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MUTATION INDUCTION BY HEAVY-ION BEAMS WITH DIFFERENT **LET** IN YEAST *SACCHAROMYCES CEREVISIAE*

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Индукция мутаций пучками тяжелых ионов с различными значениями ЛПЭ у дрожжей *Saccharomyces cerevisiae*

Одноклеточные эукариотические дрожжи являются объектом интенсивных радиобиологических и генетических исследований. Проведен анализ летального и мутагенного эффектов ионизирующей радиации с различной линейной передачей энергии (ЛПЭ) у гаплоидных дрожжей *Saccharomyces cerevisiae*. Летальный эффект, который оценивали по наклону кривой доза–эффект, зависел от ЛПЭ. Наибольший летальный эффект среди тестируемых пучков наблюдался для пучка ионов азота ^{15}N с ЛПЭ 108 кэВ/мкм. Этот пучок ионов был в 3 раза эффективнее, чем гамма-лучи с низкой ЛПЭ (0,2 кэВ/мкм). Мутагенный эффект оценивали по частоте мутаций резистентности к канаванину, реверсий мутаций сдвига рамки считывания и замен ГЦ–АТ. Наиболее эффективно ионизирующая радиация индуцирует замены пар оснований, комплексные мутации и менее эффективно — потерю одного нуклеотида. Как правило, комплексные мутации представляют собой комбинацию замены и небольшой делеции. Было показано, что тяжелые ионы эффективно индуцируют как небольшие делеции ($<$ 100 п. о.), так и точечные мутации. Интересно, что тот же пучок ионов азота ^{15}N с ЛПЭ 108 кэВ/мкм проявлял и наибольший мутагенный эффект.

Работа выполнена в Лаборатории радиационной биологии ОИЯИ.

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Koltovaya N., Zhuchkina N., Shvaneva N. E19-2021-19

Mutation Induction by Heavy-Ion Beams with Different LET in Yeast *Saccharomyces cerevisiae*

Unicellular eukaryotic yeast has been subjected to extensive radiobiological and genetic investigations. We analyzed the lethal and mutagenic effects of different linear energy transfer (LET) ionizing radiation in haploid yeast *Saccharomyces cerevisiae*. The lethal effect, which was assessed by the slope of dose-effect curve, was dependent on the LET. The most lethal effect of tested radiation was for the ^{15}N ion beam with LET of 108 keV/μm. This ion beam had a three-times higher lethal effect than low-LET (0.2 keV/μm) γ rays. The mutagenic effect was assessed by the frequency of canavanine resistant mutations, reversions of frameshift and GC–AT substitution. More efficiently the ionizing radiation induces base substitutions, complex mutations and less efficiently — single nucleotide loss. As a rule, complex mutations are combination of substitution and small deletion. It was shown that heavy ions induced efficiently both small deletion ($<$ 100 bp) and point mutations. It is interesting that the ^{15}N ion beam with LET of 108 keV/μm has also shown the most mutagenic effect.

The investigation has been performed at the Laboratory of Radiation Biology, JINR.

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INTRODUCTION

Heavy charged particles present the greatest danger in space. The induced long-term damages, for example, mutations, can give rise to cancer and may be helpful for risk quantification. Until now, the mutation induction has been insufficiently studied presumably because of greater experimental difficulties.

Historically, mutation induction by heavy charged particles was studied in microorganisms. Yeast work with light ions was first started by R. Mortimer and coworkers in Berkeley (USA) [1–3]. The study concerned the evaluation of the relative biological effectiveness (RBE) of several particle beams (D^+, ^{2+}He, ^{3+}Li, ^{5+}B, ^{6+}C, ^{10+}Ne) with energy < 10 MeV/u and LET up to 600 keV/μm inducing mutation and lethality in yeast diploid strains [1]. A strain with *his5-2* and *trp1-1* homozygous alleles was used. In these cases, the nature of reversion was unknown. Now it is known that these mutations are nonsence, ochre, and amber, respectively. Prototrophs arise from various possible mechanisms, such as back-mutation or suppression (as a result of mutation in tRNA which can bind to a termination codon on mRNA or a mutation on the ribosome decreasing the effect of a termination codon). The shape of the mutation curves was complex. Initial section up to 300 Gy was linear and reproducible. At higher doses, in some cases, the curves rushed up, and the reproducibility between experiments was poor. The slope of the initial section of the curves served as characteristic of mutation induction efficiency. The most surprising feature of these experiments was the similarity of the RBE versus LET curves for the lethal and mutagenic effects. For all effects, the curves rise to a maximum of 2–3 times in the efficiency at low LET and then decrease rapidly at higher LET. Moreover, the influence of oxygen effects was also studied [1]. For radiation with low LET, the oxygen effect was ~ 2 and decreased with increases in LET. For haploid yeast cells, the relation between RBE and LET also parallels closely for lethal and mutagenic effects [6, 7]. The maximum sensitivity for budding cells occurs at approximately the same LET (130–150 keV/μm) as that for nonbudding cells [3]. For induction of diploid lethality, the relation with LET was similar to that observed for induction of chromosome effects [1]. For mammalian cells, the RBE–LET dependence of heavy ions was also described by the curve with a local maximum: for survival it was at $\sim 80-200$ keV/μm [8].

Later, the work devoted to very heavy ions was continued by group in Giessen in cooperation with GSI (Germany) [4, 5]. Forward mutations in the *CAN1* gene were induced by heavy ions (^{40}Ar to ^{238}U) of energies 1–10 MeV/u and LET-range from 1200 up to $\sim 15\,000$ keV/μm in haploid

yeast [4, 5]. The mutant frequencies had linear dependence on doses in all cases, and survival was always exponential. There was no dependence of mutation efficiency on either LET or Z^{*2}/β^2, but a prominent influence of ion specific energy on results of the action of long-ranging δ electrons.

However, these studies cannot indicate the nature of mutation induced by heavy-ion beams. There was the general notion that ionizing radiations caused mainly large deletions and only few, if any, point mutations [9]. The molecular structure can now be studied in better detail by applying the polymerase chain reaction (PCR). Unicellular eukaryotic yeast has been subjected to extensive genetic investigations and now provides a suitable basis for analysis of mutations induced by heavy ions. So, Japanese colleagues examined the spectrum of forward mutations in short gene *URA3* [20]. Mutations were induced by carbon ions with LET of 13–107 keV/μm (see Sec. 3). In the present study, we selected simple yeast genetic systems to investigate the mutagenic effects including different molecular events (lethality, frameshift mutation, base pair substitution, and forward *CAN1* gene mutation). Special assay detects GC–AT transition in the *TRP5* gene (*trp5*-A149G allele) [10], (–1) frameshift reversion assay detects mutation that reverts a 4-base insertion in the *LYS2* gene (*lys2-Bgl* allele) [11], and a forward gene mutation assay detects any mutations inactivating the arginine permease gene *CAN1* (resistance to canavanine, $\mathrm{Can^R}$). We studied the lethal and mutagenic effects of heavy-ion beams with LET of 43–177 keV/μm. The experiments demonstrated that heavy ions induced efficiently different types of mutations such as point mutations — base pair substitution or one nucleotide deletion. More efficiently the ionizing radiation induces base substitutions (predominantly GC–AT transition), complex mutations and less efficiently — single nucleotide loss. As a rule, complex mutations are combination of substitution and small deletion.

