LACK OF CHEMICALLY INDUCED MUTATION IN REPAIR-DEFICIENT MUTANTS OF YEAST

LOUISE PRAKASH

Department of Radiation Biology and Biophysics,
University of Rochester School of Medicine and Dentistry,
Rochester, New York 14642

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ABSTRACT

Two genes, *rad6* and *rad9*, that confer radiation sensitivity in the yeast *Saccharomyces cerevisiae* also greatly reduce the frequency of chemically-induced reversions of a tester mutant *cyc1-131*, which is a chain initiation mutant in the structural gene determining iso-1-cytochrome *c*. Mutations induced by ethyl methanesulfonate (EMS), diethyl sulfate (DES), methyl methanesulfonate (MMS), dimethyl sulfate (DMS), nitroquinoline oxide (NQO), nitrosoguanidine (NTG), nitrogen mustard (HN2), β-propiolactone, and tritiated uridine, as well as mutations induced by ultraviolet light (UV) and ionizing radiation were greatly diminished in strains homozygous for either the *rad6* or *rad9* gene. Nitrous acid and nitrosoimidazolidone (NIL), on the other hand, were highly mutagenic in these repair-deficient mutants, and at low doses, these mutagens acted with about the same efficiency as in the normal *RAD* strain. At high doses of either nitrous acid or NIL, however, reversion frequencies were significantly reduced in the two *rad* mutants compared to normal strains. Although both *rad* mutants are immutable to about the same extent, the *rad9* strains tend to be less sensitive to the lethal effect of chemical mutagens than *rad6* strains. It is concluded that yeast requires a functional repair system for mutation induction by chemical agents.

THE repair capacity of organisms not only determines their survival after exposure to agents which damage their DNA, but also influences their response to the mutagenic effects of these agents. In prokaryotic and eukaryotic micro-organisms, radiation-induced mutations have been shown to depend in part on a functional repair system. *Escherichia coli* strains carrying mutations at the *uvrA* or *uvrB* loci, i.e., defective in the ability to excise ultraviolet light (UV)-induced pyrimidine dimers, give much higher yields of mutations at doses which cause no detectable effect in the corresponding wild-type strains (HILL 1965; WITKIN 1966; BRIDGES and MUNSON 1968; KONDO *et al.* 1970). As in *E. coli*, excision-defective mutants of the yeast *Saccharomyces cerevisiae* show greatly increased UV-induced reversion frequencies for several different loci tested (NAKAI and YAMAGUCHI 1969; RESNICK 1969; ZAKHAROV, KOZINA and FEDOROVA 1970; AVERBECK *et al.* 1970; LAWRENCE *et al.* 1974). Recombination-deficient strains of *E. coli* having a mutation at either the *exr* (or *lex*) locus (WITKIN 1967; BRIDGES, LAW and MUNSON 1968) or the *recA* locus (WITKIN 1969a; KONDO *et al.* 1970), on the other hand, are refractory to UV-induced mutation. UV mutagenesis is now thought to result from errors in post-replication repair,

via recombinational repair, of gaps occurring opposite unexcised pyrimidine dimers (WITKIN 1967; BRIDGES, DENNIS and MUNSON 1967; RUPP *et al.* 1971).

Strains of yeast which do not respond to UV-induced mutation (*rev*) are also known (LEMONTT 1971). Two of the three *rev* loci, *rev1* and *rev3*, reduce UV-induced mutability at all loci tested (LEMONTT 1972). Although the *rev* mutants are somewhat sensitive to UV and X-ray, they show normal frequencies of meiotic recombination (LEMONTT 1971). They are therefore not recombination-deficient mutants and are not comparable to the *recA* mutants of *E. coli.* Nevertheless, a recombinational type of repair process may still be involved in UV mutagenesis in eukaryotic organisms.

Although a functional repair system is required for radiation mutagenesis, the role of repair in chemically induced mutations has not been clearly defined. Mutations induced by the so-called "radiomimetic" chemical mutagens such as methyl methanesulfonate (MMS) and nitroquinoline oxide (NQO), seem to depend on the same repair systems required for radiation-induced mutations. Excision-defective *E. coli* strains show increased sensitivity and increased mutability to both UV and NQO (KONDO *et al.* 1970). *E. coli rec* mutants show increased sensitivity and decreased mutability to UV, NQO, X-ray and MMS (WITKIN 1969a,b; KONDO *et al.* 1970). However, none of the known repair-deficient *E. coli* mutants, including the *rec* and *uvr* mutants, exhibit altered responses to mutation induction by non-radiomimetic chemical agents such as nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) (KONDO *et al.* 1970).

In this paper, we provide evidence that a functional repair system is indeed required for the induction of mutations by non-radiomimetic chemical mutagens, including EMS and NTG. We find that strains of the yeast *S. cerevisiae* carrying either the mutant genes *rad6* or *rad9*, two genes conferring radiation sensitivity (COX and PARRY 1968), show greatly reduced frequencies of induced mutation compared to the normal *RAD* strain. Both the *rad6* and *rad9* genes are involved in chemically-induced mutation since there was a great diminution in the reversion of a tester mutant, *cy1–131*, a chain initiation mutant in the structural gene determining iso-1-cytochrome *c* (STEWART *et al.* 1971), after treatment with most of the chemical mutagens tested, including EMS, NTG, and NQO. Of the eleven chemical mutagens tested, only nitrous acid and nitrosoimidazolidone (NIL) under some conditions, had the same effect on the *rad6*, *rad9* and the normal *RAD* strain; at low doses, revertibility in all three strains was virtually the same, while at high doses, mutability of the *rad6* or *rad9* strains was greatly reduced compared to the normal *RAD* strain. Thus, these two *rad* genes, which are not comparable to any of the genes described so far in *E. coli,* are required for chemically-induced mutations.

