**INTRODUCTION**

Ataxia telangiectasia-like disorder (ATLD) was identified recently in four patients who presented with many of the clinical features of ataxia telangiectasia (A-T), including progressive cerebellar degeneration, increased levels of spontaneously occurring chromosome aberrations in peripheral lymphocytes, and increased sensitivity to ionizing radiation at both the cellular and chromosomal level (1). Although the clinical presentations of the four ATLD patients (in two families) were consistent with A-T, one notable difference was that none of the patients exhibited ocular telangiectasia (2,3). At the cellular level ATLD cells were found to be similar to A-T cells in that, in addition to hypersensitivity to ionizing radiation, they also exhibited radio-resistant DNA synthesis and failed to induce stress-activated protein kinases following exposure to ionizing radiation. However, ATLD cells were found not to exhibit the deficiency in p53 accumulation following irradiation which is a hallmark of A-T cells. At the genetic level no evidence could be found of mutations affecting the A-T gene region (2). The genetic basis of the A-T phenotype in one of the two ATLD families did not map to the ATM gene region (2). The genetic basis of the A-T phenotype in the ATLD patients was found to be mutation of the *hMRE11* gene (1).

The hMre11 protein is involved in the repair of DNA double strand breaks, as part of a complex of proteins which includes the hRad50 and Nbs1 proteins. There is growing evidence that this complex acts in the same pathway as the ATM protein (1). The ATM protein has been shown to phosphorylate the Nbs1 protein following γ-irradiation (4–7), and hyperphosphorylation of hMre11 in response to ionizing radiation is Nbs1-dependent (8). The hMre11/hRad50/Nbs1 complex rapidly associates in foci at the site of DNA double strand breaks, remaining at the sites until the damage has been repaired (9,10). It is also suggested that a significant component of S phase DNA damage recognition and checkpoint activation is dependent upon the DNA damage response pathway defined by hMre11/hRad50/Nbs1 and ATM (11). The complex plays an important role in many cellular functions including non-homologous end joining, homologous recombination, meiotic recombination, DNA damage response and telomere maintenance (12).

In this report we describe the genomic structure of the *hMRE11* gene and identify a promoter region that appears to be responsible for the divergent transcription of *hMRE11* and the adjacent gene. Knowledge of the exon structure of the *hMRE11* gene allowed us to investigate the basis of an apparent null allele in two ATLD patients in the same family.
The amplified fragments were aligned with the hMRE11 coding sequence-specific primers. The sequences of PCR using combinations of vectorette-specific primers and fragments spanning exon/intron boundaries were amplified by (PAC) DNA was ligated to vectorettes, and genomic DNA restriction enzyme-digested P1-derived artificial chromosome determined from genomic DNA using a vectorette strategy (13).

The exon/intron structure of the hMRE11 exon structure

The exon/intron structure of the hMRE11 gene (Table 1) was determined from genomic DNA using a vectorette strategy (13). Restriction enzyme-digested P1-derived artificial chromosome (PAC) DNA was ligated to vectorettes, and genomic DNA fragments spanning exon/intron boundaries were amplified by PCR using combinations of vectorette-specific primers and hMRE11 coding sequence-specific primers. The sequences of the amplified fragments were aligned with the hMRE11 cDNA sequence to determine the exon/intron boundaries. Primers were subsequently placed in the introns to facilitate the amplification of the exons. Each putative exon was sequenced to determine whether it was a single exon or two exons separated by a small intron. Approximate intron sizes were determined by PCR amplification between adjacent exons. The total size of the gene, including all exons and introns, was determined to be ~75 kb. The second exon was found to contain the start codon for mRNA transcription. The highly diverse 5' sequences identified earlier (14,15) were found to be incomplete representations of the first exon, which has two alternative donor splice sites, giving rise to two alternative 5' non-coding exons 1a and 1b. Nineteen coding exons were identified in the hMRE11 gene, the last exon containing both coding sequence and 3'-untranslated region (3'-UTR). The boundaries of all introns were found to have the consensus dinucleotides GT at their donor splice sites and AG at their acceptor splices, with the exception of the donor splice site for exon 1a, which had the variant dinucleotide GC.

