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ARTICLE

# The human *EZH2* gene: genomic organisation and revised mapping in 7q35 within the critical region for malignant myeloid disorders

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**The *EZH2* gene is a homolog of the *Drosophila Polycomb* group (*PcG*) gene enhancer of zest, a crucial regulator of homeotic gene expression. Several lines of evidence suggest a critical role for the EZH2 protein during normal and perturbed development of the haematopoietic and central nervous systems. Indeed, the EZH2 protein has been shown to associate with the Vav proto-oncoprotein and with the XNP protein, the product of a mental retardation gene. The *EZH2* gene was previously reported to be located on chromosome 21q22 and was proposed as a candidate gene for some characteristics of the Down syndrome phenotype. We report here the genomic structure and fine mapping of the *EZH2* gene. We demonstrate that the functional gene actually maps to chromosome 7q35 and that the sequence previously isolated from a chromosome 21 cosmid corresponds to a pseudogene. Finally, the nature of the EZH2 protein and its mapping to the critical region for malignant myeloid disorders lead us to propose the *EZH2* gene is involved in the pathogenesis of 7q35–q36 aberrations in myeloid leukaemia.** *European Journal of Human Genetics* (2000) **8**, 174–180.

**Keywords:** *EZH2*; genomic structure; 7q35; pseudogene; malignant myeloid disorders

## Introduction

The Polycomb group (*Pc-G*) and the Trithorax group (*trxG*) genes were originally identified in *Drosophila* as being responsible for the maintenance of expression boundaries of homeotic genes.[1] Their gene products are thought to act in multiprotein complexes at the level of chromatin structure, where PcG proteins maintain inactive homeotic genes in repressed state whereas trxG proteins ensure maintenance of the active state. Currently, 11 *PcG* genes have been isolated[2] and among these, enhancer of zest (*E(z)*) seems to play a central role. Firstly, it is one of the very few *PcG* genes conserved in lower eukaryotes[3] and, secondly, according to the phenotype analysis of loss-of-function mutations, *E(Z)* appears to be a pleiotropic gene with function in chromatin architecture, gene regulation and growth control.

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Mammalian homologs of both *PcG* and *trxG* genes have also been identified and a remarkable degree of functional conservation have been demonstrated by different groups.[4,5] The human E(Z) homolog EZH2 was initially isolated in a search for proteins that associate specifically with Vav, a human proto-oncogene product involved in lymphocyte development and activation.[6] This interaction suggests involvement of the EZH2 protein in signal-dependent T-cell proliferation. The same gene sequence was also identified in an exon-trapping experiment performed with cosmid DNA from a human chromosome 21 specific library in order to isolate genes from the so-called Down syndrome critical region.[7] The latter data led the authors to map the *EZH2* gene to human chromosome 21q22.2 and to suggest that over-expression of this gene in trisomy 21 may be associated with some of the clinical features of Down syndrome. More recently, we reported that the EZH2 protein is also involved in an interaction with the XNP protein,[8] a gene involved in several X-linked mental retardation conditions, sometimes associated with α-thalassaemia, an association which may

reflect an important role in central nervous system development and haematopoietic development.

To elucidate the potential role of the *EZH2* gene in either Down syndrome or in the syndrome associated with the deletion of 21q22, we decided to determine the genomic structure of the *EZH2* gene. In this report we demonstrate that the human *EZH2* gene is divided into 20 exons. More importantly, we found that *EZH2* maps to chromosome 7q35 and that the *EZH2* copy previously assigned to chromosome 21 corresponds to a pseudogene.

## Materials and methods

### In silico analysis

Blast searches were conducted either at the Washington University Genome Sequencing Center (http://genome.wustl.edu/gsc/blast/blast—servers) against a human genomic database (St Louis project only) or at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/cgi-bin/BLAST/) against the non-redundant Genbank + EMBL + DDBJ + PDB sequences. The EZH2 sequence used for this purpose was the one corresponding to Genbank entry U61145. Sequence alignments were performed using the TBLASTN program[9] (version 2.0.8, Jan-05-1999).

### Southern blot analysis

Six μg of total human DNA and 1 μg of YAC DNA were digested with PstI. The DNAs were transferred to a nylon membrane using standard methods.[10] Hybridisation was performed at 65°C overnight. The probe used to detect the *EZH2* gene was a 2 kb long PCR product obtained by amplifying *EZH2* cDNA using primers $EZH_2$(1) 5' CAGGATGGTACTTTCATTG3' and $EZH_2$(4) 5'GCCTTCTCACCAGCTGCAA 3' corresponding respectively to position 508–566 and 2490–2509 of the cDNA sequence.

