model when the active fault height reaches 140 m, which would occur at 2 m of deep strike-slip motion if G were 13 GPa rather than 10 GPa (Fig. 3*b*).

Because such a high proportion of the total earthquake population was selected as belonging to multiplets, it seems most plausible that we are imaging the boundary of a locked fault being eroded by creep at greater depth, rather than formation of a new fault. The extreme concentration of seismicity begs the question of whether similar behaviour might be observed surrounding locked patches of major strike-slip faults elsewhere. An essential ingredient of the proposed model is that the initial tractions on the fault be relatively uniform as well as far from failure. The most plausible time for encountering such stress states on faults outside volcanic regions would be following major earthquakes. Whether the residual stresses following such events could be sensibly uniform is currently a topic of considerable interest[21–24]. Thus searching for similar patterns of seismicity along major strike-slip faults could shed light on the tectonic earthquake cycle. Within Kilauea's rifts the 'earthquake cycle' is likely to be reset by dyke intrusion before fault slip reaches the surface. □

Methods

The measure of similarity of two traces is the average coherency, in the frequency range 4–13 Hz, of a 1.28 s (128 time samples) window containing the P wavetrain. Two events are considered similar if this average coherency averages more than 90% at four nearby stations. A multiplet is defined as a family of earthquakes in which each event is similar to at least n others. The multiplets described in this study are the three satisfying the requirement that each event is similar to at least 15 others. Although each event need not to be similar to all other events in the mutliplet, at some stations (in general those lying within the middle of the quadrants of the focal sphere) the traces are coherent across an entire multiplet. Relaxing the multiplet standards increases the size of the multiplets but decreases (on average) the accuracy with which events can be relocated; in our case decreasing n to 10 generates a single multiplet containing 425 events that spans the entire box straddling the UER in Fig. 1. Using the method of VanDecar and Crosson[25], which estimates cross-correlation errors from the internal consistency of all the redundant delay measurements, the average accuracy of the time delays for the three multiplets ranges from 0.8 to 1.7 ms (0.08 to 0.17 samples) at the nine best stations. This is 25–50 times the accuracy with which individual P-wave arrival times can be measured. For a P-wave velocity in the source region of 5 km s^{-1} (ref. 26) this is consistent with a relative location accuracy of 5–10 m in horizontal position and 10–20 m in depth. The r.m.s. differences between the measured time delays and those computed from the three relocations range from 3.9 to 4.4 ms. Most of this misfit is likely to come from errors in the assumed take-off angles and violation of the assumption that the azimuth and take-of angle from every member of the multiplet to a given station is identical[3].

Received 12 June; accepted 16 October 1996.

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ACKNOWLEDGEMENTS. We thank J.-L. Got for supplying us with a copy of his relocation code; the staff of the Hawaiian Volcano Observatory for their help in collecting the data; T. Árnadóttir, G. Beroza and P. Delaney for comments and suggestions. The figures were produced using the GMT software provided by P. Wessel and W. Smith. This work was supported by the US National Science Foundation, US Geological Survey, NASA, and a Bergmann research grant from the US-Israel Binational Science Foundation (A.R.).

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High intrinsic rate of DNA loss in *Drosophila*

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PSEUDOGENES are common in mammals but virtually absent in *Drosophila*[1]. All putative *Drosophila* pseudogenes show patterns of molecular evolution that are inconsistent with the lack of functional constraints[2–5]. The absence of bona fide pseudogenes is not only puzzling, it also hampers attempts to estimate rates and patterns of neutral DNA change. The estimation problem is especially acute in the case of deletions and insertions, which are likely to have large effects when they occur in functional genes and are therefore subject to strong purifying selection. We propose a solution to this problem by taking advantage of the propensity of retrotransposable elements without long terminal repeats (non-LTR) to create non-functional, 'dead-on-arrival' copies of themselves as a common by-product of their transpositional cycle[6–8]. Phylogenetic analysis of a non-LTR element, *Helena*, demonstrates that copies lose DNA at an unusually high rate, suggesting that lack of pseudogenes in *Drosophila* is the product of rampant deletion of DNA in unconstrained regions. This finding has important implications for the study of genome evolution in general and the 'C-value paradox'[9] in particular.

