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Genetics

Early postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain

(insertional mutagenesis/developmental arrest/human growth hormone gene)

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ABSTRACT **Insertional mutagenesis in a transgenic mouse strain (HUGH/3) was caused by integration of plasmid DNA containing the human growth hormone gene and pBR322 plasmid sequences. From this study, which includes another instance of mutagenesis, and from other reports, it is apparent that insertional mutagenesis occurs fairly frequently during DNA integration in the mouse egg and that it is not specific for the exogenous DNA employed. The mutation in HUGH/3 is recessive and results in death of embryos homozygous for the donor sequences shortly after implantation, at the egg cylinder stage on days 4–5 of gestation. Restriction mapping of the insert and of the flanking DNA regions indicates that integration must have involved a series of complex events. Approximately five copies of plasmid sequences are arrayed in tandem but are interrupted at least twice by mouse cellular sequences. In addition, the mouse flanking DNA shows extensive rearrangements, probably including a deletion of at least 10 kilobases. The rearrangements may reflect an initially unstable DNA structure followed by attainment of a more stable conformation.**

The integration of plasmid or proviral DNA into the mammalian genome has the potential for causing mutational changes. In the case of DNA transferred into somatic cells in culture, the donor DNA as well as the cellular sequences may experience duplications, deletions, rearrangements, or base substitutions, apart from the possible physical interruption of native by foreign sequences (1, 2). When the recipient cell is the fertilized egg, the change can be transmitted to progeny through the germ line and may ultimately appear in the homozygous state, so that even if recessive it can affect any cell type in which it is expressed. Transgenic mice produced by microinjection of DNA into a zygote pronucleus appear often to have rearrangements involving host flanking DNA sequences, as judged solely from Southern blot patterns in many published reports (e.g., refs. 3 and 4). Evidence in support of this conclusion is the occurrence of numbers of fragments capable of hybridizing to the plasmid probe that are greater than expected.

Insertional mutations in transgenic mice may thus shed light on the events accompanying DNA integration. They may also enable genes affecting development to be isolated and understood. We therefore undertook a search for insertional mutants among mice derived from eggs injected with phGH plasmid DNA. This plasmid contains the human growth hormone gene subcloned as a 2.6-kilobase (kb) *Eco*RI fragment into the 4.3-kb pBR322 vector (5). Six mice positive for the foreign sequences in the heterozygous state (*HGH/+*) all had unique integration patterns and became the founders of six new strains designated HUGH/1 through HUGH/6 (6). After backcrossing to wild type, followed by brother–sister matings between heterozygotes, two of the strains—HUGH/3 and HUGH/4—failed to produce any *HGH/HGH* postnatal homozygotes. Homozygosity was therefore lethal at some stage before birth. The offspring of interstrain crosses between heterozygotes of the two lethal-bearing strains included normal animals with the hybridization patterns of both strains in Southern blots of DNA digests. Thus, the defects in HUGH/3 and HUGH/4 are probably different and are due to different integrations.

We describe here developmental and DNA aberrations in the HUGH/3 strain. (HUGH/4 is the subject of a separate study.) Homozygosity for the phGH insertion in HUGH/3 results in embryo lethality shortly after implantation in the egg cylinder stage. Restriction mapping of mouse DNA regions flanking the insertion reveals a complex structure in which cellular DNA interrupts the insert. The flanking DNA has also undergone extensive rearrangements probably including deletion of some sequences.

MATERIALS AND METHODS

Screening for Lethal Embryos. Embryos from matings of *HGH/+* × *HGH/+* were examined, in comparison with normal controls, on days 3–12 of gestation (counting the vaginal plug date as day 0). Preimplantation embryos on day 3 were flushed out of the uterine horns and examined under 500× magnification. On day 5, 17 implantations in 2 litters were examined microscopically without sectioning; the number of abnormal embryos on this day was therefore not tabulated. On day 6, 19 embryos in 2 litters were serially sectioned *in utero* (8-μm-thick sections) and stained with hematoxylin and eosin; an additional litter with 7 decidual sites was examined without sectioning. Embryos at later stages (days 7–12) were dissected free of the uteri and inspected under 40× magnification.