### Fluorescence *in situ* hybridisation

Metaphase spreads were prepared from phytohaemagglutinin-stimulated human lymphocytes, cultured at 37°C for 96 h 5-bromodeoxyuridine was added for the final 7 h of culture (60 μg/ml of medium) to ensure a chromosomal R-banding of good quality. The 803 g 1 YAC clone, containing an insert of 1000 kb, was biotinylated by random priming with biotin-16–dUTP, as outlined by the Life Technologies (Bio Prime Kit) protocol. Hybridisation to chromosome spreads was performed using a standard protocol.[11] For each slide, 400 ng of biotinylated DNA was used. The hybridised probe was detected by means of fluorescence isothiocyanate-conjugated avidin. Chromosomes were counterstained and R-banded with propidium iodide diluted in antifade solution pH 11.0 as previously described.[12]

### RT-PCR and expression analysis

RNA was extracted from the various sources using the QuickPrep mRNA purification kit, according to the instructions of the manufacturer (Pharmacia, Courtabeuf, France). Reverse transcription of 500 ng of mRNA was performed in 50 μl of 1X Superscript reaction buffer (Gibco BRL, Life Technologies, Cergy Pontoise, France) containing 3 ng of $(dN)_6$/μl, 40 units of Rnasin (Promega), 1 mM dNTP, and 200 units of Superscript II reverse transcriptase. A 1/10 volume of template was used in 50 μl of PCR mixture, containing 0.2 mM deoxynucleosides triphosphates, 1.5 mM $MgCl_2$ and 1 U of Taq polymerase. The cycling program was 4 min at 96°C, followed by 35 cycles of 40 s at 94°C, 40 s at 58°C and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR amplifications were performed with the primers $EZH_2$(4) and $EZH_2$(8): 5'TACATGTGCAGCTTTCTGTTC 3' or $EZH_2$(9): 5'AGCTAAGGCAGCTGTTTCAGA 3' corresponding respectively to nucleotides 2067–2088 and 2352–2372 of the EZH2 cDNA sequence. 25 μl of PCR products were then digested by the enzyme AluI for 2 h at 37°C. Digested and undigested PCR products were loaded on a 4% gel and visualised after ethidium bromide staining.

### Patients

Paired samples were obtained from 20 patients with myeloid disorders (*de novo* acute myeloid leukaemia (AML) $n = 11$; acute transformation of myelodysplastic syndrome (MDS), $n = 9$). Blast cells were isolated at the time of diagnosis either from blood or from bone marrow samples using a ficoll (Life Technology, Cergy Pontoise, France) gradient.[13] T-lymphocytes were purified from blood obtained in remission using Dynal beads (Dynal, Compiègne, France).[14]

### Genotyping studies

Four simple tandem repeat (STR) markers were used: D7S688, D7S505, D7S642, D7S483. Primers sequences and percentages of heterozygosity were obtained from the Genome Data Base (http://gdb.infobiogen.fr)

Polymerase chain reaction (PCR) was performed with a 5'fluorescein-labelled upstream primer. Five microlitres of cellular lysate was amplified in a total volume of 25 μl containing 10 pmol of 5' and 3' primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.2 U of Taq polymerase (Gibco, Life Technologies, Cergy Pontoise, France). Taq polymerase was added after a denaturation step of 5 min at 96°C. The PCR cycling parameters were 20 s at 95°C, 20 s at 50°C, and 5 s at 72°C for 30 cycles performed in a UNO-Thermoblock (Biometra, Göttingen, Germany).

After denaturation, the PCR products were analysed using a fluorescent automated laser DNA sequencer (ALF; Pharmacia LKB Biotechnology, Uppsala, Sweden). Electrophoresis was achieved on a 6% long-ranger gel containing 8 mol/L urea in a 0.6X Tris-borate–EDTA buffer. The PCR products obtained from the blasts cells were compared with those obtained from normal cells of the same patient obtained during remission. Areas under the curve of the peaks were determined using the software Fragment Manager (Pharmacia). If A1 and A2 represent the two alleles in a heterozygous

patient, the ratio of signal A1/A2 was calculated for normal and blast cell samples. The relative copy number of alleles in blast cells samples was then calculated as $R$ = [(A1/A2) blasts]/[(A1/A2) remission]. For each patient results obtained with blast cell DNA were compared with corresponding matched normal cells. A loss of heterozygosity (LOH) usually appears as a decrease rather than a total disappearance of the lost allele because of the persistence of 1–30% normal marrow cells in samples of blast cells. When both alleles were present in the blast cell population, the allelic ration was close to 1. Samples were considered to have undergone LOH when ratios were less than 0.30.[15]

