Chlorambucil-induced mutations in mice recovered in homozygotes

(chemical mutagenesis/inbreeding schemes/reeler/polycystic kidneys/neurological defects)

L. Flaherty*[†], A. Messer*[†], L. B. Russell[‡], and E. M. Rinchik[‡]

*Molecular Genetics Program, Wadsworth Center for Laboratories and Research, Empire State Plaza, P.O. Box 509, Albany, NY 12201; [†]Department of Biomedical Sciences, State University of New York, Albany, NY 12201; and [‡]Biology Division, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8077

ABSTRACT Chlorambucil induced a number of recessive visible mutations in the mouse. Induction of these mutations was studied in two mating schemes, each designed to recover mutations after two intercrosses. In scheme 1, 10 mutations were detected in 82 mice; in scheme 2, 1 mutation was detected in 19 mice. We have estimated that the proportion of gametes carrying a recessive visible mutation may be as high as 25% after a dose of 10 mg of chlorambucil per kg to early spermatids. Seven of these mutations caused morphologically distinct abnormalities, including (i) a cerebellar abnormality similar to that expressed in homozygotes for the reeler (rl) mutation; (ii) open eyelids at birth; (iii) a rostral head hemangioma; (iv) abnormally small spleens, anemia, and umbilical hemorrhages; (v) immobility at birth; (vi) polycystic kidneys; and (vii) a circling behavior. Four additional mutations resulted in growth retardation and a runting syndrome. Because, in earlier studies, all molecularly characterized mutations induced by chlorambucil in poststem cells have proved to be deletions, these recessive visible mutations are probably deletions as well. These mutations may be useful in isolating and characterizing the genes responsible for the observed phenotypes.

Analyses of chromosomal rearrangements have proved extremely useful in a number of functional- and physical-mapping studies of the mammalian genome. Deletions, translocations, and inversions have been used to locate and define genes of importance in disease processes, including the genes controlling Duchennes muscular dystrophy and neurofibromatosis (1–5). Because deletions can be easily detected and mapped to specific locations on the chromosome, they have become one of the tools of choice in regional mapping studies in the mouse (6). For example, the deletions encompassing the albino locus have been successfully used to map several nearby embryonic lethal genes (7–9).

Recent work has shown that chlorambucil (CHL) effectively induces deletion mutations in mouse germ cells (10). The frequency of induced mutations after an exposure of 10 mg of CHL per kg is 1.3×10^{-3} per locus, the highest known rate for chemicals inducing mutations in the postmeiotic stem-cell stages. For example, CHL is four times as effective as x-rays at a dose of 3 Gy in inducing these mutations. In specific-locus tests, deletions vary in size from small ones encompassing <2 centimorgans to ones spanning 10–15% of a chromosome (10, 11). Multilocus deletions are common. A similar mutagenic effect has been described in Drosophila, where CHL induces a high frequency of recessive lethal mutations as well as translocations (12). Thus, CHL may be particularly useful in inducing additional mutations in the mouse that are caused by some type of molecularly discernible chromosomal rearrangement.

Potentially, CHL may be useful for inducing visible mutations as well. With such a high mutation rate, it would seem logical to assume that small deletions causing visible phenotypes in animals surviving past birth would also be induced. In previous specific-locus tests, at least two of the recovered mutations were homozygous viable (11). Conceivably, these recessive viable (or postnatally lethal) deletion mutations could then serve as useful tools in locating and analyzing genes that are important in mammalian genetic diseases. For example, deletions in genes causing morphological abnormalities in the mouse would be useful tools for studying, mapping, and identifying the corresponding genes in other species, especially humans.

Thus, we have begun several mating systems designed to recover and predict the frequency of postnatally morphologically distinguishable recessive mutations induced by CHL. Two of these systems are described in this report.

MATERIALS AND METHODS

CHL Treatment. CHL was obtained from Radian (Austin, TX) or Sigma. CHL was dissolved in 70% ethanol and subsequently diluted with a phosphate buffer solution, pH 6.8, as described (10). The solution was always prepared fresh and was injected i.p. into mice within 30 min of preparation (solution was kept on ice in the interim).

