ELECTRONIC LETTER

Induction of instability of normal length trinucleotide repeats within human disease genes

L Fernàndez-López, E Piñeiro, R Marcos, A Velázquez, J Surrallés

J Med Genet 2004;41:e3 (<http://www.jmedgenet.com/cgi/content/full/41/1/e3>)

Myotonic dystrophy type 1 (DM1) is the most frequent cause of adult muscular dystrophy. It is a progressive multisystem disorder with an incidence of 1 in 8000 newborns. Its molecular basis is an expansion of a CTG repeat in the 3' untranslated region (UTR) of the *DMPK* gene on chromosome 19.¹ Fragile X syndrome is the most common inherited cause of mental retardation. It is caused by an expansion of a CGG repeat in the 5' UTR of the *FMR1* gene.^{2,3} The full mutation appears in approximately 1 in 4000 males and 1 in 9000 females. The number of trinucleotide repeats (TNRs) in most unaffected individuals is in the range of 5–35 repeats in DM1 and around 14–35 repeats in fragile X syndrome. However, pathogenic loci bear expanded alleles of up to several hundred or even thousand repeats in severe cases.^{1,2,4,5}

As with many other tandemly repeated microsatellite sequences, pathogenic TNRs are highly unstable in both the somatic and germ cell lineage, leading to expansions and contractions in the length of the repeats in successive cell or individual generations.^{6–8} Expansions in the number of TNRs are known to be a molecular basis of genetic anticipation,⁹ the progressively earlier age of onset of a disease with increasing severity in successive generations. An important consequence of genetic anticipation is that the genetic transmission is interrupted and, therefore, new pathogenic alleles have to be generated from unexpanded alleles. Little is known, however, about the mechanism(s) that cause a stable, non-expanded allele to become unstable and pathogenic, probably because normal length alleles are thought to be stable compared with expanded pathogenic alleles. In addition, much of the evidence for the mechanisms that are thought to produce repeat expansion in pathological TNRs are dismissed as irrelevant for normal alleles. Therefore, understanding the factors influencing the instability of normal unexpanded alleles is of critical importance.

A number of *trans* and *cis* acting factors have been proposed to play a modulating role in the instability of expanded, disease related TNRs.¹⁰ These factors are not mutually exclusive and include the purity and length of the repeated sequence,^{7,11} age of individuals,^{6,7} mitotic drive,¹² the relative position of the TNR with respect to adjacent origins of DNA replication,¹³ and mutations in genes involved in DNA repair, replication, and recombination.^{14–30} Mutagenic agents are known to interfere with DNA metabolism by inducing DNA lesions and repair, blocking transcription and replication, and/or generating recombinational events to bypass or repair the induced DNA lesions.³¹ Therefore, we have hypothesised that mutagenic stress could result in an induction of instability of unexpanded TNR sequences, leading to an increase in the frequency of spontaneously occurring contractions/expansions in repeat number. To test this hypothesis, we analysed normal length TNR sequences at a single repeat resolution by radioactive PCR and denaturing polyacrylamide gels, using DNA from multiple single cell clones expanded in the presence or absence of mitomycin-C

Key points

- The objective of this study was to explore the effect of the mismatch repair genes *MLH1* and *MSH2/MSH6*, and mutagenic stress in the instability of normal length (CTG)_n and (CGG)_n repeats within the myotonic dystrophy and fragile X syndrome genes, respectively.
- Multiple single cell clones expanded in the presence or absence of mutagenic stress (mitomycin-C) show that normal length alleles can be destabilised with a clear bias towards repeat contractions. The data also suggest two mechanisms of mitomycin-C induced repeat instability: a major mechanism independent of mismatch repair and a minor mechanism dependent on *MLH1* deficiency.
- The major mechanism of mitomycin-C induced trinucleotide repeat instability acts in *trans*, as the clones presenting instability induced by mitomycin-C at the CTG locus also presented instability at the CGG locus, but not in a concurrently analysed mononucleotide repeat. Our data therefore indicate that (a) normal length trinucleotide repeats can be destabilised in their natural genomic context and (b) that mitomycin-C induces trinucleotide repeat contractions at normal length alleles, through a major mismatch repair independent *trans* acting mechanism.