DNA Preparation and Molecular Cloning. High molecular weight DNA from the tails, spleens, livers, or kidneys of HUGH/3 heterozygotes (*HGH/+*), and of controls of the C3H, C57BL/6, and BALB/c inbred mouse strains (Icr sublines), was isolated as described (7, 8).

Partial libraries of HUGH/3 DNAs were constructed using standard techniques (9). Genomic DNA was digested to completion with *Eco*RI and fractionated on an agarose gel. Fractions containing possible sequences flanking the inserted phGH DNA were eluted from the gel and ligated to λgtWES *Eco*RI arms. Clones were screened using a nick-translated phGH probe (5, 6). Restriction fragments from the positive clones (λHUGH/3 series) were further subcloned into the *Eco*RI site of pUC9 (pHUGH/3 series). A total genomic library prepared from BALB/c embryo DNA (a gift from Jonathan Seidman) was screened for wild-type sequences corresponding to the cloned sequences flanking the inserted phGH DNA in the HUGH/3 strain.

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Abbreviation: kb, kilobase(s).

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Restriction Analysis, Blotting, and Hybridizations. Restriction endonuclease-digested DNA was electrophoresed through 1% agarose gel and transferred to nitrocellulose under standard conditions (10). All hybridizations were carried out at 42°C in a solution containing 50% (vol/vol) formamide (11) and approximately 10^7 cpm of ^{32}P-labeled nick-translated DNA (specific activity $\approx 10^8$ cpm/μg).

RESULTS

Developmental Arrest of Homozygous Embryos. The numbers of preimplantation embryos recovered from *HGH/+* × *HGH/+* matings on day 3 were normal (23 embryos in 3 litters), and their appearance was indistinguishable from normal controls; most were in the blastocyst stage, and a few were late morulae. At all stages examined after implantation, through day 12, the numbers of uterine decidual sites, indicative of occurrence of implantation, remained normal, with an average of 8.3 per pregnant female (Table 1). Comparable litter sizes were found at birth in test matings of mice of other HUGH strains (HUGH/1, HUGH/2, and HUGH/6) in which no developmental problems had arisen from insertion of sequences of the same plasmid in the germ line (6). However, when the implantation sites in matings of HUGH/3 heterozygotes were sectioned or were opened and examined, approximately 25% in the postimplantation period through day 8 were found to lack embryos or to contain dead embryos in the process of being resorbed (Table 2). These were presumably the *HGH/HGH* homozygous segregants previously found to be absent from the postnatal population (6). Histological evidence revealed that the lethality had occurred in the egg cylinder stage soon after implantation. From the presence of only vestiges of the lethal embryos on day 6 (Fig. 1) and the detection of some abnormal embryos on day 5 (data not shown), we can conclude that the effects of the mutation were manifest in the day 4–5 period.

If only the *HGH/HGH* homozygotes died prenatally, we would expect the average normal litter size of eight to be reduced to approximately six postnatal survivors (75%). In fact, the average litter size after birth in matings of HUGH/3 heterozygotes was 2.9 (6), thereby indicating an additional prenatal loss of approximately three embryos per litter. This cannot be attributed to abnormally high transmission of *HGH* gametes because backcrosses of *HGH/+* × *+/+* yielded close to the expected 50% frequency of *HGH/+*. Although the genetic basis for the phenomenon remains puzzling, further prenatal losses were indeed observed in the period of days 10–12, thereby accounting for the reduction to a final litter size of three (Table 2). Deaths during this "second period" of lethality presented quite a different picture from those in the "first period" (early postimplantation). As seen in a characteristic case (Fig. 2), the later lethals were grossly abnormal and retarded, roughly resembling aberrant day 9 embryos, even on day 11.

Table 1. Yield of embryos from matings of *HGH/+* × *HGH/+*

Day of gestation	Number of litters	Average number of embryos or decidual sites
3	3	7.7
5	2	8.5
6	3	8.7
7	3	8.3
8	2	9.5
9	1	8.0
10	1	10.0
11	2	8.0
12	3	7.3
Totals	20	8.3*

*Average, based on a total of 166 embryos or decidual sites.