Non-LTR retrotransposable elements comprise a distinct group in the retroid lineage[10]. These elements are on average 4–5 kilobases (kb) long, have no introns, lack terminal repeats, have a dA-rich tail at the 3′ end, and contain 1–2 open reading frames, one of which encodes the reverse transcriptase. Transposition of these elements often results in the creation of 5′-truncated copies[6–8]. Such copies lack a promoter and do not encode functional proteins. These 'dead-on-arrival' copies are expected to manifest patterns of neutral evolution because they are not subject to selection for the ability to transpose; they are effectively pseudogenes.

We have studied the evolution of a particular non-LTR retrotransposable element, *Helena* (Fig. 1), which was originally cloned as one of several transposable elements activated in hybrid dysgenesis in *D. virilis*[11]. We have sequenced 18 different copies of *Helena* from 8 species in the *D. virilis* group. The sequenced region comprises 363 base pairs (bp) at the 3′ end of the putative reverse transcriptase. Alignment of these sequences revealed a low amount of sequence divergence and an unexpectedly large number of deletions (Fig. 2*a*). We have observed no internal insertions relative to the consensus. All deletions are unique; in fact, no two deletions share the same breakpoint. Figure 2*b* shows the distribution of the deletion sizes: deletions range from 1 to 75 bp and average 24.3 ± 23.1 bp.

To gain insight into the evolutionary history of the sampled copies of *Helena*, we have performed phylogenetic analysis using maximum parsimony[12] with the nucleotide sequence alignment.

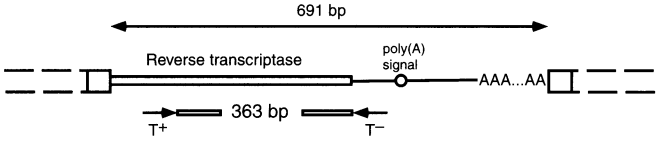

FIG. 1 Sequence organization of the insertion of *Helena* in the *singed* locus in *D. virilis*. It is 691 bp long and trucated at the 5′ end. It contains an open reading frame encoding a part of the putative reverse transcriptase, followed by a poly(A)-addition signal and a 41-bp stretch of poly(A). T^+ and T^- are the primers used to obtain sequence from multiple copies of *Helena*.

Figure 3*a* shows the resulting tree. The tree can be used to classify all substitutions into shared substitutions, which map to the internal branches of the tree, and mostly unique substitutions, which map to the terminal branches. The internal-branch substitutions can be interpreted as having occurred in the active lineages of *Helena*. Accordingly, the terminal-branch substitutions would be those that arose either in the active lineage that gave rise to a single sampled copy, or in the sampled copy itself since its insertion. When the number of active lineages is small and the sampling is relatively dense, the majority of terminal-branch substitutions will have occurred after transposition. This pattern is expected because most of the active lineages will have given rise to multiple sampled sequences, and the substitutions in active lineages will appear as shared among members of the sample and will map to the internal branches.

These assumptions can be tested by examining the patterns of purifying selection in the distribution of terminal-branch and internal-branch substitutions. The internal-branch substitutions, if they truly correspond to the substitutions in the active lineages, should show evidence of purifying selection at the amino-acid level, because the sequenced region corresponds to a functionally important and constrained protein, reverse transcriptase[13,14]. On the other hand, the terminal-branch substitutions should show a more or less random pattern of substitutions. As most of the substitutions in the third position of codons are synonymous, and the substitutions in the first and second position are usually non-synonymous, we expect that most of the internal-branch substitutions will map to the third positions and that the terminal-branch substitutions will map randomly to all three positions. This is exactly what we observed (Fig. 3*b*): there is an excess of third-position substitutions along internal branches (χ^2 test, $P = 5 \times 10^{-9}$), but substitutions along terminal branches are uniformly distributed among first, second and third positions (χ^2 test, $P = 0.2$).

The operation of purifying selection on internal branches, but not on terminal ones, is consistent with a model in which the tree's internal branches trace the evolution of active, functional lineages, whereas external branches are generated by neutrally evolving copies. This conclusion is supported by the frequency distribution of deletions, all of which are unique. Presumably, deletions in the reverse transcriptase are incompatible with transposition and are eliminated by purifying selection in active lineages. Furthermore, the observed positive and monotonic correlation (sign test, $P = 0.043$) (Fig. 3*c*) between the number of terminal-branch substitutions and the number of deletions in a sequence indicates that deletions and terminal-branch substitutions have accumulated after transposition. This relation is expected because both deletions and substitutions should accumulate with time. An alternative explanation is that *Helena* reverse transcriptase generates substitution errors at an extremely high rate (up to 7×10^{-2} mutations per nucleotide per cycle) and also produces multiple internal deletions, both of which are unlikely[6–8,15].

