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Rapid detection of allelic recombination at the *gal1* locus in yeast by assay of the recombinant gene product

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SUMMARY

A diploid heteroallelic at the *gal1* locus and producing little residual galactokinase activity was chosen. Following either γ- or UV-irradiation, recombination occurred leading to the formation of wild-type *GAL*1 genes and an increase in detectable galactokinase. An enhanced level of enzyme was first detectable 2 h after irradiation and reached a maximum within 12–14 h. With increasing doses of irradiation, more enzyme was produced and this increase superficially resembled that obtained for plated recombinants. However, far more enzyme was actually synthesized than expected from the number of viable recombinants in the cells assayed. This suggests that recombination must have occurred in cells without colony-forming ability, indeed it may have occurred more frequently in these cells than in viable cells.

1. INTRODUCTION

A drawback to studying recombination by conventional plating experiments is that the recombinants are scored some days after the actual process of recombination has occurred. This presents difficulties in, for example, timing the occurrence of recombination in the meiotic cycle or in examining physiological parameters directly affecting recombination. A more immediate measure of recombination can be obtained by the use of a diploid containing heteroalleles at a particular locus and then detecting wild-type recombinants by directly assaying the gene product of that locus. The advantages of this were first appreciated some years ago by Pritchard (1963) who used β-galactosidase in *E. coli*. Subsequently Joshi & Siddiqi (1968) used alkaline phosphatase to follow the kinetics of recombination after conjugation in the same organism, and Holliday (1971) developed a system employing nitrate reductase in the smut fungus *Ustilago maydis*.

The yeast *Saccharomyces cerevisiae* is increasingly used as a eukaryotic model for molecular biological studies and such a system could be successfully used in this organism to address a number of questions. The *gal1* locus, coding for galactokinase, has been chosen for this purpose, as the detection of this enzyme

is simple and the genes for galactose utilization have been subjected to extensive genetic analysis (for example: Douglas & Hawthorne, 1964, 1966; Nogi *et al.* 1977). A general characterization of this system is presented here.

2. MATERIALS AND METHODS

(i) *Strain*

Z65 has the genotype:

$$\frac{\alpha}{a}\ \frac{gal1\text{-}1}{gal1\text{-}4}\ \frac{lys2\text{-}1}{lys2\text{-}2}\ \frac{tyr1\text{-}1}{tyr1\text{-}2}\ \frac{his7\text{-}2}{his7\text{-}1}\ \frac{ade1}{+}\ \frac{+}{ade2}\ \frac{+}{ura1}$$

and originated from Dr D. C. Hawthorne.

(ii) *Media and cultural conditions*

Liquid medium was 1% DIFCO yeast extract, 2% Bacto peptone (YEP) containing 2% glycerol and 0·2% galactose (YPGG). Cultures were incubated with vigorous shaking.

Solid media contained 2% agar and consisted of: YPD, YEP plus 2% glucose; EB-gal, YEP containing 2% galactose and 20 μg/ml ethidium bromide (Nogi *et al.* 1977); HIS medium which was Wickerhams synthetic minimal medium (Wickerham, 1951) supplemented with 40 μg/ml of lysine, tyrosine, adenine and uracil.

All incubation was at 30 °C.

(iii) *Irradiation of cells*

Mid-log phase cells grown in YPGG were centrifuged, washed twice in 0·9% saline and resuspended in the saline at 10^7 cells/ml for ultraviolet light (UV) irradiation and 2×10^7 cells/ml for γ-irradiation. UV-irradiation was carried out at room temperature with a Hanovia germicidal lamp (254 nm) and at a dose rate of 5·5 J/m^2/sec, while the suspension of cells was at a depth of 1 mm and they were shaken during the irradiation. For γ-irradiation the cells were held on ice and oxygen was vigorously bubbled through the suspension. The γ-source was a ^{60}Co Gamma beam 650 (Atomic Energy of Canada Limited) and the dose rate was 60 krad/min. After irradiation a sample of the cells was resuspended in YPGG and incubated for 15 h. The UV-irradiated cells were incubated in the dark.