1. MATERIALS AND METHODS

1.1. Strains and Media. Strain 1663 (*MAT*α *his3-*Δ*200 ura3-52 leu2-*Δ*1 trp5*-A149G) was obtained from Dr. G. F. Crouse (Emory University, Atlanta, Georgia, USA). It was derived from the S288C derivative SJR828a (constructed by Sue Jinks-Robertson) [10]. Strain RDKY3023 (*MAT*a *his3-*Δ*200 ura3-52 leu2-*Δ*1 trp1-*Δ*63 ade2-*Δ*1 ade8 hom3-10 lys2-Bgl*) obtained from Prof. R. D. Kolodner (University of California, California, USA) was also isogenic derivative of S288C. A frameshift allele *lys2-Bgl* was constructed by insertion GATC in *BglII*-site at N-terminus of *LYS2* gene [11]. Yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose). Minimal medium $\mathrm{MM_{300}}$ and synthetic medium (SM) were described in [12]. SM with bases and amino acids (Sigma) was $\mathrm{MM_{300}}$ supplemented with adenine, arginine, histidine, methionine, tryptophan, uracil at 20 mg, tyrosine, leucine, lysine at 30 mg, and threonine at 200 mg, per 1 l $\mathrm{MM_{300}}$. Omission medium was SM minus one of the amino

acids (arginine, tryptophan or lysine). Canavanine (Sigma) was added at a concentration of 60 mg/l in SM-arg. Solid media contained 2% agar.

1.2. Irradiation. The source of γ rays was ^{60}Co (the dose rate 0.7 Gy/min) at radiation therapy unit ROKUS-M of the Dzhelepov Laboratory of Nuclear Problems (JINR, Dubna). Doses ranged between 5–100 Gy. Yeast cells were grown for 5 d on YPD agar (strain 1663) or overnight at liquid YPD (strain RDKY3023). Cell cultures were irradiated in Eppendorf tubes and kept in ice for prevention of double-strand break (DSB) repair.

Proton exposure was performed at the clinical proton beam facility of the Medico-Technical Complex (Dzhelepov Laboratory of Nuclear Problems, JINR, Dubna). Cells were irradiated with unmodified 150-MeV proton beams (LET 0.54 keV/μm) [13, 14]. Dose rate was 0.6 Gy/min. Doses ranged between 5–30 Gy. Yeast cells were grown for 5 d on YPD agar (strain 1663) or overnight at liquid YPD (strain RDKY3023). Cell cultures were irradiated in Eppendorf tubes and kept in ice.

Table 1. **Characteristics of charged particles**

Beam	Z	E_0, MeV/u	E_{target}, MeV/u	LET$_\infty$, keV/μm	Dose rate, Gy/s	$D_{\max}$, Gy
p^+	—	171	150	0.539	0.55	30
^{11}B	5	36	33.8	43	5–10	100
^{11}B	5	36	33	43	1–4	100
^{11}B	5	33	22.01	61	0.1–0.2	100
^{15}N	7	46	45.4	67	0.4–0.7	80
^{15}N	7	46	30.22	92	0.02–0.67	80
^{15}N	7	46	38.2	108	0.3–1.1	80
^{15}N	7	46	20.8	124	0.02–0.67	80
^{15}N	7	46	13.5	177	0.05–0.2	80
^{18}O	8	35	35	171	0.05–0.2	100
^{20}Ne	10	50	49.2	126	0.5–2.0	1000

* Z — atomic number; E_0 — energy of ion beam; E_{target} — particle energy on the sample surface

In the case of accelerated heavy ions, mono- or thin layers of yeast cells were irradiated with four kinds of monoenergetic beams (^{11}B, ^{15}N, ^{18}O, and ^{20}Ne) generated in the U-400M cyclotron (Flerov Laboratory of Nuclear Reactions, JINR, Dubna) with doses of 15–100 Gy. Characteristics of the different forms of radiation used are listed in Table 1. In several cases, the ions were degraded in energy by means of aluminum foils to correspond to LET values of 61 keV/μm (^{11}B), and of 92, 108, 124, and 177 keV/μm (^{15}N). Yeast 7-days cultures in stationary phase were grown on YPD agar at 28°C, harvested, washed and resuspended in water to a concentration of 10^9 cells/ml. The percentage of budding cells was less than 3%. For irradiation, dry 4-% agar in aluminum plate with cap was prepared and covered by mylar membrane filter with diameter of 20 mm. The 50-μl

samples were pipetted onto the filters for ion exposure. Plates were kept in ice before and after irradiation. Immediately before irradiation, plates without caps were fixed on the cassette disk. Beam monitoring and automatic change of cell patterns nested on the disk container were provided by the special automatic irradiation facility Genome-M [15]. The total ion range was less than 100 μm in cell culture. The mean LET of the radiations studied did not vary appreciably throughout the target culture. Immediately after irradiation, the cells were resuspended by putting filters to a tube containing sterile water (1 filter/1 ml H_2O).

Nonirradiated and irradiated suspensions after serial dilutions were plated on YPD or appropriate selective omission media to evaluate cell survival and mutagenesis, respectively. Colonies arising on YPD and appropriate selective media plates were counted after 3–5 d of incubation at 28°C.

1.3. DNA Sequencing. Yeast genomic DNA was isolated by a miniprep method [16]. The PCR amplification of genes was performed using GenPak@PCR Core (Isogene Laboratory, Moscow) and primers on Bio-Rad Termal Cycler T100. Primers were synthesized by Syntol company (Moscow).

Approximately 86% of the spontaneous and heavy-ion induced mutants had been sequenced using primers for amplification of the *CAN1* gene: CANF (5'-tct-gtc-gtc-aat-cga-aag-3'), CANR (5'-ttc-ggt-gta-tga-ctt-atg-agg-gtg-3'); and for sequencing: can1-1F (5'-ttc-tgt-gtg-gtt-tcc-ggg-tg-3'), can1-1R (5'-att-gac-cca-cgt-ctg-tgg-tg-3'), can1-2F (5'-cgc-cga-cat-aga-gga-gaa-gc-3'), can1-2R (5'-att-tca-ccc-aag-gac-tgc-gt-3'), can1-3F (5'-atc-cac-acc-tct-gac-caa-cg-3'), can1-3R (5'-gaa-tcc-aac-tgg-gcc-ggt-aa-3'), can1-4F (5'-gtt-acc-ggc-cca-gtt-gga-tt-3'), can1-4R (5'-gcc-aaa-tgc-agc-agt-aac-ga-3'), can1-5F (5'-cca-cca-aag-gtg-gtg-ttc-ca-3'), can1-5R (5'-gag-aat-gcg-aaa-tgg-ctg-gg-3'). The remainders of the samples ($\sim$10%) were amplified using primers can1-1F, CANR, and can1-5R.

Primers for amplification of the *LYS2*-fragment were LYS2-9F (5'-gct-ttg-agt-gta-tgg-gct-gc-3'), LYS2-9R (5'-ata-cac-ccc-acg-aaa-tcg-ca-3').

