The *RAD24* (= R^s_1) Gene Product of *Saccharomyces cerevisiae* Participates in Two Different Pathways of DNA Repair

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ABSTRACT

The moderately UV- and X-ray-sensitive mutant of *Saccharomyces cerevisiae* originally designated r^s_1 complements all *rad* and *mms* mutants available. Therefore, the new nomination *rad24-1* according to the *RAD* nomenclature is suggested. *RAD24* maps on chromosome *V*, close to *RAD3* (1.3 cM). In order to associate the *RAD24* gene with one of the three repair pathways, double mutants of *rad24* and various representative genes of each pathway were constructed. The UV and X-ray sensitivities of the double mutants compared to the single mutants indicate that *RAD24* is involved in excision repair of UV damage (*RAD3* epistasis group), as well as in recombination repair of UV and X-ray damage (*RAD52* epistasis group). Properties of the mutant are discussed which hint at the control of late steps in the pathways.

IN the yeast *Saccharomyces cerevisiae*, three different repair processes have been proposed to act on UV-light-induced DNA damage in the dark (for reviews, see Haynes and Kunz 1981; Game 1983). Evidence for three processes, or pathways, has largely come from genetic studies which have lead to the establishment of three "epistasis groups" of radiation-sensitive mutants (Cox and Game 1974). The studies involve comparing the phenotypes of double and triple mutants with those of the parental single mutants (Khan, Brendel and Haynes 1970; Game and Cox 1972, 1973; Brendel and Haynes 1973; Game and Mortimer 1974). For simplicity, the three epistasis groups are named by a prominent locus in each, *e.g.*, the *RAD3*, *RAD6* and *RAD52* group, or according to their postulated biological characteristics, excision repair, error-prone repair and recombination repair, respectively. Due to the pleiotropic effects which *rad* mutations may have on mutation, recombination, sporulation, etc., the mutants belonging to one group frequently have some phenotypic similarities in common. Thus, newly isolated mutants which show radiation sensitivity in addition to other phenotypes can be associated with one of the groups by their phenotypic characteristics, as well as by their interactions in double mutant combinations (Lawrence and Christensen 1976; Lemontt 1977; Game, Johnston and von Borstel 1978; Prakash and Prakash 1979; Cassier *et al.* 1981; Siede and Brendel 1982; Lawrence, Das and Christensen 1985).

This paper deals with the r^s_1 mutant, which was one of the first radiation-sensitive mutants isolated and characterized (Laskowski and Averbeck 1968; Laskowski *et al.* 1968), but was never given a *RAD* locus number (Game and Cox 1971) or assigned to one of the repair pathways. We report here the results of genetic tests which reveal that r^s_1 is not allelic with any previously designated *rad* mutant, and we propose renaming the r^s_1 locus *RAD24*. The r^s_1 mutant is sensitive both to UV light and ionizing radiation, and we describe radiation survival characteristics of double mutant strains involving r^s_1 and mutants in each of the three epistasis groups. We also describe the effects of r^s_1 on UV-induced mutation. Based on these results, we discuss the probable role of the *RAD24* gene in the repair of UV and ionizing radiation damage.

MATERIALS AND METHODS

Strains: All strains used for the pathway analysis are listed in Table 1.

Media: The media employed have been described in Eckardt and Haynes (1977). For experiments involving sensitivity to methyl methanesulfonate (MMS), fresh YPD plates were used which contained 0.02 or 0.04% MMS added to cooled medium immediately before pouring.

Radiation sources: For UV-irradiation a HNS mercury vapor lamp was used with a dose rate of 2.0 J m^{-2} s^{-1}. A ^{60}Cobalt-γ-source with a dose rate of 97.3 Gray min^{-1} was used. The irradiations were carried out on plates.