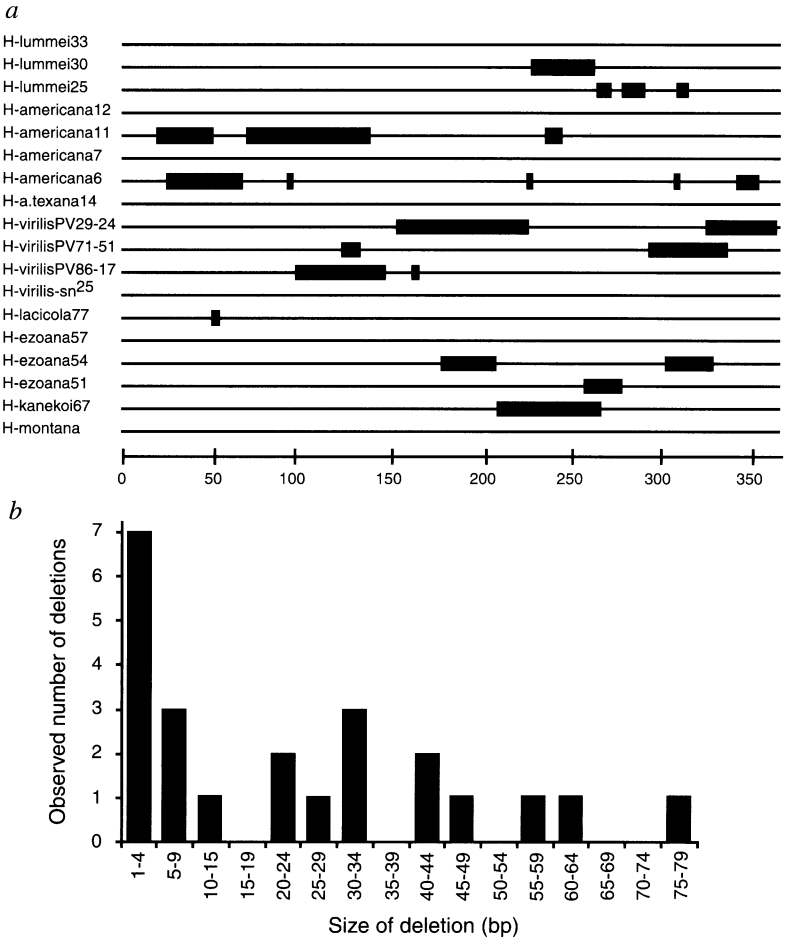

FIG. 2 Location and size of deletions in *Helena*. *a*, Location of deletions in the 18 aligned sequences of *Helena*; deletions are shown as bars. We have not observed any internal insertions relative to the consensus of the sequenced region; the only insertion we found is located at the very end of one of the sequences (H-*americana* 6) and is accompanied by a partial duplication of the primer sequence. *b*, Size distribution of the deletions.

The positive and monotonic relationship between the number of deletions and the number of terminal-branch substitution allows us to assess the deletion rate relative to the rate of nucleotide substitution. Because we cannot assume that the numbers of terminal-branch substitutions and deletions are drawn from a bivariate normal distribution, we use the approach of maximum likelihood to estimate the regression of number of terminal-branch substitutions (corrected for the length of each sequenced copy) on the number of deletions (Fig. 3*c*, legend), which gives us the relative rate of 0.16 deletions per substitution (the 95% confidence interval is 0.09–0.26 deletions per substitution). (Note that this estimate of the deletion rate is conservative, because a small proportion of the terminal-branch substitutions may have occurred when the sampled element was still actively transposing and under purifying selection. A slight, albeit non-significant, excess of third position substitutions on the terminal branches supports this interpretation.)