## Results

### Genomic organisation and chromosomal localisation of the *EZH2* gene

To characterise a genomic clone containing the *EZH2* gene, we performed TBLASTN searches using the EZH2 protein sequence against the St Louis genomic sequences database. We have identified a PAC clone (dJ1151MO5, accession No. AC006323) containing regions with 100% nucleotide identity to the entire *EZH2* cDNA sequence. We conclude that this PAC covers the *EZH2* structural gene locus. Genomic organisation of the *EZH2* gene was then determined by sequence alignment between the cDNA sequence and the sequence of clone dJ1151MO5. As indicated in Figure 1A and Table 1, the *EZH2* gene spans approximately 40 kb and is composed of 20 exons. The exons range in size from 41 to 323 bp and the introns from 0.15 to 17.7 kb. Sequences at the intron–exon junctions are given in Table 1. They all follow the gt/ag rule[16] and score highly in terms of the derived consensus sequences at these sites.[17]

Analysis of the PAC dJ1151MO5 sequence indicated that it also contained markers D7S688 (within *EZH2* intron 2), D7S2419 and the gene *Hs-cul-1*.[18] These data suggested that the gene mapped to chromosome 7q35 rather than to chromosome 21 as previously assigned. To verify by Southern blot and FISH mapping the location of the *EZH2* gene, a YAC clone (803 g 1) containing marker D7S688 was identify by searching the CEPH-Généthon database (www.cephb.fr/cgi-bin). A Southern blot containing human DNA or DNA from