(MMC), a mutagenic agent able to induce interstrand DNA cross links, strand breaks, and replication blockage. In addition, and in order to uncover a potential role of a functional mismatch repair (MMR) system in mutagen-induced TNR instability, all experiments were done in MMR proficient (SW480) or deficient cell lines (HCT116 and LoVo), as well as in the HCT116 cell line corrected for MMR deficiency by microcell mediated chromosome 3 transfer. Here we show that normal length alleles are not as stable as previously believed and that mutagenic stress induces TNR instability through an MMR independent *trans* acting mechanism.

MATERIALS AND METHODS

Cell lines

The human (male) colon cancer fibroblast cell lines SW480 (MMR proficient), HCT116 (hMLH1 deficient), and LoVo (hMSH2 and hMSH6 deficient) (kindly provided by Dr M A Peinado, Institut de Recerca Oncològica, Hospital Duran

Abbreviations: DM1, myotonic dystrophy type 1; MMC, mitomycin-C; MMR, mismatch repair; TNR, trinucleotide repeat; UTR, untranslated region

Table 1 Sequence and size of the trinucleotide repeats in the cell lines studied

Cell line	Myotonic dystrophy		Fragile X*	
	Sequence (CTG)	Size (no. of repeats)	Sequence (CGG)	Size (no. of repeats)
SW480	Pure	12/13	10+AGG+8+AGG+9	29
HCT116	Pure	11/12	9+AGG+1+AGG+19	31
LoVo	Pure	10/12	19+AGGAGG+8	29

HCT116+chr.3 was identical to HCT116 with the exception that the 11 CTG repeat allele was absent.

*All cells are male and therefore contain a single fragile X syndrome allele.

i Reynals, L'Hospitalet, Barcelona, Spain) were maintained in RPMI medium supplemented with 15% fetal bovine serum, 1% pyruvate, 1% L-glutamine, and (1%) penicillin-streptomycin as described previously.³² The HCT116 cell line corrected for MMR deficiency by microcell mediated chromosome 3 transfer (HCT116+chr.3 cell line; generously provided by Dr T Kunkel, National Institute on Environmental Health Sciences, Research Park, NC, USA) was maintained under G418 (400 µg/ml) selection in the same culture medium. The length and sequence of the repeats in the different cell lines are summarised in table 1.

Single cell cloning and culturing

Single cell clones were isolated from confluent cultures by limiting dilution into 96 well plates. The cells were diluted in culture medium at a concentration of 1 cell per 5 µl. Multiple 5 µl drops were deposited in Petri dishes and visualised using an inverted microscope. Only drops with one cell were individually plated into 96 well plates, in 100 µl of culture medium (with or without mutagen, see below). The clones were allowed to grow in the well and the medium was changed once a week. The expanded clones were transferred to 25 cm² flasks and maintained until confluence, when DNA was extracted by standard phenol-chloroform precipitation. Because of the limitations of the PCR to amplify rare alleles, only those contractions or expansions generated during the first population doublings can be detected; however, the clones were expanded until confluence to increase the yield of DNA.

Treatment of single cell clones

MMC was diluted to a concentration of 1 mg/ml and stored at 4°C. The concentration used in the microsatellite instability assays (0.005 µg/µl) induced a similar reduction of 30–50% of cell viability in all the cell lines analysed (data not shown). Mutagenic treatment was continuous in all cases. The medium with mutagen, at the indicated concentration, was replaced once a week. Parallel single cell cultures were left untreated to control for spontaneous instability.