Mice. All mice used were either produced at the Oak Ridge National Laboratory, produced at the Wadsworth Center for Laboratories and Research, or purchased from The Jackson Laboratory. The C3Hf/Rl, 101, and Tester (T) stocks have been described (10, 13, 14). The T stock contains homozygous recessive alleles at the a (nonagouti), b (brown), c^{ch} (chinchilla), p (pink-eyed dilution), d (dilute), se (short ear), and s (piebald) loci. The C57BL/6Fla (B6) and BALB/cByFla (BALB/c) strains were produced at the Wadsworth Center. The C3H/HeOuJ (C3H) and B6C3Fe-a/a-rl strains were purchased from The Jackson Laboratory.

Breeding Scheme. Two schemes for detecting mutations were used. In the first scheme, (101 × C3Hf/Rl)F₁ males (hereafter called H males), treated with 10 mg of CHL per kg, were each mated to two T-stock females, at weekly or biweekly intervals. Only offspring conceived during the third week were collected for these experiments; these offspring were designated TH. In the second scheme, B6 male mice were treated with 7.5 mg of CHL per kg. At 1 week after injection, each of these males was mated to two BALB/c females. At 2 weeks, the BALB/c females were discarded and replaced with C3H females. These females were allowed to mate with the mutagenized males for 7 days. The males were then removed and discarded. Offspring from the C3H females were saved and are designated (C3H × B6)F₁ mice.

Abbreviations: CHL, chlorambucil; T, Tester; H males, (101 × C3Hf/Rl)F₁ males; TH mice, offspring of T-stock females × H males.

For scheme 1, 48 pairs of TH mice were intercrossed. At least three brother–sister pairs of each resulting TH-G_2 progeny were mated. The offspring of these pairs (TH-G_3) were observed both at birth and at weaning for any morphological abnormalities. Because several coat-color genes were segregating in this cross, it was impossible to detect additional coat-color mutations. Breeding pairs were discarded after 15 progeny were recorded or after the breeding pair failed to produce any more offspring in a 3-month period.

For scheme 2, 21 (C3H × B6)F_1 female offspring of mutagenized B6 males were backcrossed to untreated B6 male mice. (Female F_1 mice were used for these backcrosses because they would enable us to detect X chromosome-linked lethal mutations as well as recessive viable ones.) At least eight brother–sister pairs of each resulting backcross progeny were mated; the offspring of these backcross mice were observed both at birth and at weaning for any morphological abnormalities. Breeding pairs were discarded after 20 progeny were recorded or after the breeding pair failed to produce any more offspring for 3 months.

RESULTS

Breeding Scheme. Two breeding schemes were used to determine the number of visible and viable recessive mutations produced by CHL. In the first scheme, randomly chosen progeny (TH) of mutagenized males were intercrossed (Fig. 1). The offspring from these intercrosses (TH-G_2) would carry mutations derived either from the mutagenized maternal grandfather or from the mutagenized paternal grandfather; however, no two mice would carry the same mutation. The TH-G_2 mice were then intercrossed in brother–sister combinations. If a viable recessive mutation were induced in the spermatids of the original mutagenized males and transmitted to a TH male or female, then the progeny of this TH mouse (called TH-G_2) would have a 50% chance of carrying this mutation. Thus, in matings between TH-G_2 brother–sister pairs, there is a 25% chance that both parents would carry the same mutation, such that one-fourth of the TH-G_3 mice would show signs of this mutation (presuming complete penetrance and expressivity).
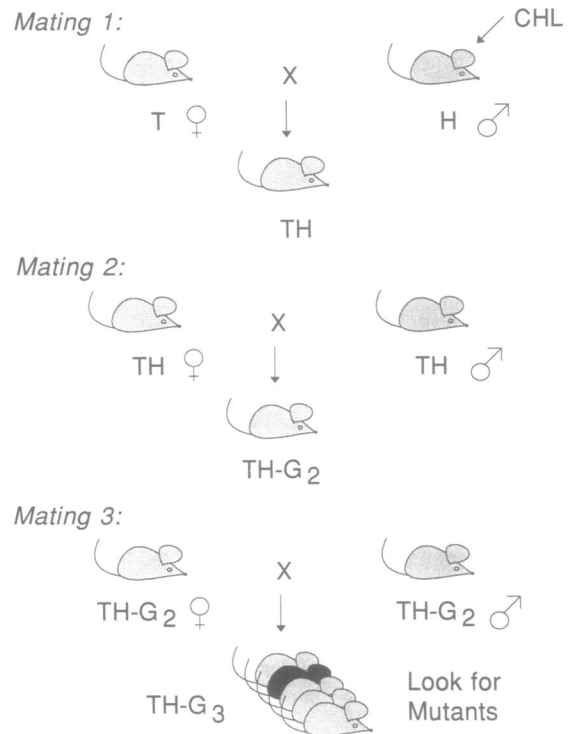