### RESULTS

**hMRE11 exon structure**

The exon/intron structure of the hMRE11 gene (Table 1) was determined from genomic DNA using a vectorette strategy (13). Restriction enzyme-digested P1-derived artificial chromosome (PAC) DNA was ligated to vectorettes, and genomic DNA fragments spanning exon/intron boundaries were amplified by PCR using combinations of vectorette-specific primers and hMRE11 coding sequence-specific primers. The sequences of the amplified fragments were aligned with the hMRE11 cDNA sequence to determine the exon/intron boundaries. Primers were subsequently placed in the introns to facilitate the amplification of the exons. Each putative exon was sequenced to determine whether it was a single exon or two exons separated by a small intron. Approximate intron sizes were determined by PCR amplification between adjacent exons. The total size of the gene, including all exons and introns, was determined to be ~75 kb. The second exon was found to contain the start codon for mRNA transcription. The highly diverse 5' sequences identified earlier (14,15) were found to be incomplete representations of the first exon, which has two alternative donor splice sites, giving rise to two alternative 5' non-coding exons 1a and 1b. Nineteen coding exons were identified in the hMRE11 gene, the last exon containing both coding sequence and 3'-untranslated region (3'-UTR). The boundaries of all introns were found to have the consensus dinucleotides GT at their donor splice sites and AG at their acceptor splices, with the exception of the donor splice site for exon 1a, which had the variant dinucleotide GC.

**Promoter region**

In a search for the promoter region of the hMRE11 gene we developed and sequenced a 4 kb contig of vectorette products extending out from the 5' end of the alternative exons 1a and 1b of the hMRE11 gene (GenBank accession no. AF303379). Several expressed sequence tags were identified in this contig but we were not able to PCR amplify between them and hMRE11 sequences using cDNA, indicating that they were not transcribed as part of the hMRE11 gene. Interestingly however, a 350 bp sequence 2.7 kb 5' of the hMRE11 gene was also homologous to the sequence FLJ20189, the human homologue of the mouse fetal globin-inducing factor gene (16). The 350 bp sequence was found to be in reverse transcriptional orientation relative to the hMRE11 gene and to contain the translation start site for the fetal globin-inducing factor gene (Fig. 1). The first 32 bp at the 5' end of the FLJ20189 sequence were also homologous to the sequence extending out of the 5' end of the hMRE11 gene, but they were separated from the 350 bp sequence in genomic DNA, being only 159 bp 5' to the end of the first hMRE11 exon. Analysis of the genomic sequence identified a donor splice site sequence match at the end of the 32 bp region of homology, and a splice acceptor consensus sequence match immediately 5' of the 350 bp region of homology (data not shown). This
Figure 1. The organization of the 5’ regions of hMRE11 and the fetal globin-inducing factor gene. The 5’ ends of the mRNA transcripts from these genes are indicated by square brackets. The first and second exons of each gene are identified; those of the fetal globin-inducing factor gene are shown by numbers in outline. The first and second exons of the fetal globin-inducing factor gene correspond to the 32 and 350 bp segments, respectively, of the FLJ20189 sequence referred to in the text. The first exon of the hMRE11 gene is subdivided to show the alternative splice donor sites used by exon 1a (shaded sequence referred to in the text. The first and second exons of each gene are inducing factor gene. The 5’ mutation (exon 5, 350A → G, N117S) in the affected children suggested that the 32 bp sequence represented a short 5’-UTR of the human fetal globin-inducing factor gene, and indicated that the hMRE11 and fetal globin-inducing factor genes were in close proximity to each other (Fig. 1) and transcribed divergently from a shared promoter.