Molecular analysis of microsatellite instability

A total of 32–57 (40 in most cases) single cell clones per treatment point and cell line were analysed for instability of the CTG repeats at the *DMPK* locus (chromosome 19), CGG repeats at the *FMR1* locus (X chromosome), and the polyA mononucleotide repeat BAT-25 (chromosome 1), which is known to be unstable in MMR deficient backgrounds and was included as an internal control of microsatellite instability. Microsatellite instability was analysed by radioactive PCR amplification and subsequent sequencing gel electrophoresis to detect changes in the length of the alleles at a single repeat resolution. The number of repeats in the amplified sequences was checked using a 30–330 bp ladder (GibcoBRL®) labelled with [γ-³³P]dATP. All PCR reactions

were performed in duplicate and run in parallel lanes of the gels to replicate the results. The reactions were carried out in a total volume of 25 µl with 1 µg of DNA using 3 µCi [α-³³P]dCTP or dATP. Amplification of the DM1-CTG repeats was performed as described by Cobo and co-workers³³ but using the primers 101 and 102 described previously by Brook *et al.*¹ The amplification of the FMR1-CGG repeat was performed using the PCR conditions and primers (C and F) described by Fu *et al.*² The BAT-25 mononucleotide microsatellite was analysed basically as previously reported,³⁴ but using the primers described by Parsons and co-workers.³⁵ The reaction products were separated by denaturing electrophoresis in 6% polyacrylamide gels containing 8 mol/l urea and visualised by autoradiography, essentially as described previously.^{36–38}

Criteria for microsatellite instability quantification

Under the experimental design and conditions used, only those mutant alleles generated during the first divisions of the single cell clonal culture could be detected. Those mutations that occur in later divisions remained non-amplified because the normal pre-existing alleles were preferentially amplified in the competitive PCR reaction. However, those mutant alleles generated in the first divisions were predominant enough to be detected because they were clonally expanded. Each clone was derived from a single cell and, therefore, any clone presenting alleles with repeat sizes shorter (contracted) or longer (expanded) than the progenitor allele was counted as an unstable clone bearing mutant alleles. Thus, the mutation frequency can only be measured at the single clone level. All mutant clones presented new or additional alleles with a gain or loss of only one or two repeat units.

Statistics

Statistical analysis of mutagen induced instability of microsatellite sequences was performed using Fisher's exact test comparing treated and untreated cultures. The degree of coincidence in the profile of genomic instability of the three markers, in multiple single cell clones of each cell line, was performed either by analysing contingency tables and performing the χ² test, or by the p value of Fisher's exact test, depending on the number of unstable clones.

RESULTS

The instability of three microsatellites (BAT-25, DM1-CTGn, and FMR1-CGGn) has been studied in multiple single cell clones expanded under the effect of a biologically effective subtoxic concentration of MMC. A total of 32–57 (40 in most cases) single cells derived from MMR proficient (SW480 and HCT116+chr.3) and deficient (HCT116 and LoVo) cell lines were clonally expanded in the presence or absence of MMC. All single cell clones were analysed for instability of each of the three microsatellite loci by radioactive PCR, sequencing gels, and autoradiography. All single cell clones were PCR amplified twice and electrophoresed in consecutive lanes of the gels to confirm the results. PCR reactions and analyses were repeated when PCR products of replicated reactions gave non-coincident band patterns in consecutive lanes of the autoradiographs. Some examples of the banding patterns obtained in the present investigation are shown in fig 1.

Table 2 and fig 2 summarise the results on the spontaneous and MMC induced instability of the mononucleotide repeat BAT-25 and the DM1 and FMR1 TNRs in multiple single cell clones of MMR proficient (SW480 and HCT116+chr.3) and deficient (HCT116 and LoVo) cell lines. We found elevated levels of spontaneous instability of the microsatellite BAT-25 in HCT116 (11 clones with expansions or contractions in the repeat number out of 33 clones analysed, or 11/33 clones) and