Tetrad analysis: Standard techniques for tetrad analysis were used. The UV and X-ray sensitivities of 3-day-old spore clones were determined in a semiquantitative droplet test. Droplets of each spore clone were irradiated with UV (48 J m^{-2}) and X-rays (240 and 480 Gray) on YPD plates. After incubation at 30° for 1 day, radiation-resistant spore clones grew up as a lawn, moderately sensitive clones as distinct colonies and highly sensitive clones showed very little colony formation. In most cases the parental ditype

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TABLE 1

List of strains

No.	Genotype	Source
2110-23	*α rad24-1 ade2-1 lys2-1 his3 ura1*	S. Kowalski
2110-222	**a** *rad24-1 ade2-1 lys2-1 his3 ura1*	S. Kowalski
2110-3C	**a** *rad24-1 ade2-1 lys2-1 his3*	F. Eckardt
2110-5A	*α rad24-1 ade2-1 lys2-1 his3*	F. Eckardt
G 557-2b	**a** *rad24-1* prototrophic	J. C. Game
2105-178	*α rad2-20 ade2-1 lys2-1 his3 ura1*	S. Kowalski
HT9-14B	*α rad2-20 ade2-1 lys2-1 his3 ura1*	S.-J. Teh
HT12-6B	**a** *rad3-2* prototrophic	S.-J. Teh
ED36B-3C	*α rad3-2 ade2-1*	E. Dowling
XV423-2a	*α rad3-12 ade2-1 hom3-10 his1-7 lys1-1 trp5-48*	R. C. v. Borstel
HT18-2C	*α rad4-4 ade2-1 lys2-1 his3*	S.-J. Teh
F467	*α rad6-1 ade2-1 leu1-12 met1-1 arg4-17 trp5-48*	C. Lawrence
271	*α rad6-1 ade2-1*	C. Lawrence
2206-1B	**a** *rad6-1 ade2-1 lys2-1 his3*	F. Eckardt
2206-3B	**a** *rad6-1 ade2-1 lys2-1 his3*	F. Eckardt
2206-22C	*α rad6-1 ade2-1 lys2-1 his3*	F. Eckardt
2121-78	*α rad9-4 ade2-1 lys2-1 his3 ura1*	S. Kowalski
478/3D	*α rad18-2 his4-ABC*	B. S. Cox
2218-5D	*α rad18-2 ade2-1 lys2-1 his3*	F. Eckardt
	α rad51-1	J. C. Game
6160/2B	*α rad52-1 ade2-1 arg4-17 arg9 trp1 lys2-1 his5-2 ilv3 leu1(2)*	B. S. Cox
HT4-21A	*α RAD ade2-1 lys2-1 his3*	S. J. Teh

(PD), nonparental ditype (NPD) and tetratype (T) tetrads could be easily identified from the UV and X-ray sensitivities of the spores. When this was not the case, complementation tests were carried out to determine the genotypes.

Complementation analysis: Diploids were isolated from crosses of appropriate r^s_I strains with the various *rad* mutants and confirmed as such by sporulation ability. The UV and ionizing radiation sensitivities of these diploids were tested by means of survival curves and compared to the homozygous *RAD* wild type and *rad* mutant diploids.

Mutation experiments: The reversion tests (*his3* → *HIS*, *lys2-1* → *LYS*) were carried out as described in Eckardt and Haynes (1977).

RESULTS

Complementation analysis and locus designation: In order to determine if the r^s_I mutation was allelic with any previously designated *rad* mutant (Game and Cox 1971; Game and Mortimer 1974; McKnight, Cardillo and Sherman 1981), complementation tests were carried out between it and mutants in each of *rad1* through *rad22* and *rad50* through *rad57* genes (except for *rad13*, which is no longer available), using the criterion of UV-light or X-ray sensitivity as appropriate. Similarly, since r^s_I confers sensitivity to MMS, we carried out complementation tests on MMS plates between it and most of the MMS-sensitive mutants described by Prakash and Prakash (1977) which define the genes *MMS1–MMS22*. Complementation between r^s_I and all the *rad* mutants was observed, and between r^s_I and all of the *mms* mutants, except *mms5-1*, *mms6-1*, *mms7-1* and *mms14-1*, which either failed to mate or in our hands failed to display a significantly MMS-sensitive phenotype. The occurrence of complementation does not definitely rule out allelism, since intragenic complementation can occur, but this has not so far been reported for any *RAD* locus. In addition, the map position of the r^s_I mutation rules out allelism with the many *RAD* genes that have been mapped, including *RAD23*, for which no complementation data are available. Therefore, we consider that the r^s_I mutation defines a separate locus and propose the symbols *RAD24* for the gene and *rad24-1* for the r^s_I mutant allele, since r^s_I strains are sensitive both to UV light and X-rays.