Table 2. Frequency of lethal HUGH/3 embryos from matings of *HGH/+* × *HGH/+*

Day of gestation	Defectives/ total*	% defective
3	0/23	0
6	7/26	27
7	5/25	20
8	5/19	26
9	3/8	38
10	7/10	70†
11	9/16	56†
12	14/22	64†

*Defectives are abnormal or dead embryos or a decidual site lacking an embryo. Of the 166 total in Table 1, the day 5 group (17 cases) is omitted here.

†Extrapolated average litter size at birth, based on embryo on embryo survival to these gestational ages, is 3.0 (18 total embryos in 6 litters).

Clones of the Flanking Regions. We have shown (figure 10 in ref. 6) that the integration pattern of phGH sequences in HUGH/3 is complex. Genomic DNA digested with *Eco*RI hybridizes to the phGH probe in eight fragments from HUGH/3 (Figs. 3 and 4). The 2.6- (hGH) and 4.3-kb (pBR322) *Eco*RI fragments that hybridize to phGH most intensely contain multiple copies of the plasmid sequences. The other six fragments are likely to contain the junctions between mouse and phGH plasmid sequences and comprise the flanking DNA. The nomenclature chosen to designate these fragments (HUGH/3-1 through HUGH/3-6) is shown in Fig. 3.

Fractions of *Eco*RI-digested HUGH/3 genomic DNA containing the putative flanking sequences were ligated to λgtWES *Eco*RI arms. Each partial library (approximately 5×10^5 clones) was screened with a nick-translated phGH probe, and four positive clones were selected for further characterization. These were designated λHUGH/3-1, -3, -4, and -5. Each of the four clones contained a number of *Eco*RI fragments, one of which hybridized to phGH. Each hybrid-

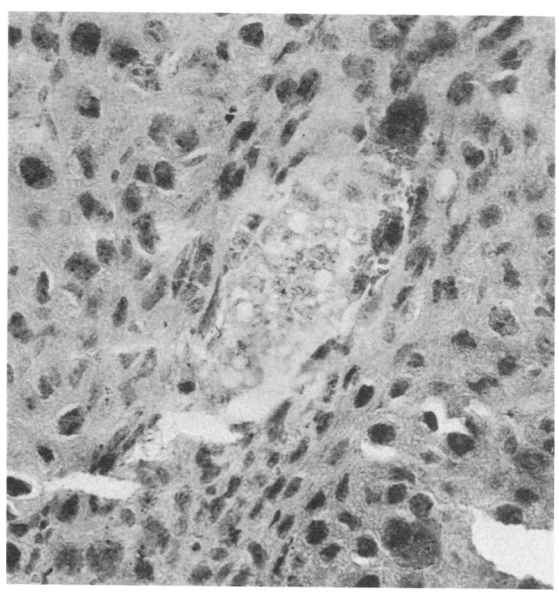

FIG. 1. Histological section of a HUGH/3 embryo being resorbed *in utero*, from a mating of *HGH/+* × *HGH/+*. The embryo, found on day 6 of gestation, has been dead for at least a day and was arrested in the egg cylinder stage shortly after implantation. (Hematoxylin and eosin stain, 8-μm section.)

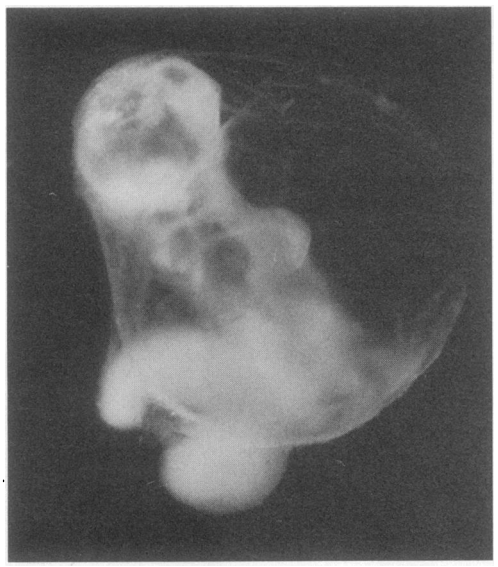

FIG. 2. Abnormal HUGH/3 embryo from a mating of *HGH/+* × *HGH/+*. Although the embryo was found on day 11 of gestation, it corresponds roughly to an aberrant day 9 embryo.