(iv) *Assay for galactokinase activity*

This was a slight modification of the method of Parks *et al.* (1971). 5×10^7 cells were removed from the culture, centrifuged and the pellet was placed on ice. The cells were then permeabilized by the method of Adams (1972). One ml of 40% (v/v) dimethyl sulphoxide in distilled water was added and the cells were incubated at

30 °C for 20 min. They were then washed twice with 50 mM Tris-HCl pH 7·8 by centrifugation and resuspended in the same buffer at 10^8 cells/ml.

50 μl of this cell suspension was then added to 50 μl of reaction mix to give final concentrations of 50 mM Tris-HCl pH 7·8, 4 mM ATP, 5 mM-$MgCl_2$, 3 mM-NaF, 1 mM dithiothreitol, 2 mM-[^{14}C] galactose (specific activity 200–500 cpm/nmol). After incubation at 30 °C for the required time, the reaction was stopped by heating the mixture in a boiling-water bath for 90 sec and then placing it on ice. 70 μl of the reaction mixture was placed on a 2·4 cm square of DEAE-cellulose paper (Whatman DE-81) which was held on a stainless-steel wire grid, and this was then washed by four successive 15 min immersions in deionized water. The papers were dried under a stream of warm air and the retained radioactivity was determined using a toluene-based scintillant.

3. RESULTS

The genes for galactose utilization are inducible and in these experiments strains were therefore routinely grown in the presence of small amounts of galactose so that they were fully induced for galactokinase at all times, glycerol providing the source of carbon for growth. The assay for galactokinase measures the phosphorylation of galactose and in this paper a fixed number of cells was used in each assay. Thus, where appropriate, the activity of the enzyme is expressed as nmoles galactose phosphorylated/5×10^6 cells/unit time.

(i) *The selection of a suitable strain*

Firstly combinations of various *gal1* alleles were assayed for residual galactokinase. All produced low but detectable amounts of enzyme, presumably due to leakiness of the mutations and/or weak interallelic complementation. The alleles present in Z65 have a relatively low background, producing from 0·1 to 0·2 % of the enzyme produced by a fully induced *GAL1* recombinant. Furthermore the addition of known numbers of recombinants to Z65 showed that with this background the assay was sufficiently sensitive to detect 5–10 recombinants in 10^5 cells. Finally, when cells of Z65 were irradiated and spread on EB-gal plates to determine induced allelic recombination, frequencies well in excess of this were obtained, so this strain was chosen for study.

(ii) *The kinetics of enzyme production after irradiation*

Plating experiments of this sort do not provide any information about when recombination occurs after irradiation and Fig. 1 shows the kinetics of enzyme production in this system. In this experiment the first increase in functional enzyme occurred 1–2 h after irradiation, although there was slight variability in this since in some experiments it occurred as late as 3 h after irradiation. The amount reached a maximum 6–12 h later, depending on the dose. The use of UV-

or of different doses of γ-irradiation gave broadly similar kinetics and in all cases examined, the maximum amount of enzyme was produced by 12–14 h. Therefore before assaying for galactokinase, cells were routinely incubated for 15 h after irradiation.