FIG. 1. Outline of scheme 1 matings. Mating 1: CHL-treated H male mice were mated to T-stock females. Mating 2: TH mice were randomly selected from a stock derived from ≈50 treated males. These mice were randomly paired and mated. Mating 3: Brother–sister TH-G_2 siblings from mating 2 were mated, and offspring were observed for abnormalities.

Out of 48 original TH breeding pairs, 44 were fertile and produced TH-G_2 offspring. With several pairs, litter sizes, fertility, and fecundity were low, making recovery of a significant number of progeny impossible. Table 1 shows the results of these matings.

In scheme 2, mutagenized B6 males were mated to C3H females (Fig. 2). F_1 female progeny were then backcrossed to

Table 1. Summary of mutation data

Average litter size for mating-2 pairs,* range	Pairs in range, no.	Litter size for range, average	Litter sizes of brother–sister mating-3 pairs,† grand average	Mating-2 pairs with n successful test crosses,‡ no.									Recessive mutants,§ no.
				0	1	2	3	4	5	6	7	8	
Scheme 1													
0	4			4									
0.1–4.0	7	2.89	4.03	3	3	1							2 (2)
4.1–6.0	8	4.50	5.18		2	2	4						0
6.1–12.0	29	7.78	7.58		5	8	16						8 (2)
Scheme 2													
0	2			2									
0.1–4.0	1	3.14	6.09		1								0
4.1–6.0	1	4.83	5.25							1			0
6.1–12.0	17	9.56	7.89				1			2	3	11	1

*In scheme 1, mating-2 pairs are TH × TH. In scheme 2, mating-2 pairs are (C3H × B6)F_1 × B6. Average litter size for each pair was calculated by dividing the total number of offspring by the total number of litters (usually >8); litters lost during pregnancy were not counted.

†The grand averages of litter sizes were calculated by first averaging litter sizes of each mating-3 pair (brother–sister pair derived from mating-2) and then using these average litter sizes to calculate an average litter size of all mating-3 pairs derived from each mating-2 pair. These values were then averaged within each range.

‡A successful test cross for scheme 1 is any brother–sister pair that yielded 15 or more offspring. A successful test cross in scheme 2 is any brother–sister pair that yielded 20 or more offspring. Numbers in table represent the number of mating-2 pairs yielding the indicated number n (at top of column) of successful test crosses. For example, in the 4.1–6.0 range of scheme 1, two mating-2 pairs yielded one successful test cross; two mating-2 pairs yielded two successful test crosses; and four mating-2 pairs yielded three successful test crosses.

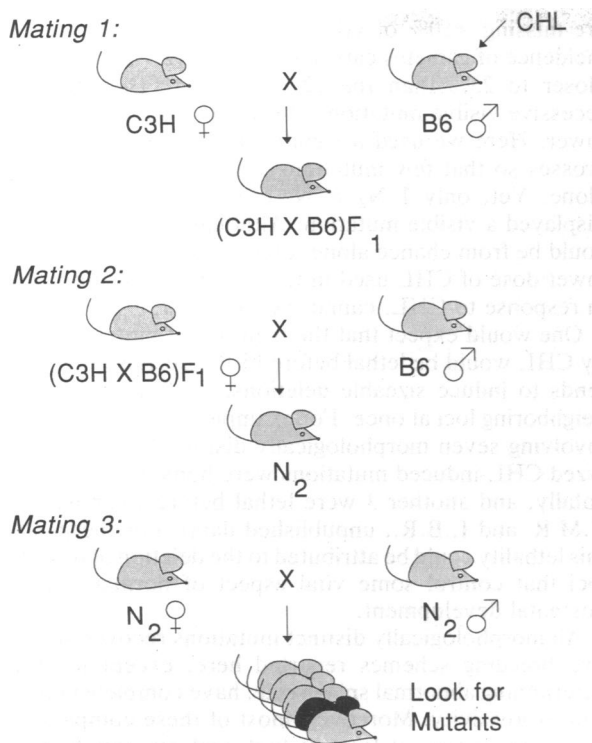§Number in parentheses indicates number of mutations that resulted in a "runting syndrome" phenotype.