The PCR protocol was as follows: initialization step — 93–94°C, 3 min; denaturation step — 94°C, 30 s; annealing step — 58°C, 30 s; extension/elongation step — 72°C, 2 min (with the exception of: annealing step — 59.5°C, extension step — 72°C, 1 min, for *LYS2*-fragment). We performed 35 cycles of this reaction. Amplified fragments were sequenced on MiSeq Illumina by Syntol (Moscow). Obtained results were analyzed using software for DNA sequencing — CodonCode Aligner and BLAST. Reference strain was S288C.

2. RESULTS

2.1. Sensitivity of Yeast Cells to Ionizing Radiation. Yeast cells of two haploid strains 1663 and RDKY3023 were irradiated with γ rays, protons, and four kinds of ion beams with different LET values (Table 1). The dose responses of the survival fractions were determined (Figs. 1 and 2). A dose-dependent decrease was observed for all radiation types. The survival

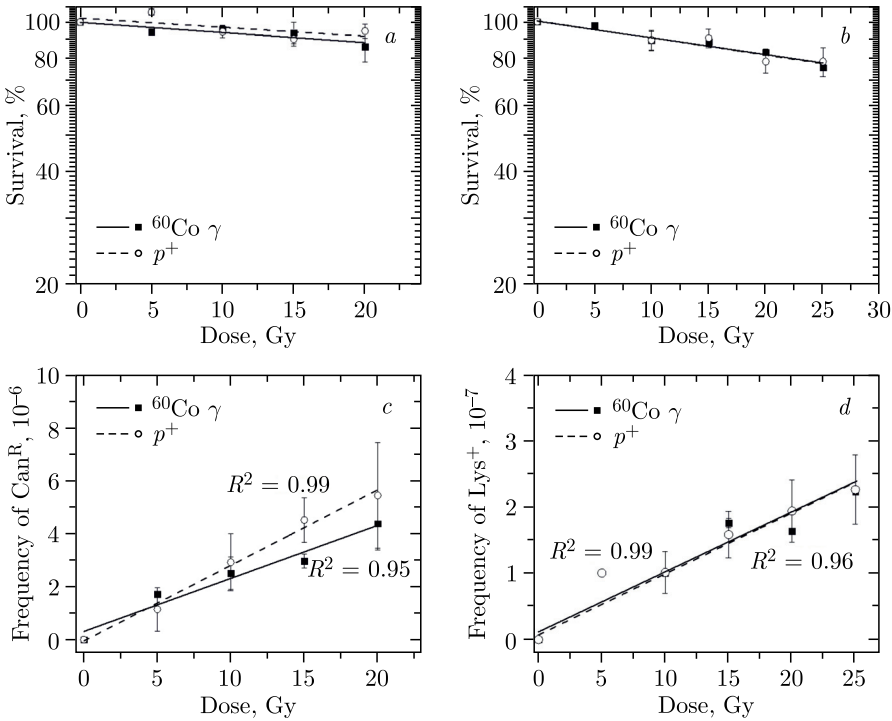

Fig. 1. Survival and mutation frequency of 1663 (a, c) and RDKY3023 (b, d) strains exposed to low-LET radiation ^{60}Co γ rays (squares) and high energy protons (circles)

(Fig. 1, a, b) was very similar for low-LET radiation (photons and high energy protons) at the used doses up to 25 Gy. Survival curves obtained with a variety of accelerated charges of high-LET particles with doses up to 100 Gy are shown in Fig. 2, a and b. Cell survival was fit by exponential function for both yeast haploid strains. For each curve in Fig. 2, a and b, the coefficient of determination R^2 was calculated and ranged between 0.90–0.99.

An important parameter to evaluate the difference in the biological effects of radiation is relative biological effectiveness (RBE). Values of RBE were determined using the ratio of the fitting curve slopes (Fig. 2, a, b). As shown in Fig. 2, c and d, the survival was influenced by LET values. The dependence of RBE of cell inactivation on LET was the curve with maximum at LET of about 100 keV/μm. For strain 1663, ^{15}N ion beam (124 keV/μm) was 3.3 ± 0.7 times more effective than γ rays, but ^{20}Ne (127 keV/μm) was more efficient (3.9 ± 0.7) than light nuclei ^{11}B and ^{15}N at the same LET (Fig. 2, c). For RDKY3023, the maximum value of RBE was 3.3 ± 0.4 at 67 keV/μm (^{15}N) or 3.0 ± 0.5 at 126 keV/μm (^{20}Ne) (Fig. 2, d) which fits well with the earlier results obtained by C. A. Tobias [6] with haploid *Saccharomyces cerevisiae*. It was shown that at low LET, the radiosensitivity was approximately constant,