Reportor gene constructs containing a 1244 bp hMRE11 promoter region sequence (GenBank accession no. AF303379), which extended into the first intron of the fetal globin-inducing factor gene from a site within hMRE11 exon 1a (Fig. 1), were analysed for promoter activity. The construct pHMRE11F, which contained the 1244 bp promoter region sequence in the hMRE11 forward direction (relative to the direction of transcription of the hMRE11 gene), was found to have a level of promoter activity that was similar to that of the reference ATM gene promoter (pHATMPF), and ~3-fold stronger than that of the construct pHMRE11R, which contained the promoter in the hMRE11 reverse orientation (Fig. 2). It appeared, therefore, that the 1244 bp region contained promoter elements for both the hMRE11 gene and the fetal globin-inducing factor gene.

Mutation analysis of an apparent null allele

In an earlier study of two ATLD families we were only able to identify one of the two mutations, that of the paternal allele, in family 2 (1). The mutated maternal allele appeared not to be expressed (1), raising the possibility that this allele was deleted either in whole or in part. However, genomic analysis using a combined CA-repeat and single nucleotide polymorphism (GenBank accession no. AF307007) which we developed in intron 13 showed that the mother in family 2 had two hMRE11 alleles represented at this point (data not shown). Additionally genomic sequence analysis across the region of the father’s mutation (exon 5, 350A→G, N117S) in the affected children identified the mutated base from the father’s allele and a normal base derived from the maternal allele, again indicating the presence of two alleles. The mother was also found to be heterozygous for a single base polymorphism (G/T) 115 bp 5’ to the 5’ end of exons 1a and 1b in the promoter region. The evidence of two alleles at three points in the hMRE11 gene indicated that the mutated maternal allele did not contain a deletion that encompassed the entire gene.

The maternal genomic hMRE11 DNA was then sequenced exon by exon and a single base change C→T was found in exon 15, corresponding to nucleotide 1714 in the cDNA sequence. In the mRNA this would change the normal codon CGA (Arg) to the translational stop codon TGA. This mutation was found in the genomic DNA of patients ATLD3 and ATLD4 (Fig. 3), whilst their unaffected sibling was found to have inherited the normal maternal allele since only the normal base was found at nucleotide 1714 (data not shown), as seen in the father (Fig. 3). This contrasted with sequence analysis across the region of the maternal mutation (1714 C→T) in cDNA from ATLD3 and 4, which showed no clear evidence of expression of the maternal allele, bearing in mind the high T background in the father (Fig. 3). The only prominent peak in the sequence at this position was the C base derived from expression of the father’s allele, the sequence of which was normal in this region. Similarly, the only prominent peak at this position in cDNA from the mother was the C base derived from her normal allele. The identification of a truncating point mutation at the genomic level suggested the possibility that the apparent absence of maternally derived mutant mRNA transcripts could be due to NMD.

The in-frame stop codon identified in the mutated maternal allele was predicted to result in truncation of the hMre11 protein from 680 amino acids to 571, a change in the predicted size of the hMre11 protein from 81 to 65 kDa. However, no truncated protein was apparent in western blots of protein extracts from cells from the mother or either of her affected sons (1 and G.S. Stewart, unpublished data). To determine whether NMD was affecting the stability of transcripts from
the mutated maternal allele, we used the protein truncation test (PTT) on cDNA derived from cells that had been incubated in the presence of the protein synthesis inhibitor anisomycin before mRNA isolation. This method had been shown previously to stabilize mutant mRNA which would otherwise be susceptible to NMD (17). Truncated hMre11 protein of ∼65 kDa was found in the PTT products from the mother and ATLD3 and 4 (Fig. 4), using cDNA derived from cells that had been treated with the protein synthesis inhibitor anisomycin. No truncated protein was evident in the PTT products from the father. Consistent with these observations, sequencing of cDNA isolated from anisomycin-treated cells showed the presence of the mutated nucleotide at position 1714 in mRNA derived from cells from the mother and both ATLD3 and 4, but not from the father (Fig. 3). These results demonstrated that the absence of detectable truncated hMre11 protein in lysates of cells from the mother and ATLD3 and 4 was a function of mRNA instability caused by NMD. In a further unrelated family with ATLD from Italy, two affected children were found to be compound heterozygotes for hMRE11 mutations. By genomic screening we identified the mutation 1714C→T, producing a stop codon which was exactly the same as the mutation in family 2. As in family 2, the mutation was not apparent at the cDNA level, although following protection of the transcript from NMD it could be detected.