FIG. 2. Outline of scheme 2 matings. Mating 1: CHL-treated B6 male mice were mated to C3H females. Mating 2: (C3H × B6)F$_1$ mice were randomly chosen from a stock derived from ≈15 treated males. These mice were mated to B6-stock mice. Mating 3: Brother–sister N$_2$ siblings were mated, and offspring were observed for abnormalities.

B6 males. Backcross mice (called N$_2$ mice) were then intercrossed in brother–sister combinations, and their offspring were observed for the production of morphological abnormalities. Again, if the mutagenized male transmitted a mutation to its F$_1$ offspring, this mutation would have a 50% chance of being transmitted to the N$_2$. Thus, in random matings between sibling N$_2$ backcross mice, there would be a 25% chance that both parents would carry the mutation and that mutation would be detectable in one-fourth of their offspring.

Litter Sizes. Among the original breeding pairs in both schemes 1 and 2, the average litter sizes ranged from 1.7 to 12.3. In the pairs exhibiting litter sizes of 6 or below, offspring of these pairs also show significantly lower than normal litter sizes (see Table 1). The most likely explanation for the genetic inheritance of low litter sizes is that these mutated mice possessed gross chromosomal abnormalities, such as translocations, that led to genetic imbalances and death in a fraction of their offspring. Previous work has shown that CHL frequently induces translocations (10, 11). Perhaps significantly, only pairs with litter sizes of greater than six yielded morphologically distinct recessive mutations (Table 1). The two transmissible traits derived from pairs with an average litter size of less than six were characterized as portraying a runting syndrome. This growth deficiency was either noted at birth and persisted until weaning or was noted at ≈3 weeks of age as a lag in growth with respect to littermate controls.

Recessive Mutations. The scheme-1 and scheme-2 breeding protocols allowed the recovery of 11 recessive viable mutations (Table 1). All of these mutations have been sibling- or progeny-tested for transmissibility; all of these mutations are inherited in a Mendelian fashion. Of these 11, 4 mutations resulted in a runting syndrome seen either at birth or at weaning. The other 7 mutations were detected on morphological or behavioral criteria. Allelism and linkage tests have

been done on some of these mutations, especially where they resemble known mutations in mouse. These mutants are described as follows.

Reeler. At 3 weeks of age, ≈25% of the mice from TH-G$_2$ × TH-G$_2$ mating 22 were seen to have an unsteady gait and to tremble when held by the tail. These mice were often smaller than their normal litter mates. Homozygous females were fertile; however, very few homozygotes survived past 2 months of age. (No homozygous male survived long enough to test for fertility.) On autopsy, these mice displayed an abnormally small cerebellum with a small and disorganized granule cell layer (Fig. 3). Normally, the Purkinje cells are found at the edge of the granule layer; however, in these mutants they appeared throughout the layer and were sparse in number. In addition, there were other areas of the brain that appeared disorganized. From descriptions of cerebellar mutants in the mouse, this abnormality most closely resembles that seen in the reeler mutant (*rl*) (15, 16). Therefore, allelism tests were done with a known reeler mutation (*rl*) maintained at The Jackson Laboratory. When *rl/rl* homozygotes were mated to known heterozygotes bearing this CHL-induced mutation, 50% of the progeny showed the characteristic unsteady gait and cerebellar abnormalities. Thus, this CHL-induced mutation appears to be an allele of *rl*. We have called this allele, *rl^{alb}*.