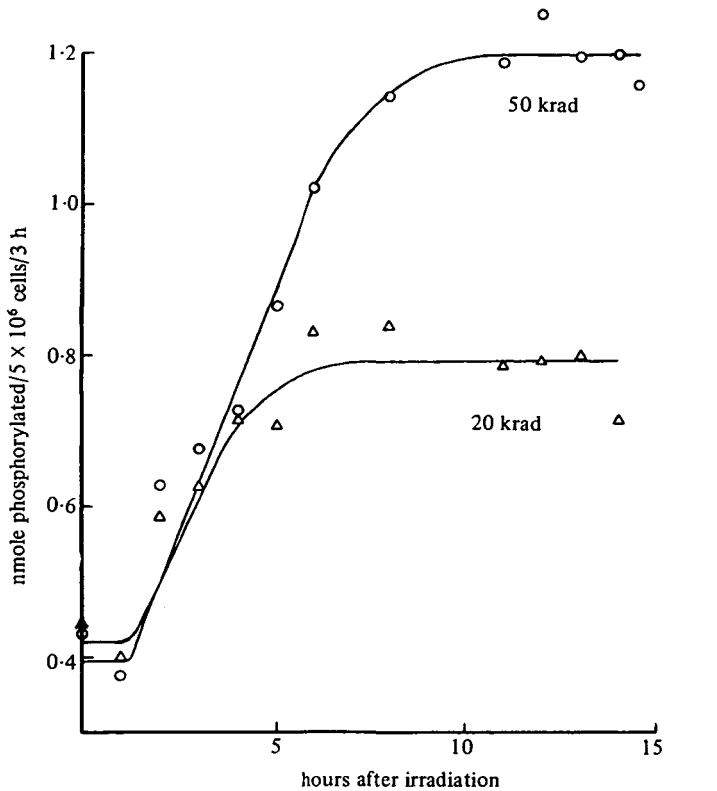

Fig. 1. The kinetics of γ-irradiation induced synthesis of galactokinase. After irradiation cells were incubated in YPGG, samples were removed at the intervals shown and assayed for galactokinase. Each point is the average of triplicate samples of 5×10^6 cells incubated for 3 h in reaction mix.

(iii) *The synthesis of galactokinase after γ-irradiation*

With increasing doses of irradiation there was an increase in the number of plated recombinants and in the production of detectable galactokinase. Figure 2 shows the survival of Z65 after γ-irradiation, while Fig. 3 gives the plated recombinants at the *gal1* locus and also, for comparison, at *his7*. There was an increase in recombination at both loci up to approximately 80 krad, followed by a plateau to at least 140 krad. Little change in the proportion of recombinants in the population occurred after the 15 h incubation (Fig. 3), so that this period of growth in YPGG was not unduly perturbing the situation.

When these irradiated cells were assayed for galactokinase, after a slight lag,

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straight-line plots were obtained (Fig. 4), the slopes of which were taken as a measure of recombination at each particular dose. Using these slopes a dose-response curve for enzyme production was constructed (Fig. 5A). As the purpose of this was to observe induced enzyme the background present in unirradiated cells was subtracted from the irradiated cells. There was an increase in detectable enzyme up to 80 krad but higher doses had no additional effect. Control experiments

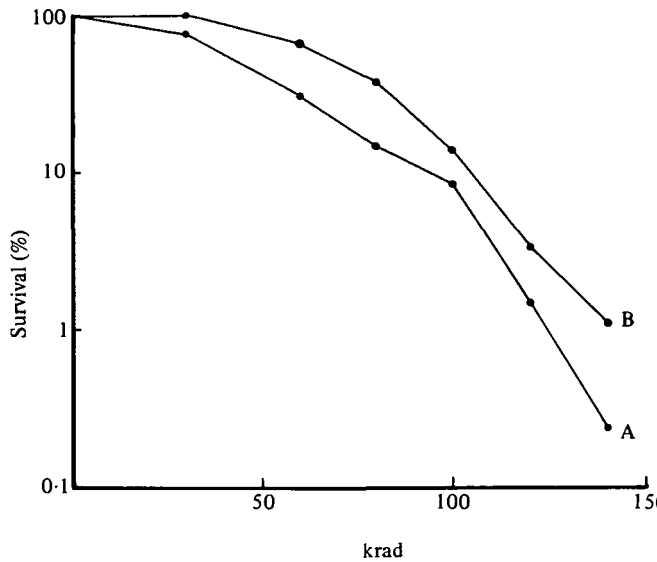

Fig. 2. The survival of Z65 after γ-irradiation. Irradiated cells were diluted and spread on YPD plates (A) immediately after irradiation and (B) after 15 h incubation in YPGG. Colonies were counted after 5 days incubation.

showed that this production of functional enzyme was not due to irradiation-induced reversion of either of the two *gal1* alleles used. When homoallelic diploids were constructed from spore clones of Z65 the maximum increase in galactokinase activity observed after either γ- or UV-irradiation was only 0·04 nmole/$5 \cdot 10^6$ cells/h, while the comparable figures for the heteroallele after γ- or UV-irradiation were 1 nmole (Fig. 5) and 7 nmole (Fig. 8) respectively.