**DISCUSSION**

In this study we defined the genomic structure of the hMRE11 gene, including its promoter region, which it shares with that of the adjacent fetal globin-inducing factor gene. The characterization of the hMRE11 genomic structure enabled us to define the genetic basis of an apparent null hMRE11 allele. The mutation was found to be a point mutation that created a premature termination codon, as does the mutation (1897C→T; R633X) in the homozygous state in patients ATLD1 and 2 in ATLD.
family 1 (1). Although both of these nonsense mutations are in the last quarter of the coding sequence, exon 15 for patients ATLD3 and 4 and the Italian patients and exon 17 for patients ATLD1 and 2, their effects on hMRE11 mRNA stability appear to be different. The nonsense mutation in exon 17 allowed expression of truncated protein while the nonsense mutation in exon 15 caused NMD and no truncated protein was produced. We could also detect the exon 17 mutation in cDNA from the parents of ATLD1 and 2, whereas we could not clearly detect the exon 15 mutation in cDNA from patients ATLD3 and 4 or their mother without resorting to treatment of cells with anisomycin before mRNA isolation.

We have performed extensive mutational analysis of the ATM gene, and have identified many nonsense mutations by analysing cDNA with no suggestion of NMD operating. However, it is possible that NMD does operate for the ATM gene, but at a level at which sufficient mRNA is made to allow mutation detection in cDNA, without the need to treat the cells with a protein synthesis inhibitor before mRNA isolation. Truncated ATM protein is never found, indicating that if transcripts with premature termination codons are translated, the resulting proteins are very unstable and rapidly degraded. In contrast, we have found that one of two nonsense hMRE11 alleles is expressed as truncated protein. Complete absence of ATM is not lethal, whereas complete absence of hMre11 protein appears to be lethal, as demonstrated by the fact that the hMRE11 gene is essential for normal cell proliferation and embryonic survival in the mouse (18). Consistent with the findings in mice is the observation that the nonsense mutation in exon 17 in patients ATLD1 and 2, and the paternal missense mutation inherited by ATLD3 and 4, are hypomorphic mutations that are expressed as at least partly functional hMre11 proteins (1). Thus hMRE11 null alleles, which in the homozygous state are lethal in the mouse, are present in the human population and appear to occur in ATLD patients only in the presence of a second mutant allele encoding hMre11 protein with residual function.

The involvement of hMre11 in the repair of DNA double strand breaks has raised the possibility that mutation of the hMRE11 gene might contribute to the development of some cancers. In a recent screen of 159 unselected human primary tumours for hMRE11 alterations, three missense mutations, one in a lymphoma and two in breast carcinomas, were identified (19). Matched normal tissue was available for only one missense mutation which was found to be a somatic mutation. In two of the tumours, under-representation of the sequence from the wild-type allele indicated loss of heterozygosity in the chromosomal region which includes hMRE11. An aberrant transcript with an insertion of intronic sequences was found in a third breast carcinoma, but no sequence alteration was evident at the genomic level in either the tumour or matched normal tissue (19). The inserted sequences used the authentic splice donor and acceptor sites at the 3′ end of exon 19 and the 5′ end of exon 20, respectively, but translation into the inserted sequence would have encountered a premature termination codon. Transcripts that include this insertion might be expected to be subject to NMD, since the premature termination codon is 5′ to the final splice junction. However, no wild-type transcripts were found in the tumour and normal tissue did not express the aberrant mRNA. In the absence of expression of a wild-type allele in the cells expressing the aberrant transcript, it is unclear whether mRNAs containing this insertion would be subject to NMD.