MATERIALS AND METHODS

Strains: The *cyc1–131* mutant was isolated by the chlorolactate procedure (SHERMAN *et al.* 1974) and contains an alteration of the initiation codon of the structural gene determining iso-1-cytochrome *c* (STEWART *et al.* 1971). The *cyc1–131* mutant requires a G:C to A:T transition in

order to yield true revertants and has been shown to revert with high frequencies with mutagens that show specificity for inducing G:C to A:T transitions as well as with moderate frequencies with non-specific mutagens (PRAKASH and SHERMAN 1973). The *rad6* and *rad9* mutations were isolated by COX and PARRY (1968). The *rad6* gene maps near *ole1* on chromosome VII (LAWRENCE, unpublished results.

Diploid strains used in these experiments were constructed from haploid stocks by standard procedures of yeast genetics. Meiotic segregants from the cross B-651 (a *cyc1–131 lys2 his1 trp2 RAD6+*) × CL21–4A (*α CYC1 rad6*) were used to generate the diploids LC-0 (*cyc1–131/cyc1–131 RAD6+/RAD6+ RAD9+/RAD9+*) and LC-6 (*cyc1–131/cyc1–131 rad6/rad6 RAD9+/RAD9+*). B-651 can^R (a canavanine-resistant derivative of B-651) was crossed to *α rad9 ade2–1*, kindly provided by B. S. COX. Haploid segregants obtained from random spores were used to generate diploid LC-9 (*cyc1-131/cyc1-131 RAD6+/RAD6+ rad9/rad9*). The three diploids thus were derived from segregants of related pedigrees. The diploid LC-9A was constructed by using the same a (LP23–90) as was used in the cross to generate LC-9. The *α* parent was obtained by crossing LP23–90 to *α cyc1–131 ilv3* (LP74–12), to yield the strain *α cyc1–131 ilv3 rad9* (LP118-ID). The full genotype of heterozygous markers in these strains is given in Table 1. The diploid strains are each homozygous for the *cyc1–131* allele, which is the site of reversion in the mutation experiments that follow. LC-0 is homozygous for the wild-type alleles of *rad6* and *rad9* and will be referred to as the *RAD* diploid, for the sake of brevity. LC-6 is homozygous for the *rad6* mutation but wild-type at the *RAD9* locus and will be referred to as the *rad6* diploid. Likewise, LC-9 and LC-9A, both homozygous for the *rad9* mutation but wild-type at the *RAD6* locus, will be referred to as the *rad9* diploids (Table 1).

Source of chemical mutagens: The mutagens were obtained from the following sources: methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), Eastman Organic Chemicals; diethyl sulfate (DES); Fisher Scientific Co.; dimethyl sulfate (DMS) and *N*-methyl-N′-nitro-*N*-nitrosoguanidine (NTG), Aldrich Chemical Co.; *β*-propiolactone (*β*-PL), trade name "Betaprone", Fellows Medical Mfg. Co., Oak Park, Mich.; nitrogen mustard (HN2), trade name "Mustargen", Merck, Sharp and Dohme; 1-nitrosoimidazolidone-2 (NIL), DRS. F. K. ZIMMERMANN (Technische Hochschule, Darmstade, Germany) and R. PREUSSMAN (Forschegruppe Präventivmedizin, Freiburg, Germany); [5-^{3}H] uridine ([^{3}H]-U), Schwarz-Mann; 4-nitroquinoline-1-oxide (NQO), DRS. Y. KAWAZOE and T. SUGIMURA (National Cancer Center, Tokyo, Japan) and was purchased from T. Schuchardt GMBH and Co. (Munich, Germany).

Since commercially available preparations of the monofunctional alkylating agents EMS, MMS, DES and DMS often contain impurities which increase toxicity but do not affect mutagenicity (LOPPES 1968; STRAUSS, personal communication), these chemical agents were repurified by vacuum distillation at 10 to 15 mm Hg before use.

Media and quantitative reversion frequencies: The selective media and methods used for determining reversion frequencies of *cyc1* mutants have been described in detail by PRAKASH and

TABLE 1

Strain numbers and genotypes

Common name	Strain no.	Genotype
RAD	LC-0	a *cyc1–131 RAD6+ RAD9+ his1 TRP2+* *α cyc1–131 RAD6+ RAD9+ HIS1+ trp2*
rad6	LC-6	a *cyc1–131 rad6 RAD9+ HIS1+ lys2* *α cyc1–131 rad6 RAD9+ his1 LYS2+*
rad9	LC-9	a *cyc1–131 RAD6+ rad9 HIS1+ lys2 TRP2* *α cyc1–131 RAD6+ rad9 his1 lys2 trp2*
rad9	LC-9A	a *cyc1–131 RAD6+ rad9 ILV3+ lys2* *α cyc1–131 RAD6+ rad9 ilv3 LYS2+*

SHERMAN (1973) and by PRAKASH, STEWART and SHERMAN (1974). Mutagenized and untreated cells were plated on lactate medium and the revertants were scored after 7 to 14 days of incubation at 30°. Viability before and after mutagenic treatment was determined by plating cells on glycerol medium and scoring after three days of incubation at 30°. A solution of filter-sterilized 10% sodium thiosulfate was used to inactivate the chemical mutagens at the end of the desired time, except in the cases decribed below. It should be pointed out that ρ^- strains (cytoplasmic "petites"), which may be induced by certain mutagens, do not grow on either glycerol medium or lactate medium, while the *cyc1* strains grow on glycerol medium but not on lactate medium.

Mutagenic treatments

1) Diethyl sulfate (DES): cells suspended in 0.05 *M* potassium phosphate buffer, pH 7.1 (phosphate buffer) were incubated for 20 min at 30° with 0.1 to 1.0% (v/v) DES.