The increase in galactokinase after irradiation (Fig. 5A) superficially resembled the increase in plated recombinants (Fig. 3*A*). In fact, however, considerably more galactokinase was synthesized than expected from the number of plated recombinants. These plated recombinants (Fig. 3*A*) can be translated into the enzyme expected from them by assaying cells containing a known proportion of recombinants and so establishing the amount of enzyme synthesized by given numbers of recombinants. Two curves were then constructed, the first was based on the assumption that recombination frequencies were the same in all cells assayed, both viable and non-viable (Fig. 5B). The other assumed that recombination had occurred solely in the viable cells (Fig. 5C) and was based on the actual

viability of the cells assayed (Fig. 2B). For these to be comparable with the enzyme actually produced, the background of plated recombinants was subtracted in each case.

Clearly, more enzyme was actually synthesized (curve A) than expected from

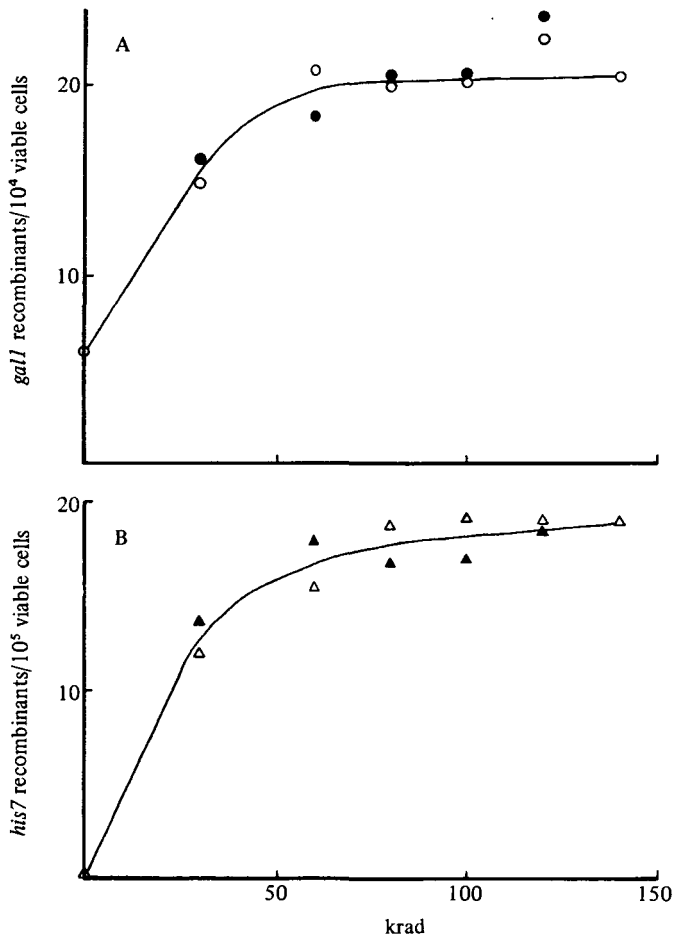

Fig. 3. Recombination (A) at the *gal1* and (B) *his7* loci after γ-irradiation. After suitable dilution irradiated cells were spread on EB-gal plates or on HIS plates, either immediately after irradiation or after 15 h incubation in YPGG. ●——●, *gal1* recombinants, immediate plating; ○——○, *gal* recombinants, after incubation; ▲——▲, *his7* recombinants, immediate plating; △——△, *his7* recombinants, after incubation.

the number of plated recombinants particularly when curve C is considered, the enzyme expected if only the viable cells in the population were recombining. This increased synthesis is not simply the result of over-production of the enzyme due to a radiation-induced perturbation in the control of galactokinase expression in either the viable or the dead cells which have recombined. Control experiments

showed that γ- or UV-irradiated cultures of a Z65 *GAL*1 recombinant produced the same amount of enzyme as unirradiated cultures. Therefore cells without colony forming ability must be capable of recombining and expressing the recombined gene.