To compare the observed rate of accumulation and the size of deletions in *Helena* with that in mammals, we have analysed a dataset of human and murine processed pseudogenes compiled by Graur *et al.*[16]. Deletions in *Helena* are 3.3 (1.6 to 6.6) times more frequent relative to the rate of nucleotide substitutions, and are on average more than 7 times larger (24.3 ± 23.1 bp compared with 3.2 ± 4.6 bp) than deletions in the mammalian processed pseudogenes. Such a high rate of deletion and the large size of those deletions combine to eliminate DNA 25 times faster in 'dead-on-arrival' copies of *Helena* than in mammalian pseudogenes relative to the nucleotide substitution rate.

Such a high incidence of deletions could be a result of an increased rate of DNA deletion in *Drosophila* or, alternatively, of direct selection for a smaller genome. The latter model predicts a correlation between the total length of deleted DNA and the number of terminal branch substitutions, which is not supported by our data.

Is such high rate of DNA loss a general feature of *Drosophila* or merely a property of a particular stretch of 363 bp of DNA in a particular transposable element? Nothing about the position of deletions or their surrounding sequences suggests that the deletions were generated by a mechanism specific to the sequence of *Helena*. There are no hotspots of deletions: all deletions are unique and evenly distributed along the sequence. The breakpoints in about half (11 out of 23) of the deletions contain short direct repeats from 2–7 bp in length. In each case, the repeat is different, and there is no correlation between the presence of direct repeats and the length of the deletion. The presence of repeats suggests that at least some of the deletions were generated by a homology-dependent mechanism, such as recombination or DNA replication slippage[17,18], but, because an arbitrary sequence would contain a large number of random 2–7-bp repeats (for instance, a 1,000-bp stretch of DNA would be expected to have more than 2,000 2-bp direct repeats separated by 0–75 bp), any random stretch of DNA would be prone to multiple deletions by such a mechanism.

If such a high rate of large deletions is a general feature in *Drosophila*, this would have profound consequences for *Drosophila* genome evolution. We expect, based on the estimates of the neutral nucleotide substitution rates in *Drosophila* and mammals[19], that a pseudogene in *Drosophila* would lose its DNA much more quickly than a mammalian pseudogene (average time to loss of 50 per cent of the DNA is 11.8 million years in *Drosophila*, compared with over 800 million years in mammals) and will quickly become unrecognizable by both DNA hybridization and polymerase chain reaction. Quick elimination of pseudogenes would affect the steady-state number of pseudogenes in the genome of any *Drosophila* species and reduce the probability of detecting a homologous pseudogene in diverse species of *Drosophila*. It would also explain why all studied putative pseudogenes in *Drosophila* have been found not to evolve in a strictly neutral fashion[2–5]. Purifying selection, gene conversion or some other mechanism must have preserved their integrity, since true pseudogenes would be obliterated by multiple large deletions and could not be detected.

Differences in deletion rate may also contribute to the divergence in genome size among taxa, the so-called 'C-value paradox'[9]. Two reports[20,21] find a positive correlation between genome size and intron size in a variety of taxa. In addition, the reduction in the intron size in birds, whose genome size is smaller than that of other tetrapods, has been inferred to be due to multiple separate deletions scattered along the introns[21]. It is noteworthy that pseudogenes are much rarer in birds than in mammals[1]. These results argue that differences in genome size among related organisms may be determined primarily by the variation in the