2) Dimethyl sulfate (DMS): cells suspended in phosphate buffer were incubated for 12 min at 30° with 0.01 to 0.05% DMS (v/v).

3) Ethyl methanesulfonate (EMS): cells suspended in phosphate buffer were incubated for up to 5 hr at 30° with 0.5% EMS which was prepared by adding 0.1 ml EMS to 20 ml cells.

4) Methyl methanesulfonate (MMS): cells suspended in phosphate buffer were incubated for up to 40 min at 30° with 0.1% MMS (v/v).

5) Nitrogen mustard: (di-(2-chloroethyl)methylamine) (HN2); HN2 solutions were made just before use by dissolving the 10 mg content of a vial of "Mustargen" in phosphate buffer and diluting in the same buffer to the desired concentrations. Cells suspended in phosphate buffer were treated for 1 hr at 30° with 0.02 to 0.2 mg HN2/ml.

6) 4-nitroquinoline-1-oxide (NQO):NQO solutions were made just before use by dissolving to 1 mg NQO/ml in acetone and then diluting in phosphate buffer to the desired concentrations. Cells suspended in phosphate buffer were treated for 30 min at 30° with 0.1 to 1.0 μg NQO/ml.

7) Nitrosoimidazolidine (NIL): NIL solutions were made just before use by dissolving NIL as 10 mg/ml in phosphate buffer and diluting in the same buffer to the desired concentrations. Cells suspended in phosphate buffer were incubated with 0.05 to 0.4 mg NIL/ml for 30 min at 30°.

8) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG: NTG solutions were made just before use by dissolving to 10 mg/ml in acetone and then diluting in phosphate buffer to the desired concentrations. Cells suspended in phosphate buffer were treated for 40 min at 30° with 10 to 40 μg NTG/ml.

9) Nitrous acid (HNO_2): in order to obtain more reproducible results, the cells were starved by suspending in sterile distilled water and storing at 4° for 1 to 3 days. Starved cells were washed with sterile distilled water and resuspended in 0.5 *M* sodium acetate buffer, pH 4.8 (acetate buffer). HNO_2 was made just before use by dissolving sodium nitrite in acetate buffer to the desired concentrations. Cells suspended in acetate buffer was treated for 20 min at 30° with 0.1 to 0.5 mg HNO_2/ml. The reaction was terminated by the addition of an equal volume of a solution of 2.7% $Na_2HPO_4 \cdot 7H_2O$/1% yeast extract.

10) β-propiolactone (β-PL): cells suspended in phosphate buffer were incubated at 30° for 1 hr with 0.004 to 0.02% β-PL (v/v) prepared just before use in sterile water. The reaction was terminated by centrifugation and washing the cells with water.

11) Tritiated-uridine([5^3-H]-U): described in detail in PRAKASH and SHERMAN (1973). Cells were grown for 16 hours in a synthetic glucose medium containing 50 μCi[5-^{3}H]-U/ml. The next day, cells were washed several times in distilled water. resuspended in 0.05 M KH_2PO_4 and stored at 4°C. Cells were plated for viability and number of revertants both immediately and after 10 days of storage. A control culture was treated as described above, except that the [^{3}H]-U was omitted from the medium.

RESULTS

A special set of mutants containing identified altered codons in the iso-1-cytochrome *c* gene of yeast has been used as tester strains for determining mutagenic

specificities (PRAKASH and SHERMAN 1973). One of the mutants, *cyc1–131*, contains a GUG codon in place of the normal chain initiation codon AUG and requires a G:C to A:T transition to yield the normal protein (STEWART *et al.* 1971). This mutant was reverted preferentially and with a high frequency by EMS, DES, HNO_2, NTG, NIL, [^{3}H]-U, NQO and β-PL and it was therefore concluded that these agents, to varying degrees, selectively induce G:C to A:T transitions, at least at the *cyc1–131* site (PRAKASH and SHERMAN 1973; PRAKASH, STEWART and SHERMAN 1974). In addition, UV, X-rays, HN2, MMS and DMS were found to revert all the tester strains with about the same efficiency or without any dependence on simple types of base-pair changes and it was concluded that these mutagens were relatively non-specific.

Since the *cyc1–131* tester reverted well with all eleven chemical mutagens and with ionizing and UV radiation, it was used in studies designed to determine the genetic control of mutation induction using a wide variety of mutagens (PRAKASH, unpublished results). This tester was coupled to over 20 different *rad* genes and diploids were constructed which were homozygous for *cyc1–131* as well as a particular *rad* gene. Initial studies concerning the effect of these *rad* genes on chemically-induced reversion of *cyc1–131* (PRAKASH, in preparation) indicated that both the *rad6* and *rad9* diploids were relatively refractory to mutation induc-

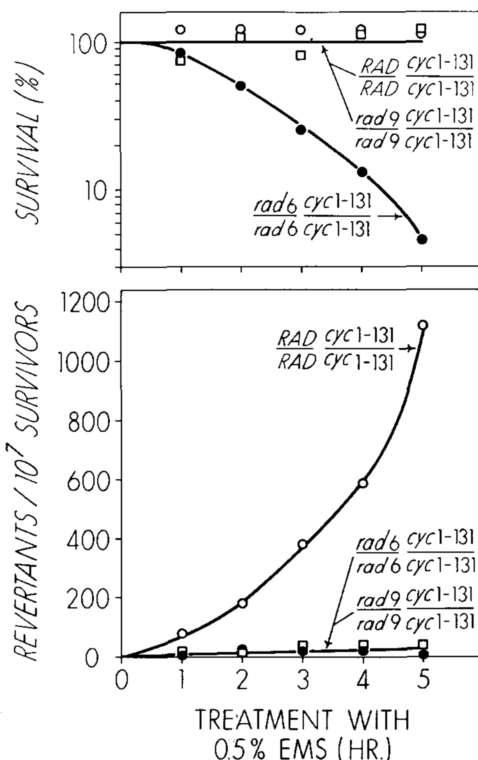

FIGURE 1.—Survival and *cyc1-131* reversion dose-response curves for LC-0 (○), LC-6 (●), and LC-9 (□) treated with EMS.

tion. These strains were therefore further characterized by testing a wide variety of mutagens. Complete dose response curves were obtained for the *RAD* and *rad6* diploids with each mutagen.

