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Cellular Response to DNA Damage Is Enhanced by the pR Plasmid in Mouse Cells and in *Escherichia coli*

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The pR plasmid, which enhances the survival of *Escherichia coli* C600 exposed to UV light by induction of the SOS regulatory mechanism, showed the same effect when it transformed mouse LTA cells (*tk*$^-$, *aprt*$^-$). With Tn*5* insertion mutagenesis which inactivates UV functions in the pR plasmid, we recognized two different regions of the plasmid, uvp1 and uvp2. These pR UVR$^-$ mutants exhibited the same effect in LTA transformed cells, demonstrating that resistance to UV light, carried by the pR plasmid, was really due to the expression of these two regions, which were also in the mouse cells. Statistical analysis showed that the expression of the uvp1 and uvp2 regions significantly increased ($P < 0.01$) the survival upon exposure to UV light in mouse cells and bacteria. These results might suggest the presence of an inducible repair response to DNA damage in mouse LTA cells.

The pR plasmid is a 23-kilobase plasmid derived from a *Hin*dIII fragment of TP120 (11). This plasmid, similar to pKM101 (18), enhances the survival of *Escherichia coli* C600 exposed to UV light (UV survival) by interacting with the SOS regulatory mechanism.

The multifunctional response to DNA damage, termed SOS repair pathways, is induced in *E. coli* and other bacteria by a variety of adverse conditions. DNA degradation products or single-stranded DNA activate a DNA protease (RecA protein). The RecA protease activity in turn destroys a repressor molecule (LexA product), causing the cell to make more RecA and to unmask many other genes involved in repair, recombination, mutagenesis, and delay of cell division (19, 27).

The SOS response has two major characteristics: (i) it is induced by the agents that interfere with DNA replication and (ii) it induces genetic variation within the bacterial population (point mutation, general and site-specific recombination mutagenesis, and delay of cell division), and perhaps genetic rearrangements (30).

A major area of interest is the question of whether a comparable regulatory mechanism also exists in higher organisms. To date no one has yet isolated and characterized mammalian RecA and LexA proteins comparable to those of bacteria, and for this reason it is difficult to study directly an SOS-like stress response system based on a common pathway in mammalian and in bacterial cells. The most important data that document an SOS-like system in mammalian cells are the studies on mutagen-induced reactivation of simian virus 40, herpes simplex virus, and adenovirus (2, 6, 15, 25) and the data derived by split dose experiments (23). More controversial than the UV light-enhanced reactivation of damaged mammalian viruses is the evidence for induction of a mutagenic activity in mammalian cells (5, 20, 24). All the data are based on the response of mammalian cells to the insults of the same agents that cause SOS response in *E. coli*.

Elli et al. (10) have reported the surprising result that when the pR plasmid is introduced into LTA mouse cells their UV survival is enhanced, suggesting the presence of an inducible repair system in these cells. This trait is maintained in these cells for several generations, indicating the existence of a stable genetic change. In this paper we demonstrate that enhanced resistance to UV light (UV resistance) is due to the expression of the UVR function of the pR plasmid since pR UVR$^-$ mutants lose enhanced UV resistance both in *E. coli* and mouse cells.

MATERIALS AND METHODS

Plasmids and cell lines. The pR (Ap$^+$ UV$^+$) plasmid is an *Hin*dIII fragment (23 kilobase pairs) of TP120 (11). ptk1 (Ap$^+$ *tk*$^+$) (kindly supplied by S. Bacchetti) contained a selectable *tk*$^+$ marker. It is derived from a 3.4-kilobase pair fragment of herpes simplex virus type 1 cloned into the *Bam*HI site of pBR322 (13). LTA cells (*tk*$^-$ *aprt*$^-$) used as wild type for UV light sensitivity were obtained from S. Bacchetti and maintained in α-minimal essential medium (α-MEM) plus 10% fetal calf serum (FCS) (12).

LA-TU and LA-D cell lines (*tk*$^+$ UV$^+$) were LTA cells transfected with both ptk1 and pR plasmids and maintained in α-MEM containing hypoxanthine (0.1 mM), aminopterine (1 μM), and thymidine (40 μM) (HAT) plus 10% FCS (Difco Laboratories, Detroit, Mich.). LA-TU is an uncloned cell line, and the LA-D cell line is a cloned derivative of LTA (*tk*$^+$ UV$^+$) cells (10).