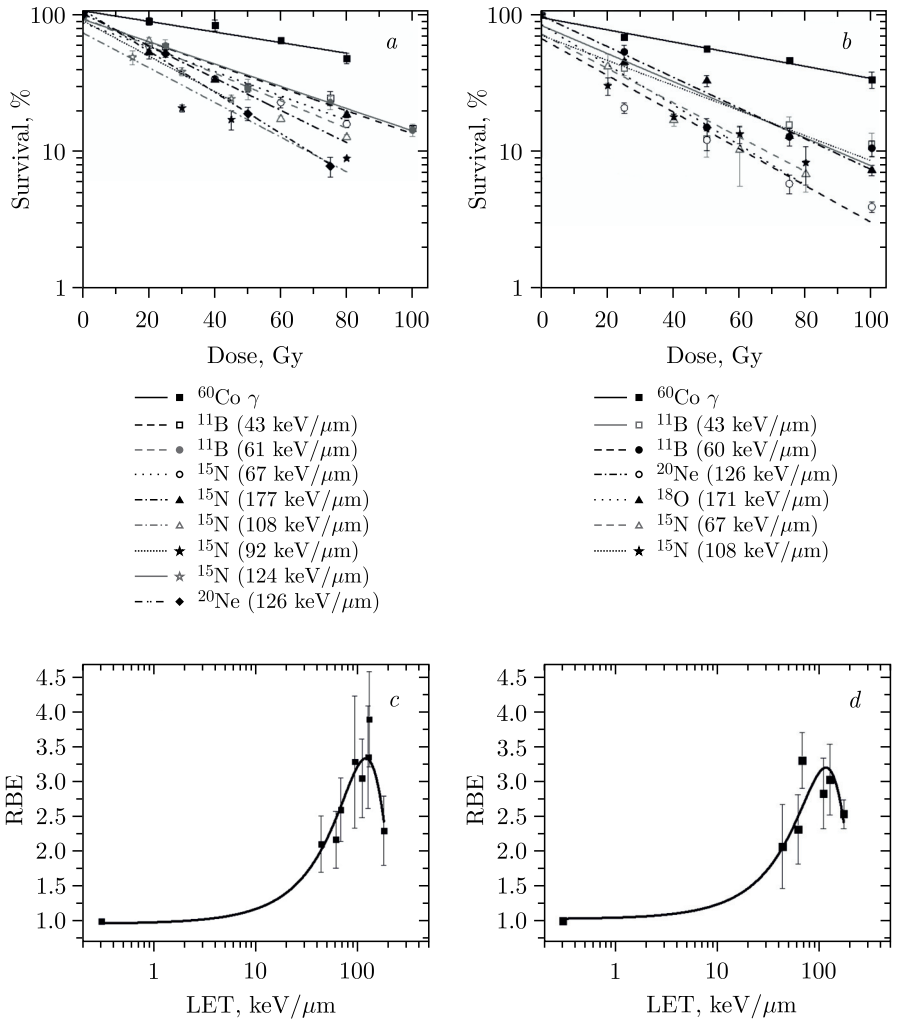

Fig. 2. Survival and mutation frequency of yeast strains exposed to ^{60}Co γ rays and ionizing radiation with different LET listed in Table 1. Representation of a mean of 1–3 experiments and standard errors. *a*) Survival of strain 1663. *b*) Survival of strain RDKY3023. *c*) The RBE for lethality as a function of LET for strain 1663. The RBE was estimated as a ratio of slope of lethality curve of γ rays to that of ion beam. R^2 was in the range of 0.90–0.99 with one exception 0.85 for ^{15}N beam with LET of 92 keV/μm. *d*) The RBE for lethality as a function of LET for strain RDKY3023. The RBE was estimated as a ratio of slope of lethality curve of γ rays to that of ion beam. R^2 was in the range of 0.90–0.98 with two exceptions 0.74 and 0.85 for ^{11}B beams with LET of 43 keV/μm and 60 keV/μm, respectively

then it was a region of increasing sensitivity with increasing LET, and finally at high-LET values, it was a region of decreasing sensitivity. The observed maximum RBE of ~ 2.0 was obtained with α particles from ^{210}Po (3.4 MeV) at LET of 120 keV/μm [6] and ~ 3.0 for carbon ions (114 MeV) [3].

2.2. Mutagenic Effect of γ Rays and Ion Beams on Yeast Cells. To investigate the mutagenic effects of heavy-ion beams in eukaryotic cells, we have used several genetic assays. The frequency of induced point mutations,

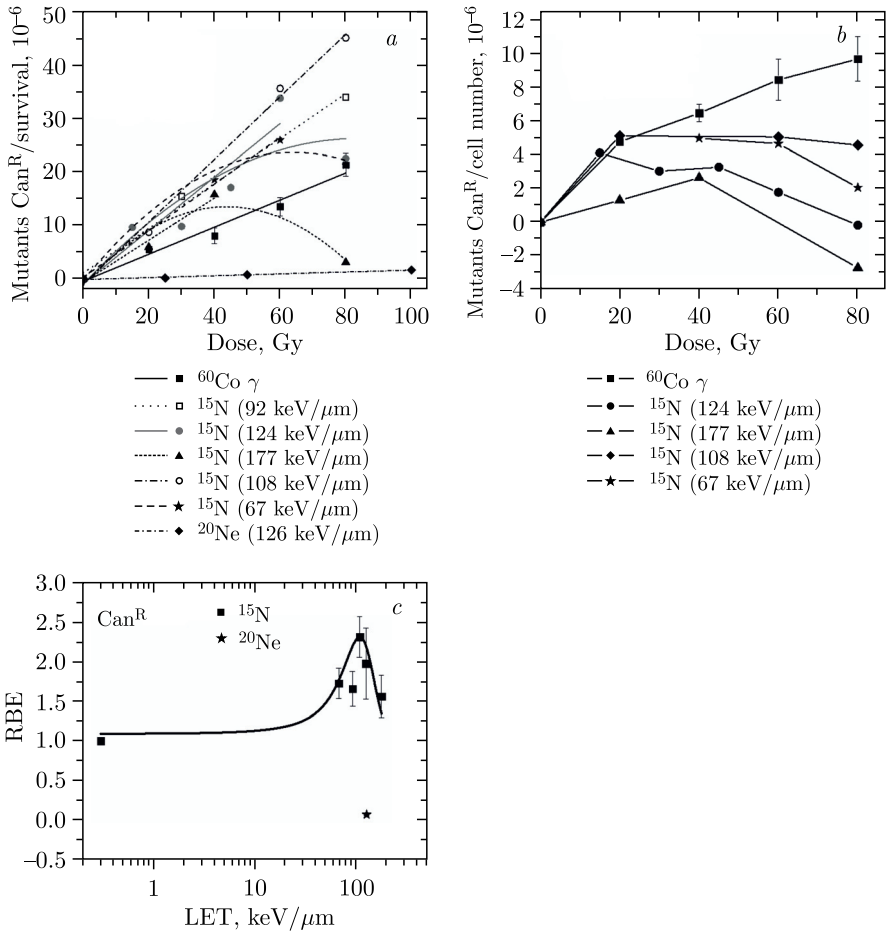

Fig. 3. Induction of Can^R by ^{60}Co γ rays and accelerated ions. Representation of a mean of 1–3 experiments and standard errors. *a*) Frequency of Can^R mutants per survival. *b*) Frequency of Can^R mutants per cell number after ^{60}Co γ rays and accelerated ^{15}N ions treatments. *c*) Efficiencies of the various radiations for point mutations which were calculated from linear part of curves presented in (*a*). R^2 was in the range of 0.96–0.992 with one exception 0.66 for ^{15}N beam with LET of 124 keV/μm

such as forward (Can^R), (−1) frameshift (Lys^+), and GC–AT base pair substitution (Trp^+) in the yeast *Saccharomyces cerevisiae* (see Sec. 1), was studied using selective medium. The mutagenic effects of heavy-ion beams were compared with those of γ-ray irradiation. The mutagenic effect (Fig. 1, *c*, *d*) was very similar for low-LET radiation (photons and high energy protons) at the used doses up to 25 Gy.

The dose-effect curves of forward gene mutations and frameshift mutations were described by the linear function for γ-ray irradiation or polynomial function for heavy-ion irradiation up to 100 Gy (Fig. 3, *a*, *b* and Fig. 4, *a*). Most often the frequency of heavy-ion induced mutation increased with increasing dose, peaked at the doses of 40–60 Gy, and decreased subsequently. However, in the case of LET $\sim$ 92–108 keV/μm, the frequency of Can^R increased consistently with increasing dose up to 80 Gy, and we could not observe plateau or decreasing when using interval of doses (Fig. 3, *a*). The maximum frequencies of Can^R and Lys^+ mutants induced by the beams with LET of 108 keV/μm were $4.5 \cdot 10^{-5}$ and $1.5 \cdot 10^{-6}$, with a background spontaneous mutant frequency of $(1.7 \pm 1.3) \cdot 10^{-6}$ and $(4.1 \pm 2.6) \cdot 10^{-8}$ (i.e., increase in 26-fold and 37-fold, respectively). Thus, the heavy ions were more effective in the induction of point mutations than the γ rays at doses up to 80 Gy, the exceptions were ^{20}Ne irradiation with LET of 126 keV/μm for Can^R mutant induction or ^{18}O irradiation with LET of 171 keV/μm for Lys^+ mutant induction less effective at all doses.

