Differential Response of Mouse Male Germ-Cell Stages to Radiation-Induced Specific-Locus and Dominant Mutations

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ABSTRACT

In an attempt to provide a systematic assessment of the frequency and nature of mutations induced in successive stages of spermato- and spermiogenesis, X-irradiated male mice were re-mated at weekly intervals, and large samples of progeny, observed from birth onward, were scored and genetically tested for recessive mutations at seven specific loci and for externally recognizable dominant mutations. Productivity findings provided a rough measure of induced dominant-lethal frequencies. A qualitative assessment of specific-locus mutations (which include deletions and other rearrangements) was made on the basis of homozygosity test results, as well as from information derived from more recent complementation studies and molecular analyses. Both recessive and dominant visibles revealed clear distinctions between spermatogonia and postspermatogonial stages. In addition, differences for both of these endpoints, as well as for presumed dominant lethals, were found among various postspermatogonial stages. It may be concluded that radiation produces its maximum rates of genetic damage in germ-cell stages ranging from midpachytene spermatocytes through early spermatids, a pattern unlike any of those that have been defined for chemicals; further, the frequency peaks for radiation are lower and broader. The difference between post-stem-cell stages overall and stem-cell spermatogonia was smaller than is generally found with chemicals, not only with respect to the frequency but also the nature of mutations.

ALMOST from the beginning of mammalian germ-cell mutagenesis in the early 1930s, investigators have attempted to make comparisons of at least broad groupings of different exposed germ-cell stages (reviewed by Russell 1954). Moderate- to high-dose irradiation of males was found to cause a period of temporary sterility that conveniently divided offspring derived from post- and presterile-period matings. The two sets of offspring, now known to be derived from exposed spermatogonial stem cells and exposed post-stem-cell stages, respectively, differed for a number of endpoints, such as reductions in size of litters sired by irradiated males (presumably related to dominant-lethal mutations), incidence of partial sterility in the progeny (presumably due to reciprocal translocations), and incidence of complete sterility among sons (now known to be also the result of certain translocations). In all respects, the offspring derived from irradiated post-stem-cell stages were more severely affected than those derived from exposed spermatogonial stem cells.

Because it became of interest to determine whether differences could also be detected among the various post-stem-cell stages, a large systematic study was initiated at Oak Ridge to determine the frequency and nature of specific-locus mutations and other genetic endpoints induced by radiation in successively sampled male germ cells. Treatments for the experiment (to be referred to as the "G experiment") were carried out from 1955 to 1957, with genetic tests continuing into the 1960s, but only parts of the results have heretofore been reported, and these only in abstracts or summaries (Russell *et al.* 1958a,b; Russell 1963, 1964; Sega *et al.* 1978). In the interim, differential radiation responses of post-stem-cell stages were demonstrated for other endpoints, such as dominant lethals induced by low-linear energy transfer (LET) radiation (Bateman 1958; Leonard 1965; Ehling 1971) or by neutrons (Russell *et al.* 1953, 1954), translocations (Auerbach and Slizynski 1956), and sex-chromosome loss (Russell 1976).

What makes publication of the G experiment of interest at this time is the large body of chemical mutagenesis results that has accumulated in more recent years. These results have revealed various germ-cell-stage response patterns not only for induced gross chromosomal endpoints (Ehling 1974; Ehling 1977; Lyon 1981; Searle 1981) but also for both the frequency and nature of recessive mutations scored by the specific-locus method (Russell *et al.* 1990). Here, we show that the pattern observed with radiation is unlike any of those that have

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Dedicated to Jan Drake, who has enriched the literature of genetics and mutagenesis both with his own fine publications and with so many others for which, as editor of Genetics, he helped bring about a healthy birth.

been defined for chemicals. Not only are the stages of maximum response different, but the magnitude of the difference between stages is less extreme.

MATERIALS AND METHODS

The specific-locus test (Russell 1951) was used to detect recessives at seven marked loci, and the same progenies were also scored for dominants at miscellaneous loci. Male (101/Rl × C3H/Rl)F_1 mice, aged 13–16 wk, were randomized into irradiated and control groups, each containing 120 males per replicate experiment. Whole-body irradiation to a dose of 3 Gy consisted of 250 kVp X rays (half-value of 0.4 mm copper) delivered at ~0.84 Gy/min, as measured on the rotating scatter block on which the males were exposed in Lucite holders.

Immediately after treatment, each of the 120 irradiated and 120 control males of a given replicate was mated to two T-stock females, with cages that housed irradiated and control males alternating on the shelf, and the females randomized among those cages. The non-inbred, multiple-recessive T stock is of genotype *a/a; b/b; p* c^{ch}*/p* c^{ch}*; d se/d se; s/s* [*a = nonagouti* (*agouti* locus, chromosome *2*), *b = brown* (*brown*, now *tyrosine-related protein 1*, locus, chromosome *4*), *p = pink-eyed* (*pink-eyed dilution* locus, chromosome *7*), c^{ch} *= chinchilla* (*albino*, now *tyrosinase*, locus, chromosome 7, 14 cM distal to *p*), *d = dilute* (*dilute*, now *myosin Va*, locus, chromosome *9*), *se = short-ear* (*short-ear*, now *bone morphogenetic protein 5*, locus, chromosome 9, 0.16 cM distal to *d*), and *s = piebald spotting* (*piebald*, now *endothelin receptor type B*, locus, chromosome *14*)].