The potent mutagen EMS reverts the *RAD* diploid very efficiently over a dose range resulting in virtually 100% survival (Figure 1). However, the presence of either the *rad6* gene or the *rad9* gene greatly reduces EMS-induced reversion frequencies (Figure 1). Even at non-lethal doses of EMS, the reversion frequency is about 10 times greater in the *RAD* diploid than in the *rad6* diploid. Survival in the *rad6* diploid decreases with increasing time of exposure to EMS, whereas in the *rad9* diploid, there is no loss in viability over the dose range tested. Nevertheless, EMS mutability in the *rad9* diploid is quite low.

The reduced reversion frequencies in the *rad6* and *rad9* diploids extend to other chemical mutagens including NQO, NTG, MMS, DMS, HN2, β-PL, [^{3}H]U, and DES (Table 2). NQO and NTG mutability are reduced 50 to 100-fold compared to the normal *RAD* diploid (Figures 2 and 3). The reduction occurs even at doses resulting in high survival in the *rad6* and *rad9* diploids, as

TABLE 2

Frequency of induced cyc1–131 → CYC1 *mutations (expressed as revertants/10*^{7} *survivors) with various mutagens in normal and radiation-sensitive diploid yeast*

Mutagenic treatment	Strains		
	RAD	*rad6*	*rad9*
HNO_2 (0.35 mg/ml, 20 min)	142 (100%)	151 (78%)*	218 (100%) [344 (93%)]†
NIL (0.4 mg/ml, 30 min)	1588 (89%)	541 (100%)*	470 (81%)†
EMS (0.5%, 5 hr)	1124 (80%)	0 (4.6%)	37 (100%)
DES (0.5%, 15 min)	226 (94%)	29 (57%)	9 (100%)
MMS (0.1%, 40 min)	5 (94%)	0 (1.8%)	<1 (87%) [0 (100%)]†
DMS (0.05%, 12 min)	24 (89%)	0 (0.25%)	<1 (1.8%) [0 (3.6)%]†
HN2 (0.2 mg/ml, 1 hr)	10 (80%)	0 (0.07%)	0 (55%)
NQO (1 μg/ml, 1 hr)	395 (78%)	0 (1.6%)	34 (34%)
NTG (40 μg/ml, 40 min)	1506 (76%)	0 (8.5%)	10 (20%)
β-PL (0.02%, 1 hr)	16 (100%)	0 (3%)	
[^{3}H]-U (10 days decay)	14 (98%)	0 (89%)	
UV (25 Joules/m^2)‡	15 (96%)	0 (0.12%)	8 (53%)

Numbers in parenthesis represent percent survival.
Reversion frequencies are expressed as the number of revertants obtained after treating cells minus those that occurred spontaneously, except for treatments with [^{3}H]-U. Reversion frequencies for cells grown in the presence of [^{3}H]-U are expressed as the number of revertants obtained after 10 days of storage at 4°, minus the number obtained after no storage. In most experiments, spontaneous reversion frequencies were less than 1 per 10^7 cells. An entry of 0 indicates that no revertants were found out of 2 to 8 × 10^7 total cells plated, or that the difference between the induced and spontaneous reversion frequency was 0.
* Reversion rates decrease at higher doses. See Figures 4 and 5.
† LC-9A.
‡ The UV survival and reversion data were kindly provided by Dr. C. Lawrence.
No entry indicates that the strain was not tested with the mutagen.

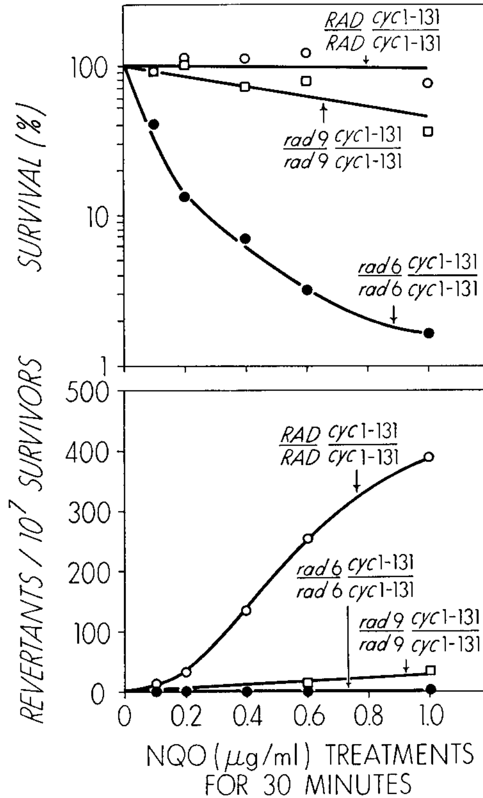

FIGURE 2.—Survival and *cyc1-131* reversion dose-response curves for LC-0 (○), LC-6 (●), and LC-9 (□) treated with NQO.

was observed for EMS-induced reversions. The three mutagens EMS, NQO and NTG, which are efficient in reverting *cyc1–131* in the *RAD* diploid, essentially lose their mutagenicity for that site in the *rad6* or *rad9* diploids.