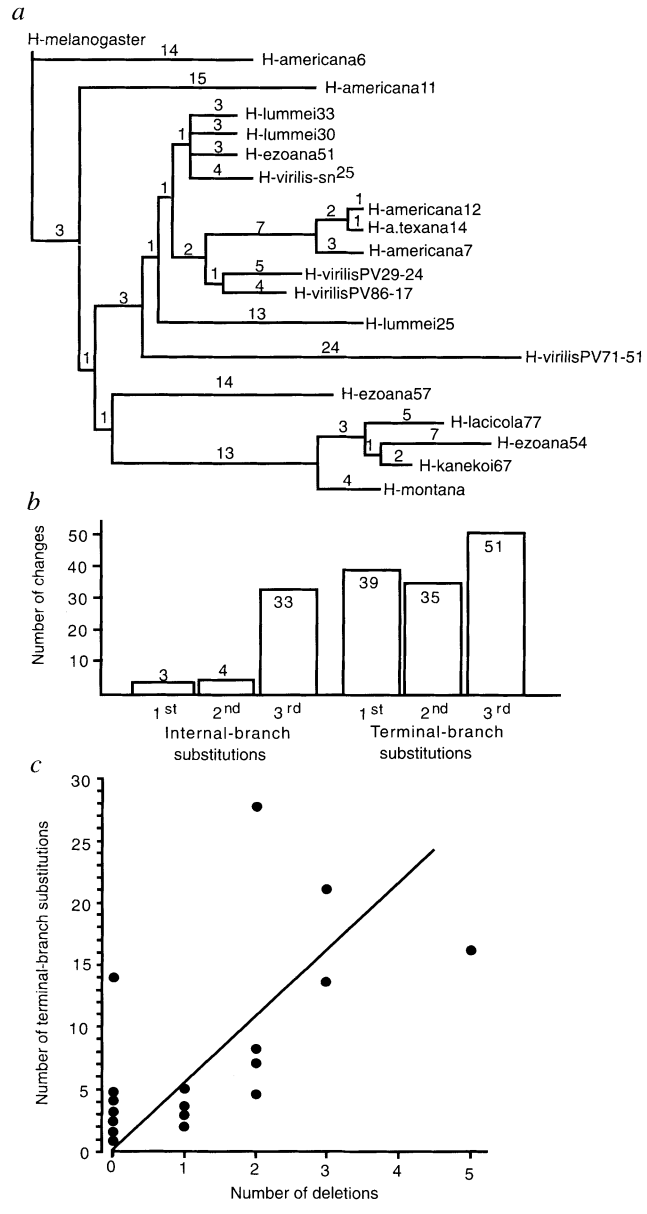

FIG. 3 Phylogenetic analysis. *a*, Maximum parsimony tree using all characters in the nucleotide alignment at equal weight. Each sequence is labelled with the species name and a unique number (see Methods). A *D. melanogaster Helena* sequence was used to root the tree. The number of unambiguous substitutions is shown above each branch. This tree is one of 75 equally parsimonious trees which differ in the placement of the branches H-*virilis* PV71-51, H-*americana* 6, H-*americana* 11 and H-*lummei* 25. Different placements of these branches introduce minor changes into the distribution of substitutions along the internal and the terminal branches. *b*, Distribution of substitutions along the internal and terminal branches. *c*, Relationship between the number of deletions and the number of terminal-branch substitutions (corrected for the length of each sequence). The solid line is the maximum-likelihood regression line.

genome-wide deletion rate, and not, for instance, by different rates of insertion of transposable elements. The wide phylogenetic distribution of non-LTR retrotransposable elements[10] may permit the application of the approach used here to other taxa, to determine whether or not there is a correlation between deletion rate and genome size. □

Methods

Cloning. A single strain was used to generate *Helena* sequences for each species. Each copy is designated with a unique number and the name of the species from which it was isolated. H-*virilis*-*sn*[25], H-*montana* and H-*italmelanogaster* sequences were cloned directly using λ libraries. The rest were obtained by TA cloning (Invitrogen) of the products of the PCR reaction using primers T^+ (5′-CAACAACTGCGGTGGCTCAAC-3′) and T^- (5′-GATTTAATGCGGGTGGTCTT-3′). These primers amplify a 363-bp stretch of DNA from the very 3′ end of the putative *Helena* reverse transcriptase. The sequences H-*virilis* PV71-51, H-*virilis* PV86-17 and H-*virilis* PV29-24 were obtained using the respective P1 clones as template (for instance, DNA of the P1 clone PV71-51 was used to amplify H-*virilis* PV71-51); the rest were obtained using genomic DNA as template. Cloning in λ libraries, DNA hybridization and other standard protocols were carried out as described[11].

Sequencing. Preparation of the sequencing templates used a transposon-facilitated protocol[22]. DNA sequencing was carried out on a ABI373A automated DNA sequencer (Perkin Elmer) with the *Taq* Cycle Sequencing Dye-Primer or DyeDeoxy Terminator kits. All sequences have been deposited in the GenBank data base under accession numbers U65653 and U62715–U62731.

Phylogenetic analysis. Sequence alignment was done with the aid of Sequencher 2.0 (GeneCodes). Maximum parsimony analysis was carried out using the PAUP[12] software package. It used all the characters in the nucleotide alignment at equal weight. Deletions were treated as missing data. We also used the MacClade[23] software package to aid with analysis and manipulation of the *Helena* gene trees.