Isolation and restriction enzyme analysis of pR insertion mutants. Tn*5* insertions in pR were isolated by infecting *E. coli* C600 cells harboring plasmid pR with the λ phage vector carrying Tn*5* transposon (λ *B*221 *c*I857 Tn*5* *kan*). The transductants were selected on agar plates containing ampicillin and kanamycin (1). pR::Tn*5* DNAs were isolated from cultures of *E. coli* as described by Humphrey et al. (14), digested with restriction endonucleases under the conditions recommended by the supplier (Boehringer GmbH, Mannheim, Federal Republic of Germany), and electrophoresed on 0.8% agarose gels in Tris-borate-EDTA buffer. Molecular weights were determined with a λ DNA marker digested with *Hin*dIII enzyme.

In vitro construction of pLM54 (deletion derivative of pR).

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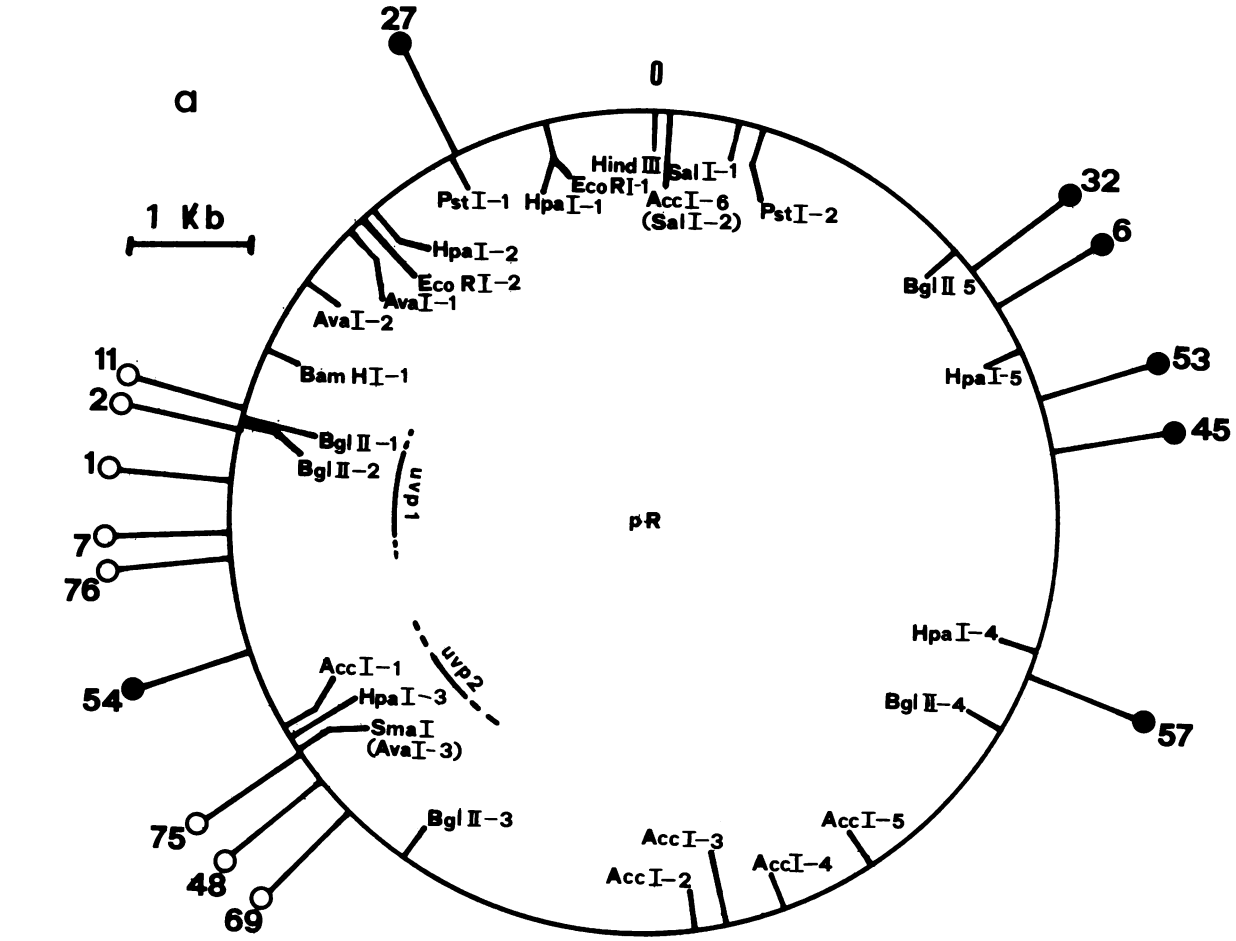