The reduced mutability of *cyc1–131* observed in the *rad6* diploid is due to the *rad6* gene and not to variation in genetic background of the *RAD* and *rad6* diploids. Three other diploids homozygous for both *cyc1–131* and *rad6* were constructed by crossing meiotic segregants from the same cross used to generate LC-6. The four *rad6* diploids, representing six independent tetrads, were tested for revertibility of *cyc1–131* by EMS and DES by placing 20 μ liter of each mutagen on a filter disc placed in the center of a petri plate seeded with the appropriate strain. All four diploids homozygous for *rad6* showed reduced reversion with EMS and DES, whereas the *RAD* diploid and three diploids heterozygous for *rad6* showed normal reversion. The reduction in mutability is thus a property of the *rad6* gene.

Of the eleven chemical mutagens tested, only HNO_2 and NIL reverted the *rad6* and *rad6* diploids (Table 2). In fact, reversion frequencies are virtually the same in the normal *RAD* diploid strain as in the radiation-sensitive mutants

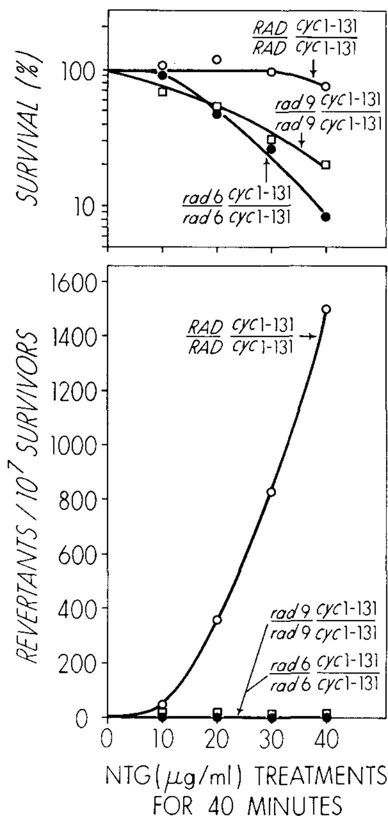

FIGURE 3.—Survival and *cyc1-131* reversion dose-response curves for LC-0 (○), LC-6 (●), and LC-9 (□) treated with NTG.

when low doses of either HNO_2 or NIL are used (Figures 4 and 5). NIL-induced revertants arise initially with the same kinetics in the *RAD* and the *rad6* diploid for concentrations up to 0.2 to 0.3 mg/ml. However, as the dose increases, the frequency of induced revertants rises in the *RAD* diploid and falls in the *rad6* diploid. It should be noted that since there is no loss in viability in either strain over the dose range used, the decreased frequency of revertants observed in the *rad6* diploid is a reflection of an absolute decrease in the number of revertants. In the presence of the *rad9* gene, the frequency of NIL-induced revertants increases with increasing dose, as it does in the *RAD* diploid, but at a reduced rate (Figure 4). The kinetics of HNO_2-induced mutation in the *rad6* diploid (Figure 5) is similar to the kinetics of NIL-induced mutation in that strain (Figure 4). At low doses, there is an increase in reversion frequency, a leveling off and then a decrease in frequency with increasing dose. In the *rad9* diploids, on the other hand, the frequency of revertants increases with increasing HNO_2 concentration, within the dose ranges used (Figure 5). There is no apparent difference in reversion frequency at low doses of HNO_2 in *RAD*, *rad6* and *rad9*

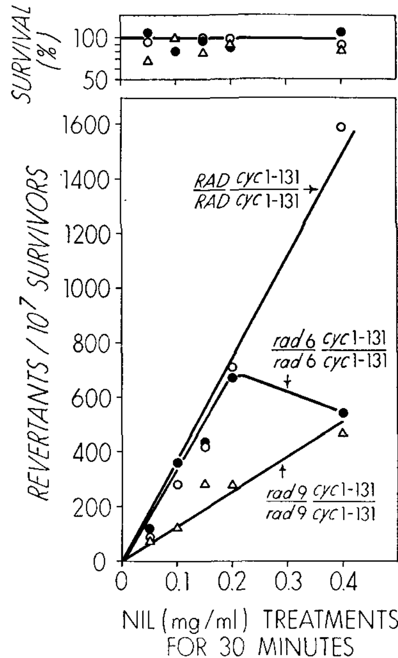

FIGURE 4.—Survival and *cyc1-131* reversion dose-response curves for LC-0 (○), LC-6 (●), and LC-9A (△) treated with NIL.

diploids. In the *rad6* diploid, however, there is a decrease in viability at higher HNO_2 doses and a corresponding decrease in numbers of revertants.

All or almost all of the large revertant colonies on lactate medium are the result of intragenic mutations and not the result of extragenic suppressors. Intragenic and extragenic reversion of *cyc1* mutants can be differentiated on the basis of colony size and cytochrome *c* content (see SHERMAN *et al.*, 1974). Most intragenic revertants have normal or near normal amounts of cytochrome *c* and have normal colony sizes on lactate medium, while nearly all of the extragenic revertants have below normal amounts of cytochrome *c* and form smaller colonies on lactate medium. Extragenic revertants of *cyc1–131* can be due to suppressors that usually cause increased levels of iso-2-cytochrome *c*. These can be distinguished clearly from intragenic revertants, since most of them still have far below the normal amounts of total cytochrome *c*. Even the rare revertants having normal amounts of cytochrome *c* that is entirely iso-2-cytochrome *c* can be conveniently identified by the slightly different spectral properties of the c_α-band. Over 50 nitrous acid- and NIL-induced revertants of the *RAD*, *rad6* and *rad9* diploids were picked and subcloned, and the resulting strains were examined for cytochrome *c* content and growth on lactate medium. From these results, we are confident that the colonies scored on lactate medium are truly the result of intragenic mutations. Thus, most of the HNO2- and NIL-induced revertants expressed in the results of Figures 4 and 5 represent intragenic revertants. Extragenic sup-