Map position of the *RAD24* gene: The *RAD24* locus is very closely linked to the *RAD3* gene which maps on the right arm of chromosome *V* (Snow 1967; a genetic map of yeast can be found in Mortimer and Schild 1985). Data from diploids in which both genes were heterozygous gave 112 PD:0 NPD:3 T tetrads for *rad3-2* and *rad24-1*, corresponding to a map distance of 1.3 cM (Perkins 1949). There is currently no information about gene order of *RAD3* and *RAD24* with respect to outside markers.

The cloned *RAD3* gene isolated by and obtained from Naumovski and Friedberg (1983) does not complement *rad24-1*, since a *rad24-1* strain transformed with a multicopy plasmid containing *RAD3* (pNF 3000) shows an X-ray sensitivity similar to that of the untransformed strain (K. C. Sitney, J. C. Game and R. K. Mortimer, unpublished results). This provides further evidence that *rad24-1* defines a locus separate from the *RAD3* gene.

UV-light epistasis relationships of the *RAD24* gene: Double-mutant interaction studies with UV-light-sensitive mutants in yeast have revealed that most such mutants fall into one of three different epistasis groups (Game and Cox 1973; Haynes and Kunz 1981; Game 1983). In order to determine the UV-epistasis groups (if any) to which *RAD24* belongs, we analyzed the UV survival of double mutant strains we constructed which carried *rad24-1* in combination with representative mutations from each of the three groups. The results are shown in Figures 1A–3A. It can be seen that *rad2-20*, *rad3-2* and *rad3-12* are epistatic to *rad24-1*, and epistasis was also observed between *rad4-4* and *rad24-1* (data not shown). These mutants fall into the excision repair group (Game and Cox 1972; Prakash 1977; Reynolds and Friedberg 1981), and this result implies that the *RAD24* gene also plays a role in excision repair. As a single mutant, its UV sensitivity is comparable to that of some other excision-defective mutants, but much less than the maximal sensitivity seen in alleles of *RAD1*, *RAD2* and *RAD3*, although leakiness of the *rad24-1* allele cannot be excluded. It also differs from these mutants in

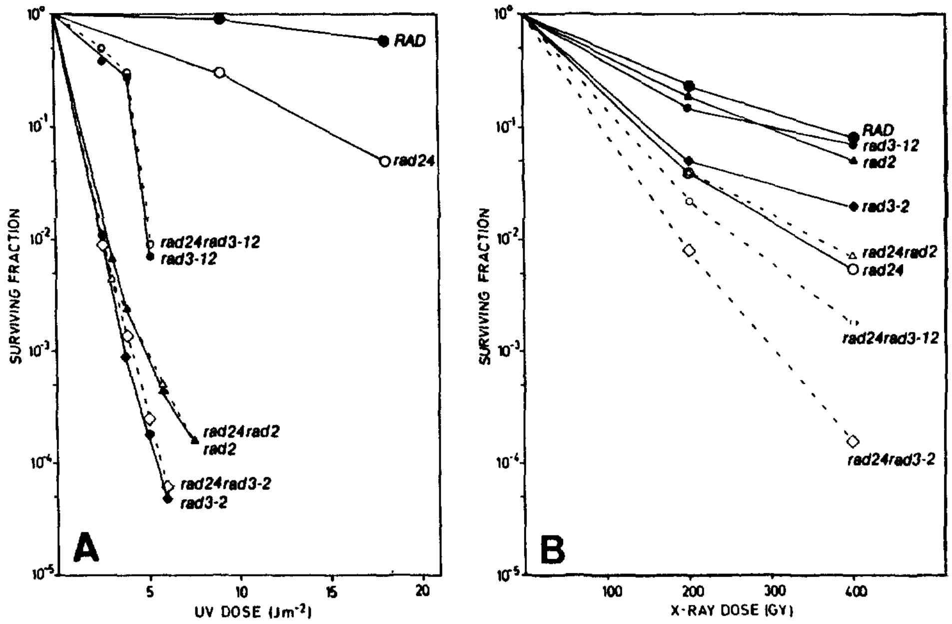

FIGURE 1.—The figure shows survival data for UV (A) and X-rays (B) for double mutants constructed with *rad24* and representative genes of the excision repair pathway. The data are averaged from seven tetrads of two independent crosses *rad24* × *rad2-20*, one tetrad of the cross *rad24* × *rad3-12* and from repeated experiments with the only double mutant available of two independent crosses *rad24* × *rad3-2*, g631-14a, for which the genotype was ascertained by complementation.