FIG. 1. Restriction maps of pR and pLM54. (a) pR restriction map showing the position of 15 Tn*5* insertions and the regions of pR DNA essential for expression of the UVR phenotype. Symbols: ○, Tn*5* insertions that inactivate UV resistance; ●, Tn*5* insertions that do not inactivate UV resistance. The isolation numbers of Tn*5* insertions are shown next to the symbols. (b) Restriction map of pLM54 (deletion derivative of pR). The thick line represents the inverted repeat of Tn*5*. Restriction enzymes cleavage sites are numbered according to their counterclockwise order from the *Hin*dIII site. The regions of DNA essential for expression of the UVR phenotype are uvp1 and uvp2.

One of the pR::Tn*5* plasmids, named pR54R, was chosen to construct the pLM54 plasmid, a deletion derivative of pR. pR54R DNA was digested with *Hin*dIII enzyme and incubated with T4 ligase (Boehringer) in 20 μl of ligation buffer. The condition of ligation favored the circularization rather than polymerization of fragments (7). *E. coli* C600 was transformed with the ligated DNA by the method of Cohen et al. (4). The transformants were selected on agar plates containing ampicillin (20 μg/μl) and replicated onto plates containing kanamycin (30 μg/μl). The Ap^+ Kan^- colonies were tested for UV light sensitivity. The DNA of pLM54, constructed by this procedure, was extracted, digested, and electrophoresed as described above.

Transformation, cotransformation, and selection. Transformation and cotransformation of LTA tk^- cells with ptk1 only and with ptk1 and various uncleaved pR DNA mutants were carried out by the calcium phosphate technique (12, 29) as previously described (10). The transformants were selected

in HAT medium plus 10% FCS. After 2 weeks in selective medium (HAT), tk^+ clones were picked from independent plates grown into mass cultures and maintained in HAT medium plus 10% FCS, including during UV light and 4-nitroquinoline-*N*-1-oxide (4NQO) treatment. The generation time of all cell lines tested was similar (24 to 25 h).

DNA extraction and dot blot hybridization. High-molecular-weight DNA from LTA transformed cells was prepared as described by Wigler et al. (29). For dot blot hybridization, 10 to 30 μg of mouse cell DNA was denatured by incubation in 0.2 M NaOH, neutralized in 1 M ammonium acetate (final pH 8.5 to 9), and spotted onto nitrocellulose membrane filters (17). Filters were dried in vacuo at 80°C, prehybridized in 10× Denhardt solution–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate at 65°C for 2 h and then hybridized for 12 h at 65°C in the same solution containing α^{32}P-nick-translated pR DNA as a probe (Amersham Corp. nick translation kit) at 10^7 cpm/ml with specific activity $>10^8$ cpm/μg. After hybridization, filters were washed extensively three times with successive 500-ml portions of 2× SSC, 1× SSC, and 0.1× SSC at 65°C. Filters were exposed to Fuji X-ray films for autoradiography.

UV light and 4NQO treatment. Bacterial and mouse cells were irradiated as previously described (10, 11). The 4NQO (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol (10^{-2} M) and then diluted in α-MEM to give the desired final concentration.

LTA mouse cells (6×10^2) were seeded in 60-mm petri dishes and, after 24 h, irradiated or incubated at 37°C for 1 h with various concentrations of 4NQO.

Colonies were stained and counted after 15 days of growth in α-MEM plus 20% FCS (LTA cells) or in α-MEM plus HAT and 20% FCS (transformed tk^+ cell lines). Surviving fractions for each dose were obtained from an average of two plates.