Open eyelids. Approximately 25% of the mice from TH-G$_2$ × TH-G$_2$ mating 46 were observed to have open eyelids at birth. Subsequently, these mice developed an opaque cornea and were unable to see. Both homozygous males and females were fertile, and there was complete penetrance of the defect.
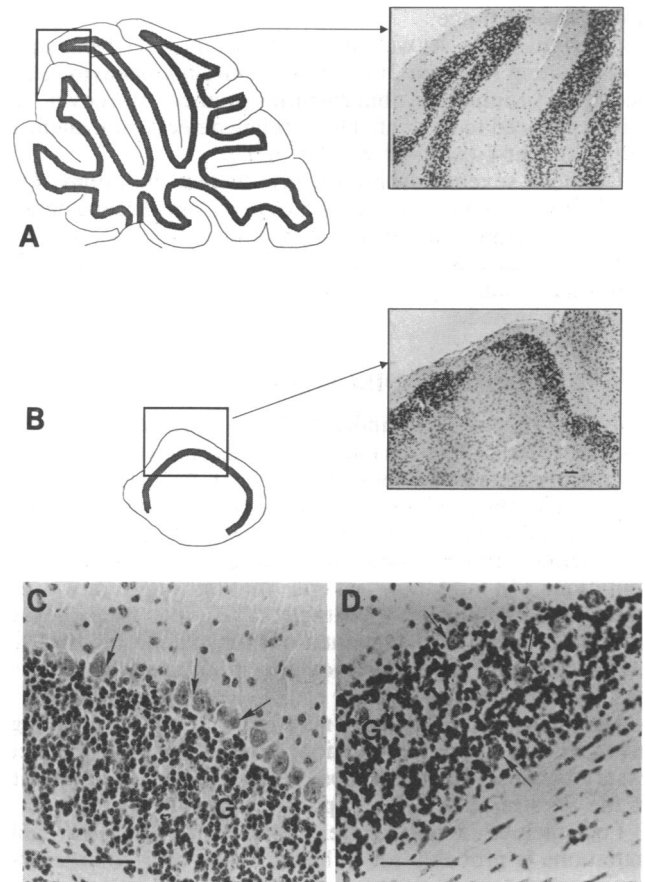

FIG. 3. Comparison of normal and *rl^{alb}/rl^{alb}* cerebella. (*A Left* and *B Left*) Representative comparative drawings of normal (*A*) and *rl^{alb}/rl^{alb}* (*B*) cerebella. (*A Right* and *B Right*) Hematoxylin/eosin-stained slides from the approximate area indicated in box. (*C*) Granule cell layer from normal mouse. (*D*) Granule cell layer from mutant. Arrows in *C* and *D* indicate Purkinje cells. Bar = 100 μm.

This abnormality is similar to several other mouse mutations, including *oe*, open eyelids (chromosome 11), *gp*, gaping lids, and *lg*, lid gap (14, 17). Allelism tests with these known mutations have not yet been done.

Head hemangioma. At birth, ≈25% of the mice from TH-G$_2$ × TH-G$_2$ mating 37 had a hemangioma in the central dorsal surface of their nostrum. No other hemangiomas or other abnormalities were noted. Subsequently, these mice developed normally and showed complete viability and fertility. At weaning, the only noticeable defect was a slight bluntness of the nostrum.

Abnormally small spleen. At birth, ≈25% of the mice from TH-G$_2$ × TH-G$_2$ mating 6 were seen to be pale and cyanotic with multiple hemorrhages in the skin around the umbilical cord. On autopsy, these animals had rudimentary spleens closely clinging to the stomach wall. No other abnormalities of these mice were noted. The homozygous mutants normally died within 3 days of birth. Also noted from this same breeding pair was one mouse with no anus and a shortened abdominal cavity.

Immobile at birth. At birth ≈25% of the mice from the TH-G$_2$ × TH-G$_2$ mating 19 were observed to lack the normal mobility of newborns. These mice would remain relatively immobile and were unable to turn or suckle. They soon died, presumably from lack of nourishment. On autopsy, no gross abnormalities were noted.

Polycystic kidneys. At ≈1–2 weeks of age, ≈25% of the mice from TH-G$_2$ × TH-G$_2$ mating 31 were seen to have an abnormally large hind region often characteristic of kidney disease. On autopsy, these mice exhibited bilateral polycystic disease of the kidneys. All affected mice died between 1 and 3 weeks of age.