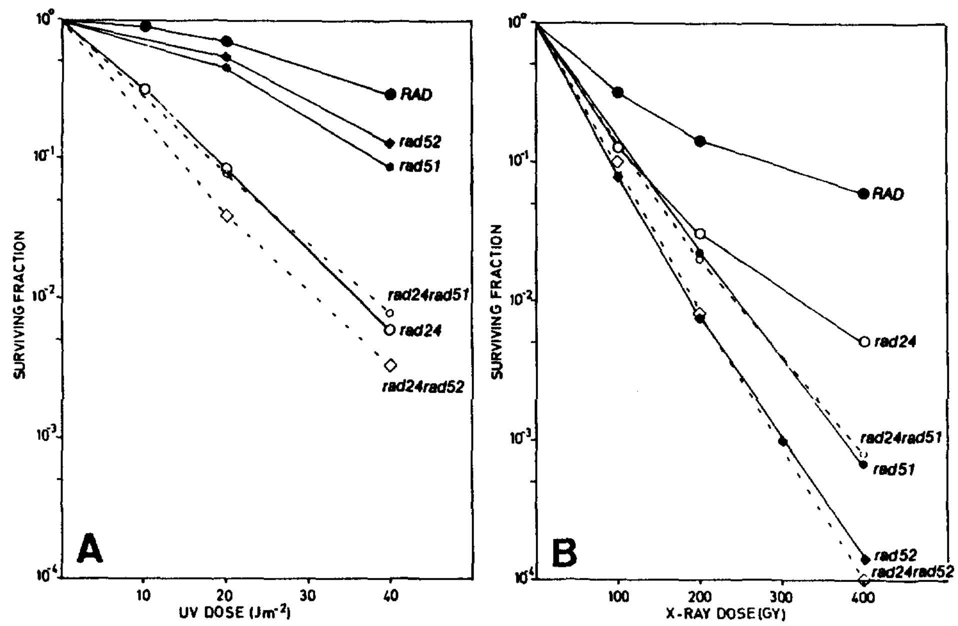

FIGURE 2.—The figure shows survival data for UV (A) and X-rays (B) for double mutants constructed with *rad24* and representative genes of the error-prone pathway. The data are averaged from ten tetrads of three independent crosses *rad24* × *rad6*, six tetrads of the cross *rad24* × *rad9*, four tetrads of the cross *rad24* × *rad18* and four tetrads of two independent crosses *rad6* × *rad18*.

showing an epistatic interaction with *rad51-1*. Thus, the *RAD24* gene product may function in the UV repair mediated by mutants in the *RAD52* epistasis group as well. The very slight increase in UV sensitivity seen in some *rad52-1 rad24-1* double mutant strains (Figure 2A) may result from epistasis with variation in background genotypes, but is also consistent with additivity. However, there is clearly no significant synergistic action between these mutants.

The interaction of *rad24-1* with mutants of the *RAD6*, or "error-prone" UV-epistasis group was tested in combinations with *rad9-1*, *rad18-2* and *rad6-1* (Figure 3A). It can be seen that for *rad9-1 rad24-1* there is, at most, a marginal increase in sensitivity, suggesting an epistatic interaction and arguing for a role of the *RAD24* gene in this repair pathway. However, there is an apparent synergistic interaction between *rad24-1* and *rad18-2* and *rad6-1*, respectively (Figure 2A), with these double mutants showing the same sensitivity as *rad6-1 rad18-2* strains. Results with respect to *rad6-1* are complicated by the fact that there is a wide variation in UV sensitivity of different *rad6-1*