RESULTS

Localization of two regions responsible for UV resistance in the pR plasmid. To localize in the pR genome the regions involved in UV resistance, we isolated a number of independent insertion mutants with the Tn*5* transposon. The pR restriction map and the localization of 15 Tn*5* insertions in plasmid pR, obtained by a standard restriction technique, are shown in Fig. 1. *E. coli* C600 cells transformed by Tn*5* insertion derivatives of pR were tested for UV light sensitivity (Table 1). In the mutants which lost the ability to enhance UV survival (i.e., wild type resistance), the insertion of Tn*5* was localized to two different regions. The other mutants, which maintained UV resistance, have the Tn*5* insertion outside these two regions (Fig. 1a). In one of these mutants (pR54R), Tn*5* was localized between these two regions, named uvp1 and uvp2. The particular insertion of Tn*5* in plasmid pR54R and the presence of one *Hin*dIII site in each inverted repeat of Tn*5*, have allowed us to construct a deletion derivative of pR. For this purpose, pR54R DNA was cleaved with *Hin*dIII, and ligated with T4 ligase, and ampicillin-resistant transformants were then selected (see Materials and Methods). This procedure yielded a derivative

TABLE 1. Characterization of Tn*5* insertion derivatives of pR

Plasmid	Insertion point (kb)[a]	Inactivated region[b]	UV phenotype[c]
pR1S	5.5	uvp1	UVR⁻
pR2S	5.0	uvp1	UVR⁻
pR6R	19.4		UVR
pR7S	5.9	uvp1	UVR⁻
pR11S	4.8	uvp1	UVR⁻
pR27R	1.6		UVR
pR32R	19.7		UVR
pR45R	18.0		UVR
pR48S	8.3	uvp2	UVR⁻
pR53R	18.4		UVR
pR54R	7.0		UVR
pR57R	15.9		UVR
pR69S	8.7	uvp2	UVR⁻
pR75S	8.0	uvp2	UVR⁻
pR76S	6.1	uvp1	UVR⁻

[a] Position in counterclockwise order from pR *Hin*dIII site. kb, Kilobase.
[b] Inactivation by Tn*5* insertion into either of the two regions (uvp1 and uvp2) involved in UV resistance.
[c] The UV phenotype was determined by UV survival curves performed as previously described (11). UVR⁻, UV resistance was not significantly different from that of wild-type *E. coli*. (The D_0 values ranged from 25 to 27 J/m²). UVR, Significant enhancement of UV resistance. (The average of the D_0 values was 55 J/m².)

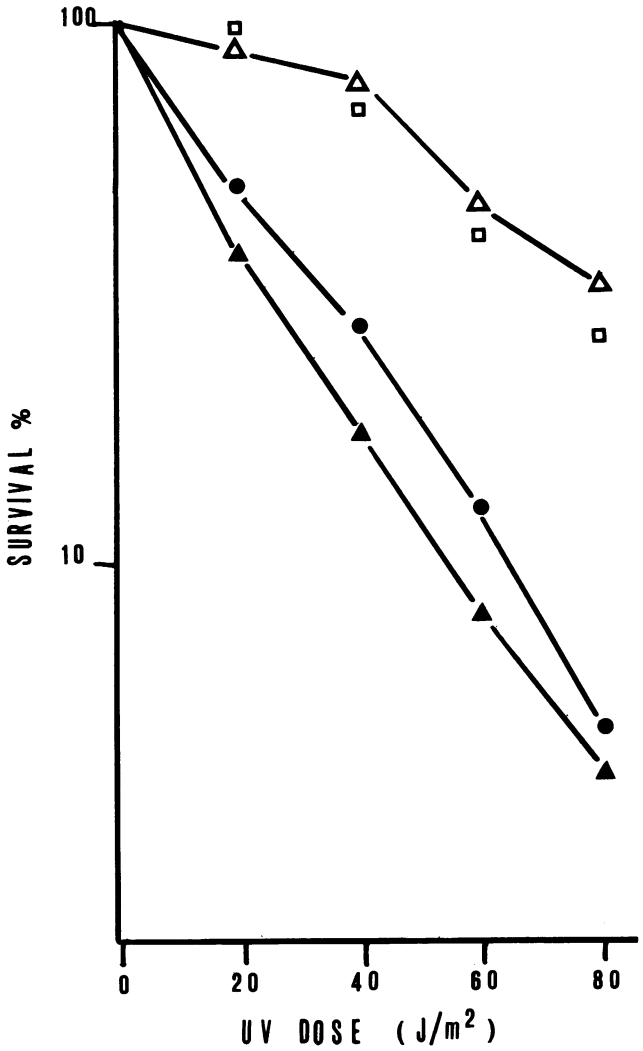

FIG. 2. UV survival curves of *E. coli* C600 transformed with pR, pLM54 (deletion derivative), or p54R (insertion derivative). Cells were irradiated with UV light at a dose rate of 5 J/m² per s. Symbols: ●, *E. coli* C600; □, *E. coli* C600 plus pR; ▲, *E. coli* C600 plus pLM54; △, *E. coli* C600 plus pR54R.