The determination of the hMRE11 gene organization was vital to our identification of the mutated maternal allele inherited by ATLD3 and 4, but in addition showed that the hMRE11 gene is transcribed divergently from a promoter region that also appears to be used by the fetal globin-inducing factor gene. That the hMRE11 gene shares its promoter region with another gene is reminiscent of the organization of the promoter regions of other genes involved in the response to double strand DNA breaks, i.e. the ATM gene with the NPAT gene (20,21), PRKDC with MCM4 (22) and BRCA1 with M17S2 (23). Our promoter analysis suggests that the promoter for the fetal globin-inducing factor gene is situated, at least in part, in the 159 bp separating it from the hMRE11 gene. The genomic sequence 5′ of the 5′-UTR of the fetal globin-inducing factor gene does not match the consensus splice acceptor sequence (data not shown), suggesting that an additional 5′-UTR derived from a promoter within an intron of the hMRE11 gene or beyond the 3′ end of the hMRE11 gene is unlikely. The 1244 bp fragment we analysed for promoter activity included the 159 bp intergenic region and 998 bp of the intron of the fetal globin-inducing factor gene. This DNA sequence had promoter activity in the hMRE11 orientation, but we did not determine whether the entire 1244 bp was required for optimal promoter activity.

The exon/intron structure of the hMRE11 gene was determined using hMRE11-specific PAC DNA. Before using the PACs we performed fluorescence in situ hybridization (FISH) analysis to check that they mapped to chromosome 11q21, and found that whilst five of them did map as expected, two others mapped to chromosome 3q25. Further investigation demonstrated that the chromosome 3q25-specific PACs contained an hMRE11-processed pseudogene (GenBank accession no. AF307006, data not shown). Another hMRE11-hybridizing sequence on chromosome 7q11.2–q11.3 had been identified earlier by FISH, using a 14 kb sequence which encompassed the 5′ region of the hMRE11 locus (14). However, we suspect that the hybridization to chromosome 7 was due to the fetal globin-inducing factor gene, because database searches with the sequence for this gene identified closely related sequences on chromosomes 5q and 7q (data not shown). Northern blotting analysis had previously identified two hMRE11 transcripts, one of 2.5 kb and one of ~6 kb (14), raising the possibility that there was an additional hMRE11-related gene that was transcriptionally active. We have performed genomic walking out of the known 3′ hMRE11 sequence and found that subsequent cDNA PCR back to the penultimate exon (across a 10 kb intron) identified some transcripts with final exons that were much longer than previously determined. These transcripts probably account for the larger hMRE11 mRNA species found by northern blotting. Our investigation indicates that there is only one functional hMRE11 gene and that the pseudogene on chromosome 3 is not expressed (S. Pitts, unpublished data).

The determination of the hMRE11 exon structure described here will aid future mutation analysis of the hMRE11 gene in suspected ATLD patients. Our finding that NMD affects mutated transcripts from mutant hMRE11 genes has important implications for cDNA mutation screens of the hMRE11 gene. It is likely that a proportion of mutations will be missed if it is
not possible to culture cells in the presence of a protein synthesis inhibitor prior to mRNA isolation. Additionally, the fact that the hMRE11 pseudogene will hamper mutation detection in cDNA if the possibility of genomic DNA contamination is not obviated should be borne in mind when deciding the mutation screening strategy.

**MATERIALS AND METHODS**

**Determination of the hMRE11 exon structure**

Human genomic clones representing hMRE11 were obtained by screening a PAC library (Pieter de Jong, Roswell Park Cancer Institute, Buffalo, supplied by the UK HGMP Resource Centre) using fragments of hMRE11 cDNA representing the 5′ and 3′ ends of the coding sequence. The PACs were mapped chromosomally by FISH. The hMRE11-specific PAC clones 131I1, 184D4, 264J21, 281L17 and 316C18 were found to detect chromosome 11q21, whilst the hMRE11-specific clones 99M15 and 127F3 were found to map to chromosome 3q25. The promoter region of hMRE11 was amplified from PAC DNA using primers 22306 (5′-CAAGAAGGTACACCACATGAGTA-3′) and 19234 (5′-GAACGTCATATTGATGCCCTCG-3′), both of which incorporated sites for the restriction enzyme KpnI. The resulting 1244 bp fragment was cloned into the KpnI site of pGL2-Basic vector (Promega) in both forward and reverse orientations, with the resulting constructs designated pHMRE11F and pHMRE11R, respectively. Promoter activity assays were performed as described previously (24). In brief, each test construct (10 µg) was mixed with 2 µg of pCMV-β-gal and electroporated into DG75 cells. Relative luciferase activity was determined 48 h after electroporation and expressed as a percentage of the activity of pSV40-LUC. Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample. An ATM promoter construct pHATMPF (24) was used as a reference for promoter strength in some experiments.