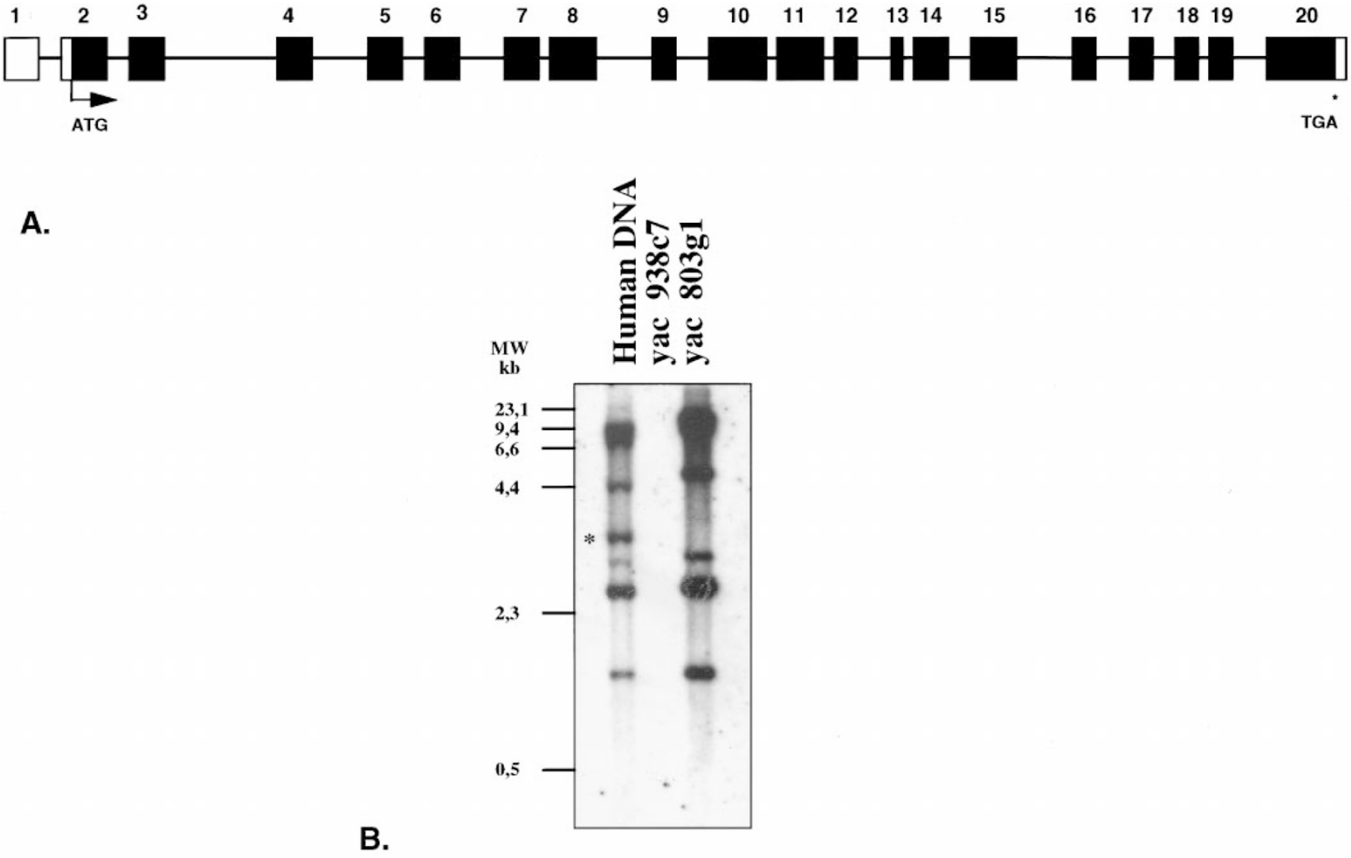


**Figure 1** **A** Schematic illustration of the genomic structure of the *EZH2* gene. The human EZH2 gene contains 20 exons (boxes). The open reading frame (black boxes) is distributed over 19 exons. The positions of the initiation (ATG) and termination (TGA) codons are indicated. **B** Comparative Southern blot analysis of human genomic DNA and DNA from YAC 803 g 1. Pst I digests were size-fractionated on a 1% agarose gel and analysed as described under Materials and methods. YAC 938 c 7, located elsewhere on chromosome 7, was used as a control.

**Table 1** Intron/exon boundaries of the human *EZH2* gene. Exonic sequences are in upper case and intronic sequences in lower case. The 5'gt and 3'ag ends of introns are indicated in bold. ND: not determined

| Exon | Size (bp) | 5' splice donor | Intron size (bp) | 3' splice acceptor |
|---|---|---|---|---|
| 1 | ND | ND | ND | ctttt**ag**AATAAT |
| 2 | 124 | GTAAAG**gt**ataatt | 577 | ttaa**ag**AGTATG |
| 3 | 128 | AGGGAG**gt**tggtt | 13720 | gttttt**ag**TGTTCG |
| 4 | 117 | TTTATG**gt**atgta | 2785 | ttttt**ag**GTGGAA |
| 5 | 121 | ATAGAG**gt**gagcc | 846 | gtttc**ag**AATGTG |
| 6 | 141 | GAGATG**gt**atgcc | 1473 | tgttt**ag**ATAAAG |
| 7 | 103 | GGAAAA**gt**aagaa | 533 | atgtc**ag**ATATAA |
| 8 | 164 | TACATC**gt**aagtg | 6781 | tttgc**ag**CTTTTC |
| 9 | 92 | CATTTG**gt**aagac | 1477 | ttcgt**ag**GAGGGA |
| 10 | 242 | CCTCTG**gt**aagac | 484 | tttgt**ag**AAGCAA |
| 11 | 170 | AGACAG**gt**aaga | 443 | ttgtc**ag**GTGTAT |
| 12 | 95 | ACACCG**gt**gagtc | 1140 | tttgc**ag**GTTGTG |
| 13 | 41 | AAAAGG**gt**tagca | 466 | tactc**ag**ACGGCT |
| 14 | 126 | CAGAGT**gt**aagta | 776 | tctga**ag**GTCAAA |
| 15 | 179 | AAAAAG**gt**gagca | 2238 | tctct**ag**CATCTA |
| 16 | 96 | GGAGAG**gt**aaggc | 1210 | ttttt**ag**ATTATT |
| 17 | 82 | ACAATG**gt**atgtt | 943 | cttttt**ag**ATTTTG |
| 18 | 81 | CAAAAG**gt**aggta | 154 | tttgc**ag**TTATGA |
| 19 | 85 | TTACAG**gt**tgtga | 1365 | gtttc**ag**ATACAG |
| 20 | > 323 | | | |

yeast cells containing this YAC was then probed with the complete *EZH2* cDNA sequence. As shown in Figure 1B, the results confirm that the YAC 803 g 1 contains the *EZH2* gene since a total of 5 DNA fragments were detected. When compared with the banding pattern obtained with human genomic DNA, an additional approximately 3.3 kb-long PstI fragment, absent from YAC 803 g 1, was detected suggesting that the sequence corresponding to the probe cross-hybridises with another locus in the human genome. FISH mapping was also performed using the YAC 803 g 1 as a probe. As observed in Figure 2, this YAC is located in chromosomal band 7q35. Altogether, these experiments demonstrate unambiguously that the *EZH2* sequence is located in 7q35.