Figure 1 Representative images of the analysis of microsatellite instability by radioactive PCR and sequencing gels. Examples are shown on the microsatellite BAT-25 in untreated SW480 (A) and LoVo (B) cells, DM1 CTG repeat in untreated HCT116 cells (C) and MMC treated SW480 cells (D), and the fragile X syndrome CGG repeat in untreated (E) and MMC treated (F) SW480 cells. Eight (A,B) or seven (C–F) single cell clones are shown in each case, with two repeated PCR amplicons in each consecutive lane. The length of repeated sequences and the number of alleles in each cell line are shown in table 1.

especially in the LoVo cell line (25/35) compared with the MMR proficient cell lines SW480 (6/39) or HCT116+Chr.3 (0/40). This is consistent with the mutator phenotype

Figure 2 Spontaneous instability of the mononucleotide repeat BAT-25 and the trinucleotide repeats (CTG)_n and (CGG)_n in MMR proficient (SW480 and HCT116+chr.3) and deficient (LoVo and HCT116) cell lines.

characteristic of MMR deficiency and confirmed the suitability of our experimental design to detect microsatellite instability.

Although a statistically non-significant tendency towards higher instability of TNRs in MMR deficient cells (especially the CGG repeat in the LoVo cell line and the CTG repeat in the HCT116 cell line) was observed, both trinucleotides presented a low spontaneous instability compared with the mononucleotide repeat, irrespective of the MMR genetic background (table 2, fig 2). This result indicates that, in the absence of mutagenic stress, a functional MMR plays a secondary role in human cells to maintain the stability of unexpanded TNRs associated with human diseases in their natural genomic context.

MMC dramatically increased the frequency of unstable clones in the SW480 cell line and, to a lesser extent, in the HCT116 cell line (table 2, fig 3). DNA sequencing analysis of the mutants confirmed that changes in the size of the PCR products were to changes in the repeat number, rather than to deletions elsewhere in the flanking regions (data not shown). MMC induced an 18-fold increase in the frequency of CTG repeat mutations (from 2.6 to 45% in untreated and MMC treated cells, respectively; $p<0.001$) and a >20-fold increase in the frequency of CGG repeat mutations (from 0.0 to 53.8%; $p<0.001$) in the SW480 cell line. In addition, all MMC induced mutations had a contraction of the repeat sequence (fig 3). This increase was not observed in the MMR proficient HCT116+chr.3 cell line, indicating that the

Table 2 Spontaneous and mutagen induced instability of the mononucleotide repeat BAT-25 and the myotonic dystrophy type 1 (CTG) and fragile X syndrome (CGG) trinucleotide repeats in multiple single cell clones of MMR proficient (SW480 and HCT116+chr.3) and deficient (HCT116 and LoVo) cell lines

		Cell line			
		SW480	LoVo	HCT116	HCT116+ch3
Treatment	Marker	MC/TC (%)	MC/TC (%)	MC/TC (%)	MC/TC (%)
Control	BAT-25	6/39 (15.4)	25/35 (71.4)	11/33 (33.3)	0/40 (0.0)
	CTG	1/39 (2.6)	0/36 (0.0)	3/32 (9.4)	1/40 (2.5)
	CGG	0/41 (0.0)	6/57 (10.5)	1/44 (2.3)	0/40 (0.0)
	BAT-25	15/40 (37.5)	27/38 (71.0)	23/40 (57.5)	0/40 (0.0)
	CTG	18/40 (45.0)***	1/40 (2.5)	8/40 (20.0)	3/40 (7.5)
	CGG	21/39 (53.8)***	2/40 (5.0)	10/40 (25.0)**	0/40 (0.0)

MC/TC (%), mutant clones/total clones (percentage of mutant clones).
The difference between control and MMC treated cells is statistically significant with ** $p<0.01$ or *** $p<0.001$.

