (6 h) and 77% (24 h), while for *xrs*1 this fraction was 21% (6 h) and 77% (24 h), suggesting that there is no difference in the number of cells taking up plasmid DNA.

Molecular analysis of recombination. High-molecular-weight DNA from independently isolated transformants of CHO, *xrs*1, or *xrs*7 cells carrying recombined plasmids (pΔ2 plus pΔ3) was cut with *Hin*dIII and *Bam*HI. The DNA was separated by gel electrophoresis, blotted onto nitrocellulose, and hybridized to a probe from the *E. coli gpt* gene region. If the cells had correctly recombined the deletion plasmids, a 1.9-kilobase (kb) *Hin*dIII-*Bam*HI fragment should hybridize to the probe (see Fig. 1). A representative autoradiograph of CHO and *xrs*1 transformant DNAs is shown in Fig. 4A. The 1.9-kb fragment was present in every transformant. However, additional fragments hybridized to the *gpt* probe in most transformants, suggesting that a number of other (unrecombined) plasmids have integrated. These additional plasmid molecules may have integrated separately into the genome or may be present in one location as a contiguous group. To check this, high-molecular-weight DNA from transformants was cut with enzymes which do not have a restriction site in the pSV2*gpt* sequence. If a number of separate bands were still seen, then it is likely that the plasmids were integrated separately. However, as shown in Fig. 4B, only one relatively large fragment was found to hybridize in these conditions, suggesting that a group of plasmid molecules, including those recombining correctly, were integrated together.

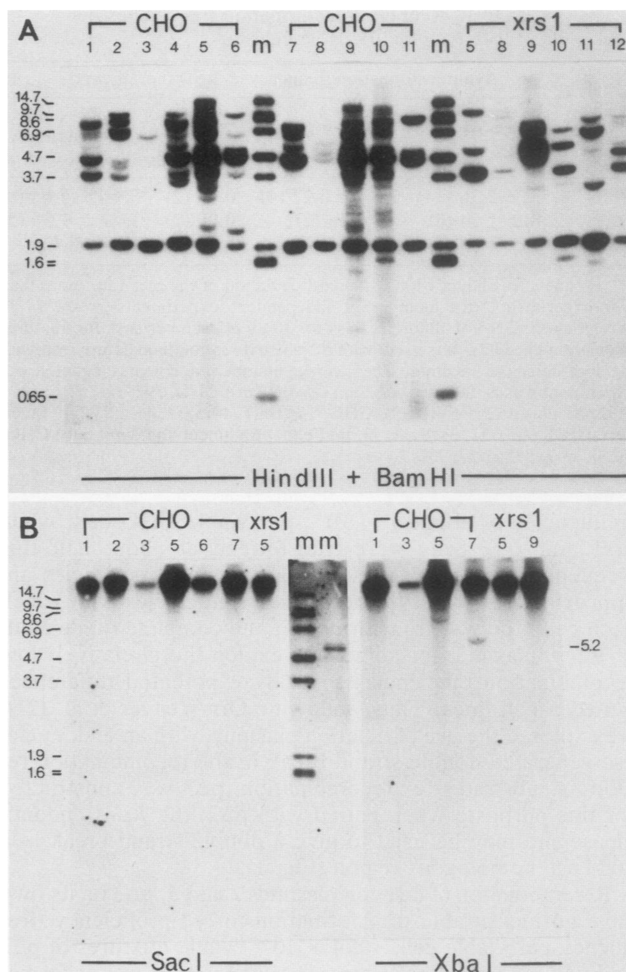

FIG. 4. Southern blot hybridization of high-molecular-weight DNA (10 μg per lane) from independent pΔ2-plus-pΔ3-transformed cell lines (CHO or *xrs*1; numbered arbitrarily) to a *gpt* gene probe. (A) DNA cut with *Hin*dIII and *Bam*HI; (B) DNA from some of the same cell lines cut with *Sac*I or *Xba*I (noncutters in pSV2*gpt*). Lanes m, Molecular weight markers; sizes are shown to the left (λ marker series) (in kilobases) or right (linear pSV2*gpt*, panel B only).

DISCUSSION

We have shown that CHO cells can efficiently recombine the *gpt* gene from transferred pairs of plasmids carrying nonoverlapping deletions. The recombination efficiency may be expressed arbitrarily as the transformation frequency after exposure to the deletion plasmids relative to that after exposure to the complete pSV2*gpt* plasmid, in each case corrected for cell viability at the time of selecting for *gpt* gene activity. On this basis, pairs of plasmids linearized at a site well outside their region of *gpt* gene homology gave recombination efficiencies of about 6%; this efficiency is within the range reported recently by others with pairs of deletion plasmids used to measure recombination of the *neo* gene in mouse and human cells (13, 35). The introduction of a double-strand break into the homologous region of pΔ3 and cotransfer with pΔ2 gave recombination efficiencies of about or >100% (Table 1). However, we should note that in these calculations we have chosen to compare pSV2*gpt* transformation frequencies at half the total DNA concentration used for the pairs of deletion plasmids to keep the number of complete *gpt* gene sequences constant. For both pSV2*gpt* and pΔ2 plus pΔ3, the transformation frequency for CHO cells had an approximately linear dependence on plasmid DNA concentration (Fig. 2). (The steep decline in pΔ2 plus pΔ3 transformation frequency above 15 μg of each per flask is not understood, although it may represent selective lethality of high DNA concentrations on cells integrating plasmid

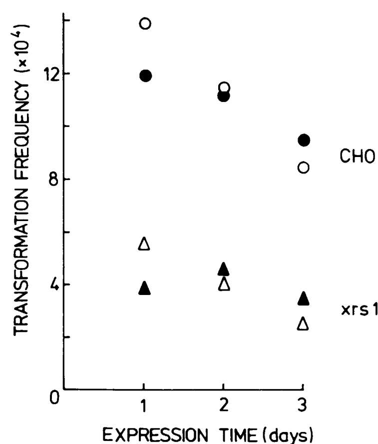

FIG. 3. Effect of posttransfer interval (expression time) before respreading cells into selective medium on the transformation frequency per 10^4 viable cells. Closed symbols, pSV2*gpt* transfers; open symbols, pΔ2 plus pΔ3 transfers. Conditions of transfer as given in Table 1, footnote *a*.