izing fragment was inserted into the *Eco*RI site of pUC9. The subclones are designated pHUGH/3-1, -3, etc. The *Eco*RI fragments were analyzed by hybridization to reveal that each indeed contained murine DNA sequences with either hGH or pBR322 sequences adjacent to one *Eco*RI site. Restriction maps of the flanking regions and their tentative arrangement in HUGH/3 genomic DNA are shown in Fig. 3.

The presence of repetitive sequences in some clones was confirmed when nick-translated wild-type genomic DNA was hybridized to restriction fragments from λHUGH/3-1, -3, -4, and -5 immobilized on nitrocellulose (data not shown). The hybridization conditions only permitted detection of sequences present at more than 100 copies per genome (12). Fragment HUGH/3-1 lacks repetitive sequences within its 5–6 kb of mouse DNA. HUGH/3-3 contains a member of the B1 family of repetitive DNA (13) identified by cross hybridization to a clone (a gift from Robert Perry) of the B1 family (data not shown). Fragments HUGH/3-4 and -5 contain unidentified repetitive sequences. The locations of the repetitive sequences are indicated in Fig. 4.

The 3.2-kb *Stu* I–*Eco*RI fragment from pHUGH/3-3 lacks repetitive sequences and was inserted into pUC9 to generate pHUGH3-3Δ.

Rearrangement of Wild-Type Mouse Sequences During Integration of Plasmid DNA. To analyze the locus at which phGH sequences were integrated in HUGH/3, Southern blots of digested wild-type genomic DNA from C3H, C57BL/6, and BALB/c mice were probed with cloned fragments flanking the integrated phGH sequences.

The mouse DNA cloned in pHUGH/3-1 (Fig. 4) contains single internal *Hin*dIII and *Pvu* II sites and should, therefore, hybridize to two *Hin*dIII and two *Pvu* II fragments in wild-type DNA. However, wild-type DNAs of C3H and

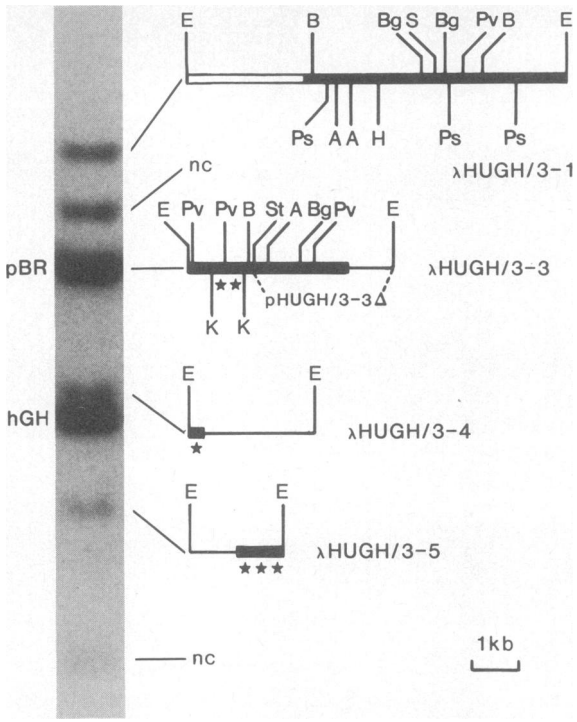

FIG. 4. Origin of four clones containing mouse flanking sequences. The Southern blot displays the *Eco*RI pattern of HUGH/3 DNA hybridized with nick-translated phGH. Of the six *Eco*RI flanking-sequence fragments represented by bands, two were not cloned (nc). Restriction maps for the four cloned fragments are shown, with the location of repetitive sequences (stars). pHUGH/3-3Δ is a subclone (from λHUGH/3) that lacks repetitive sequences. Restriction sites within hGH or pBR322 are omitted. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal* I; A, *Ava* I; Ps, *Pst* I; Bg, *Bgl* II; Pv, *Pvu* II; St, *Stu* I; K, *Kpn* I.