In each of 10 successive replicates (initiated over a span of 2 yr), each of the 240 males was re-mated at weekly intervals to a new set of two T females, for a total of 6 wk postirradiation. The females were housed in individual cages following the week of cohabitation with the male. In two of the 10 replicates (Groups 6 and 7), the weekly re-mating cycle was continued for an additional 7 wk. In two replicates (Groups 6 and 8), males were separated from females after 13 wk (Group 6) or 6 wk (Group 8) of weekly re-mating, respectively, and made to resume the cycle at some later time, namely for wk 14–20 (Group 8) or 37–40 (Group 6). The irradiated germ-cell stages being sampled during different weeks are shown in Table 1.

F_1 offspring were recorded at birth (except on weekends), at 10 days of age and at weaning age (days 18–26), at which time all were discarded, except those showing a visually detectable aberrant phenotype that might indicate a recessive mutation at a specific locus or a dominant elsewhere. Those animals were paired at weaning with appropriate test mates.

Presumed specific-locus mutants were first tested for allelism with the appropriate marker, after which crosses were initiated to make the mutant allele homozygous. Unless the mutant allele was distinguishable from the marker allele (*e.g., c vs.* c^{ch}) or had a closely linked marker (*e.g., d* for *se*, and vice versa), the procedure consisted of outcrossing the primary mutant (which may be generically designated *m*/*m**, where *m** is the new allele) to wild type to derive 12 +/*m* or +/*m** offspring (six in the case of *p*, which is loosely linked to *c*), each of which became the founder of an outcross/backcross line (for details, see Rinchik *et al.* 1994). If all of these lines yielded viable homozygotes that were indistinguishable from marker-allele homozygotes (*m*/*m*), it was concluded that a repeat mutation had been induced; because the experiment was conducted prior to the identification of molecular markers, *m** could not be isolated and propagated. If one or more of the outcross/backcross lines failed to yield viable homozygotes, or yielded *m**/*m** animals that were distinguishable from *m*/*m*, the new mutation was recovered from the founder or from a carrier animal within the outcross/backcross line and propagated in a breeding stock. Many of these stocks, along with those of *c*, *d*, and *se* alleles, were still available in later years when it became possible to further characterize the mutations through complementation experiments and/or molecular studies.

Presumed mutants that did not involve one of the specific-locus markers were outcrossed to wild type, usually (101 × C3H)F_1, to test for transmission of a dominant or X-linked trait. In the case of mottled or variegated mutants, crosses were also made to appropriate specific-locus recessives to determine whether the presumed mutant might be (1) mosaic for a new allele at one of the specific loci (Russell 1964; Russell and Russell 1996) or (2) a carrier of a chromosome rearrangement, such as a *T(X;A)* causing position-effect variegation (Russell 1983). If a transmitted mutation was found, it was propagated in a breeding stock wherever feasible.

TABLE 1

Germ-cell stages sampled in matings made during successive postirradiation intervals

Mating week[a]	Germ-cell stages irradiated[b]
1	Spermatozoa
2	Late spermatids
3	Early spermatids
4	Meiotic divisions; diplotene; mid- to late pachytene
5	Early pachytene; leptotene; preleptotene
6, 7	Differentiating spermatogonia
8–40	Stem-cell (A_s) spermatogonia

[a] Each male was paired with two females immediately after irradiation and paired with two fresh females at 7-day intervals thereafter.

[b] From Oakberg 1984.

RESULTS

Productivity

Table 2 summarizes productivity parameters for irradiated and control males for the various mating intervals. Altogether, 65,887 and 115,655 offspring were born in 13,276 and 17,356 litters sired by irradiated and control males, respectively. In Table 3, the results for irradiated sires are expressed as percentages of the concurrent controls, and some of these percentages are plotted in Figure 1. A breakdown for the wk 9–20 interval (not shown) indicated very little fluctuation between the various mating weeks. For one of the replicates (Group 7), a daily check for vaginal plugs was conducted during wk 5, an interval during which the number of litters per irradiated sire was less than one-half that for controls. The number of definite plugs was found to be virtually identical, 204 and 202, respectively, indicating that the reduction in litter number was not the result of decreased libido.

The nearly complete absence of offspring from matings made during wk 7 presumably results from the killing of early differentiating spermatogonia, a germ-cell stage

TABLE 2

Productivity parameters for irradiated and control sires

	3 Gy				Control			
Week	No. sires	Litters born	Offspring born	Offspring weaned	No. sires	Litters born	Offspring born	Offspring weaned
1	1,199	1,978	10,915	9,281	1,200	2,090	13,860	12,237
2	1,199	1,975	10,052	9,412	1,200	2,028	13,626	12,306
3	1,199	1,577	4,582	4,266	1,200	2,028	13,798	12,657
4	1,199	1,283	3,749	3,499	1,200	1,982	13,535	12,243
5	1,198	878	3,348	3,132	1,199	1,947	13,316	12,072
6	1,198	1,096	4,623	4,284	1,199	1,966	13,091	11,939
7	239	17	41	35	240	431	2,833	2,518
8	239	115	676	635	240	357	2,233	2,055
9–20	2,381[a]	3,681	23,692	20,768	2,390[b]	3,809	24,825	21,774
37–40	116	676	4,209	3,737	120	724	4,538	4,091

[a] Weeks 9 and 10, 239 each; weeks 11–13, 358 each; weeks 14–16, 119 each; weeks 17–20, 118 each.
[b] Weeks 9 and 10, 240 each; weeks 11–13, 359 each; weeks 14–16, 119 each; weeks 17–20, 119 each.

that is extremely sensitive to radiation (Oakberg 1984). Higher radiation doses produce a period of complete sterility that continues beyond wk 7, during which time spermatogonial regeneration occurs from stem cells prior to the resumption of differentiation into more advanced stages (Oakberg 1984). In the G experiment, this regeneration was apparently complete by wk 9 postirradiation.