**NMD detected by PTT analysis**

The NMD detection procedure was based on that described by Bateman et al. (17). The complete hMRE11 coding sequence was amplified in one PCR product, such that PTT analysis was performed on full-length hMre11 protein. The cDNA substrates for PTT were made from mRNA isolated from lymphoblastoid cell lines (5 × 10⁶ cells) of the individuals to be analysed. The cells were either untreated or treated for 4 h with the protein synthesis inhibitor anisomycin (Sigma) at a final concentration of 0.1 mM. The cells were washed twice in phosphate-buffered saline to remove residual anisomycin, before RNA was extracted using the Ultraspec RNA Isolation system (Biogenesis). The RNA was resuspended in 100 µl of DEPC-treated water (Gibco BRL) and single-stranded cDNA was synthesized by reverse transcription using the Reverse-IT kit (AB Gene), primed using an oligo-dT primer. A single round of PCR was performed using the primer 25931 (5′-GCTATATACACCTACTATAGAAACAGACCCATGAGTACTGAGATGACGAGGTGACACTTGTGATGTCG-3′), which contains a T7 RNA polymerase site, and primer 19234 (see above). The PCR conditions were 35 cycles of denaturation at 94°C for 10 s, annealing at 57°C for 30 s and extension at 68°C for 2 min using Expand Long Template Taq polymerase (Roche). PTT analysis was performed using the TNT T7 Coupled Reticulo-cyte Lysate System (Promega) and [³⁵S]methionine; in vitro translation products were separated according to size by SDS–PAGE on 8% polyacrylamide gels. Gels were fixed with a solution of 20% methanol and 10% acetic acid for 30 min and then treated with Amplify (Amersham Pharmacia Biotech) for an additional 30 min. Dried gels were exposed to scientific imaging film (Kodak) for 24–72 h and developed.

**DNA sequencing**

PCR products were purified and 50 ng was sequenced in 20 µl reactions containing 2 µl ABI Prism Big Dye Terminator (PE biosystems), 6 µl buffer (200 mM Tris–HCl, 5 mM MgCl₂, pH 9.0) and 1 pmol primer. The PCR conditions were 25 cycles of 96°C for 10 s, 60°C for 2 s and 60°C for 4 min. Sequencing reactions were subsequently ethanol-precipitated and then resuspended in formamide/dye (5:1) loading buffer. Samples were loaded onto sequencing gels and run on the ABI Prism 377 DNA sequencer according to ABI protocols. The primers 19239 (5′-GAACGTCATATTGACGCTGATGGTTCTGC-3′) and 19242 (5′-CTTCCACATCTGTGATCTGATACG-3′) were used specifically for cDNA sequence analysis of the hMRE11 mutation at nucleotide 1714 described here.

**Database information**

Accession nos for data in this article are as follows (GenBank, http://www.ncbi.nlm.nih.gov/Genbank) for hMRE11 promoter region and exon/intron sequences, accession nos AF303379–
Table 2. Primer sequences for exon amplification and sequencing

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Primers indicated in bold can be used for exon sequencing.

AF303395; hMRE11 pseudogene sequence, accession no. AF307006; hMRE11 intron 13 polymorphic marker, accession no. AF307007; complete hMRE11 cDNA sequence, accession nos NM_005590, NM_005591 and AF073362; FLJ20189, accession no. NM_017704; M. musculus fetal globin-inducing factor gene, accession no. NM_019683.

ACKNOWLEDGEMENTS

We would like to thank the Cancer Research Campaign, the Kay Kendall Leukaemia Fund, the A-T Society and the Institute for Cancer Studies for their continued support.

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