C57BL/6 (the parental strains for the HUGH mice) contain only one strongly hybridizing band for each of these enzymes (Fig. 5, *Left*, lanes H and Pv). Moreover, DNA digested with *Pst* I contains two hybridizing bands (1.9 and 4.8 kb) even though the pHUGH/3-1 probe has three *Pst* I sites in the mouse DNA and thus should hybridize to four *Pst* I fragments in the wild-type DNA, including one 2.6 kb and one 1.3 kb long. In all cases examined, the restriction patterns of C3H, C57BL/6, and BALB/c mice were identical, thereby making it unlikely that restriction-site polymorphism could explain the discrepancies between the actual and predicted results. Furthermore, similar results are observed when pHUGH/3-3Δ is used as a probe on an identical filter (Fig. 5, *Right*).

The *Hin*dIII restriction pattern showed polymorphism among C3H, C57BL/6, and BALB/c (data not shown); however, in all remaining cases the restriction patterns for these strains were identical.

When the filter from Fig. 5 that was probed with fragment HUGH/3-1 was exposed for a long period, additional weakly hybridizing bands were observed (data not shown). The

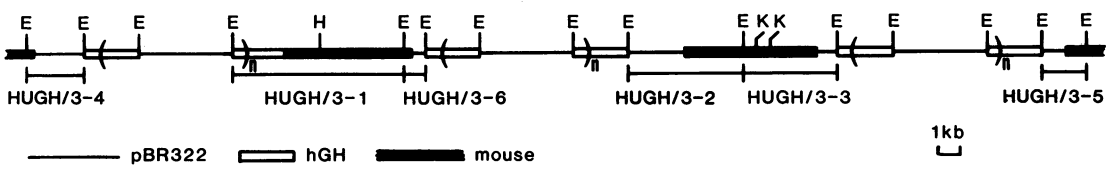

FIG. 3. phGH integration map of HUGH/3, based on Southern blot analyses of single and double digests of DNA hybridized with labeled hGH or pBR322 sequences. Approximately five copies of phGH are distributed in three discrete regions (in parentheses). Relative locations of six fragments (HUGH/3-1 through HUGH/3-6) containing mouse and foreign sequences and bordered by *Eco*RI restriction sites are indicated. Definitive positions of the flanking *Eco*RI fragments were not determined. E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I.

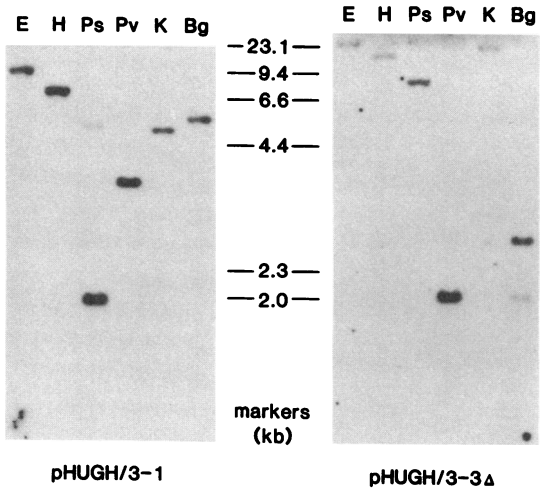

FIG. 5. Southern blot analyses of digests of wild-type genomic DNA hybridized with the flanking-sequence probes pHUGH/3-1 (*Left*) and pHUGH/3-3Δ (*Right*). Note that the observed patterns are not predicted by the maps of λHUGH/3-1 or HUGH/3-3 (see Fig. 4); and that the two probes used here did not yield the same size bands. E, *Eco*RI; H, *Hin*dIII; Ps, *Pst* I; Pv, *Pvu* II; K, *Kpn* I; Bg, *Bgl* II.

pattern of bands sometimes varied between C3H and C57BL/6 DNAs, thus indicating restriction fragment length polymorphism.