Depression of average litter size is likely to be a measure of induced dominant-lethal mutations and is already found during wk 1 and 2 when there is no cytotoxicity (litter number essentially normal; also, see Oakberg 1984). When littersize reduction comes to a peak (wk 3 and 4), it may also contribute to the drop in litter number, *i.e.*, litters of zero may be expected when *average* litter sizes are only 2.9. However, the continued drop in litter number, even after the average litter size has begun to climb (wk 5 and 6), probably indicates some direct killing of late differentiating gonia and of early spermatocytes (though not as severe as that of early differentiating spermatogonia) (Oakberg 1984; Cattanach *et al.* 1990).

Specific-locus mutations

Frequency of specific-locus mutations: When offspring are examined at birth, the only recognizable phenotype presumably indicative of specific-locus mutations is reduction in eye pigment. Of 15 such animals, seven did not survive to weaning age, all having died before day 10, and most of them within a day or two after birth. Many of the non-wild-type hair-pigment phenotypes are detectable at the time of the day-10 observation, and all phenotypes, including short-ear, are clearly evident by the time of the third observation, at weaning. Because the number of offspring diminishes between birth and weaning (Table 2), with most of the deaths occurring in the first week of life, and because it is not known how many mutants, other than those affecting eye pigment, died before being recog-

TABLE 3

3 Gy productivity results as percentage of concurrent controls

Week	Litters born (per sire)	Offspring born (per sire)	Average litter size at birth	Survival to weaning (%)
1	94.7	78.8	83.3	100.7
2	97.5	73.8	75.7	100.3
3	77.8	33.2	42.8	94.3
4	64.8	27.7	42.8	95.1
5	45.1	25.2	55.7	95.4
6	55.8	35.3	63.4	95.1
7	4.0	1.5	—	96.1
8	32.3	30.4	94.1	102.1
9–20	97.0	95.8	98.8	99.9
37–40	96.6	95.9	99.4	98.5

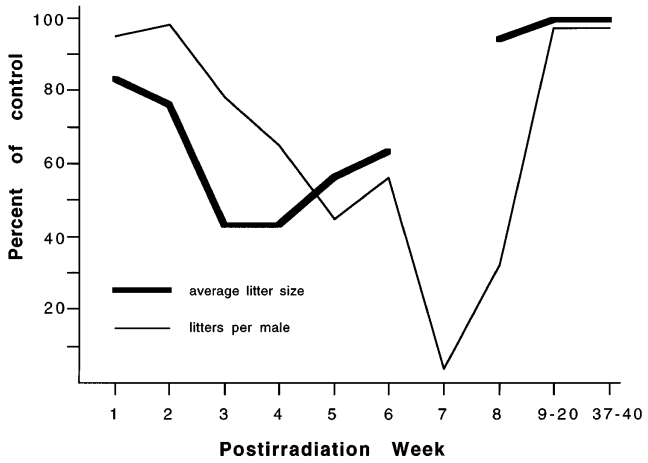

Figure 1.—Productivity results in successive postirradiation weeks, expressed as percentages of concurrent controls. No average litter size value is plotted for postirradiation wk 7 because the number of litters was so small (probably as a result of spermatogonial killing) as to make such a value unreliable.

nized, we have computed total observed specific-locus mutation frequencies as composites of (1) the incidence of nonsurviving presumed mutants recognized at birth among total offspring counted at birth, and (2) the incidence of mutants surviving to weaning (plus four presumed *s* mutants that died before weaning; see footnote *f*, Table 4) among total offspring counted at weaning (Table 4). Although observations at birth helped include some of the early-dying mutants, the total observed mutation rate computed by the composite is still an underestimate of the actual mutation frequency because of the inability to recognize hair-pigment and ear-length phenotypes until later. The calculated frequencies include 10 presumed *s*-locus mutants that were not allelism-tested. This seems legitimate because of 13 presumed *s*-locus mutants that were tested, 12 in fact carried a mutation at *s*, while not one of nine confirmed dominant-spotting mutants had been classified originally as an *s*-locus mutant.

Induced specific-locus mutations: The experimental specific-locus mutation frequency (last column, Table 4) for all irradiated postspermatogonial stages (namely, 49 mutations from summed wk 1–5, a frequency of 1.64×10^{-3}) is almost three times as high as, and differs significantly from, that for differentiating and stem-cell spermatogonia (namely, 21 mutations from summed wk 6–40, a frequency of 0.60×10^{-3}); $P = 0.001$ by Fisher's exact test. Within the period when irradiated postspermatogonial stages are being sampled, wk 3 and 4 exhibit the highest frequencies, but each value has relatively wide confidence limits. The distribution does not differ significantly from one that assumes the overall average

TABLE 4

Frequency of presumed specific-locus mutations conceived at different intervals after 3 Gy irradiation

	No. of presumed mutants			Frequency (10^{-3})		
	Not surviving to weaning					
Week	Recognized at birth[e] (1)	Recognized day ≥ 10[f] (2)	Surviving to weaning[a] (3)	Column (1)[b]	Columns (2) + (3)[c]	Total[d]
1	0	3	12	0	1.62	1.62 (0.87, 2.56)
2	1	0	11	0.10	1.17	1.27 (0.71, 2.15)
3	0	0	9	0	2.11	2.11 (1.05, 3.93)
4	2	1	5	0.53	1.71	2.24 (0.92, 4.19)
5	1	0	4	0.30	1.28	1.58 (0.62, 3.52)
6	0	0	3	0	0.69	0.69 (0.19, 1.88)
7	0	0	0	0		
8–40	3	0	15	0.10	0.60	0.70 (0.43, 1.08)

No whole-body specific-locus mutants were found in the control. Not shown in the table are three specific-locus mosaics, one of these in an irradiated group but all three of probable spontaneous origin (see text). The table excludes two specific-locus clusters found in irradiated groups, but resulting from spontaneous mutations in the prior generation (see text).