Circling defect. At weaning, ≈25% of the mice from an N$_2$ × N$_2$ mating (scheme 2) exhibited a circling behavior characteristic of inner ear abnormalities in mice. Moreover, the mice appeared to be deaf. This circling defect is not allelic to either shaker-1 (*sh-1*) or whirler (*wi*).

Of these seven morphologically distinct mutations, four (reeler, open eyelids, polycystic kidneys, and circling defect) have been retained for further study and genetic analysis. The mutations causing the runting syndromes, head hemangioma, abnormally small spleen, and immobility at birth have not been propagated.

DISCUSSION

Previous results have indicated that CHL is a powerful inducer of lethal deletion mutations in the mouse (10, 11). The results presented here indicate that CHL is also an effective mutagen for the production of recessive mutations that do not kill prenatally. From 41 tested pairs in scheme 1, we found six well-defined transmissible morphological and neurological mutations. In addition, several runting syndromes were noted and found to be heritable. Thus, the frequency of observed recessive viable mutations for scheme 1 is 10/82 or ≈12%. For scheme 2, the frequency of recovered mutations has the lower value of 1/19 or 5%. That this frequency is lower than that in scheme 1 may be from the lower dosage given in this strain combination. (The lower dose was necessary because the higher dose of 10 mg/kg causes sterility in the B6 strain; E.M.R., unpublished results.)

For scheme 1, actual frequency of induced recessive viable mutations is probably much higher than reported here. Because our detection system was not maximal and only based on visual observation, many recessive viable mutations could be missed. Moreover, use of only three test crosses decreases the chances of finding recessive mutations. For example, the probability that three test crosses is sufficient for detection (i.e., at least one of the test crosses contains two heterozygous parents) is 0.58. Thus, we estimate that in scheme 1 we

are missing ≈50% of visible mutations and that the actual incidence of gametes carrying a recessive visible mutation is closer to 25% than the 12% reported. The incidence of recessive visible mutations in scheme 2 appears to be much lower. Here we used a significantly higher number of test crosses so that few mutations would be missed by chance alone. Yet, only 1 N$_2$ × N$_2$ cross out of 19 tested (5%) displayed a visible mutation. Although this lower incidence could be from chance alone, other explanations, such as the lower dose of CHL used in this scheme or strain variations in response to CHL, cannot be excluded.

One would expect that the majority of mutations induced by CHL would be lethal before birth. Because this mutagen tends to induce sizeable deletions, it often deletes several neighboring loci at once. For example, in specific-locus tests involving seven morphologically distinct loci, 10 of 16 analyzed CHL-induced mutations were homozygous lethal prenatally, and another 3 were lethal before weaning (ref. 11; E.M.R. and L.B.R., unpublished data). For the most part, this lethality could be attributed to the deletion of neighboring loci that control some vital aspect of normal prenatal or postnatal development.

All morphologically distinct mutations recovered from the two breeding schemes reported here, except for the one determining abnormal spleen size, have complete penetrance and expressivity. Moreover, most of these completely penetrant mutations, at least at first analysis, involves only a single characteristic. Therefore, each one probably represents the disruption of a single locus. Because all molecularly analyzed mutations previously induced by CHL in post-stem-cell stages have been deletions [so far 10 of 10 are confirmed deletions (ref. 11; E.M.R. and L.B.R., unpublished data)], it is likely that the recessive visible mutations described here are deletions as well. Only one viable, postmeiotic CHL-induced mutation [at the brown (*b*) locus] has been molecularly analyzed, and it, too, was confirmed as a deletion (E.M.R. and L.B.R., unpublished data).

If these recently recovered recessive visible CHL-induced mutations do represent deletions, they could serve as useful tools for isolating and characterizing genes that influence disease processes. One of the foremost problems in isolating and characterizing disease-causing genes is the difficulty in identifying the gene at the DNA level once the linkage relationships have been established. When disruption of the disease-causing gene involves only a single-base-pair change, the gene is extremely difficult to find. However, when the mutation is a deletion or some other type of chromosomal rearrangement, characterization of the disrupted gene becomes more amenable to molecular genetic analysis. Use of these deletions, together with recently developed positional-mapping and walking techniques, should allow localization and identification of the genes responsible for a number of mouse developmental abnormalities. For example, several laboratories have been characterizing a multitude of additional polymorphic markers based on dinucleotide repeats or inverted sequences recognized by random 10-mer primers (18–22). Moreover, yeast artificial chromosome (YAC) libraries are now available for the mouse, and recent procedures have yielded long stretches of contiguous mouse DNA contained within these YACs (23, 24). As the mouse map becomes more and more saturated with markers and as walking techniques become more refined, defining the extent of these induced deletions and, thereby, identifying the underlying genetic defects will become easier.