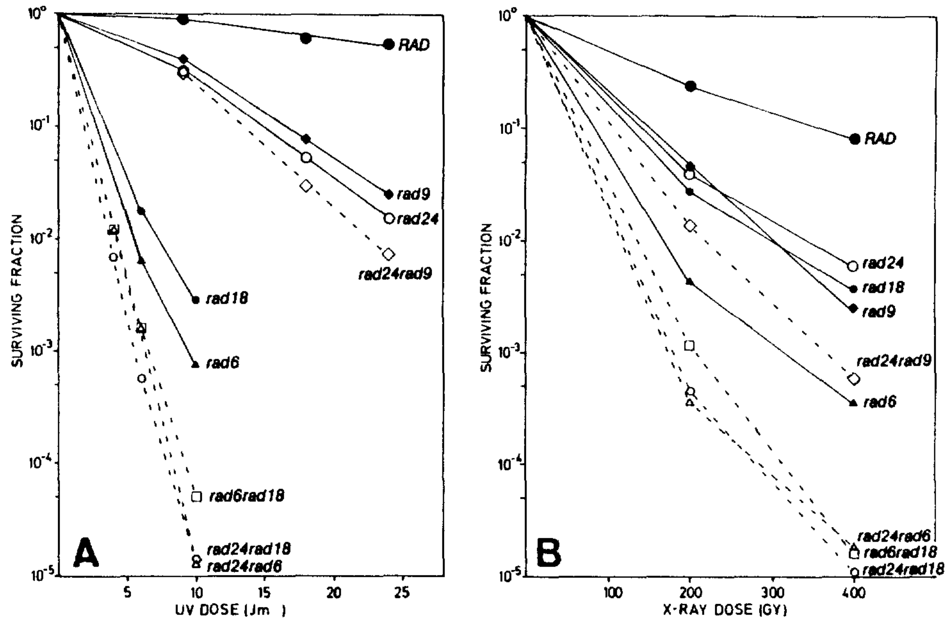

FIGURE 3.—The figure shows survival data for UV (A) and X-rays (B) for double mutants constructed with *rad24* and representative alleles of the recombination repair pathway. The data are averaged from five tetrads of the cross *rad24* × *rad51* and three tetrads of the cross *rad24* × *rad52*.

single mutant strains, probably reflecting the susceptibility of this allele to translational suppressors and other genetic modifiers (LAWRENCE *et al.* 1974; LAWRENCE and CHRISTENSEN 1979; TUITE and COX 1981). In this study, the most sensitive (and hopefully not suppressed) *rad6-1* spore from the various tetrads has always been used for reference. It seems unlikely though that *RAD24* is involved in the *RAD6/RAD18* repair pathway for UV damage.

In summary, *RAD24* does not seem to fall exclusively into any one UV-epistasis group. Mutants with a major block in excision repair are clearly epistatic to *rad24-1*, but there is also evidence of epistasis with some, but not all, mutants in the other two groups.

Ionizing radiation epistasis relationships of the *RAD24* gene: The single and double mutant strains, for which UV sensitivity is discussed above, were also monitored for X-ray sensitivity. For the *rad3-2 rad24-1* double mutant (Figure 1B) there is a slightly more than additive increase in sensitivity compared to the single mutants, in contrast to the epistatic response seen for UV light. This effect was confirmed by transforming a *rad3-2 rad24-1* double mutant strain with a clone of the *RAD3* gene obtained from L. NAUMOVSKI and E. FRIEDBERG. The *RAD3* transformants were significantly more X-ray-resistant than the untransformed strain, and the increased resistance was dependent on the presence of the plasmid containing the *RAD3* gene (K. SITNEY and J. GAME, unpublished observations). The difference in response for UV light and X-rays may indicate separate functions for the *RAD3* gene in these two processes. The other excision defective mutants we studied here, *rad2-20*, *rad3-12* (Figure 1B) and *rad4-4* (data not shown) differ from *rad3-2* in not being significantly X-ray-sensitive, and in double mutant combinations with *rad24-1* no additional X-ray sensitivity is conferred by them (Figure 1B).