[a] All confirmed by allelism test, except for one presumed *a*-, one presumed *p*-, and six presumed *s*-locus mutants, which were sterile or died young (see Table 6).

[b] Based on number of offspring born (see Table 2).

[c] Based on number of offspring weaned (see Table 2).

[d] 95% confidence limits in parentheses (based on weighted average between numbers born and weaned, where appropriate).

[e] Characterized by absence of eye pigment (presumed *p*-locus mutants) or reduced eye pigment (one case, a presumed *p*-, *b*-, or *c*-locus mutant).

[f] All were presumed *s*-locus mutants. Most mutants at other loci, except for *se*, would also be recognizable at that age.

frequency for each of the 5 wk ($P = 0.7$). The induced mutation frequency (experimental rate minus historical Oak Ridge control, Russell and Russell 1996; 95% confidence limits in parentheses) is 6.40 (4.45, 8.64)/locus/0.01 Gy for postspermatogonial stages (wk 1–5) and 2.65 (1.60, 4.28)/locus/0.01 Gy for spermatogonia (wk 6–40). (Because historical controls do not include observations made at birth, only weaning-age data for the G experiment were included in these calculations.) The latter rate is identical to that for a larger sample (40 mutations) published earlier (Russell 1963). The induced rate for the sum of postspermatogonial stages is 2.4 times the spermatogonial rate.

Spontaneous specific-locus mutations: No whole-body specific-locus mutants were observed among offspring of untreated males. However, among several mottled offspring recovered from experimental and control progenies, there were two, and possibly three, that were revealed by progeny tests to be mosaics (of ~50:50 composition) for specific-locus mutations. Such visible mosaics are thought to have their origin in the $(101 \times C3H)F_1$ genome as single-strand mutations occurring during the perigametic interval, *i.e.*, between the last premeiotic mitosis and the first postmeiotic one (Russell and Russell 1996). One of the confirmed mutants, mosaic for the p^{nl} mutation 116G (Russell *et al.* 1995) (*i.e.*, of genotype $p/+///p/p^{nl}$, where the /// symbol separates the components of the mosaic), was found in the progeny of an irradiated male but was almost certainly of spontaneous origin. This mutant was conceived during wk 6 postirradiation, when irradiated differentiating spermatogonia (rather than cells in the perigametic interval) are being sampled. The confirmed 50:50 mosaic in the controls was of genotype $c^{ch}/+///c^{ch}/c^{jl}$, and the possible mosaic was of presumed genotype $d/+///d/d$* (unfortunately not tested for *se*).

Additionally, two mutant clusters were observed, one for a d^{pl} mutation (Russell and Russell 1996), the other for an a^{jv} (now renamed a^{ev}) mutation (Russell and Russell 1997). Although found in irradiated progenies, these mutations must have occurred spontaneously in either the 101 or C3H parent of the irradiated $(101 \times C3H)F_1$ male (Russell and Russell 1996) and thus do not enter into mutation-rate calculation based on the latter. Two *p*-locus mutants sired by irradiated males who were siblings were found to carry different mutations, one a juvenile-lethal, the other a viable, allele.

Nature of specific-locus mutations: The distribution of mutations among the specific loci is shown in Table 5 for offspring conceived at different intervals postirradiation. Because the number of mutants for any given interval is relatively small, comparisons among individual weeks are not meaningful. The sum of the results for postirradiation wk 1–5, which sample the postspermatogonial stages, may, however, be compared with the locus-spectrum for spermatogonia X-irradiated at similar dose rates (Russell 1964). The distributions are significantly different ($P < 0.01$, by chi-square), the greatest contribution to the difference deriving from the *Df(d se)* mutations, which contributed 11.4% of the mutants recovered from postspermatogonial stages but none of those from stem-cell spermatogonia (in subsequent experiments, however, from which only partial

TABLE 5

Distribution of mutations among the specific loci

Germ-cell stage/postirradiation week	*a*	*b*	*c*	*p*	*d*	*se*	*d se*	*s*
Postspermatogonial stages								
1	1	2	1	1	1	1	3	5
2	0	1	0	4	1	1	2	2
3	2	1	0	1	1	0	0	4
4	0	1	0	2	0	0	0	3
5	0	1	1	0	0	0	0	2
Differentiating spermatogonia								
6	0	0	1	0	0	0	0	2
7	0	0	0	0	0	0	0	0
Stem-cell spermatogonia								
8–40	0	4	3	0	3	0	0	5
Total, postspermatogonial stages	3	6	2	8	3	2	5	16
Total, spermatogonia, this experiment	0	4	4	0	3	0	0	7
Spermatogonia, other data[a]	2	32	15	22	24	2	0	69

Excludes presumed mutants recognized by reduced eye pigment at birth but not surviving past 10 days (see text).

[a] Oak Ridge data from several experiments conducted with X-irradiation of similar dose rate as that used in the G experiment, the data from which are included (W. L. Russell 1964).