Wild-Type Clones Related to HUGH/3 Flanking Sequences. Using pHUGH/3-1 and pHUGH/3-3Δ as probes, clones were isolated from a wild-type genomic library. Three clones (A1, B4, and C2) from the pHUGH/3-1 probe were arranged in a linear restriction map; however, an additional one (C1) proved not to be homologous to any of the other three (Fig. 6). C1 seems to be rare in wild-type mouse DNA, contrasting with the clear homology to HUGH/3-1 flanking sequences in clone pHUGH/3-1 and HUGH/3 strain DNA. On the other hand, one clone related to pHUGH/3-3 (E1) shows no

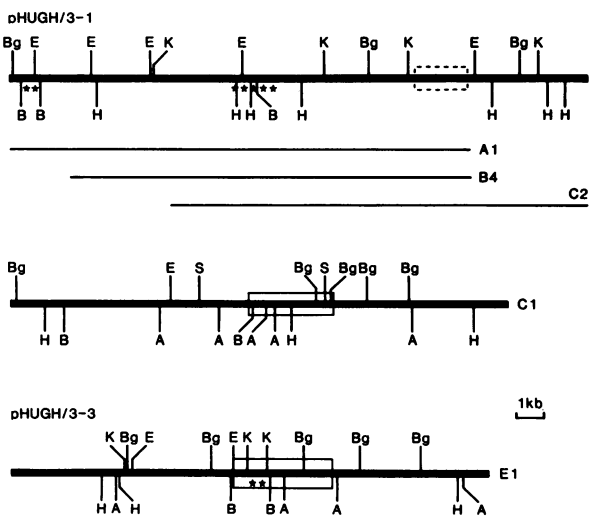

FIG. 6. Restriction map of wild-type target sequences. Four wild-type genomic clones (A1, B4, C1, and C2) were isolated using pHUGH/3-1 as a probe, and one (E1) with pHUGH/3-3Δ. A1, B4, and C2 have overlapping sequences and can be aligned in a single physical structure. The dashed box indicates the homology to pHUGH/3-1 and corresponds to the region between *Bam*HI and *Eco*RI sites (Fig. 4). C1 has a region (box) homologous to the *Bam*HI/*Bam*HI region in pHUGH/3-1 (Fig. 4) and does not show homology to any of the other three clones. E1 contains most of the sequences (box) of pHUGH/3-3 and shows no homology to any of the clones isolated with pHUGH/3-1. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; K, *Kpn* I; S, *Sal* I; Bg, *Bgl* II; stars, repetitive sequences.

homology to pHUGH/3-1 wild-type clones. In general, the restriction maps of these clones correlate closely to the patterns shown in Fig. 5. These data suggest a deletion of at least 10 kb and/or an inversion within complex rearrangements. Not surprisingly, no gross abnormalities were seen in metaphase spreads (from *HGH*/+).

DISCUSSION

The results reported here indicate that the integration of phGH sequences into the mouse genome after injection into a zygote pronucleus must have entailed complex changes during which mouse DNA sequences in the target region of HUGH/3 were deleted and/or rearranged. It is also possible that endogenous DNA from other regions or chromosomes may have inserted into the target area, along with the phGH sequences.

A partial reconstruction of the wild-type target area was made, in relation to cloned fragments flanking the inserted sequences (Fig. 6). Restriction pattern discrepancies between flanking-sequence hybridizing probes and the wild-type genomic isolates clearly document rearrangements. Moreover, the possibility of deletions in the flanking mouse DNA is supported by hybridization tests in which *Hin*dIII and *Eco*RI digests of wild-type DNA probed with pHUGH3-1 or pHUGH3-3Δ revealed no bands of equal size. In contrast, the HUGH/1 strain, in which *HGH*/*HGH* homozygotes are normal (6), does not have large rearrangements at the site of foreign DNA integration (data not shown). Discrepancies were also observed between wild-type and flanking-region DNA from HUGH/4—another transgenic mouse strain in which integration of phGH sequences resulted in early developmental lethality of *HGH*/*HGH* homozygotes (6). However, insertional mutagenesis in HUGH/3 and HUGH/4 are independent events involving different chromosomal regions and rearrangements (ref. 14, and unpublished data).