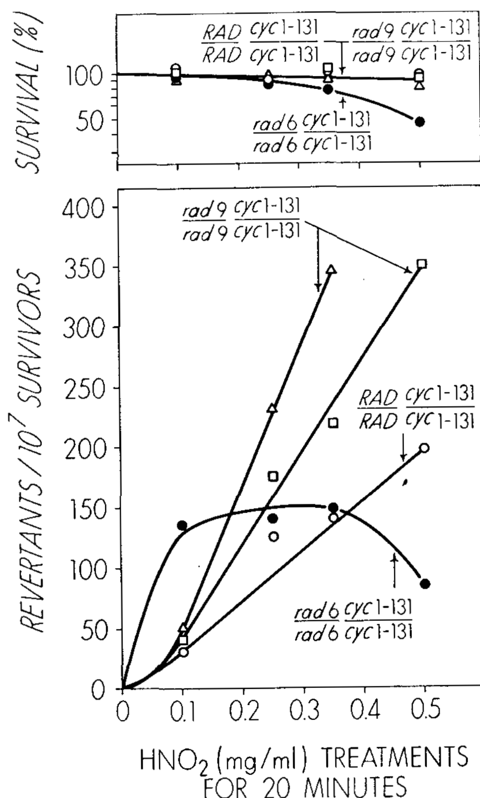

FIGURE 5.—Survival and *cyc1-131* reversion dose-response curves for LC-0 (○), LC-6 (●), LC-9 (□) and LC-9A (△) treated with HNO_2.

pressors occurred with very low frequencies. It should be stressed that all of the HNO_2- and NIL-induced revertants of the *rad6* diploid retained their UV sensitivity and could therefore not have arisen as a result of simultaneous reversion of the *rad6* locus.

In summary, both *rad6* and *rad9* are similar in their response to mutability with chemical agents. Both *rad6* and *rad9* greatly reduce reversion of *cyc1–131* with all chemical agents tested, except for NIL and HNO_2, when low doses of these agents are used. At high doses, however, reversion frequencies are reduced in *rad6* and *rad9* strains, even with these agents (see Figures 4 and 5). Both *rad6* and *rad9* were originally selected for on the basis of their sensitivity to the lethal effects of UV (COX and PARRY 1968); however, *rad6* is much more sensitive to UV and x-ray than is *rad9*. Perhaps correlated with this observation is the fact that the UV mutability of *cyc1–131* is reduced to a much greater extent in *rad6* than it is in *rad9* (Table 2; LAWRENCE, personal communication).

It is evident from the results presented here that in the *rad6* or *rad9* diploids, chemically-induced and radiation-induced reversion of *cyc1–131* is greatly diminished compared to the *RAD* strain carrying the wild-type allele of both of those genes.

DISCUSSION

It is now fairly well-established that mutations induced by both ionizing radiation (such as X-ray and γ-ray) and UV depend on genes conferring sensitivity to these agents and involved in repair of damage caused by these agents (BRIDGES 1969; BRIDGES, LAW and MUNSON 1968; KONDO *et al.* 1970; WITKIN 1969). Correlations exist between sensitivity of organisms to radiation and sensitivity to certain chemical agents. Excision-defective, UV-sensitive strains of *E. coli* are also sensitive to bifunctional alkylating agents such as nitrogen and sulfur mustards (BRIDGES and MUNSON 1966). In addition, such strains are also sensitive to the lethal effects of NQO (KONDO *et al* 1970). The correlation between a defect in excision-repair and sensitivity to UV and NQO is also found in *Bacillus subtilis* (FELKNER and KADLUBAR 1968; LUMBACK and FELKNER 1972; TANOOKA and TAKAHASHI 1972), *Salmonella typhimurium* (YAMAMOTO, FUKADA and TAKEBE 1970) and cultured cells from patients with xeroderma pigmentosum (TAKEBE *et al.* 1972; STICH, SAN and KAWAZOE 1973. Increased rates of mutation caused by both UV and NQO occur in *E. coli* mutants unable to excise pyrimidine dimers. Such strains are not sensitive to the lethal effects of X-ray or the monofunctional alkylating agent MMS and show normal responses to mutation induction by X-ray and MMS (KONDO *et al.*, 1970). *E. coli* strains carrying a mutation at the *recA* or *exr* locus show greatly reduced mutability induced by X-ray and MMS (KONDO *et al.* 1970) as well as UV and NQO (KONDO *et al.* 1970). In yeast correlations have been found for sensitivity to UV and HN2 on the one hand and X-ray and MMS on the other hand (BRENDEL, KHAN and HAYNES 1970; BRENDEL and HAYNES 1973). These findings certainly suggest that mutation induction by at least the "radiomimetic" chemical agents such as NQO and MMS should depend partly on the same genes which control UV and X-ray mutagenesis.

The results presented in this paper indicate that mutations induced by the non-radiomimetic chemical agents also depend on genes involved in repair. A mutation at either the *RAD6* or *RAD9* locus substantially reduces the frequency of reversion of *cyc1–131* induced by a wide variety of chemical agents as well as with UV (Table 2). The reduced mutability is reflected over a wide range of concentrations in the dose-response curves (Figures 1 to 5). Even at doses resulting in comparable survival in all three strains, very low reversion frequencies were obtained in the radiation-sensitive mutants with EMS, NQO, NTG, HN2, β-PL, [^{3}H]-U decay and DES. NIL and HNO_2 were the only chemical mutagens tested which, at low doses, induced similar reversion frequencies in all three strains (Table 2; Figures 4 and 5). It is not readily apparent why the frequencies of NIL- and HNO_2-induced revertants should decrease in the *rad6* diploid and increase in the *RAD* diploid at doses resulting in such high survival in both strains. Since the *rad6* mutant is an amber-containing nonsense mutant (LAWRENCE *et al.* 1974; B. S. Cox, personal communication), one possible explanation for the observed revertibility of the *rad6* diploid with nitrous acid and NIL is that these two mutagens are efficient inducers of amber suppressors. However, over 50 NIL- and nitrous acid-induced *CYC1* revertants of the *rad6* diploid were

tested to determine the nature of the revertants. All of them were shown by spectroscopic examination of intact cells to be intragenic revertants which retained their UV sensitivity. Therefore, the high frequency of reversions induced by low doses of NIL and HNO_2 in the *rad6* diploid cannot be accounted for by simultaneous reversion of *rad6* to *RAD6+* or by the induction of an extragenic suppressor of the *rad6* amber site. In addition, the synthetic lactate medium used to score for *cyc1* revertants tends to inhibit the appearance of amber suppressors (SHERMAN *et al.* 1973).