TABLE 6

Characterization of genetic lesion for specific-locus mutants originating from irradiated postspermatogonial or spermatogonial stages

	Postspermatogonial stages	Differentiating spermatogonia	Stem-cell spermatogonia
Large lesion documented[a]	21	1	6
Large lesion suspected[b]	11	2	2
Null allele[c]	3	—	4
Intralocus (documented or presumed)[a]	5	—	2
Not tested[d]	10	—	4

[a] Documented by complementation and/or molecular evidence (see text).
[b] Primary mutant was sterile or near-sterile (one *p*, five *s*), died soon postweaning (one *a*, one *s*), was small and/or produced small litter sizes (one *a*, one *d*), gave evidence of poor transmission of the mutant allele (one *b*, one *p*, two *s*), or transmitted mutant allele inseparably from translocation (one *se*).
[c] *b*-viable and d^{op} alleles; no evidence for deletion of flanking sequences.
[d] Three *b* mutants were tested for allelism but not for survival of homozygotes. Seven and four presumed *p* and *s* mutants, respectively, died shortly after birth.

spectra have been reported, a small number of such deficiencies were recovered from treated stem cells, *e.g.*, Russell 1971). Another fairly large contributor to the significance of the difference is the *a* locus, which appeared almost six times more mutable in postspermatogonial stages than in stem-cell spermatogonia. It has been noted elsewhere (Russell and Russell 1996, 1997) that *spontaneous* mutations that apparently occur in the perigametic interval (between the last premeiotic mitosis and the first postmeiotic one) have a higher proportion of *a*-locus mutations than do those occurring at other times.

In the decades following completion of the G experiment, much has been learned not only about the genes marked in the specific-locus test but also about regions of the genome that surround them. Extensive complementation studies involving *d*, *se*, and *d se* mutants (Russell 1971), *c* mutants (Russell *et al.* 1982), *b* mutants (Rinchik *et al.* 1994), *p* mutants (Russell *et al.* 1995), *s* mutants (O'Brien *et al.* 1996), and *a* mutants (L. B. Russell, unpublished results) have served to localize functional units flanking the markers and to identify the phenotype of mice in which the marked gene (but no known flanking functional unit) was completely ablated. Several of the mutant alleles generated in the G experiment (identified by an allele symbol ending in G) were included in some of the complementation studies. For others, complementation results obtained with other alleles were applicable (*e.g.*, because mice with only the *tyrosinase* gene ablated were revealed to be fully viable, any *c*-locus mutant that was homozygous lethal or only poorly viable, had to be a multilocus, or "large," lesion—similarly for several of the other loci). Deletion breakpoints could be mapped, at least relative to other functional units. A number of the G-experiment alleles have also been analyzed by molecular techniques (Rinchik *et al.* 1986; Rinchik *et al.* 1993; Rinchik 1994; Johnson *et al.* 1995; R. Miltenberger, personal communication), serving in some cases to map applicable deletion breakpoints more accurately and, in others, to provide evidence for small intralocus changes. At least four of the specific-locus mutants carried translocations; in three of these, one translocation breakpoint was at, or very closely linked to, one of the marked loci.

Evidence derived from all these various sources is summarized in Table 6. For mutations induced in postspermatogonial stages, the proportion of documented or suspected large lesions is 80%. Among the relatively limited sample of spermatogonial mutants from the G experiment, there was also an appreciable, though somewhat smaller, proportion of presumed large lesions. More meaningful comparisons can be made by pooling spermatogonial mutants from several experiments, as has been done for certain specific loci. Thus, for spermatogonia exposed to low-LET irradiation (excluding 24-hr fractionation regimes, which change the spectrum), the frequency of large lesions was 31.7% among 41 *c*-locus mutations and 14.3% among 63 mutations in the *d se* region (Russell and Rinchik 1993); counting only the documented large lesions and documented or presumed intralocus mutations, the frequency of the former was 50% among 22 spermatogonial *c*, *d*, and *se* mutations as compared to 80.7% among 26 postspermatogonial G-experiment mutations (Table 6).

Mutations at other loci

Offspring of the T × (101/Rl × C3H/Rl)F_1 cross that had abnormalities visible to the naked eye and recognizable at any time during the first 3–4 wk of life were saved at weaning age, and attempts were made to test any surviving mice genetically, as described in materials and methods. Altogether, 170 such variants were recorded: 93 and 77 in experimental and control groups, respectively, with 76.3 and 75.3%, respectively,

TABLE 7

Distribution of miscellaneous variants among offspring conceived at different intervals postirradiation and among controls

Postirradiation week	No. of offspring weaned	No. of variants[a] that were:				Proportion of surviving variants with probable genetic basis[d] (%)	Frequency (10^{-3}) of variants that:	
		Transmitting[b]	Sterile	Died young[c]	Not transmitting		Transmitted	Probably had genetic basis[d]
1	9,281	5 + 2?	2	4	6	60.0	0.54	0.97
2	9,412	7	1	4	5	61.5	0.74	0.85
3	4,266	3 + 1?	2	1	1	85.7	0.70	1.41
4	3,499	8	2	6	3	76.9	2.29	2.86
5	3,132	1	2	1	2	60.0	0.32	0.96
6 + 7	4,319	1 + 1?	1	2	1 + 1[e]	60.0	0.23	0.69
1–5	29,590	24 + 3?	9	16	17	67.9	0.81	1.22
6–40	29,459	1 + 3?	2	6	12 + 1[e]	31.6	0.03	0.20
Control	115,655	6 + 3?	4	19	43 + 2[e]	22.4	0.05	0.11

[a] Animals that were small, without another associated phenotype, were recorded only for Groups 1 and 2. Extrapolation from the distribution among the various subclassifications of these 22 "smalls" indicates that—had this type been recorded in all 10 replicates—the frequency of variants for wk 1–5 and wk 6 + 7 would each approximately double, but the frequency for controls would be unaffected. No extrapolation can be made for wk 8–40 because these weeks were not included in Groups 1 and 2.