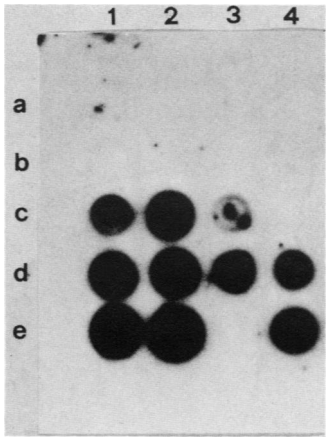

FIG. 3. Presence of pR DNA in different mouse cells lines as detected by dot blot hybridization. DNA isolated from the clones shown in Table 2 was spotted onto a nitrocellulose filter and hybridized with α-^{32}P-labeled pR. Lane 1, DNA from LTA cells transformed with pR54R. (a) Blank; (b) LA540; (c) and (d) LA541; (e) LA542. Lane 2, DNA from LTA cells transformed with pR69S. (a) LA691; (b) LA692; (c) through (e) LA690. Lane 3, DNA from LTA cells transformed with pR76S and DNA from LTA cells. (a) LA761; (b) LA762; (c) and (d) LA760; (e) LTA. Lane 4, DNA from LTA cells transformed with ptk1 and DNA from LTA cells transformed with pR wild type. (a) blank; (b) LAtk0; (c) LAtk1; (d) LA-D; (e) LA-TU.

of pR, called pLM54 (8 kilobase pairs), in which DNA containing the uvp2 region was deleted (Fig. 1b). The survival curves of pLM54 and pR54R are shown in Fig. 2. The comparison of the survival curves shows that pLM54 lost the ability to enhance UV survival in *E. coli* C600. Therefore, we can conclude that the two regions, uvp1 and uvp2, are not contiguous but are both responsible and necessary for the expression of UVR phenotype in bacteria.

Expression of UV resistance of pR mutants in mammalian cells. The correlation between UV resistance and presence of pR DNA sequences has been ascertained in all cell lines cotransformed by pR and ptk1 DNA as demonstrated in a previous work (10). The expression of UV resistance in two of these cell lines (LA-TU and LA-D), characterized at passages 15 to 18 as previously described, was analyzed at passages 35 to 40 to verify the stability of the expression of this genetic trait. The results of dot blot hybridization analysis showed the presence of the plasmid also after passage 40 (Fig. 3, lanes 4d and e), and the analysis of the survival curves (Table 2) shows the characteristic enhancement of UV survival (the D_0 value of LA-TU and LA-D was about two times that of the untransformed parental line). To analyze the expression of the uvp1 and uvp2 regions in mammalian cells, appropriate and representative pR::Tn*5* mutants were selected and used in cotransformation experiments. For this purpose, LTA cells were cotransformed with plasmid ptk1 as a selectable marker and with the following pR mutants: pR76S (uvp1$^-$ uvp2$^+$), pR69S (uvp1$^+$ uvp2$^-$) in which only one of the two regions was inactivated, and pR54R (uvp1$^+$ uvp2$^+$) in which the transposon insertion was outside the two regions. For each cotransformation experiment, three independent clones were picked from separate plates and put into mass cultures under selective pressure (HAT medium). The cell lines obtained were LA760, LA761, LA762, LA690, LA691, LA692, LA540, LA541, and LA542. In addition, two clones were also isolated from LTA cells transformed with ptk1 only, and the cell lines thus obtained were named LAtk0 and LAtk1. All these cell lines were screened both for the presence of pR DNA sequences and for UV and 4NQO survival. The analysis of randomly isolated LTA *tk*$^+$ transformants by dot blot hybridization with pR as probe showed that clones LA760, LA690, LA541, and LA542 were positive for the presence of pR DNA sequences (Fig. 3). The other transformed *tk*$^+$ LTA cells, i.e., LA761, LA762, LA691, LA692, and LA540, did not show hybridization with a pR DNA nick-translated probe; therefore, these last cell lines, selected in HAT medium, were transformed only with ptk1 DNA.