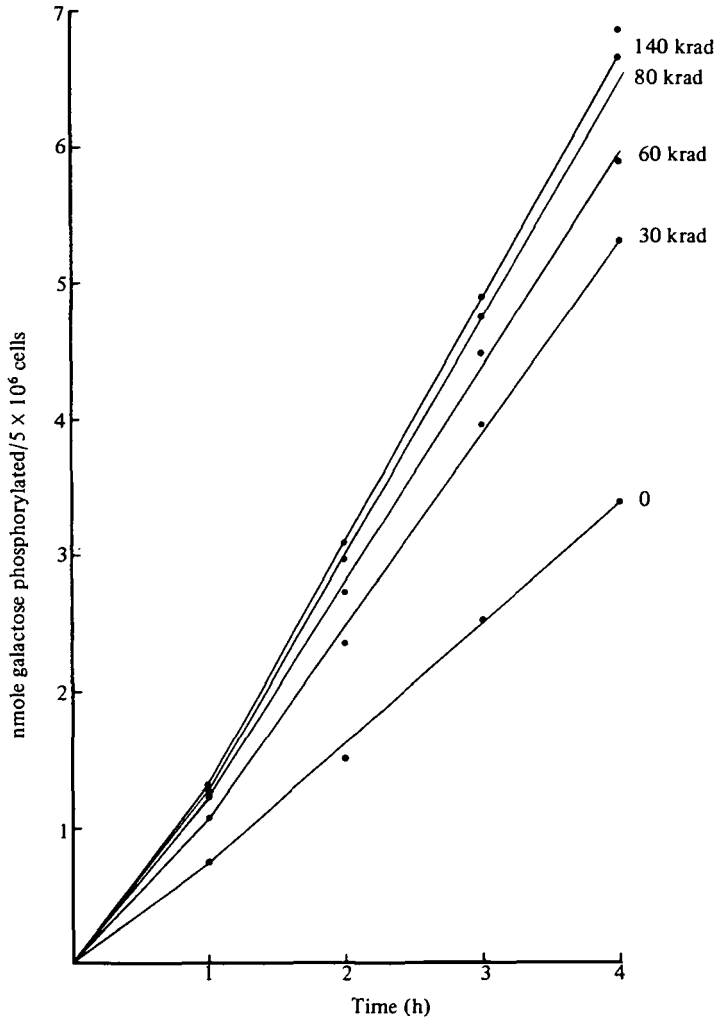

Fig. 4. Synthesis of galactokinase after various doses of γ-irradiation. Cells were irradiated with the doses shown, incubated for 15 h in YPGG and assayed for galactokinase. Each point is the average of duplicate samples removed from the assay at the times shown.

Moreover, for doses greater than 80 krad the enzyme actually produced (curve A) was approximately twice as much as expected, even if recombination had occurred equally in all cells, viable and non-viable (curve B) suggesting a slight preferential occurrence of the process in dead cells. This result may, however, be

slightly distorted by the 15 h incubation since the proportion of recombinants in the population and the ratio of live-to-dead cells could be altered during this period of growth. This is unlikely, however, to be a marked effect as there was little increase in viable cell numbers at any dose (Fig. 2), there was also little change in the proportion of recombinants in the population during this time (Fig. 3) and anyway curve B is based on the recombinants obtained after the incubation. Thus the excess production of galactokinase probably represents a genuine preferential occurrence of recombination in the non-viable cells.

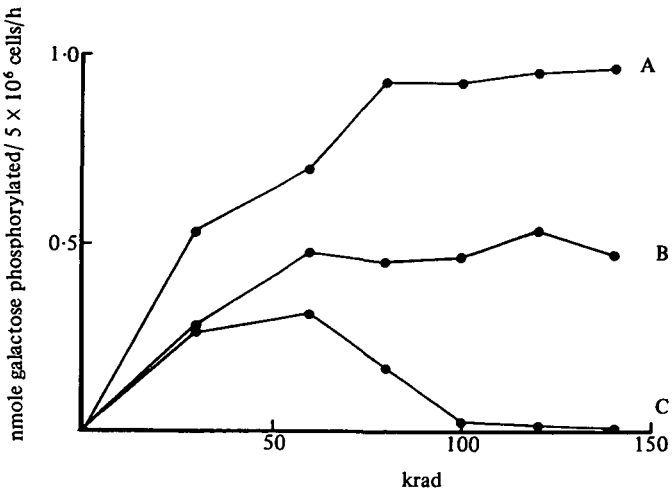

Fig. 5. The actual amount of galactokinase produced after γ-irradiation compared with that expected from the plated recombinants. Curve A is the amount of galactokinase actually produced. Curve B is the amount of enzyme expected if recombination had occurred equally in all the cells assayed, both viable and non-viable. Curve C is the amount of enzyme expected if recombination had occurred only in the viable cells of those assayed. See text for an explanation of how the curves were constructed.