[b] Number of followed "?" indicates probable, but not stringently proved, transmission (*e.g.*, abnormally high percentage of progeny that died early, rather than exhibiting the specific phenotype).

[c] Approximately one-quarter of these animals died by 35 days of age and were not mated. The remainder were paired at weaning with one or more test mates but died shortly thereafter.

[d] Clearly and probably transmitting, plus sterile variants.

[e] Mottled variants shown to be mosaic for a mutation at one of the seven marked loci (see text).

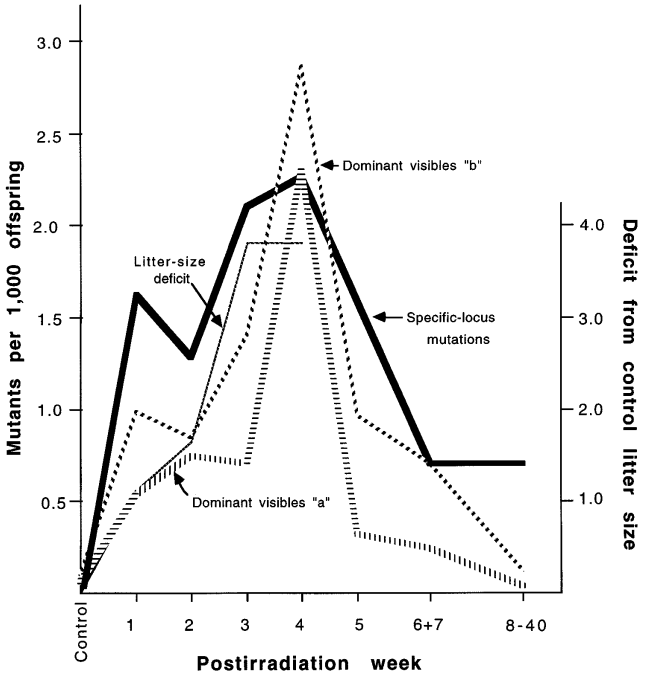

Figure 2.—Mutant frequencies in successive postirradiation weeks for specific-locus mutations and for dominant visibles as classified by criteria "a" (proved transmissibility) or "b" (clear or possible transmissibility, or sterility; see text and Table 7). Also shown are deficits from control average litter sizes (scale on right), a rough indicator of dominant-lethal mutations (because of the complication of germ-cell killing, results are plotted only through wk 4 postirradiation). All controls are contemporary (from the G experiment), except for the specific-locus control, which is historical.

surviving to reproductive age. Numbers of variants represent a summation of all 10 replicate groups, except that in Groups 9 and 10 some variants (primarily those with tail anomalies) were not recorded (with no bias to particular postirradiation weeks), and except that mice that were classified only as "small," without any other obvious phenotype (22 cases), were recorded and tested only for Groups 1 and 2. Other categories of phenotypes (some of which were also associated with small size) were as follows: lighter fur (8), darker fur (1), skin defects (4), mottling (20), abnormal legs and/or feet (10), ears (14), eyes (6), or tail (51), non-piebald white spot(s) (29), absence of anal and/or genital openings and tail, with death at birth (10), and miscellaneous (2). (Note that the total number of phenotypes slightly exceeds the number of variants because a few variants combined abnormalities, most commonly abnormal tail and small ventral spot.) The proportion of variant phenotypes that were shown to be transmissible was lowest for leg/foot (0 + 14.3%?) and tail abnormalities (5.7 + 11.4%?), and highest for skin anomalies (100%), lighter fur (83.3%), and uncomplicated small size (50 + 20.0%?) (percentages followed by "?" indicate probable, but not stringently proved, transmission). The low transmissibility of tail phenotypes had already been observed by Russell (1951). Among clearly transmissible anomalies, there is no evidence for anything but random distribution of different phenotypes among the various groups, except that of altogether only six dominant visibles observed in controls, two were "lighter" (tested to be *Sl*, now *Mgf*, alleles) and two were characterized by scaly skin.

Among the 31 clearly transmissible dominant visibles, six were associated with balanced translocations. Of these, four were X-linked, with three of them [*T(X;4)1Rl*, *T(X;7)2Rl*, and *T(X;7)3Rl)*] exhibiting variegation for a specific-locus marker resulting from X inactivation in a linked autosomal segment, and one [*T(X;12) 13Rl*] exhibiting small body size. Another X-linked dominant (found in the control group) was the result of a mutation in the *Mo* gene (now, *Atp7a*). Two autosomal translocations [*T(10;17)11Rl* and *T(10;15)20Rl*] produced a mutant phenotype at the *Sl* locus on chromosome 10. One of the dominant-spotting mutations turned out to be a *W* (now Kit^W) allele. The remaining dominants, all of which were autosomal, were not tested for allelism with known genes. All of the sex-linked mutations enumerated above were recovered in females.

Table 7 summarizes the frequencies of all types of variants conceived at different intervals after irradiation and in controls. In view of the reduced penetrance that has been noted for many dominants (Selby 1990), frequencies calculated for transmissible traits are probably underestimates, although it should be noted that considerably more than 20 offspring per variant were examined in most cases. Further, overall frequencies would undoubtedly have been considerably increased had the record for Groups 9 and 10 been more complete, and had small animals with no associated phenotypes been recorded and tested for all replicates instead of for Groups 1 and 2 only (where 50% of the cases turned out to be heritable). Extrapolation to what the total frequencies might have been had small animals been recorded in all replicates are given in footnote *a* of Table 7, but the body of Table 7 includes only the actually scored cases.