The cell lines positive for the plasmid by blot hybridization were then analyzed for their sensitivity to UV light and 4NQO. Although the damage induced by these two mutagens is different (UV light induces pyrimidine dimers, whereas 4NQO produces quinoline purine adducts), the lesions are supposed to be repaired by a process in common,

TABLE 2. Characterization by dot blot hybridization and survival quantitative parameters of transformed LTA cells[a]

Transforming DNA	Cell line	Physical presence of pR sequences	$\bar{D}_0 \pm$ SE (J/m^2)	$\bar{D}_q \pm$ SE	$\bar{n}$
None	LTA	−	3.0 ± 0.4	2.3 ± 0.3	2
ptk1	LAtk0	−	3.2 ± 0.4	2.1 ± 0.3	1.9
ptk1	LAtk1	−	2.8 ± 0.2	2.7 ± 0.2	2.5
ptk1 plus pR wild type	LA-TU	+	6.3 ± 0.2	5.5 ± 0.1	2.3
ptk1 plus pR wild type	LA-D	+	5.8 ± 0.3	3.8 ± 0.2	1.9
ptk1 plus pR76S (uvp1$^-$ uvp2$^+$)	LA760	+	3.1 ± 0.2	1.2 ± 0.8	1.4
ptk1 plus pR76S (uvp1$^-$ uvp2$^+$)	LA761	−	2.8 ± 0.1	2.3 ± 0.07	2.2
ptk1 plus pR76S (uvp1$^-$ uvp2$^+$)	LA762	−	3.3 ± 0.02	1.8 ± 0.01	1.7
ptk1 plus pR69S (uvp1$^+$ uvp2$^-$)	LA690	+	3.1 ± 0.03	2.8 ± 0.02	2.4
ptk1 plus pR69S (uvp1$^+$ uvp2$^-$)	LA691	−	2.7 ± 0.1	2.4 ± 0.1	2.3
ptk1 plus pR69S (uvp1$^+$ uvp2$^-$)	LA692	−	2.7 ± 0.03	2.0 ± 0.02	2.1
ptk1 plus pR54R (uvp1$^+$ uvp2$^+$)	LA540	−	2.6 ± 0.4	1.6 ± 0.4	1.8
ptk1 plus pR54R (uvp1$^+$ uvp2$^+$)	LA541	+	5.6 ± 0.3	4.2 ± 0.2	2.1
ptk1 plus pR54R (uvp1$^+$ uvp2$^+$)	LA542	+	6.6 ± 0.1	3.4 ± 0.07	1.6

[a] The value of $\bar{D}_0$ and $\bar{D}_q$ was derived by irradiation experiments as previously described (10). From an operational point of view, the $\bar{D}_0$ value is the dose reducing the surviving fraction by a factor of 1/e, measured on the exponential portion of the survival curve fitted by the least square method. Parameters: $\bar{n}$, intercept on the ordinate obtained by back extrapolation of the exponential portion of the curve; $\bar{D}_q$; intercept on the abscissa resulting from back extrapolation of the exponential portion of the curve. $\bar{D}_q$ was calculated from the relationship $\bar{D}_q = \bar{D}_0 \ln \bar{n}$ (9). The results for each cell line were obtained from two to four experiments, each performed on different passages.