The *rad6* mutation has been shown to decrease the reversion of other *cyc1* alleles and its effect is therefore not restricted to the *cyc1–131* allele. A diploid homozygous for *cyc1–115*, a missense mutant which reverts by G:C transitions and transversions, shows high revertibility induced by NTG, whereas NTG-induced reversion of this same site is barely detectable when the strain is simultaneously homozygous for the *rad6* mutation (PRAKASH, unpublished results).

While both *cyc1* alleles, *cyc1–131* and *cyc1–115*, revert by an alteration of a G:C base-pair, the effect of *rad6* is not restricted to G:C sites. The UAA (ochre)-containing nonsense mutant *cyc1–9* (STEWART *et al.* 1972) is highly UV-revertible (STEWART *et al.* 1971; SHERMAN and STEWART 1973; LAWRENCE *et al.* 1974). Although any amino acid whose mRNA codon differs from UAA by a single base change is compatible with function at that position, UV induces predominantly A:T to G:C changes of the first base in the UAA codon, resulting in insertion of glutamine (STEWART *et al.* 1972; SHERMAN and STEWART 1974). This change is obviously not a change involving a G:C base-pair, nor are any of the other reversional events, since the UAA codon lacks G:C base-pairs. This highly UV-revertible ochre mutant also shows a drastic reduction in UV-induced revertibility when coupled with the *rad6* gene (LAWRENCE *et al.* 1974). The specificity of UV-induced reversion of *cyc1–9* is changed by the *rad6* mutation. Glutamine is no longer the predominant amino acid found in intragenic revertants of *cyc1–9* when the *rad6* mutation is present; instead, various amino acid replacements occur (LAWRENCE *et al.* 1974). When the *rad6* mutant is reverted either by intragenic reversion or by a suppressor mutation, then the reversion frequencies in these strains approach the values obtained in the corresponding *RAD* strain (LAWRENCE *et al.* 1974). Also, *rad6* reduces γ-ray-induced reversion of *cyc1–9* (LAWRENCE *et al.* 1974).

In addition, the effect of the *rad6* gene extends to all loci tested so far. In the presence of *rad6*, UV-induced back mutation of *his1* to HIS is reduced (MOUSTACCHI 1972) as are UV-induced back reversions of *arg4–17* to ARG and *lys2–1* to *LYS*, as well as forward mutation of CAN^S to can^R (LAWRENCE *et al.* 1974). Similar results have been obtained by B. S. Cox (personal communication).

The reduction in mutability observed in the presence of the *rad9* gene is also likely to be due to the *rad9* gene and not to variation in genetic background. Both *rad9* diploids (LC-9 and LC-9A) show similar responses to the lethal effect of HNO_2 and to mutability induced by HNO_2 (Figure 5). In addition, both *rad9* diploids show similar mutagenicity in response to NQO, EMS and NIL.

Although perhaps more studies involving the influence of repair defects on mutation-induction have been done in *E. coli* than in any other single organism thus far, none of the repair-deficient mutants of *E. coli* described so far are comparable in terms of their mutagenic response to either the *rad6* or *rad9* mutants in yeast (see Table 3). *RecA* mutants are perhaps most similar to *rad6* in terms of their mutability; however, EMS mutability is normal and NTG mutability only slightly reduced (KONDO *et al.* 1970).

In *B. subtilis*, an MMS-sensitive, UV-sensitive mutant was refractory to *his* reversions induced by MMS, UV and NTG, even though spontaneous revertants occurred with a normal frequency (HILL, PRAKASH and STRAUSS 1972). It was concluded that the establishment of induced mutations, including those produced by NTG, require repair functions but that different mechanisms are involved in the production of spontaneous and induced mutations. A mutant which does not respond to NTG mutagenesis has also been described in *Haemophilus influenzae* (KIMBALL, SETLOW and LIU 1971). However, neither of these two mutants were tested for revertibility with a wide variety of chemical mutagens.

None of the bacterial mutants described so far are as non-responsive to mutations induced by as wide a variety of mutagens as are the *rad6* and *rad9* mutants described in this paper. The *rad6* strain shows greater UV sensitivity than the *rad9* strain, although both are UV-sensitive (COX and PARRY 1968; LAWRENCE,

TABLE 3

Comparison of mutability in normal and radiation-sensitive strains of Saccharomyces cerevisiae *and* Escherichia coli

	S. cerevisiae			*E. coli**				
Mutagen	*RAD*(normal)	*rad6*	*rad9*	Normal	*uvrA*	*polA*	*recA*	*lex* (or *exr*)
HNO_2	+	+	+	..	..	..	..	..
NIL	+	+	+	..	..	..	..	..
EMS	+	—	—	+	+	+	+	..
DES	+	—	—	..	..	..	..	..
MMS	+	—	..	+	+	+	—	..
DMS	+	—	..	..	..	..	..	..
HN2	+	—	—	..	..	..	..	..
NQO	+	—	—	+	++++	+	—	..
NTG	+	—	—	+	+	+	±	+†
β-PL	+	—	..	..	..	..	..	..
[3H]-U	+	—	..	..	..	..	..	..
UV	+	—‡	±§	+	++++	+	—	—†
Ionizing radiation	+	—‡	..	+	+	+	—	—