Figure 3 MMC induced expansions and contractions of the trinucleotide repeats (CTG)_n (DM1 locus) and (CGG)_n (fragile X locus) in MMR proficient (SW480 and HCT116+chr.3) and deficient (LoVo and HCT116) cell lines.

observed TNR instability induced by MMC in the SW480 cell line is not dependent on a functional MMR. However, the level of MMC induced TNR contractions is higher ($p<0.001$) in the MLH1 deficient HCT116 cell line than in its genetically matched MLH1 proficient HCT116+chr.3 counterpart (fig 3), suggesting two mechanisms of MMC induced TNR instability: a major mechanism independent of MMR and a minor mechanism dependent on MLH1 deficiency. The negative results obtained in the LoVo cell lines indicate that MSH2/MSH6 deficiency plays no role in MMC induced instability.

To obtain further insights into the mechanisms of MMC induced TNR instability in the SW480 and the HCT116 cell lines, we next analysed the profile of MMC induced microsatellite instability in multiple single cell clones (fig 4). Surprisingly, we observed that most SW480 clones presenting instability in the CTG marker (black squares) also presented instability in the CGG marker but not necessarily in the mononucleotide marker which profile of instability is not coincidental. That was not the case in the HCT116 cell line after MMC treatment. The coincidence in the profile of MMC induced CTG and CGG contractions was highly significant ($p<0.001$) and cell type (SW480) specific, suggesting a *trans* acting mechanism of MMC induced TNR instability in this cell line.

DISCUSSION

Expansions of TNRs are causally related to an increasing number of human genetic diseases. Most studies have been focused on expanded (pre-) pathogenic alleles that, unlike normal length alleles, are known to be highly unstable at both the somatic and germinal levels. Mouse models and

Figure 4 Profile of MMC induced BAT-25, (CTG)_n, and (CGG)_n microsatellite instability in 40 independent single cell clones derived from the cell lines SW480 and HCT116. Grey and black boxes represent stable and unstable (with contracted or expanded alleles) clones, respectively, for each microsatellite analysed.

micro-organisms have been used to study such instability and the role of MMR deficiency, although in these genetic models the expanded pathogenic allele is out of its human natural genomic context. In addition, the results are conflicting, as MMR deficiency leads to TNR instability in

Escherichia coli and yeast,^{15–21} whereas MSH2¹⁴ and MSH3²² are required for TNR instability in mice. Other studies have predicted no role of MMR in TNR instability in yeast.²³ All these studies were performed with expanded disease causing alleles. Studies on the instability of non-expanded alleles are very scarce. Here we show that non-expanded alleles are not as stable as previously believed and can be dramatically destabilised in their natural genomic context after mutagenic stress by a *trans* acting mechanism independent of MMR. A minor role for MLH1 was also observed in our study and, therefore we concluded that there are two mechanisms of MMC induced TNR instability: a major mechanism independent of MMR and a minor mechanism dependent on MLH1 deficiency. To reach this conclusion, we have used a novel approach based on the detection of instability of normal length alleles at single repeat resolution in cell clones expanded in the presence or absence of mutagenic MMC treatment. This approach led us to conclude that MMC was able to induce a >18-fold increase in the frequency of mutant clones bearing contractions in both TNRs in the SW480 cell line only. A minor increase in MMC induced TNR contractions was also observed in the MLH1 deficient cell line (HCT116); however, such inductions were not observed in the MSH2/MSH6 deficient cell line (LoVo) or in the HCT116 cell line corrected for MLH1 deficiency by microcell mediated chromosome 3 transfer. This is in agreement with previous data suggesting that MSH2 deficiency plays a minor role in disease associated TNR instability in human cells²⁴ or yeast.²³ A previous study in cell lines also indicated that mutations in *hMLH1* or *hMSH2/6* does not result in the instability of CTG or CGG tracts to the levels observed in individuals with DM1 or fragile X syndrome.²⁵ However, the method used in that study (Southern blot) did not allowed the detection of mutant alleles resulting from expansions or deletions of single repeats.