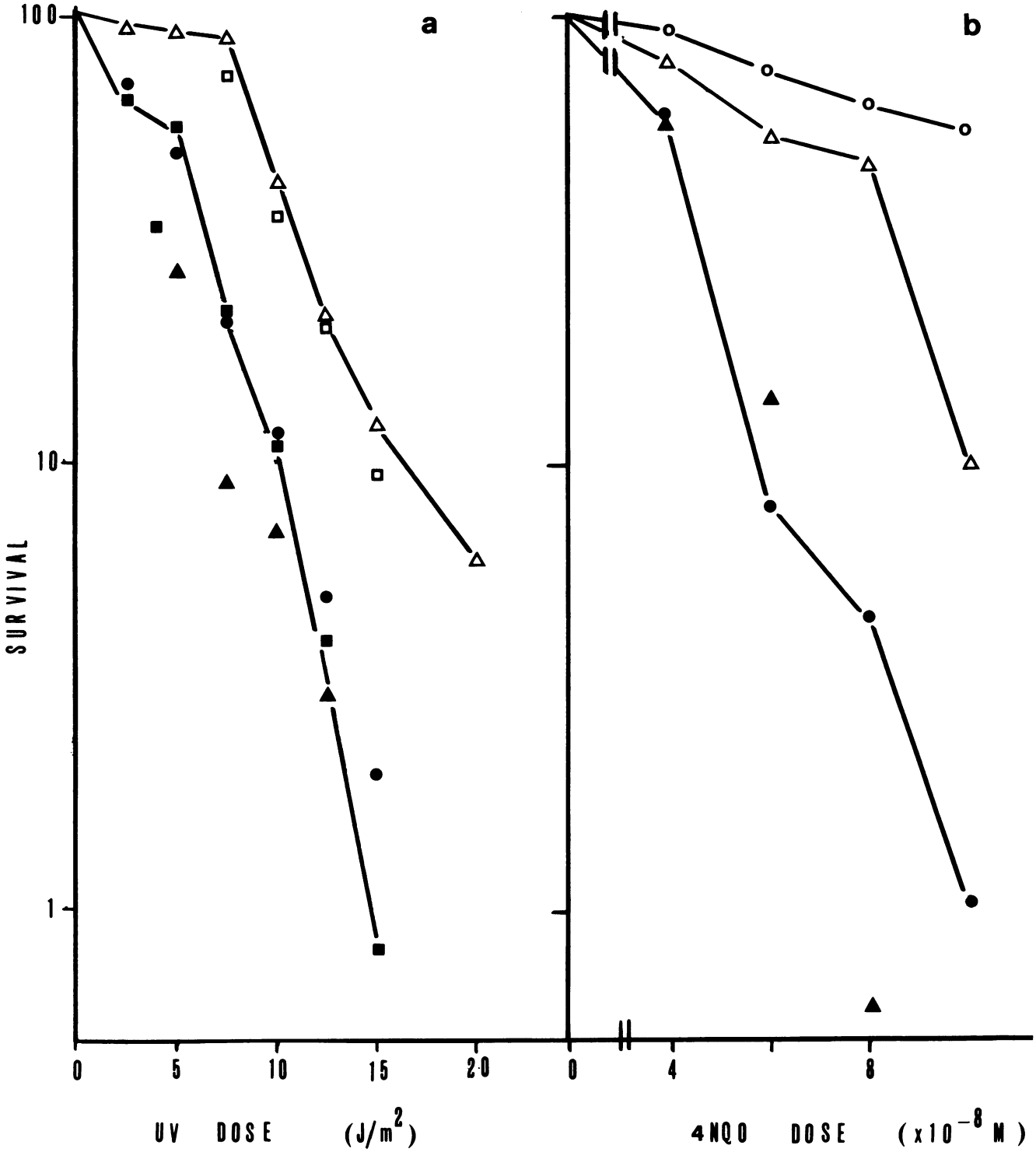

FIG. 4. (a) UV survival curves of LTA cells cotransformed with ptk1 and pR mutant DNA. Cells were irradiated with UV light at a dose rate of 1.25 J/m^2 per s. The fluences were measured with a Latarjet UV meter. The survival of the LA-D cell line was determined at passage 40 in HAT medium; for the other cell lines, there were about 35 passages in HAT medium. Symbols: △, LA541; □, LA-D line; ●, LA690; ▲, LA760; ■, LAtk1. (b) 4NQO survival curves of LTA cells cotransformed with ptk1 and pR mutant DNA. Cells were exposed to 4NQO at various concentrations for 1 h. Symbols: ○, LA-TU line; △, LA541; ▲, LA760; ●, LA690.

at least in part, to both (26). In this respect, the effect of pR on UV and 4NQO survival has been evaluated to determine a correlation between the repair pathways in bacteria and mammalian cells.

The quantitative parameters of the UV survival curves of the cell lines tested are listed in Table 2, and the survival in 4NQO is shown in Fig. 4b. As determined by the Student *t* test for comparison of slopes, the UV survival curves of

LA760 and LA690 cell lines, transformed with pR76S and pR69S respectively, are not significantly different from those of LTA, LAtk0, and LAtk1 control cell lines (Table 2, Fig. 4a). Therefore, the presence in LA690 and LA760 cells of the pR DNA from UVR$^-$ mutant plasmids confers a wild-type level of UV resistance. The survival curves of LA541 and LA542 cell lines, containing pR54R which enhances UV resistance in *E. coli*, show an increased resistance not different from those of LA-TU and LA-D cell lines containing pR wild type (Table 2 and Fig. 4a). On the average, the D_0 values of UV light-resistant cell lines were 2.1 times that of control cell lines. The remaining cell lines (LA761, LA762, LA691, LA692, and LA540), which were negative for pR sequences, exhibited wild-type UV survival. The slopes of the survival curves of UV light-resistant clones versus UV light wild-type cells were significantly different ($P < 0.01$) as determined by the Student t test.