The occurrence of insertional mutagenesis in these transgenic mice is not due specifically to phGH DNA. It may in fact take place in transgenic animals during integration of virtually any exogenous DNA, with strikingly high frequency. Dominant mutations may occur (15, 16), but those with lethal developmental effects in the heterozygous state would be likely to go undetected among routine losses of embryos after DNA injection into zygotes or infection of embryos with retroviral vectors. Detection of recessive mutations, such as those in HUGH/3 and HUGH/4, requires screening of progeny from matings between heterozygotes. In the present study, deleterious mutations experimentally caused by recombinant DNA integration were found in one-third (2/6) of the cases. Relatively little screening of this sort has been reported in the literature. A rough estimate of the overall frequency, based on available information on experimental transgenic mice and including proviral as well as plasmid DNA insertions (6, 15–19), is approximately 15–20%. In addition, there is evidence for mutations caused by spontaneous proviral DNA integration of murine leukemia retroviral sequences *in vivo* (20, 21). This high frequency has obvious implications not only in the germ line but also in gene transfers into somatic cells, for example, hematopoietic stem cells destined for replacement of cells *in vivo* for clinical cures, even though recessive mutations would be innocuous in heterozygous somatic cells. Changes in host cellular as well as transferred DNAs have been described in spontaneous cases as well as in gene transfer experiments involving cultured mammalian somatic cells (e.g., refs. 1, 2, 22–24).

The integration of plasmid (but not retroviral) DNA into the mouse egg seems generally to involve a tandem array of plasmid sequences. It is not known whether the first stage is homologous recombination before integration, as reported for cells in culture (25), or integration followed by some

version of gene amplification (26, 27). In either case, our results lead us to propose that the first product of DNA integration is a highly unstable structure; and that rearrangements, possibly involving the insert as well as the host target sequences, occur as a means of achieving a more stable conformation.

If DNA integration can be mutagenic by causing rearrangements—apart from physically interrupting host sequences—it is reasonable to speculate that some chromosome regions (unrelated to immunoglobulin loci) may be recombinatorial "hot spots." Possibly some highly mutable loci may exemplify an intrinsic tendency toward fragility (28) or local DNA rearrangements. Such regions might then be more likely to integrate DNA, especially if it has exposed ends. Linear DNA has in fact been found to be more readily incorporated than circular DNA into the DNA of the mouse egg (16).

A number of recessive early lethal genes are known in the mouse, including some that cause defects at 4–5 days of development after the embryos have elicited a normal uterine implantation reaction, as in the case of the mutation described here. These include some *t* alleles and some *albino*-locus mutations. It is of interest that DNA inversions occur in the *t* complex (29, 30) and that deletions found in the *albino* region may involve regulatory as well as structural genes (31). These mutations, along with the ones in HUGH/3 and HUGH/4 (14), may be merely examples of a much more widespread phenomenon whereby DNA rearrangements of various sorts may be at the root of a number of other developmental aberrations at different stages. These will not all ultimately prove to involve sequences for peculiarly "developmental" events, inasmuch as many *de novo* functions or products with developmental consequences may not be tissue- or stage-specific nor directly implicated in morphogenesis.

Characterization of the disrupted genetic region in HUGH/3 has proved to be greatly complicated by the presence of extensive rearrangements that reduce the feasibility of isolating a meaningful segment of the corresponding normal region, and by the small amount of material available (e.g., for RNA isolation) from the lethal segregants. Nevertheless, the high frequency of mutations caused by experimental insertion of DNA offers a realistic prospect of isolating a "developmental" gene through the use of hybridizing probes of the foreign DNA. Among other reported cases of insertional mutagenesis in mice, two that have lent themselves to fruitful molecular analyses are expressed at later stages of development. These are a mutation in the $\alpha 1$ collagen gene (19) and a defect in limb morphogenesis (18).

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