Two sets of frequencies were computed: frequency "a" for visible variants that gave clear proof of transmission and frequency "b" for clearly, plus questionably, transmitting visible variants, plus sterile variants (Table 7 and Figure 2). That many of the steriles probably had a genetic basis may be inferred from the finding that their frequency is an order of magnitude higher among variants derived from irradiated postspermatogonial stages than among variants found in controls. For mice conceived during wk 1–5 postirradiation (derived from irradiated postspermatogonial stages), both frequencies "a" and "b" are more than an order of magnitude greater than the corresponding values in the controls ($P < 0.001$). However, for mice conceived during wk 6–40 postirradiation (derived from irradiated stem-cell or differentiating spermatogonia), there is no difference

from controls for frequency "a" and a nonsignificant difference ($P = 0.17$) for "b." The difference between postspermatogonially and spermatogonially irradiated groups is clearly significant ($P < 0.001$ and $= 0.002$ for "a" and "b," respectively).

Within postspermatogonially irradiated groups, the point estimates of frequencies, both "a" and "b," are highest for wk-4 progeny. Wk 4 differs significantly from the sum of all the other postspermatogonial stages (wk 1 + 2 + 3 + 5) both for frequencies "a" ($P = 0.049$) and "b" ($P = 0.009$); in fact, frequency "a" for wk 4 is as high as the frequencies for all the other postspermatogonial stages combined. Because other genetic endpoints in the G experiment—presumed dominant lethals and specific-locus mutations—indicate highest frequencies for wk 3 and 4, calculations for the dominant visibles were also made by comparing wk 3 + 4 with the remaining wk (1 + 2 + 5). The differences were found to be significant both for frequencies "a" ($P = 0.03$) and "b" ($P = 0.02$).

Calculation of a spontaneous frequency of dominant visibles per gamete must be based on twice the total number of animals observed, because any mutation could have been derived from either parental gamete. Frequency "a" (which is probably more appropriate than "b" for comparisons with other published data) was 25.9×10^{-6}, which is about three times the spontaneous rate compiled by Searle (1974) from seven earlier publications, and over five times the rate derived from Schlager and Dickie's (1971) data. This discrepancy could have resulted from different criteria for, or acuity of, observation, or from greater survival of mutants under the conditions of the G experiment. The postspermatogonially induced (*i.e.*, observed minus control) rate per male gamete per 0.01 Gy in the G experiment was 24×10^{-7} and 36×10^{-7} for "a" and "b," respectively. A spermatogonial rate cannot be calculated for "a," because the single clearly transmitted case yields a rate no higher than the spontaneous; for "b," the rate is 3.1×10^{-7} per male gamete per 0.01 Gy, similar to the 4.7×10^{-7} derived from other published data (Searle 1974), which are, however, more comparable to "a" than "b." Thus, while the postspermatogonial rate found for dominant visibles is higher than the published spermatogonial rate (even adjusting for difference in the spontaneous rates), the G-experiment spermatogonial rate seems unusually low.

Among mutations affecting hair pigment or external-ear morphology, the mutation rate to recessives at seven specific loci was considerably greater than the rate to dominants at a presumably much larger number of loci throughout the genome. Thus, for the total of all experimental groups, there were 18 of the latter type of mutations, counting confirmed dominants, or 23 if questionable transmitters and steriles are included; by contrast, there were 62 specific-locus mutants. This finding for what were largely postspermatogonial mutations confirms early results for spermatogonial mutations (Russell 1951).

DISCUSSION

Unlike other specific-locus experiments on irradiated males, the G experiment accumulated a large sample of progeny for each of the postirradiation weeks in an attempt to provide a meaningful assessment of the response of successive germ-cell stages. All surviving presumed specific-locus mutants were not only allelism-tested but bred to homozygosity, and the results of these tests, along with information from complementation studies and molecular analyses done in subsequent years, allowed a qualitative assessment of mutations induced by radiation in different types of male germ cells. The experiment was further unique in (1) routinely scoring offspring at birth and 10 days, in addition to weaning age, thus allowing a more accurate estimate of mutation frequency, and (2) in recording all externally visible variants and testing them for transmission of dominantly inherited traits.

The most common endpoint used for the comparison of genetic sensitivities of various mouse germ-cell stages has been dominant-lethal mutations. In the case of post-stem-cell stages of the male mouse, radiation studies with X rays revealed a peak yield in progenies sired during wk 3 after 200r irradiation (early spermatids) (Bateman 1958), during wk 3 and 4 after higher doses (midspermatocytes through early spermatids) (Ehling 1971) or during days 7–20 (early and midspermatids) (Leonard 1965). For cyclotron or bomb neutrons, the yield was greater from days 19–23 (early spermatids and meiotic divisions) than from days 2–6 (spermatozoa) (Russell *et al.* 1953, 1954). In chemical mutagenesis studies, the dominant-lethal pattern varies with the compound tested, some chemicals producing peak yields from exposed spermatozoa (sometimes including late spermatids), others positive primarily in earlier post-spermatogonial stages (Ehling 1977; Searle 1981; Lyon 1981; Ehling and Neuhäuser-Klaus 1995).

Although dominant-lethal incidences were not determined directly, the data on average litter size in the very large G experiment can provide an approximate indicator of dominant lethality (while the data on average litter *number* provide some measure of germ-cell killing). Litter sizes were lowest for conceptions made in wk 3 and 4 postirradiation, but lesser reductions were also found in prior and subsequent weeks, indicating that the peak in dominant-lethal incidence is not as sharp as the peaks found in the case of several chemical mutagens. In general, the productivity results from the G experiment support earlier findings on dominant-lethal and cell-killing frequencies (see above).