In the previous study [17], we examined the differences between induction of base pair substitution by ^{60}Co γ rays in two models of haploid and diploid

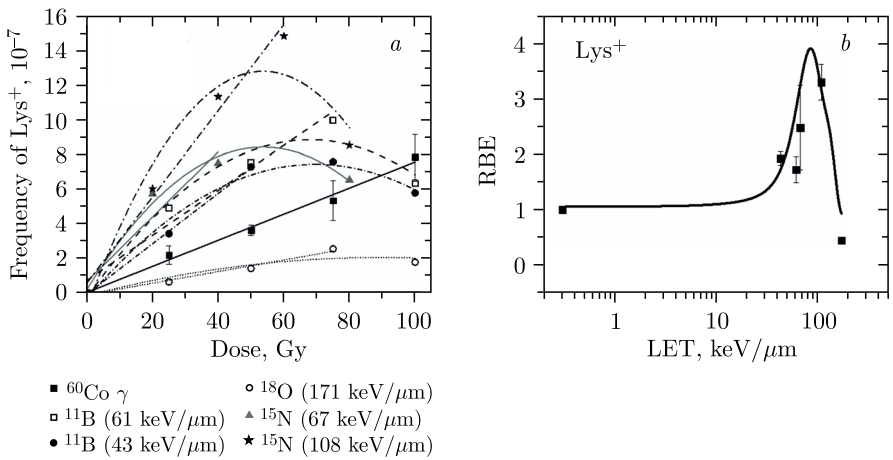

Fig. 4. Induction of frameshift Lys^+ mutations by ^{60}Co γ rays and accelerated ions listed in Table 1. Representation of a mean of 1–3 experiments and standard errors. *a*) Dose-effect curves. *b*) Efficiencies of the various radiations for point mutations which were calculated from linear part of curves presented in (*a*). R^2 was in the range of 0.95–0.998 with one exception 0.83 for ^{15}N beam with LET of 67 keV/μm

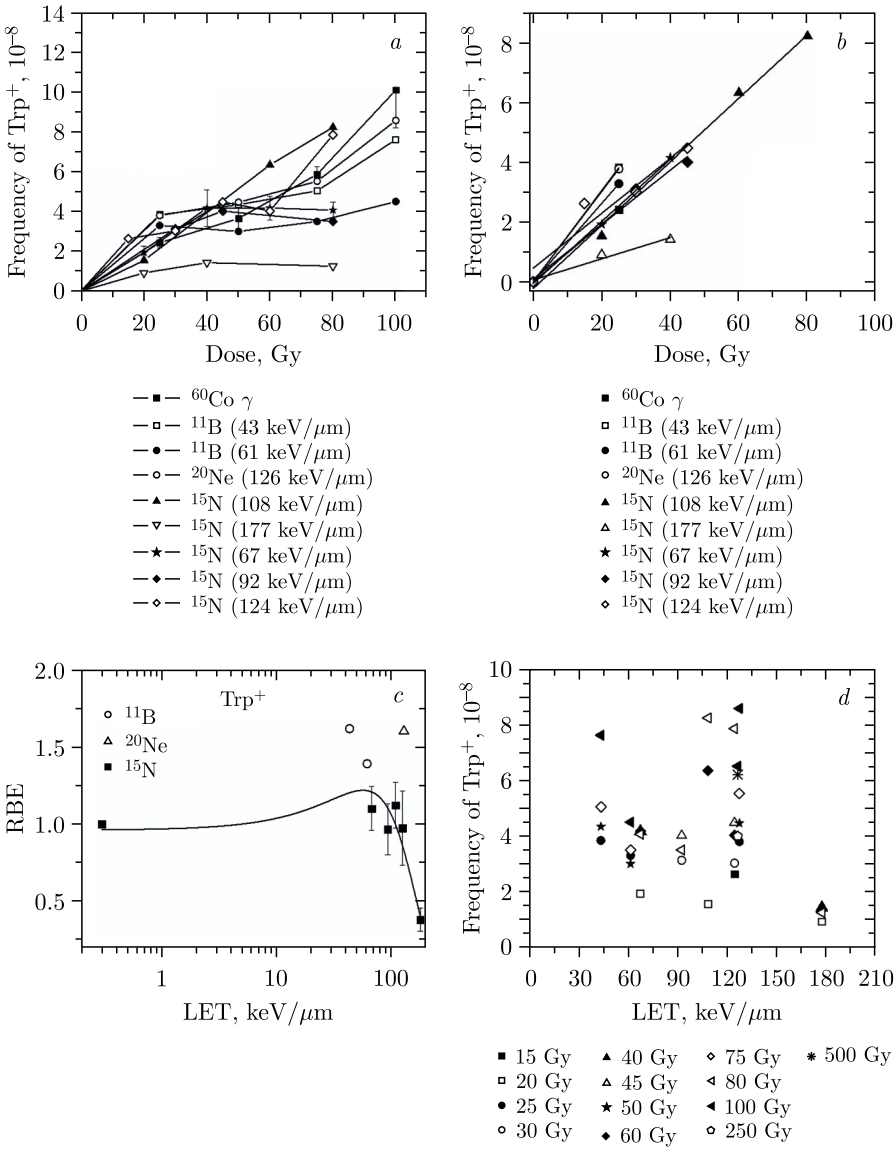

Fig. 5. Induction of GC–AT transition in strain 1663 by heavy-ion beam irradiation. Frequency of base pair substitution (*a*) and approximation (*b*) by linear functions of plots at small doses. R^2 was in the range of 0.95–0.996 with one exception 0.87 for ^{15}N beam with LET of 124 keV/μm. *c*) Efficiencies of the various radiations for base pair substitution calculated using ratio of slopes as in (*b*). *d*) Dependence of mutation frequency on LET

yeast strains. We have used a collection of 6 isogenic haploid *trp5*-strains and 14 isogenic haploid and diploid *cyc1*-strains that are specific markers of all possible base pair substitutions. These strains differ from each other only in single base substitutions within codon-50 of the *trp5* gene or codon-22 of the *cyc1* gene. In the present work, to test base pair substitution, we have used only one *trp5*-strain 1663 which detects GC–AT transition induced more efficiently by γ rays. The linear dependence of the substitution frequency on doses in the range of doses up to 100 Gy for haploid *trp5*-strains ($R^2 = = 0.52–0.90$) or up to 1000 Gy for haploid *cyc1*-strains ($R^2 = 0.83–0.998$) was observed [17]. Comparison of two haploid assays showed that GC–AT transition was induced more efficiently in *trp5*-assay (at 25–100 Gy) than in *cyc1*-assay (at 250–1000 Gy) with factor 10 if to compare the slope of curve.

In the present study, we investigate the induction of GC–AT transition by heavy-ion beams with different LETs. The dose-effect curves had a complex form including two ascending fragments with plateau between them (Fig. 5, *a*). The first maximum was reached at the doses of 25–45 Gy, except for LET of 108 keV/μm for which linear dependence ($R^2 = 0.99$) was observed. In the only experiment the cells were irradiated with doses up to 1000 Gy (^{20}Ne, LET of 126 keV/μm), and the frequency of transition $(2.9–8.3) \cdot 10^{-8}$ did not change significantly in the region of doses 250–1000 Gy [18]. Thus, for heavy ions, the frequency reached a plateau at ~ 100 Gy at which it was $\sim 8.57 \cdot 10^{-8}$ (Fig. 5, *a*) but did not increase linearly as in the case of γ rays [17].