### Sequence and expression analysis of the *EZH2* copy located on chromosome 21

Since previously published data located the *EZH2* gene on chromosome 21q22[7] and because Southern blot analysis indicated the presence of a second sequence cross-hybridising with the EZH2 probe, we searched for the corresponding genomic sequence by performing a BLASTN search with the *EZH2* cDNA sequence against all genomic sequences deposited in genbank (NCBI). This search identified two cosmid clones from chromosome 21 (No. AF015726 and AJ229042) which showed 95% nucleotide identity between a 562 bp long genomic fragment and a segment corresponding to the SET domain of *EZH2* cDNA (from exon 17 to 20) (Figure 3A). However, no sequence homologous to the 5' region of the EZH2 ORF was present in any cosmid analysed. In addition, careful analysis of the alignement revealed a surprising organisation. First, as shown in Figure 3B,

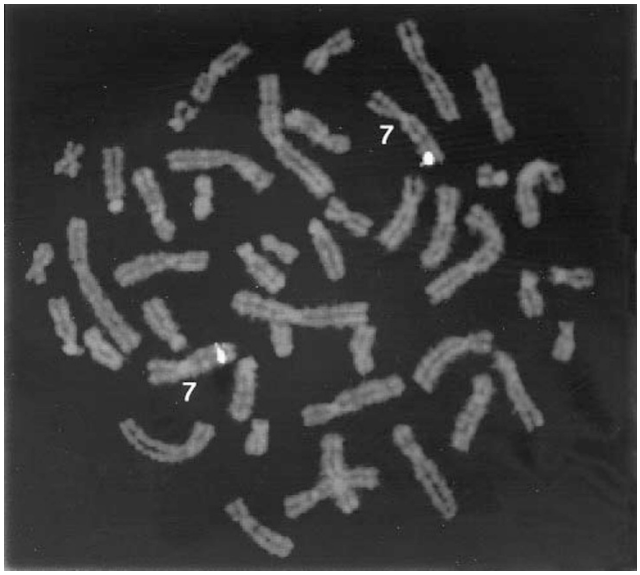


**Figure 2** FISH mapping of the *EZH2* gene. DNA from clone 803 g 1 was used as a probe for FISH analysis as described under Materials and methods. A total of 30 metaphase cells were analysed, and 98% of the cells showed specific fluorescent spots on the 7q34–q35 bands of the human genome.

introns 17, 18 and 19 are absent from the chromosome 21 *EZH2* copy and secondly, the 3'UTR is found proximal to the coding sequence. This surprising organisation was confirmed by PCR analysis presented in Figure 3C. Indeed, primers 4 and 8 were shown to lead to the amplification of an expected 2900 bp long product from genomic DNA (encompassing introns 17, 18 and 19) and to the synthesis of an expected 422 bp long fragment from cDNA. On the contrary primers 4 and 9 were shown to be unable to amplify any product from cDNA but led to the synthesis of an expected 482 bp long fragment from genomic DNA. We also examine the cosmid sequence for the presence of PstI sites around the homologous region and found that the conserved sequenced is totally contained within a 3236 bp long PstI fragment. This result strongly suggests that the additional PstI fragment detected in the Southern blot experiment originates in 21q22.

Lastly, to ensure that the chromosome 21 copy did not correspond to any expressed sequence, we took advantage of the AluI restriction site polymorphism found between both sequences: the chromosome 7 copy contains an AluI restriction site which is absent from the one in chromosome 21. A series of RT-PCR experiments followed by AluI digestion were performed and the results are shown in Figure 4. They indicate that, in RNA from either 9 week-old embryo or from foetal brain, the PCR product obtained is totally digested by AluI and that no undigested product remains. No RNA originating from the chromosome 21 copy can therefore be detected in these samples. Two other types of cell or tissues