The X-ray-sensitive mutants *rad51-1* and *rad52-1* are epistatic to *rad24-1*, as shown in Figure 2B. It is known that *rad52-1* is also epistatic to other mutants in the *RAD50* to *RAD57* series (GAME and MORTIMER 1974; MCKEE and LAWRENCE 1980), but shows an additive response in combination with *rad6* and *rad18* mutants (BRENDEL and HAYNES 1973; MCKEE and LAWRENCE 1980). Similarly, *rad24-1* appears to show an additive or synergistic interaction with *rad9-4*, *rad6-1* and *rad18-2* (Figure 3B). The results with *rad6-1*, however, may reflect variations in genetic background as discussed for the UV-interactions earlier. These may also account for observations by GAME and MORTIMER (1974) of an additive interaction in the *rad6 rad18* double mutant, in contrast to findings of MCKEE and LAWRENCE (1980), who reported an epistatic interaction for X-ray sensitivity in these mutants.

In summary, the X-ray repair process for which *RAD24* is required is clearly *RAD51* and *RAD52* dependent, but is probably at least partially independent of *RAD3*, and the genes of the "error-prone pathway," *RAD6*, *RAD9* and *RAD18*.

Absence of effects of *rad24-1* on UV-induced mutagenesis: The results described suggest that the *RAD24* gene is required for some aspect of UV-excision repair. All mutants associated with the control of excision repair so far analyzed (except *cdc8*, which has not been tested for the capacity to incise, PRAKASH, HINKLE and PRAKASH 1979) are absolutely or partly deficient in incision adjacent to a dimer (for review, see FRIEDBERG *et al.* 1983). All of them show considerably increased UV-induced mutation frequencies compared to the repair competent wild type (for review, see HAYNES and KUNZ 1981, table 1). It had been reported for the *rad24* mutant that the UV-induced reversion frequencies of the missense allele *ilv3* (formerly *is2*) were considerably enhanced (AVERBECK *et al.* 1970), whereas those of certain ochre alleles were not or only slightly increased above wild-type level (ECKARDT, KOWALSKI and LASKOWSKI 1975).

In order to determine if a selective advantage in survival of the prototrophic cells ("δ-effect," see ECKARDT and HAYNES 1977; HAYNES and ECKARDT 1979) is the cause of the putatively enhanced mutation frequencies of the *rad24* mutant, we tested the influence of the *rad24* mutation on the reversion of the *his3* allele and the ochre allele *lys2-1* (locus), as well as in the nonselective forward mutation system *ade2* → *adex ade2*. The *rad24* mutation has no effect on UV-induced forward mutation. The induced frequencies of white adenine auxotrophic double mutants are identical for *rad24-1* and wild type, and both increase linearly with dose (data not shown). Also the *LYS* and *HIS* revertants are induced linearly with dose in the low-dose region in both strains, with the *LYS* frequencies being slightly lower and the *HIS* frequencies slightly higher in the mutant compared to the wild type (Figure 4A and B). At higher doses the frequencies increase quadratically in *RAD* wild type and with an approximately third power of dose in the *rad24* mutant. This gives the impression that the *rad24* mutant exhibits enhanced mutability at the *his3* allele compared to wild type. However, this presumed enhanced mutability of the *rad24* mutant is likely to be due to a "δ-effect" that is strongly supported by calculations of the so-called "apparent survival" (data not shown), a test that has been developed by HAYNES, ECKARDT and KUNZ (1985).

Furthermore, spontaneous and UV-induced *HIS* prototrophs have been isolated and checked for their UV sensitivities. Nearly all induced (eight of ten) *HIS* clones showed increased although varying resistance (average dose reduction factor (DRF) *ca.* 1.5) as compared to the (four) *HIS* clones from the control and the *his* cell population (data not shown). Genetic analysis of some of the *HIS* clones revealed that their UV resistance was not due to a second-site suppressor which could have suppressed both *his3* and *rad24-1* (as is the case for the *rad2-20* allele, which is slightly suppressible by ochre suppressors F. ECKARDT-SCHUPP, unpublished data). At the moment it is unclear why certain *HIS* prototrophs induced in the *rad24* population have selective advantage compared to the auxotrophic cell population.