To compare the RBE of mutations at different LET, mutation effectiveness was calculated from linear part of the mutation induction curve (Figs. 3, *a*, 4, *a*, 5, *b*). Mostly, R^2 of linear parts was in the range of 0.95–0.99. The RBE showed a maximum around 100 keV/μm, both for survival and for forward (*can1*) and frameshift (Lys$^+$) mutations (Figs. 2–4). The RBE was $(2.3 \pm \pm 0.3)$ and (3.3 ± 0.3) at 108 keV/μm for forward and frameshift mutations, respectively (Figs. 3, *c* and 4, *b*). For base substitution, we did not find such a dependence (Fig. 5, *b–d*). Frequency practically did not depend on LET except for highest LET of 177 keV/μm at which it falls.

2.3. Sequencing. To investigate the molecular nature of mutations, we have used the same genetic assays. In the case of base substitutions, the nature and the site of molecular event are known. In the present study, we have tested GC–AT transition in G149 of mutated allele of the *TRP5* gene (2124 bp). In other two assays, we detected the phenotypic effect and did not know precisely a molecular event and were forced to spend sequencing.

The nature of mutations arising in the *lys2-Bgl* reversion assay is limited by two features of the *LYS2* gene (4179 bp). Frameshift mutations can arise only in the "open window" near mutation, and reversions are constrained by the nature of the sequence in this region. In addition, the protein sequence changes resulting from the frameshift mutations must yield an active protein. Earlier, D. X. Tishkoff et al. [11] using a closely related strain RDKY2672 and analyzing 46 spontaneous (24 *rad27* Lys$^+$ and 22 *msh2* Lys$^+$) mutants,

localized the "open window" between nucleotides 368–497, i.e., 129 bp. In the present study, independent 65 Lys^+-mutants were isolated. Nucleotides 171–696 of the *LYS2* gene were amplified and sequenced as described in Sec. 1. Analysis (Table 2) showed that in the case of *lys2-Bgl* mutation, the "open window" was a 182-bp (nucleotides 318–500) region bounded by the

Table 2. **Spectrum of frameshift mutations (spontaneous and induced by ^{15}N, 67 keV/μm) observed in strain RDKY3023. DNA sequence analysis of *LYS2*-fragment (nucleotides 171–696)**

Type/Dose	Number of Lys^+-mutants, %			
	0 Gy*	0 Gy	20 Gy	80 Gy
Base substitution	0	0	0	0
Frameshift	C1 → C0 1 C2 → C1 3 C3 → C2 — C4 → C3 2 A1 → A0 1 A2 → A1 — A3 → A2 2 A4 → A3 1 A5 → A4 — A6 → A5 5 T1 → T0 — T2 → T1 — T3 → T2 — T3 → T5 — T5 → T4 3 G1 → G0 — G2 → G1 1 G3 → G2 — 18**(75.0)**	C1 → C0 2 C2 → C1 1 C3 → C2 — C4 → C3 1 A1 → A0 3 A2 → A1 — A3 → A2 2 A4 → A3 — A5 → A4 — A6 → A5 7 T1 → T0 — T2 → T1 — T3 → T2 2 T3 → T5 2 T5 → T4 1 G1 → G0 — G2 → G1 1 G3 → G2 — 22**(78.6)**	C1 → C0 1 C2 → C1 2 C3 → C2 — C4 → C3 3 A1 → A0 — A2 → A1 — A3 → A2 — A4 → A3 — A5 → A4 — A6 → A5 1 T1 → T0 1 T2 → T1 2 T3 → T2 2 T3 → T5 — T5 → T4 1 G1 → G0 1 G2 → G1 1 G3 → G2 — 15**(78.9)**	C1 → C0 — C2 → C1 — C3 → C2 — C4 → C3 2 A1 → A0 1 A2 → A1 2 A3 → A2 — A4 → A3 — A5 → A4 — A6 → A5 1 T1 → T0 — T2 → T1 1 T3 → T2 — T3 → T5 — T5 → T4 3 G1 → G0 2 G2 → G1 1 G3 → G2 — 13**(72.2)**
poly 1 → 0	2	5	3	3
poly 2 → 1	4	2	5	4
poly 3 → 2	2	4	2	0
poly 4 → 3	3	1	3	2
poly 5 → 4	3	1	1	3
poly 6 → 5	5	7	1	1
poly 3 → 5	—	1	—	—
Complex	Δ1 + BS 5 Δ + BS — Δ — ins(+2) + + 2BS 1 6**(25.0)**	Δ1 + BS 4 Δ74 + BS 2 Δ — 6**(21.4)**	Δ1 + BS 2 Δ + BS — Δ25, Δ93 2 4**(21.1)**	Δ1 + BS 1 Δ + BS — Δ4, Δ7, Δ22, Δ7 4 5**(27.8)**
Total	24	28	19	18
"Open window"	368–497 (129 bp)	343–485 (142 bp)	318–500 (182 bp)	363–467 (104 bp)

* Cited from [11] for strain RDKY2672

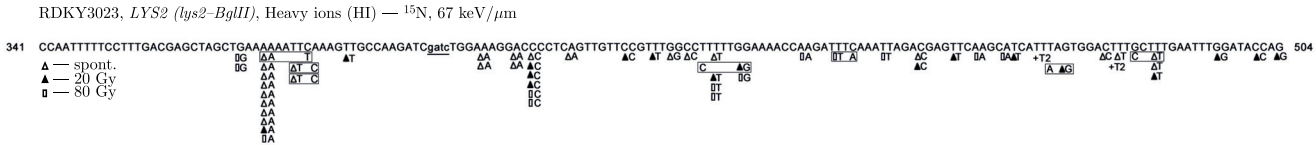

Fig. 6. Summary of Lys$^+$-revertants induced by ^{15}N (67 keV/μm) in strain RDKY3023. Nucleotide sequence coordinates 341–504 of the coding strand of the *lys2-Bgl* allele are displayed. A total of 28 background mutants, 19 mutants induced after irradiation at a dose of 20 Gy, and 18 mutants induced after irradiation at 80 Gy were analyzed. Complex mutations are given in boxes. Deletions Δ(318–412) and Δ(369–389) are not represented. Frameshift insertion *gatc* is underlined