Heritable translocations have been used to a lesser extent to explore distinctions between germ-cell stages. For X rays, Auerbach and Slizynski (1956) reported

a higher yield from spermatids than from spermatozoa. An even greater difference in the same direction was found for triethylenemelamine (TEM) (Generoso *et al.* 1982). For chemicals in general, these two stages, separately or jointly, have invariably been found to yield a higher rate than have spermatogonia (Generoso *et al.* 1980, review; Searle 1981, review). Overall, differential translocation and dominant-lethal yields have not always been parallel (Lyon 1981, review; Generoso *et al.* 1982). Paternal sex-chromosome losses, a chromosome-breakage-related endpoint, was inducible by X rays in all postspermatogonial stages, with a probable (but not very sharp) peak in early spermatids; such losses were not recovered from irradiated spermatogonia for reasons that do not necessarily indicate absence of initial damage (Russell 1976).

Using specific-locus mutations as an endpoint capable of revealing gene mutations, as well as chromosomal deletions or rearrangements, early chemical mutagenesis studies generally distinguished between stem-cell and post-stem-cell yields (Russell *et al.* 1981), but more recent ones have employed a weekly re-mating scheme and have revealed several diverse response patterns. Only a few of the several compounds that were mutagenic in later male germ-cell stages were also positive in spermatogonial stem cells, and there were at least three response patterns for post-stem-cell stages. Most of the chemicals fitted "Pattern 1," *i.e.*, peak yields observed after exposure of spermatozoa and late spermatids; two chemicals, which were efficient deletion inducers, fitted "Pattern 2," namely, a sharp peak for *early* spermatids; and two, including ENU, a point mutation inducer, defined "Pattern 3," namely, peak yield for late differentiating spermatogonia, preleptotene, and leptotene spermatocytes (Russell *et al.* 1990). Because mutagenesis studies with chemicals have revealed these various specific-locus response patterns, each defined by a sharp peak, it is of interest to examine the yield of such mutations from different germ-cell stages exposed to radiation.

The G experiment showed clear distinctions between postspermatogonial stages and spermatogonia (including differentiating gonia with stem-cell gonia), the former yielding almost three times the mutation frequency of the latter. Among the weeks that sample postspermatogonial stages, the highest point estimates were obtained for wk 3 and 4, the same two weeks in which dominant-lethal mutations appear to reach a peak, although the specific-locus distribution did not differ significantly from one assuming average postspermatogonial frequencies throughout. Dominant visibles, by contrast, yielded a significant peak in wk 4, and the combined frequencies for wk 3 and 4 were also significantly higher than those for the other weeks that sample postspermatogonial stages. From the combination of these three sets of results, it may therefore be concluded that radiation produces its maximum rates of genetic damage in germ-cell stages ranging from midpachytene through early spermatids.

The fact that the maxima for the various endpoints do not define very sharp peaks lends some support to Searle's (1981) conclusions that "the most striking difference between germ-cell responses to radiation and chemicals is one of homogeneity *vs.* heterogeneity," although "homogeneity" is clearly too extreme a characterization for the radiation response, because there is a difference not only between spermatogonial and postspermatogonial stages, but among the latter as well. If selection occurs among postspermatogonial cells, one might expect a lowered yield from the stages that were relatively less mature at the time of irradiation. On the contrary, the yield has been found to be greater from them than from spermatozoa and mature spermatids. The pattern of differential yield among postspermatogonia does not fit any of the three patterns found earlier for various chemicals (Russell *et al.* 1990) or a fourth pattern discovered recently (L. B. Russell, unpublished results). Of particular interest is the high yield for wk 4 (irradiated midpachytene through meiotic divisions), which could implicate certain postrecombination and segregation events.

Because the length of time required for development of all post-stem-cell stages of human spermatogenesis is only 0.6% of a generation time (18.6% for occupational exposures), the risk from genetic damage induced in these stages by a given mutagen is usually considered negligible compared to the genetic damage accumulated in stem cells—unless yield of mutations from one or more of the post-stem-cell stages is a high multiple of the yield from spermatogonia, as, for example, in the case of triethylene melamine (TEM; Lyon 1981). The G experiment has shown not only that post-stem-cell stages as a whole are only about three times as mutable as stem cells but also that no one of the individual stages has a drastically higher mutation rate.

A clear indication that postspermatogonial mutations differ from spermatogonial ones comes both from the specific-locus spectrum and from conclusions that can be drawn from complementation studies of the specific-locus regions and from molecular analyses of individual mutations. Because the spermatogonial sample from the G experiment alone is rather limited, additional spermatogonial data from other radiation experiments were used for both sets of comparisons, which indicated that mutations induced in postspermatogonial stages include a higher frequency of "large" lesions than those induced in spermatogonia. For those chemicals that are positive in both postspermatogonial and spermatogonial stages, the difference is in the same direction, but its magnitude is greater. Thus, for ENU (ethylnitrosourea)- and MNU (methylnitrosourea)-induced specific-locus mutations, the proportions of mutations that are large lesions are 50 and 2.6% for postspermatogonial and spermatogonial stages, respectively (Russell *et al.*

1990), while the corresponding proportions for low-LET irradiations appear to be >80% (G experiment) and ~50% (Russell 1986; Russell and Rinchik 1993), *i.e.*, the proportion of large lesions is not negligible among radiation-induced spermatogonial mutations. Thus, for the nature, as well as for the frequency, of mutations, variations among different germ-cell stages, although clearly demonstrable, are less extreme for radiation than for certain chemicals.

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