DNA into essential parts of the genome.) While there is considerable statistical variation in measuring transformation frequencies (Table 1), it may also be noteworthy that at DNA concentrations greater than 5 μg of each per flask, the frequencies found for pΔ2 plus pΔ3 were approximately double those for pSV2*gpt* (Fig. 2).

In comparison to the CHO parent cells, at total DNA concentrations in excess of 5 to 10 μg per flask, the *xrs* mutants showed much lower transformation frequencies (Fig. 2 and 3). This is particularly evident in Fig. 2, which shows that an increase in DNA concentration had little effect on *xrs*1 transformation frequency by either pSV2*gpt* or pΔ2 plus pΔ3. A comparison of the transformation frequencies of *xrs* cells by pSV2*gpt* and pΔ2 plus pΔ3 (Table 1, Fig. 2) suggests that their recombination efficiencies may be about 50% lower than those for CHO cells. However, as noted above, the variation in such data is relatively large, so that recombination efficiencies for CHO and the *xrs* mutants overlap when errors are taken into account.

We have tested the system for other variables that might influence these results. Expression times between 1 and 3 days had little effect on transformation frequencies, although occasionally, as in Fig. 3 (pΔ2 plus pΔ3 into CHO cells), some decline was seen at the longer expression times. The fraction of cells taking up DNA was high and did not vary between the CHO parent and *xrs* mutant cells (see Results). While this uptake led to transformation of only a small proportion of viable cells, it is evident from Fig. 4 that many transformed cells carried a number of tandemly integrated plasmids. Since only one correctly recombined *gpt* gene is required for transformed cells to grow in the selective medium, the presence of a number of plasmids in each cell may add some uncertainty to the estimation of recombination efficiencies. However, our molecular analyses (Fig. 4; data not shown) suggest that the parent CHO cells and the *xrs* mutants have a similar wide range of copy numbers for both the correctly recombined gene and the total *gpt* sequences hybridizing to the probe. Also in a separate series of experiments (not shown), we have found that CHO and *xrs*1 transformants show a similar wide range of stabilities, as measured by challenge with selective (XHATM) medium after growth for 20 to 30 doublings in nonselective conditions.

The reduction in transformation frequency for *xrs* cells compared with CHO cells (Fig. 2) does not, therefore, appear to follow from large differences in either the proportion of cells taking up plasmid DNA or homologous recombination efficiencies. We suggest, however, that the reduced transformation frequency observed for *xrs* mutants may result from some defect in the process by which the *gpt* gene is stably integrated into hamster genomic DNA. This deficiency could still involve recombination insofar as integration requires the breakage of genomic DNA and rejoining to plasmid DNA (or vice versa, since we cannot distinguish reduced integration from increased instability of the integrated plasmid). The *xrs* defect may therefore be in a process similar to those which are presently termed indiscriminate, illegitimate, or nonhomologous recombination (1–3, 6, 20, 22, 28–30, 34, 44). It is of interest in this context that ionizing-radiation damage is efficient at promoting illegitimate recombination (e.g., translocations between nonhomologous chromosomes) but inefficient at inducing sister chromatid exchange (presumed to require extensive homology) in cultured mammalian cells (16). In these terms, the *xrs* gene product would seem to be unlike the *S. cerevisiae* *RAD52* gene product, which is required for homologous integration of nicked or gapped DNA but not for circular plasmid integration or excision of integrated plasmids (8, 21).

Biochemical measurement of DNA double-strand break repair suggests that more than one type of rejoining process operates in irradiated hamster cells (25, 42). However, there is little molecular information on mechanisms of repair of radiation-induced strand breakage (37). A recent analysis (28) of nonhomologous recombination of endonuclease-broken simian virus 40 (SV40) molecules transferred into monkey cells concluded that two mechanisms prevail: direct ligation and repair synthesis primed by terminal homologies of a few nucleotides. Given the sequence diversity of the mammalian genome, the latter mechanism might operate to integrate foreign DNA, and indeed, some studies have implicated short homologies in the integration (33) and rearrangement (30) of viral sequences in mammalian genomes.

Experiments designed to assess strand break rejoining of damaged plasmids in the *xrs* mutants and other radiation-sensitive mutants isolated recently in this laboratory are in progress.

After the present paper was submitted, a short report on the recombination efficiency of another of the *xrs* series of mutants, *xrs*5, was published (P. D. Moore, K.-Y. Song, L. Chekun, L. Wallace, and R. Kucherlapati, Mutation Res. **160**:149–155, 1986). While the authors claim a sixfold reduction in plasmid recombination efficiency for *xrs*5, the transformation frequencies they found were very variable, and they omitted to correct for cell viability after DNA transfer. These factors make it difficult to prove a difference in recombination proficiency between CHO and *xrs* cells, as was found in the present study. In accordance with this view also, Moore et al. found no significant difference between CHO and *xrs*5 cell extracts in the ability to mediate recombination of plasmid DNA in vitro.

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