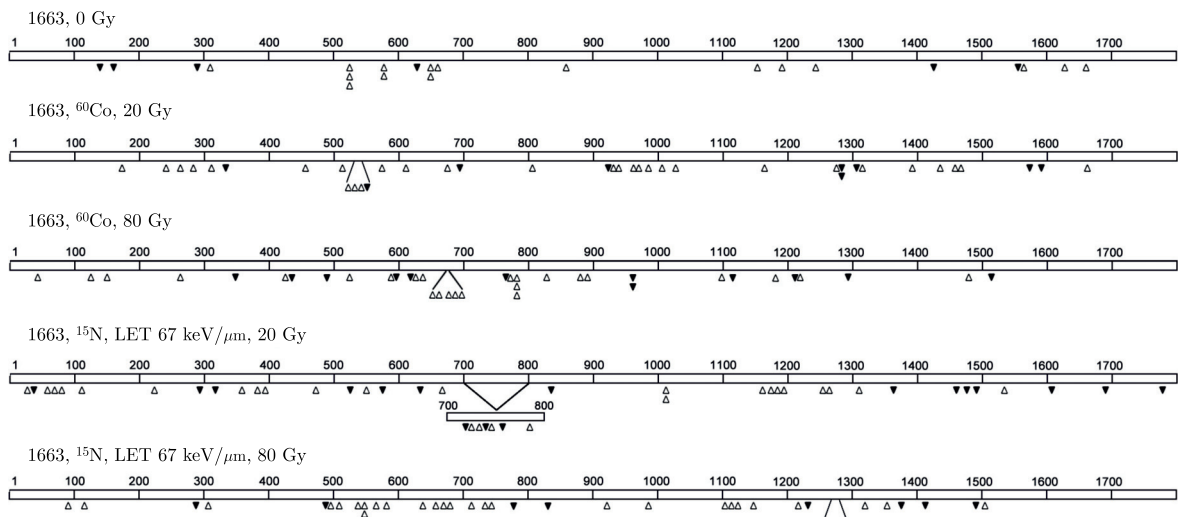

Fig. 7. Mutation spectrum spontaneous and induced by ^{60}Co γ rays (20 and 80 Gy) and nitrogen-ion beam ^{15}N with LET of 67 keV/μm (20 and 80 Gy) at irradiation of yeast strain 1663. Numbers refer to the nucleotide position of the *CAN1* gene (1773 bp). White triangles — substitute mutations; inverted black triangles — insertions/deletions

upstream and downstream termination codons flanking the *lys2-Bgl* mutation (+GATC, nucleotides 388–391) (Fig. 6).

As shown in Table 2, only 72–79% of the arising mutations were Δ1-deletions, but 21–28% were multiple mutations. One base pair deletion occurs in poly stretches more efficiently, for example, 6A, as seen from Table 2 and Fig. 6. Multiple mutations often included two adjacent mutations, more frequently Δ1 + BS, within one helical turn (mutation cluster). This spectrum was the same for spontaneous mutations and after irradiation by ^{15}N (67 keV/μm), but multiple mutations included large deletions which met more often in the last case.

To analyze the mutations that can arise in a less constrained situation, the sequences of Can^{R} mutations, which inactivate the 1.8-kb arginine permease gene, were determined. We selected spontaneous mutants and those induced by γ rays and nitrogen-ion beam with LET of 67 keV/μm at doses of 20 and 80 Gy, identified the nucleotide sequences of the *CAN1* gene of the mutant cells and analyzed the mutation spectrum. Radiation induced transitions, transversions, insertions, inversions, deletions, and multiple mutations in the *CAN1* gene. The types of mutation induced by each radiation are summarized in Table 3. Out of 23 analyzed spontaneous mutants, 16 (69.6%)

Table 3. **DNA sequence changes in *can1* mutants induced by γ rays and ^{15}N (67 keV/μm)**

Mutation type/ Irradiation (Dose)	Number, %				
	Spontaneous (0 Gy)	γ rays (20 Gy)	γ rays (80 Gy)	^{15}N (20 Gy)	^{15}N (80 Gy)
Single					
Base substitutions (BS)	16 (69.6)	16 (53.3)	16 (53.3)	17 (48.6)	12 (46.2)
Deletions 1 nt	4 (17.4)	5 (16.7)	8 (26.7)	7 (20.0)	2 (7.7)
Deletions > 1 nt	1 (4.3)	1 (3.3)	1 (3.3)	3 (8.6)	3 (11.5)
Insertion 1 nt	1 (4.3)	—	—	2 (5.7)	—
Inversion (2 nt)	—	1 (3.3)	—	—	—
Total	22 (95.7)	23 (76.7)	25 (83.3)	29 (82.9)	17 (65.4)
Complex					
2BS	—	4 (13.3)	1 (3.3)	1 (2.9)	4 (15.4)
3BS	—	—	1 (3.3)	—	1 (3.8)
BS + Δ1 nt	1 (4.3)	1 (3.3)	2 (6.6)	2 (5.7)	1 (3.8)
BS + 1 ins. 1 nt	—	—	—	1 (2.9)	—
BS + 2 ins. 1 nt	—	—	—	1 (2.9)	—
2BS + Δ1 nt	—	1 (3.3)	—	—	1 (3.8)
2BS + Δ2 nt	—	—	1 (3.3)	—	—
3BS + Δ1 nt	—	—	—	—	1 (3.8)
3BS + Δ2 nt	—	1 (3.3)	—	—	—
BS + Δ15	—	—	—	—	1 (3.8)
Δ+ inver. (2 nt)	—	—	—	1 (2.9)	—
Total	1 (4.3)	7 (23.3)	5 (16.7)	6 (17.1)	9 (34.6)
Total	23	30	30	35	26
Mutation rate	$(1.9 \pm 0.5) \times \times 10^{-6}$	$(6.9 \pm 0.5) \times \times 10^{-6}$	$(22.7 \pm 2.2) \times \times 10^{-6}$	$(14.2 \pm 6.6) \times \times 10^{-6}$	$(28.0 \pm 5.3) \times \times 10^{-6}$

Table 4. **Types of base substitutions* induced by γ rays and ^{15}N (67 keV/μm)**

Mutation type/ Irradiation (Dose)	Number, %				
	Spontaneous (0 Gy)	γ rays (20 Gy)	γ rays (80 Gy)	^{15}N (20 Gy)	^{15}N (80 Gy)
Changes at					
A·T	5 (29.4)	14 (43.8)	9 (36.0)	7 (28)	11 (36.7)
G·C	12 (70.6)	18 (56.3)	16 (64.0)	18 (72)	19 (63.3)
Transitions					
A·T → G·C	—	3 (9.4)	1 (4.0)	2 (8.0)	1 (3.3)
G·C → A·T	8 (47.1)	10 (31.3)	5 (20.0)	7 (28.0)	8 (26.7)
Total	8 (47.1)	13 (40.6)	6 (24.0)	9 (36.0)	9 (30.0)
Transversions					
G·C → T·A	1 (5.9)	7 (21.9)	7 (28.0)	11 (44.0)	10 (33.3)
A·T → T·A	2 (11.8)	9 (28.2)	8 (32.0)	4 (16.0)	7 (23.3)
C·G → G·C	3 (17.6)	1 (3.1)	4 (16.0)	0	1 (3.3)
A·T → C·G	3 (17.6)	2 (6.2)	—	1 (4.0)	3 (10.0)
Total	9 (52.9)	19 (59.4)	19 (76.0)	16 (64.0)	21 (70.0)
Total	17	32	25	25	30

* There were used all BS including those from complex mutations

were single base substitutions (SBS) and 5 (21.7%) were one-nucleotide insertions/deletions (indel). Out of 30 γ (20 Gy)-ray induced mutants, 16 (53.3%) were SBS and 5 (16.7%) were indels. Out of 30 γ (80 Gy)-ray induced mutants, 16 (53.3%) were SBS and 8 (26.7%) were indels. Out of 35 ^{15}N (20 Gy)-induced mutants, 17 (48.6%) were SBS and 9 (25.7%) were indels. Out of 26 ^{15}N (80 Gy)-induced mutants, 12 (46.2%) were SBS and 2 (7.7%) were indels. These results show a trend for nitrogen-ion beams with LET of 67 keV/μm to cause small deletions or insertions more frequently than γ rays. The same dependence was shown for single deletion > 1 bp: 3.3% vs 8.6% and 11.5%. Thus, γ rays induced base substitutions (BS) more efficiently than heavy ions which, in their turn, induced more efficiently deletions and complex mutations (Table 3).