+ = revertible.
± = revertibility somewhat lower than normal strain.
— = very low or barely detectable revertibility.
++++ = revertibility higher than in normal strain.
No entry indicates that the strain was not tested.
From * KONDO *et al.* 1970, except where indicated.
† WITKIN 1967.
‡ LAWRENCE *et al.* 1974.
§ LAWRENCE, personal communication.

personal communication). The *rad9* gene reduces the frequency of UV reversions of *cyc1–131* about twofold over a wide range of doses whereas the *rad6* reduction factor is substantially greater (LAWRENCE *et al* 1974 and LAWRENCE, unpublished results). Although *rad6* and *rad9* strains show essentially similar patterns of response in terms of mutability by chemical agents, i.e., both are fairly immutable strains, the *rad9* diploid tends to be more resistant to the lethal effect of all agents tested than is the *rad6* diploid (Tables 2 and 4). The *rad6* and *rad9* sites complement each other (COX and PARRY 1968; PRAKASH, unpublished results) and recombine with each other (COX and PARRY 1968; LAWRENCE, personal communication). Thus, they represent mutations at different genetic loci.

The precise nature of the biochemical step(s) specified by the *rad6* or *rad9* mutants has not yet been identified. Diploids homozygous for *rad9 sporulate* normally, whereas diploids homozygous for *rad6* do not sporulate (COX and PARRY 1968). Therefore, it is not possible to determine whether the *RAD6* locus has any effect on meiotic recombination. UV-induced mitotic intragenic recombination is normal in a *rad6* diploid but has not been tested in a *rad9* diploid (HUNNABLE and COX 1971). Also, excision of UV-induced primidine dimers

TABLE 4

Sensitivity to the lethal effect of various mutagens in normal and radiation-sensitive strains of Saccharomyces cerevisiae *and* Escherichia coli

	S. cerevisiae			*E. coli**				
Mutagen	*RAD*(normal)	*rad6*	*rad9*	Normal	*uvrA*	*polA*	*recA*	*lex* (or *exr*)
HNO_2	+	±	±	+	—†	..	—†	..
NIL	+	±	±	..	..	..	..	..
EMS	+	—	+	+	+	—	+‡	+‡
DES	+	—	+	..	..	..	..	..
MMS	+	—	—§	+	+	—	—	—‖
DMS	+	—	..	..	..	..	..	..
HN2	+	—	+	+	—¶	..	..	..
NQO	+	—	+	+	—	—	—	..
NTG	+	—	—	+	+	—	—	—‡
β-PL	+	—	..	..	..	..	..	..
[³H]-U	+	—	..	..	..	..	..	..
UV**	+	—	—††	+	—	—	—	—‡
Ionizing radiation**	+	—	—§	+	+	—	—	—‖

+ = same sensitivity as the normal *RAD* strain.
± = same sensitivity as the normal *RAD* strain at low doses, more sensitive than *RAD* at high doses.
— = greater sensitivity than the normal *RAD* strain.
No entry indicates that the strain was not tested.
From * KONDO *et al.* 1970, except where indicated.
† HOWARD-FLANDERS and BOYCE 1966.
‡ WITKIN 1967.
§ ZIMMERMANN 1968.
¶ BRIDGES and MUNSON 1966.
‖ MOUNT, LOW and EDMISTON 1972.
** LAWRENCE *et al.* 1974.
†† COX and PARRY 1968.

is known to occur in *rad6* strains, although at a somewhat slower rate than in normal *RAD* strains (B. S. Cox, personal communication).

In order to explain the reduction in mutagenic potential observed in strains defective in either the *RAD6* or *RAD9* loci, one has to invoke some step which is common to the pathways of mutation induction by all the diverse chemical agents tested. Although the initial lesions produced by these agents are certainly different with each mutagen, there is likely to be a mechanism common in the repair of the damage induced by the various agents. The pathway(s) of error-prone repair for mutations induced by UV, ionizing radiation, and the various chemical agents probably share some common steps. On the other hand it is possible that HNO_2 and NIL produce mutations via different error-prone repair pathway(s). These two agents revert the wild type and both radiation-sensitive mutants at low doses to about the same degree. It should be stressed that it is only at increasing doses that differences in both lethality and mutations become apparent in these three strains (Figures 4 and 5). In the *rad6* diploid, the decrease in NIL-induced revertants does not coincide with decreased survival (Figure 4). In fact, within the dose-range used, viability in all three diploids is the same. On the other hand, the reduced frequency of HNO_2 induced revertants coincides with a loss of viability in the *rad6* diploid (Figure 5). These results suggest that the pathway(s) of repair of HNO_2- and NIL-induced mutations may be different from the pathways of repair of mutation induced by the rest of the chemical agents. A common step of repair may involve some kind of repair synthesis dependent on a "repair" DNA polymerase. According to this view, a strain deficient in the hypothetical repair replication enzyme would not undergo repair synthesis, and might be fairly immutable as well as sensitive to various agents. However, preliminary results (PRAKASH, unpublished results) indicate that there is no difference in DNA polymerase activity in mitochondria-free crude cell extracts of the *RAD* and *rad6* diploids.

Our results lead us to agree with the conclusion reached by LAWRENCE *et al.* (1974), namely, that the *RAD6* locus is involved in error-prone DNA repair. It would also seem that the *RAD9* locus is involved in error-prone DNA repair. Whatever the nature of the defect in the *rad6* and *rad9* mutants, it is clear that both loci are intimately concerned with induced mutations. Whether these two loci affect the same pathway or not has not yet been determined.

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Corresponding editor: G. R. FINK