DISCUSSION

The paper deals with a radiation-sensitive mutant, r_1^s, which had previously been isolated and genetically characterized (LASKOWSKI and AVERBECK 1968; LASKOWSKI *et al.* 1968; AVERBECK *et al.* 1970), but had not yet been associated with one of the three repair

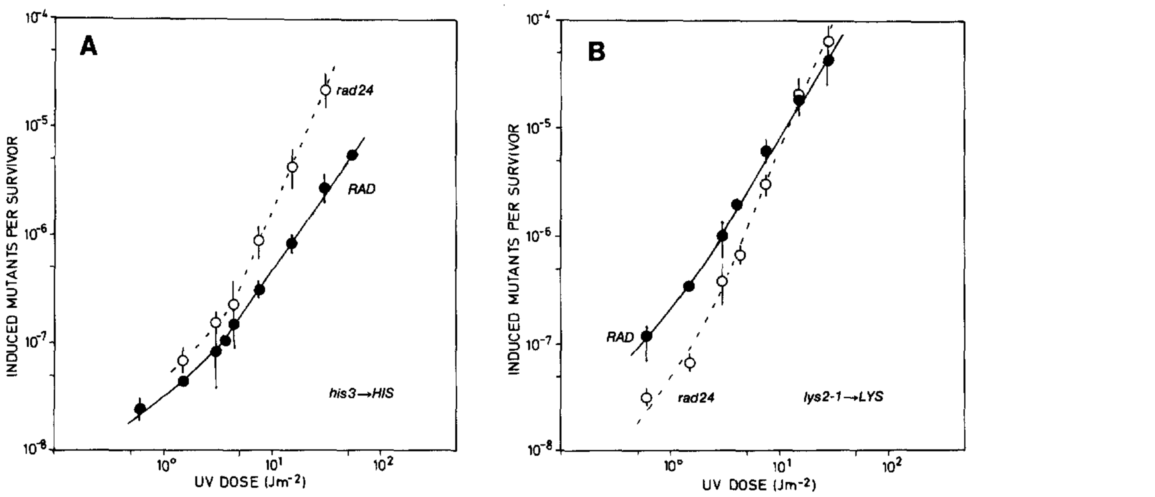

FIGURE 4.—The UV-induced mutation frequencies to *HIS* prototrophy (A) and *LYS* prototrophy (B) (locus revertants of the ochre allele *lys2-1* identified by their red pigmentation due to the unsuppressed ochre allele *ade2-1*) in the repair-competent wild type HT4-21A (●) and the *rad24* mutant 2110-222 (○) have been plotted on a double log plot in dependence of UV dose. The error bars indicate the standard deviation of three experiments.

pathways established for *Saccharomyces cerevisiae*. The r_1^s mutant has been described as moderately UV-, X-ray- and MMS-sensitive (LASKOWSKI and AVERBECK 1968; LASKOWSKI and LEHMANN-BRAUNS 1973) and with enhanced spontaneous and UV-induced mitotic intragenic and intergenic recombination (KOWALSKI and LASKOWSKI 1975). Homozygous r_1^s diploids are sporulation-deficient (AVERBECK 1970).

Complementation and mapping data presented here indicate that r_1^s is unlikely to be allelic with previously designated *RAD* or *MMS* mutants; therefore, we propose to rename this gene *RAD24*, and the r_1^s mutant allele *rad24-1*. The extremely close linkage (1.3 cM) between *RAD24* and *RAD3*, on the right arm of chromosome *V*, may be coincidental or may possibly reflect some functional relationship between the two loci, especially as mutants in each gene display pleiotropic phenotypes and a *RAD3* clone does not complement *rad24-1*. However, *rad24-1* complements and recombines with both *rad3-2* and *rad3-12*.

We measured the UV- and X-ray survival of double mutant strains to determine the epistasis group (if any) to which *RAD24* belongs. Results for UV light implicate *RAD24* in both the *RAD3*- ("excision repair") and *RAD52*- ("recombinational repair") dependent processes. Interactions of *rad24-1* with mutants of the *RAD6* group indicate independence, rather than dependence, of the genes concerned (see above). Similar results were obtained for X-ray repair, except in the case of *rad3-2 rad24-1* where, in contrast to the epistasis seen for UV light, a synergistic interaction with respect to X-ray sensitivity is observed. This suggests that *RAD3* and *RAD24* function in different repair processes handling damage induced by ionizing radiation.