The results obtained after treatment of representative transformed cell lines with 4NQO showed that uvp1 and uvp2 regions of pR conferred a significantly enhanced resistance to 4NQO compared with the survival of LA760 and LA690 cell lines (Fig. 4b). The comparison of the results obtained with both UV and 4NQO survival and by dot blot hybridization of mouse cells transformed with pR UVR$^-$ mutants showed that the ability to enhance UV and 4NQO resistance is lost in LA760 and LA690 lines, while this function is expressed in the cell lines (LA541 and LA542) transformed with pR54R in which the uvp1 and uvp2 regions are not inactivated.

DISCUSSION

By studying 15 Tn*5* insertion mutants and one deletion mutant of pR, we identified two regions of the pR plasmid involved in UV resistance.

The analysis of these mutants allows us to conclude that both regions are necessary for expression of the UVR phenotype in bacterial cells. On the basis of a comparison of the UV resistance function of the pR and pKM101 plasmids (18), we suggest that the most significant difference is that the pR plasmid carries two nonadjacent regions involved in UV resistance and that the uvp1 region maps within the region defined Rep in pKM101 plasmid. It is worth noting that all the Tn*5* insertions in the uvp1 region eliminate the ability of pR to increase UV resistance but not the ability of the plasmid to replicate autonomously. Furthermore, the Tn*5* insertions that allowed us to identify uvp2 are localized in the same position as the pKM101 *mucB* gene.

These observations allow us to make two hypotheses: (i) the genes involved in UV resistance in pR could be analogous to the *muc* pKM101 genes, but they have a different structural organization; (ii) uvp1 could be a region with a regulatory function for the expression of uvp2. Preliminary experiments seem to support the latter hypothesis (P. A. Battaglia et al., work in progress).

In the last years, several authors showed that plasmid or bacterial genes, carrying antibiotic resistance, are expressed in yeast cells (3, 16, 28). Moreover, with respect to the expression of prokaryotic genes in mammalian cells, the most suitable vectors are animal viruses: simian virus 40 and polyomavirus DNAs are among the most usual vectors (8, 21). Combining phage, plasmid, or viral DNAs allows the construction of so-called "shuttle vectors" which can be replicated (and possibly expressed) in either prokaryotic or eukaryotic cells.

The pR plasmid, wild type or UVR mutant, transforms and is expressed in mouse LTA cells although it is not cloned in a eukaryotic vector. Nevertheless, we cannot exclude at the present state of our knowledge the possibility that pR is not integrated in the mouse genome or that its expression is due to the formation of concatenamers with ptk1 DNA.

Transformation experiments carried out in mouse cells with pR and pR mutants have demonstrated that: (i) both uvp1 and uvp2 regions are essential for the expression of UVR phenotype in mouse cells as in bacteria; (ii) this plasmid confers a stable genetic change in the expression of this function in mammalian cells. This trait does not seem to be conferred by pKM101 to LTA cells; in fact, transformation experiments with pKM101 have been unsuccessful in producing expression of a stable UVR phenotype (unpublished data). Furthermore, Porter et al. (22) reported that the introduction of plasmid pKM101-associated *muc* genes in *Saccharomyces cerevisiae* determined a detectable increase in mutagenic effect but not a significant enhancement of UV survival.

The results obtained with plasmid pR both in prokaryotic and mammalian cells suggest that this plasmid may be very suitable for investigating whether an inducible repair pathway is present also in mammalian cells for the following reasons: (i) it enhanced UV survival both in bacteria and mouse cells; (ii) the uvp1 and uvp2 regions are involved in the SOS repair system in bacteria probably amplifying or triggering this process; (iii) plasmid pR, although not cloned in a eukaryotic vector, is capable of giving rise to a stable UVR phenotype in transformed mouse cells.

The possibility of making genetic and molecular investigations with plasmid pR in prokaryotic cells and the ability of pR to express UV resistance, even in mouse cells, make it possible to compare some SOS functions in bacteria with some inducible responses to DNA damage in mammalian cells.

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