Interestingly, those single cell SW480 clones showing MMC induced contractions at the DM1 repeat also presented contractions at the FMR1 repeat. This finding is probably unrelated to the number of repeats and their sequence or purity in the different cell lines, as they all have a pure 10–12 CTG repeat sequence in the DM1 locus and 29–31 CGG repeats with two interruptions in the fragile X locus. The coincidence in the profile of TNR instability in the SW480 cells is highly indicative of a *trans* acting mechanism of TNR instability in this cell line instead of a direct *cis* action of MMC on the repeated sequence at both loci in each unstable clone.

The exact mechanism(s) explaining our observation is currently unknown. MMC is an S phase dependent mutagen that induces DNA cross links that block replication. The relationship between replication and TNR expansion is well documented, especially in yeast, where mutations in genes involved in the replication apparatus have been shown to increase TNR instability.^{26–27} According to the literature, a possible candidate is FEN1, a protein involved in base excision repair and in the processing of the Okazaki fragments during replication of the lagging strand,²⁸ with an important role in TNR stability, at least in yeast.^{29–30} However, human FEN1 immunoblotting experiments indicate that FEN1 is present at normal expression levels and molecular size in SW480 cell extracts (data not shown). Another candidate gene we are now extensively investigating is *p53*, as it is known that the SW480 cell line expresses a non-functional *p53* protein.

The fact that the non-coincidental increase of CTG and CGG instability after MMC treatment is observed in the HCT116 cell line but not in the corrected HCT116+chr.3 cell line suggests a *cis* effect of MMC in TNR instability in an hMLH1 deficient background. Our results also

indicate that hMLH1 plays a role in the cellular response to MMC.

In the present study we have identified mutagenic stress as a new factor in disease associated normal length TNR instability, suggesting that normal length alleles are not as stable as previously believed. As all MMC induced altered clones had contractions, this effect is probably unrelated to the generation of (pre)pathogenic alleles from normal length alleles. Moreover, the process regulating repeat instability in somatic mitotic cells may be different to the situation in the germline in vivo. To clarify a potential role of MMC in the formation of pathogenic alleles, we are currently investigating by small pool PCR⁶ whether MMC modulates the repeat dynamics of DM1 alleles in successive cell generations.

ACKNOWLEDGEMENTS

We are greatly indebted to Professor Dr G Sutherland (Women's and Children's Hospital, Adelaide, Australia) for helpful comments and for reading the manuscript. We would also like to thank Dr L Martorell (Sant Pau Hospital, Barcelona, Spain) for helpful comments and technical advice, and Drs M A Peinado and T Kunkel for sharing the cell lines. This work was partially funded by La Fundació La Marató de TV3 (project no. 1999-85), the Generalitat de Catalunya (SGR-00197-2002) and the Spanish Ministry of Science and Technology (MCYT) (project no. CICYT, PM98-0179). L Fernández-López and E Piñeiro are supported by predoctoral fellowships awarded by the MCYT. Dr J Surrallés is supported by a "Ramón y Cajal" project entitled "Genome stability and DNA repair", awarded by the MCYT and co-financed by the UAB.

Authors' affiliations

L Fernández-López, E Piñeiro, R Marcos, A Velázquez, J Surrallés, Group of Mutagenesis, Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

The first two authors contributed equally to this work.

Correspondence to: Dr J Surrallés or Dr A Velázquez, Group of Mutagenesis, Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain; jordi.surralles@uab.es or antonia.velazquez@uab.es