Statistical methods. Maximum-likelihood estimate of relative rate of deletions versus nucleotide substitutions was found under the assumptions that each sequence, upon transposition, has no deletions or unique substitutions, that rates of deletions and substitutions are constant in time, and that, at any given time, the number of deletions and the number of substitutions follow a Poisson distribution. The confidence limits were found using the χ^2 approximation of the log-likelihood ratio. The positive correlation between the number of terminal branch substitutions and the number of deletions was ascertained using a sign test.

Received 25 July; accepted 12 September 1996.

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ACKNOWLEDGEMENTS. We thank J. Schutzman, M. Siegal, A. Berry, D. Weinrech, R. Lewontin, P. Capy and members of the laboratory for helpful discussions; D.A.P. particularly thanks A. Petrov for help with statistical analysis. This work was supported by grants from the NIH.

CORRESPONDENCE and requests for materials should be addressed to D.A.P. (e-mail: dpetrov@oeb.harvard.edu). Nucleotide alignments and information on statistical methods used in this study are available on the World-Wide Web at http://www.oeb.harvard.edu/hartl/lab/dmitri.html, or on request.

Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene

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DUCHENNE muscular dystrophy (DMD) is a severe, progressive muscle-wasting disease that causes cardiac or respiratory failure[1,2] and results in death at about 20 years of age. Replacement of the missing protein, dystrophin, using myoblast transfer in humans or viral/liposomal delivery in the mouse DMD model is inefficient and short-lived[3,4]. One alternative approach to treatment would be to upregulate the closely related protein, utrophin[5,6], which might be able to compensate for the dystrophin deficiency in all relevant muscles[7,8]. As a first step to this approach, we have expressed a utrophin transgene at high levels in the dystrophin-deficient *mdx* mouse. Our results indicate that high expression of the utrophin transgene in skeletal and diaphragm muscle can markedly reduce the dystrophic pathology. These data suggest that systemic upregulation of utrophin in DMD patients may lead to the development of an effective treatment for this devastating disorder.

A truncated utrophin transgene was modelled on the Becker dystrophin transgene, which has been shown to correct the dystrophic phenotype of *mdx* mice[9,10] (Fig. 1*A*). To generate high levels of muscle expression, the utrophin transgene was driven by the human skeletal α-actin (HSA) promoter (Fig. 1*B*). Several transgenic lines expressing the utrophin transgene were generated with differing levels of transgene expression. Immunoblot analysis of muscle samples from transgenic lines demonstrating high-level expression are shown in Fig. 1*C*. The multiple fainter bands probably result from the proteolytic breakdown of the highly expressed transgene product[11]. Line 347 also shows weak expression of the transgene in the heart. Line 261 contains, but does not express, the transgene. Further analysis of the F-3 line shows no evidence of transgene expression in heart, brain, kidney, lung, liver, intestine, skin or pancreas. To demonstrate that the utrophin transgene localized to the sarcolemma, immunofluorescence of skeletal-muscle sections from the F-3 line was performed using antibodies specific to utrophin and dystrophin. The sarcolemmal localization pattern of dystrophin (Fig. 1*D*, *a*) and the utrophin transgene (Fig. 1*D*, *b*) in tibialis anterior (TA) muscle sections demonstrate that they can colocalize to the sarcolemma *in vivo*. In contrast with the transgenics, the normal localization of utrophin in adult skeletal muscle is exclusively at the neuromuscular and myotendenous junctions and in the capillaries and nerves[12,13].

The limb muscles of the dystrophin-deficient *mdx* mouse only develop atrophy and weakness late in life. But histological and physiological analysis reveals several muscle defects in common with DMD patients, including muscle-fibre degeneration, giving rise to a dramatic elevation of serum creatine kinase (CK) level and evidence of massive myofibre regeneration, with most fibres having centrally located nuclei[14]. Thus changes in the levels of serum CK and numbers of centralized nuclei have been used to monitor the pathology of the muscle in several transgenic lines expressing dystrophin transgenes in *mdx* mice[9,10,11,15]. Male transgenic F-3 mice carrying the utrophin transgene were crossed with dystrophin-deficient female *mdx* mice. The resultant offspring were analysed (Fig. 2*a*) at approximately 5 weeks of age when the skeletal muscle is still undergoing rounds of degeneration and regeneration. The CK levels of male transgenic *mdx* mice (utro-tg