Among base pair substitutions, GC–AT transition and GC–TA and AT–TA transversions were induced more efficiently (Table 4).

The distribution of the mutation sites in the *CAN1* gene is presented in Fig. 7. A total of 178 mutation sites are localized in the *CAN1* gene. Mutations in all examined cases were located practically uniformly throughout the gene without "hotspots", but mutations are less common at terminal fragments (nucleotides 1–300 (12%), 1500–1773 (7%)) and more common in central part (nucleotides 600–900 (27%)).

3. DISCUSSION

In the present study, we have analyzed lethal and mutagenic effects of four kinds of heavy ions. It was shown that in haploid yeast *Saccharomyces cerevisiae*, heavy ions induced efficiently both small deletion (< 100 bp)

and point mutations, such as base pair substitutions or one-nucleotide deletions. More efficiently the ionizing radiation induced base substitutions, complex mutations and less efficiently — single nucleotide loss (Table 3). The GC–AT and AT–GC transitions are mainly induced by guanine oxidation and subsequent 8-oxoguanine and adenine mispairing during DNA replication. Under aerobic conditions, the frequency of mutations was higher and depended on LET [1]. The oxygen effect at low LET was ~ 2 and decreased with increasing LET. As a rule, complex mutations are combination of substitution and small deletion within a few helical turns. The source of complex mutations may be the clustered DNA damages. Ionizing radiation induces clusters of DNA damages, such as oxidized bases, abasic sites and strand breaks located on opposing strands within a few helical turns. Clustered damages can be induced by even low doses of ionizing radiation [19]. The dose responses for cluster induction can be described by a linear relationship.

Work similar to ours has been done in Japan Labs [20]. They used the *URA3* gene as a target. Yeast cells were treated by accelerated $^{12}C^{+5}$ ions with LET of 13, 25, 50 (290 MeV/u) and 107 keV/μm (220 MeV/u) at doses up to 200 Gy. Dose-dependent effect for forward mutations had a maximum at 100 Gy. At the maximum, the mutation frequency in the *URA3* gene was 100 times higher as compared to spontaneous mutation frequency. With an increase in LET, cell survival decreased, and mutation frequency increased. The PCR analysis showed that most of the transversion mutations were GC–TA, and all the transition mutations were GC–AT. It is interesting to compare spectrum of mutations in *CAN1* (1773 bp) (Tables 3 and 4) and *URA3* (804 bp) [20] genes. The types of base changes in γ-ray induced mutants included transversions (76.0% vs 66.7%), transitions (24.0% vs 20.0%), and indels (26.7% vs 13.3%). Cells were irradiated at 80 and 66 Gy, survivals were (48 ± 4)% and ~ 53%, respectively. The types of base changes in ^{15}N (67 keV/μm)- and ^{12}C (107 keV/μm)-ion induced mutants included transversions (70.0% vs 68.7%), transitions (30.0% vs 13.7%), and indels (7.7% vs 17.6%). Cells were irradiated at 80 and 100 Gy, survivals were (16 ± 1)% and ~ 48%, respectively. Thus, we have obtained comparable results. But mutations induced by carbon ions (LET of 25 and 107 keV/μm) had a strange feature. Sites of their localization were near the linker regions of nucleosomes, but mutations, spontaneous and induced by γ rays, were located evenly throughout the gene [20]. Wherein, the distribution of mutations induced by ^{12}C-ion irradiation with LET of 13 keV/μm looked like that induced by γ rays. The authors hypothesized that the locus of mutations might be concerned with the nucleosome structure. But we have not observed irregularity of distributions of *can1* mutations. The reason of such different distributions is possibly related to structural and functional specificity of proteins.

We can also compare our results obtained at analysis of one-gene sequencing to the results of whole-genome sequencing. Recently, genomics and next-generation sequencing technology have been widely used. Exome sequencing and whole-genome resequencing were applied to study the

molecular characterization of mutations on a whole-genome level [21–28]. The following are genome sizes of several organisms: yeast *Saccharomyces cerevisiae* (12.1 Mb, 16 chromosomes, 6294 genes), fungi *Aspirgillus oryzae* (37 Mb, 8 chromosomes, 12 000 genes), plant *Arabidopsis thaliana* (135 Mb, 5 chromosomes, 25–31 000 genes), rice *Oryza sativa* (420 Mb, 12 chromosomes, 32–50 000 genes), *Homo sapiens* (3200 Mb, 23 chromosomes, 18 826 genes). More advanced results were achieved by studying plants because γ rays are the most widely used mutagenic radiation in plant selection. Now, new physical mutagens, such as heavy-ion beams, are attractive for plant selection. Heavy ions can generate high LET that induces an increased proportion of DNA double-strand break causing large DNA deletions and/or rearrangements [29]. High-LET-mediated mutagenesis displays a winder mutation spectrum and a higher mutation frequency [30, 31] compared with low-LET irradiation (UV rays or X/γ rays). For example, fast-neutron irradiation of dry seeds has been traditionally considered to predominantly induce deletion mutations of 2–4 kb in size [32, 33], and γ-ray irradiation — mostly deletions, particularly small deletions [34]. However, whole-genome resequencing revealed that fast neutrons and γ rays induced a higher frequency of SBS than deletion mutations, and more indels than large deletions [21, 23, 24, 35]. Comparison of mutational spectrum induced by C-ion (LET of 30 keV/μm) and Ar-ion (LET of 290 keV/μm) irradiations revealed that both types of radiations mainly induce small mutations including SBS and small indels (< 100 bp) [26]. Furthermore, Ar ions induce chromosomal rearrangements or large deletions ($\geqslant 100$ bp) more frequently than C ions, and, in contrast, C ions induce more SBS and small indels than Ar ions [26]. However, since the analyzed mutants in the above-mentioned studies belong to different species, and the analysis methods were different, data cannot be accurately compared. The new studies of mutational effects of γ rays (250 Gy) and C-ion beams (LET of 107 keV/μm) by whole-genome resequencing in rice (*Oryza sativa*) showed that, on average, 57 SBS, 17.7 deletions, 5.9 insertions, and 0.6 structural variations (SV — including large deletions or insertions, inversions, duplications, or reciprocal translocations) were detected in each γ-ray-irradiated mutant, whereas 43.7 SBS, 13.6 deletions, 5.3 insertions, and 2.0 SV were detected in each C-ion-irradiated mutant [36]. The difference in the total number of mutations in the whole genome between γ rays and C-ion beams was mainly attributed to the nucleotide base transition, 1-bp insertion, and < 4-bp deletions. R. Yoshihara et al. [37, 38] have also revealed that γ rays tend to induce more transition and small deletions less than 2 bp. These results indicate that γ-ray irradiation induces more minor modifications or DNA damage on DNA strands than ion-beam irradiation. Our assays used do not detect large chromosome rearrangements, but we observed the rise of complex mutations induced by ^{15}N-ion beam (Table 3). Further, we are planning to publish results concerning the chromosome rearrangements induced by heavy ions in yeast.

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