Genetics: Flaherty *et al.*

Proc. Natl. Acad. Sci. USA 89 (1992)   2863

1. Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C. & Kunkel, L. M. (1987) *Cell* **50,** 509–517.
2. Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M. & Kunkel, L. M. (1986) *Nature (London)* **323,** 646–650.
3. Wallace, M., Marchuk, D., Andersen, L., Letcher, R., Odeh, H. R., Saulino, A., Fountain, J., Brereton, A., Nicolson, J., Mitchell, A., Brownstein, B. & Collins, F. (1990) *Science* **249,** 181–186.
4. Viskochil, D., Buchberg, A., Xu, G., Cawthon, R., Stevens, J., Wolff, R., Culver, M., Carey, J., Copeland, N., Jenkins, N., White, R. & O'Connell, P. (1990) *Cell* **62,** 187–192.
5. Cawthon, R., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P. & White, R. (1990) *Cell* **62,** 193–201.
6. Rinchik, E. M. & Russell, L. B. (1990) in *Genome Analysis,* eds. Davies, K. E. & Tilghman, S. M. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 1, pp. 121–158.
7. Niswander, L., Yee, D., Rinchik, E. M., Russell, L. B. & Magnuson, T. (1989) *Development* **105,** 175–182.
8. Johnson, D. K., Hand, R. E., Jr., & Rinchik, E. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8862–8866.
9. Rinchik, E. M. (1991) *Trends Genet.* **7,** 15–21.
10. Russell, L. B., Hunsicker, P. R., Cacheiro, N. L., Bangham, J. W., Russell, W. L. & Shelby, M. D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3704–3708.
11. Rinchik, E. M., Bangham, J. W., Hunsicker, P. R., Cacheiro, N. L., Kwon, B. S., Jackson, I. J. & Russell, L. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1416–1420.
12. Woodruff, R. C., Mason, J. M., Valencia, R. & Zimmering, S. (1985) *Environ. Mutagen.* **7,** 677–702.
13. Russell, W. L. (1951) *Cold Spring Harbor Symp. Quant. Biol.* **16,** 327–336.
14. Lyon, M. F. & Searle, A. G. (1989) *Genetic Variants and Strains of the Laboratory Mouse* (Oxford Univ. Press, Oxford).
15. Falconer, D. S. (1951) *J. Genet.* **50,** 192–201.
16. Goffinet, A. M. (1984) *Brain Res. Rev.* **7,** 261–296.
17. Mackensen, J. S. (1960) *J. Hered.* **51,** 188–190.
18. Aitman, T. J., Hearne, C. M., McAleer, M. A. & Todd, J. A. (1991) *Mammal. Genome* **1,** 206–210.
19. Hearne, C. M., McAleer, M. A., Love, J. M., Aitman, T. J., Cornall, R. J., Ghosh, S., Knight, A. M., Prins, J.-B. & Todd, J. A. (1991) *Mammal. Genome* **1,** 273–282.
20. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990) *Nucleic Acids Res.* **18,** 6531–6535.
21. Welsh, J., Petersen, C. & McClelland, M. (1991) *Nucleic Acids Res.* **19,** 303–306.
22. Sudweeks, J., Woodward, S. & Teuscher, C. (1991) *Fifth International Workshop Mouse Genome,* ed. Demant, P., 91.
23. Larin, Z., Monaco, A. P. & Lehrach, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4123–4127.
24. Hieter, P., Connelly, C., Shero, J., McCormick, M. K., Antonarakis, S., Pavan, W. & Reeves, R. (1990) in *Genomic Analysis,* eds. Davies, K. E. & Tilghman, S. M. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 1, pp. 83–120.