(iv) *The synthesis of galactokinase after UV-irradiation*

This occurrence of recombination in non-viable cells was somewhat unexpected, particularly as in similar experiments recombination was found to be confined to live cells in the related fungus *U. maydis* (Holliday, 1971). To confirm the result in Fig. 5 the experiment was repeated using UV-irradiation. Fig. 6 shows the survival of Z65 after UV-irradiation and plated recombinants at the *gal1* and *his7* loci after the 15 h incubation. UV-light induced more recombinants on plates than γ-irradiation for comparable levels of killing and similarly more functional galactokinase was also produced up to a dose of about 220 J/m^2 (Fig. 7). Unlike γ-irradiation, however, with higher doses of UV there was a reproducible decline in the amount of enzyme produced compared with lower doses.

These actual amounts of galactokinase synthesized were compared with the amounts of enzyme expected from the number of plated recombinants obtained

after the incubation (Fig. 8), as was done with γ-irradiation. Up to 55 J/m^2 there was a reasonably good fit between predicted and actual galactokinase but at higher doses, far more enzyme was produced than could be accounted for solely by the occurrence of recombination in the viable cells (compare curves A and C). Therefore recombination must also have occurred in the non-viable cells of the population.

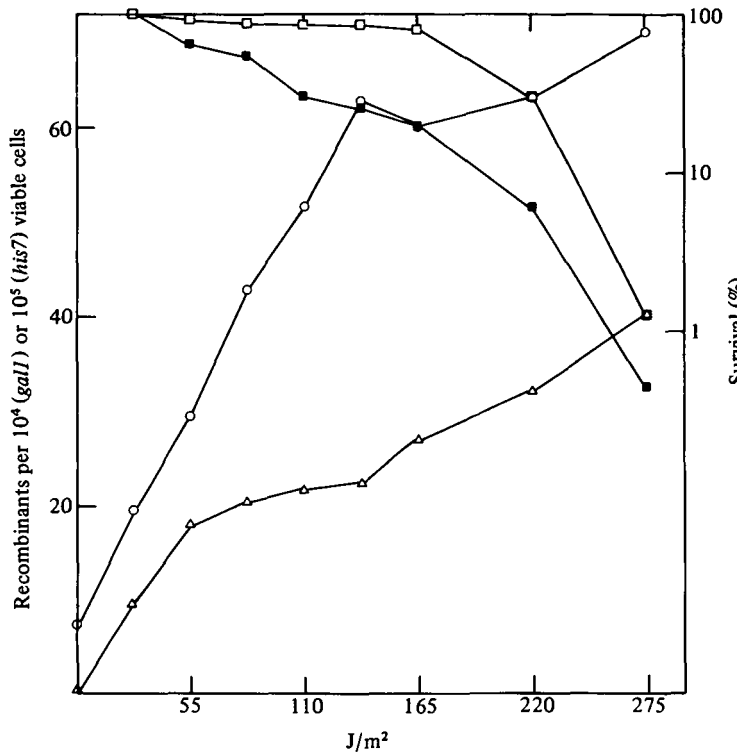

Fig. 6. Survival and recombination of Z65 after UV-irradiation. ■——■, survival, immediate plating; □——□, survival after incubation; ○——○, *gal1* recombinants, after incubation; △——△, *his7* recombinants, after incubation. Legend as in Figures 2 and 3.

Incidentally, petite induction by UV-irradiation will not have affected these results to any great extent since Z65 *GAL*1 petites are able to express galactokinase in YPGG.

On the question of whether the non-viable cells recombine more or less than the viable cells, the enzyme detected with doses of 165 and 220 J/m^2 was, respectively, 3·15- and 3·75-fold more than that expected if all cells recombined with the frequency of the viable cells (Fig. 8, compare A with B). Moreover for the dose of 165 J/m^2 non-viable cells constituted only 16·3 % of the cells assayed (Fig. 6), yet these accounted for 73·5 % of the total enzyme (cf. Fig. 8A, C). Thus there was clearly a preferential occurrence of recombination in non-viable cells, as had been observed with γ-irradiation.