Received 6 May 2003

Accepted 2 July 2003

REFERENCES

- 1 Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion JP, Hudson T, Sohn R, Zemelmann B, Snell RG, Rundle SA, Crow S, Davies J, Shelbourne P, Buxton J, Jones C, Juvonen V, Johnson K, Harper PS, Shaw DJ, Housman DE. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 1992;68:799–808.
- 2 Fu Y-F, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH, Holden JJA, Fenwick RG Jr, Warren ST, Oostra BA, Nelson DL, Caskey CT. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 1991;67:1047–58.
- 3 Kremer EJ, Pritchard M, Lynch M, Yu S, Holman K, Baker E, Warren ST, Schlessinger D, Sutherland GR, Richards RI. Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)_n. *Science* 1991;252:1711–14.
- 4 Harley HG, Brook JD, Floyd J, Rundle SA, Crow S, Walsh KV, Thibault M-C, Harper PS, Shaw DJ. Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature* 1992;335:545–6.
- 5 Longshore JM, Tarleton J. Dynamic mutations in human genes: a review of trinucleotide repeat diseases. *J Genet* 1996;75:193–217.
- 6 Monckton DG, Wong LC, Ashizawa T, Caskey CT. Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. *Hum Mol Genet* 1995;4:1–8.
- 7 Martorell L, Monckton DG, Gamez J, Johnson KJ, Gich I, López de Munain A, Baiget M. Progression of somatic CTG repeats length heterogeneity in the blood cells of myotonic dystrophy patients. *Hum Mol Genet* 1998;7:307–12.
- 8 White PJ, Borts RH, Hirst MC. Stability of the human fragile X (CGG)_n triplet repeat array in *Saccharomyces cerevisiae* deficient in aspects of DNA metabolism. *Mol Cell Biol* 1999;19:5675–84.
- 9 Richards RI, Sutherland GR. Dynamic mutation: A new class of mutations causing human disease. *Cell* 1992;70:709–12.
- 10 Richards RI. Dynamic mutations: a decade of unstable expanded repeats in human genetic disease. *Hum Mol Genet* 2001;10:2187–94.