The interaction of the *rad24* mutation with the *rad3* mutation is an interesting one. Normally, excision-deficient mutants are hardly X-ray sensitive (for review, see table 1 in HAYNES and KUNZ 1981; and the *rad2-20* strain in Figure 1); this is commonly interpreted as indicating, at most, a minor role of excision repair in the repair of damage induced by ionizing radiation (COX and PARRY 1968; RESNICK 1969; BRENDEL and HAYNES 1973). However, the *rad3* gene has a moderate X-ray sensitivity that clearly is synergistically enhanced in a *rad24* background. Obviously, *RAD3*-dependent repair of damage induced by ionizing radiation is different from that induced by UV light, and the *RAD24* gene function is involved in the control of the latter, but not of the former. *RAD3* is required for the repair of bulky lesions in DNA, as well as for pyrimidine dimer repair and, in contrast to other genes required for UV excision repair, it is essential for normal mitotic growth (FRIEDBERG *et al.* 1983; HIGGINS *et al.* 1983; NAUMOVSKI and FRIEDBERG 1983).

We found that *rad24-1* has little or no effect on UV-induced mutation frequencies. In this, it differs from many other mutants in the UV-excision repair process which enhance UV mutability (RESNICK 1969; MOUSTACCHI 1969, 1971; AVERBECK *et al.* 1970; LAWRENCE *et al.* 1974; ECKARDT, KOWALSKI and LASKOWSKI 1975). These other mutants are defective in an early step in excision repair, namely incision adjacent to a dimer (REYNOLDS and FRIEDBERG 1981; WILCOX and PRAKASH 1981). Possibly the lack of effect of *rad24-1* on UV mutability suggests a later block in the process, such that dimers would not be left accessible to other, more mutagenic, processes. The previously reported (KOWALSKI and LASKOWSKI 1975) increase in spontaneous and UV-induced mitotic recombination in *rad24-1* diploids resembles the phenotypes of excision-deficient mutants. However, although double mutant UV-survival data suggest that *rad24-1* is defective in *RAD51*-dependent UV repair, this phenotype differs from other mutants in this pathway, including *rad51-3* (MORRISON and HASTINGS 1979) and *rad52-1* (PRAKASH *et al.* 1980), which are largely defective in UV-induced recombination.

In summary, the *RAD24* gene seems to be associated with at least two different repair pathways, and its function depends on the type of damage, *i.e.*, UV- or X-ray-induced. The *RAD24* gene participates in the control of excision repair of UV damage controlled by the *RAD3* epistasis group and also takes part in the repair of ionizing radiation-induced damage which is under control of the *RAD52* epistasis group. Although this is not typical for *RAD* genes it is not unique to the *RAD24* gene either. For *cdc8*, epistasis with both *rad1* and *rad6* has been reported (PRAKASH, HINKLE and PRAKASH 1979). For the repair of damage induced by the bifunctional furocoumarin 8-Methoxypsoralen (8-MOP) the *pso1* gene is epistatic to *rad6* and *rad52*; however, with respect to damage induced by the monofunctional 3-Carbethoxypsoralen (3-CPs), *pso1* and *rad52* interact synergistically (MOUSTACCHI *et al.* 1983; HENRIQUES, DA SILVA and MOUSTACCHI 1985).

These results seem to indicate that a DNA repair "pathway" should be visualized as a dynamic complex of varying functions depending on the actual substrate, *i.e.*, the agent-specific lesion to be repaired, rather than a static, defined sequence of consecutive enzymatic reactions. The participation of one gene in the function of two repair systems may reflect an indirect role or the need for the same biochemical step in independent repair processes. For example, in *E. coli* the gene product of the $uvrD^+$ gene, probably DNA helicase II (KUSHNER *et al.* 1983; KUMURA *et al.* 1983), plays a role in the rejoining of breaks in DNA produced during the excision repair of UV-induced damage (SHIMADA, OGAWA and TOMIZAWA1968), as well as in postreplicational repair (YOUNGS and SMITH

1976), and furthermore in mismatch correction (NEVERS and SPATZ 1975). No attempt has been made so far to analyze biochemically what defect(s) in repair the *rad24* mutant exhibits and what role the *RAD24* gene product might play in repair—either directly or indirectly—*in vivo*.

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