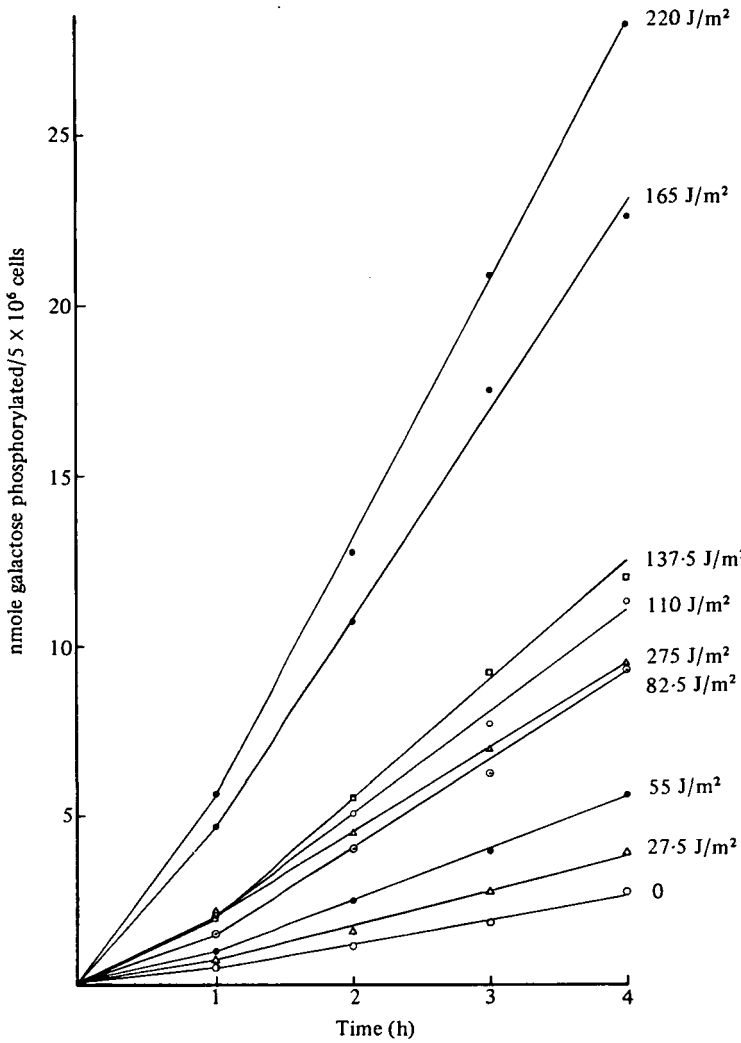

Fig. 7. Synthesis of galactokinase after various doses of UV-irradiation. Legend as in Figure 4.

Finally, similar results to those described here have been obtained with *gal1* alleles other than the two present in Z65. For instance, suitable alleles are those present in strain A364A combined with either X2928-3D-1C or X2181-1B, strains from the Yeast Genetic Stock Centre, Berkeley, USA.

4. DISCUSSION

This paper demonstrates that functional galactokinase can be produced after irradiation of a diploid heteroallelic at the *gal1* locus. Far more enzyme was produced than could be accounted for by radiation-induced reversion of the alleles

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used and most of the galactokinase produced was presumably the result of induced allelic recombination.

Induced enzyme was first detectable 2–3 h after irradiation and therefore in principle all of the recombination may have occurred by this time, the delay in reaching the maximum enzyme activity at 12 h representing the time needed for full expression of the recombinants. For example, since the cells were fully induced