- 11 **Eichler EE**, Holden JJA, Popovich BW, Reiss AL, Snow K, Thibodeau SN, Richards CS, Ward PA, Nelson DL. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. *Nature Genet* 1994;**8**:88–94.
- 12 **Khajavi M**, Tari AM, Patel NB, Tsuji K, Siwak DR, Meistrich ML, Terry NHA, Ashizawa T. 'Mitotic drive' of expanded CTG repeats in myotonic dystrophy type 1 (DM1). *Hum Mol Genet* 2001;**10**:855–63.
- 13 **Cleary JD**, Nichol K, Wang YH, Pearson CE. Evidence of cis-acting factors in replication-mediated trinucleotide repeat instability in primate cells. *Nature Genet* 2002;**31**:37–46.
- 14 **Manley K**, Shirley TL, Flaherty L, Messer A. Msh2 deficiency prevents in vivo somatic instability of the CAG repeat in Huntington disease transgenic mice. *Nature Genet* 1999;**23**:471–3.
- 15 **Jaworski A**, Rosche WA, Gellibolian R, Kang S, Shimizu M, Bowater RP, Sinden RR, Wells RD. Mismatch repair in *Escherichia coli* enhances instability of (CTG)_n triplet repeats from human hereditary diseases. *Proc Natl Acad Sci USA* 1995;**92**:11019–23.
- 16 **Kang S**, Jaworski A, Oshima K, Wells RD. Expansion and deletion of CTG repeats from human disease genes are determined by the direction of replication in *E. coli*. *Nature Genet* 1995;**10**:213–18.
- 17 **Kolodner RD**, Tishkoff DX, Filosi N, Gaida GM. A novel mutation avoidance mechanism dependent on *S. cerevisiae* RAD27 is distinct from DNA mismatch repair. *Cell* 1997;**88**:253–63.
- 18 **Jakupciak JP**, Wells RD. Genetic instabilities in (CTG.CAG) repeats occur by recombination. *J Biol Chem* 1999;**274**:23468–79.
- 19 **Moore H**, Greenwell PW, Liu CP, Arnheim N, Petes TD. Triplet repeats form secondary structures that escape DNA repair in yeast. *Proc Natl Acad Sci USA* 1999;**96**:1504–9.
- 20 **Jankowski C**, Nasr F, Nag DK. Meiotic instability of CAG repeat tracts occurs by double-strand break repair in yeast. *Proc Natl Acad Sci USA* 2000;**97**:2134–9.
- 21 **Schweitzer JK**, Livingston DM. Destabilization of CAG trinucleotide repeat tracts by mismatch repair mutations in yeast. *Hum Mol Genet* 1997;**6**:349–55.
- 22 **van den Broek WJAA**, Nelen MR, Wansink DG, Coerwinkel MM, te Riele H, Groenen PJ, Wieringa B. Somatic expansion behaviour of the (CTG)_n repeat in myotonic dystrophy knock-in mice is differentially affected by Msh3 and Msh6 mismatch-repair proteins. *Hum Mol Genet* 2002;**11**:191–8.
- 23 **Miret JJ**, Pessoa-Brandao L, Lahue RS. Instability of CAG and CTG trinucleotide repeats in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1997;**17**:3382–7.
- 24 **Goellner GM**, Tester D, Thibodeau S, Almqvist E, Goldberg YP, Hayden MR, McMurray CT. Different mechanisms underlie DNA instability in Huntington disease and colorectal cancer. *Am J Hum Genet* 1997;**60**:879–90.
- 25 **Kramer PR**, Pearson CE, Sinden RR. Stability of triplet repeats of myotonic dystrophy and fragile X loci in human mutator mismatch repair cell lines. *Hum Genet* 1996;**98**:151–7.
- 26 **Ireland MJ**, Reinke SS, Livingston DM. The impact of lagging strand replication mutations on the stability of CAG repeat tracts in yeast. *Genetics* 2000;**155**:1657–65.
- 27 **Mirkin SM**, Smirnova EV. Positioned to expand. *Nature Genet* 2002;**31**:5–6.
- 28 **Gordenin DA**, Kunkel TA, Resnick MA. Repeat expansion—all in a flap? *Nature Genet* 1997;**16**:116–18.
- 29 **Freudenreich CH**, Kantraw SM, Zakian VA. Expansion and length-dependent fragility of CTG repeats in yeast. *Science* 1998;**279**:853–6.
- 30 **Schweitzer JK**, Livingston DM. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. *Hum Mol Genet* 1999;**7**:69–74.
- 31 **Surrallés J**, Ramírez MJ, Marcos R, Natarajan AT, Mullenders LHF. Clusters of transcription coupled repair in the human genome. *Proc Natl Acad Sci USA* 2002;**99**:10571–4.
- 32 **Surrallés J**, Hande PH, Marcos R, Lansdorp P. Accelerated telomere shortening in the human inactive X chromosome. *Am J Hum Genet* 1999;**65**:1616–22.
- 33 **Cobo A**, Martínez JM, Martorell L, Baiget M, Johnson K. Molecular diagnosis of homozygous myotonic dystrophy in two asymptomatic sisters. *Hum Mol Genet* 1993;**2**:711–15.
- 34 **Hoang J-M**, Cottu PH, Thuille B, Salmon RJ, Thomas G, Hamelin R. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Res* 1997;**57**:300–3.
- 35 **Parsons R**, Guo-Min L, Longley M, Modrich P, Liu B, Berk T, Hamilton SR, Kinzler KW, Vogelstein B. Mismatch repair deficiency in phenotypically normal human cells. *Science* 1995;**268**:738–40.
- 36 **Peinado MA**, Malkhosyan S, Velázquez A, Perucho M. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc Natl Acad Sci USA* 1992;**89**:10065–9.
- 37 **Yasuda J**, Navarro JM, Malkhosyan S, Velázquez A, Arribas R, Sekiya T, Perucho M. Chromosomal assignment of human DNA fingerprint sequences by simultaneous hybridization to arbitrarily primed PCR products from human/rodent monochromosome cell hybrids. *Genomics* 1996;**34**:1–8.
- 38 **López A**, Xamena N, Marcos R, Velázquez A. Germ cells microsatellite instability. The effect of different mutagens in a mismatch repair mutant of *Drosophila* (spell) *Mutat Res* 2002;**514**:87–94.