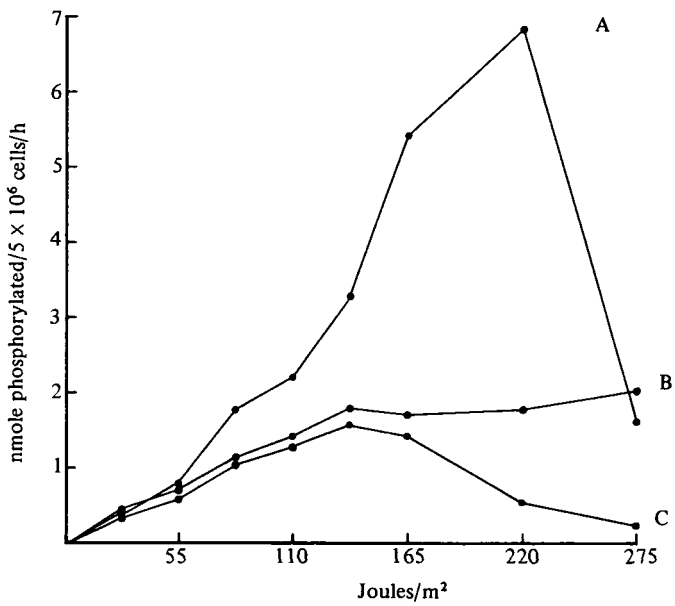

Fig. 8. The actual amount of galactokinase produced after UV-irradiation compared to that expected from the plated recombinants. Legend as in Figure 5.

before irradiation and therefore expressing defective enzyme, the remaining increase could be turnover and replacement of this by active galactokinase. This extreme case is unlikely however, since with lower doses of irradiation, maximum galactokinase was reached at 6 h and it is therefore more likely that the period of increase is due to a prolonged occurrence of recombination together with some time for full expression of active enzyme. Attempts to eliminate any increase due to turnover of defective enzyme by inducing cells only after irradiation gave complex results which did not clarify matters.

Similar kinetics of enzyme production were observed after either γ- or UV-irradiation. However, for an equivalent amount of killing more galactokinase was produced after UV-irradiation than after γ-irradiation although with high doses of UV there was a decline in induced galactokinase compared with lower doses. The reason for this is not understood, however a Z65 *GAL1* recombinant irradiated with a similar dose retained the same galactokinase activity as an unirradiated control. Therefore the decline in Z65 is probably not simply an inactivation of *gal1* itself but may be due to some inhibition of recombination. No

similar effect was observed with γ-irradiation. However using the smut fungus *Ustilago maydis*, Holliday (1971) did observe a comparable effect with γ-irradiation but only at doses higher than 450 krad, far in excess of those used here.

Holliday's system using the *Ustilago* enzyme nitrate reductase, is that most comparable to the yeast system described in this paper. One prominent difference between them, however, is that in *Ustilago* recombination does not occur in non-viable cells but is confined to viable cells. In contrast, in *Saccharomyces* recombination clearly does occur in cells without colony-forming ability, indeed it appeared to occur in them more frequently than in viable cells. This may indicate that recombination enzymes are only induced in cells which are extensively damaged, a high proportion of which are nonetheless subsequently unable to form colonies. Alternatively, recombination enzymes may be present in all cells and the non-viable cells may simply be those with more lesions in their DNA, leading to more recombination during attempted repair. A precedent does exist for this occurrence of recombination in non-viable cells, namely in *rec*B and *rec*C strains of *E. coli* (Birge & Low, 1974). It is therefore somewhat surprising that in *Ustilago maydis*, more closely related to yeast than is *E. coli*, no recombination was detected in non-viable cells. This contrast between the two organisms may be a reflexion of wide differences in DNA metabolism between them, particularly in view of the extreme radioresistance of *Ustilago* (e.g. Holliday, 1971) and its possession of only one DNA polymerase (Banks *et al.* 1976) compared to the three in *Saccharomyces* (Chang, 1977; Wintersburger & Blutsch, 1976).

This question of whether dead cells recombine is not simply an arcane aspect of necrophilia, but is relevant to several of the projects for which this yeast system was devised. For instance, it would be of interest to know whether the several yeast mutants which fail to produce plated recombinants (Game *et al.* 1980; Prakash *et al.* 1980), are in fact competent to produce functional enzyme. This could occur if recombinants died in the process of recombination and therefore it is important to establish the principle of whether dead cells are able to recombine. This question and others such as the timing of recombination in the meiotic and mitotic cycles, are being investigated at present. Indeed, preliminary results suggest that induced mitotic recombination can occur in either the G1 or G2 phases of the cell cycle.

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