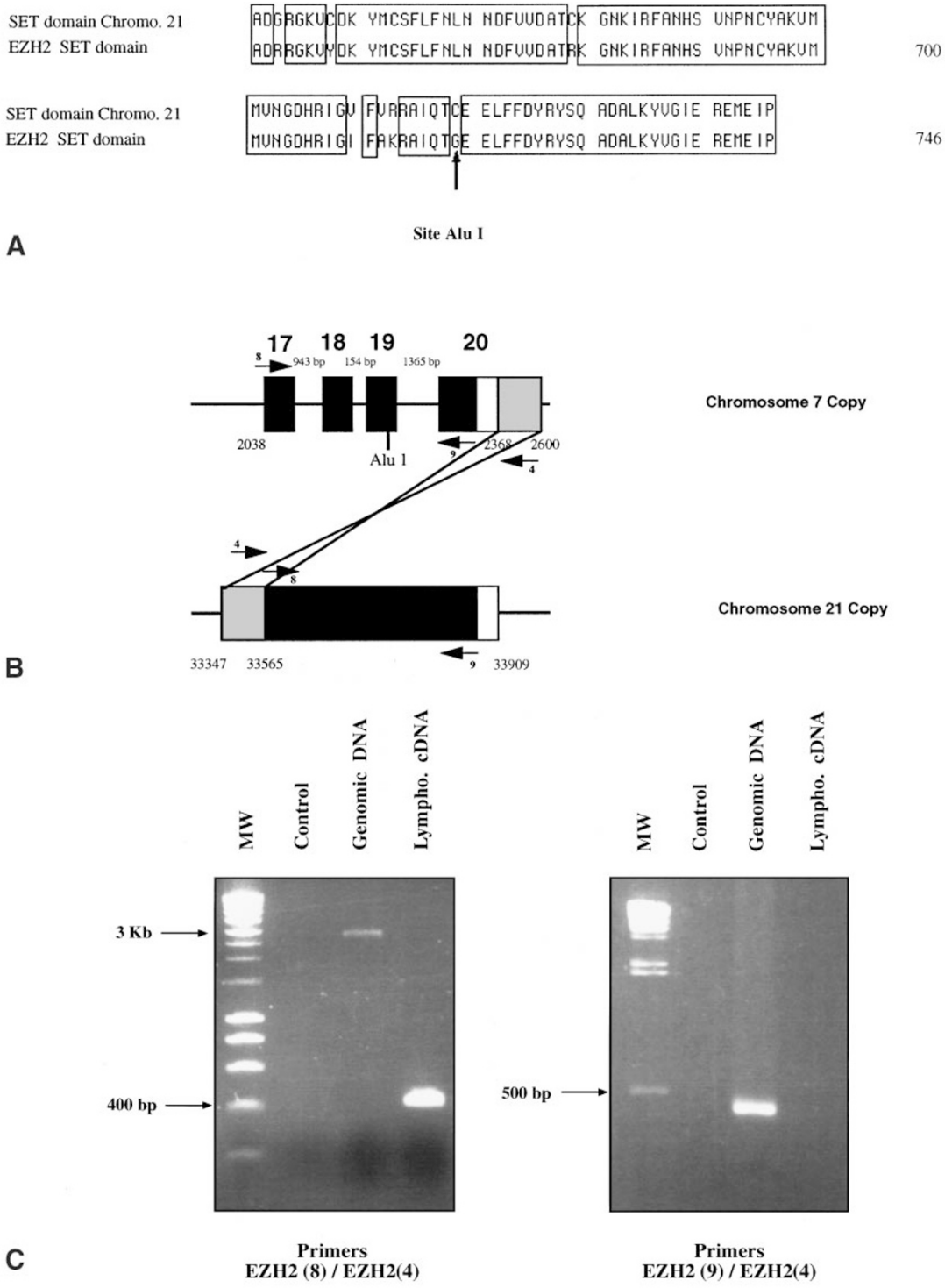


**Figure 3** **A** Amino acid sequence alignment between chromosome 7 and chromosome 21 genes. Common sequences are boxed and the position of the AluI restriction site which encompasses one of the divergent positions is indicated **B** Structural comparison of the conserved region between chromosome 7 and chromosome 21. The schematic figure compares the structures of exon 17 to 20 from the *EZH2* gene with the structure of nucleotides 33347 to 33909 from the chromosome 21 cosmid. Exons are represented by boxes. The black boxes indicate coding of the region. Primers used in RT-PCR experiments are shown as well as the position of the AluI site. The inversion of the sequence corresponding to nucleotide 2368–2600 of the *EZH2* cDNA sequence (grey box) is shown. **C** PCR results using primers EZH2 (8) and (4) or EZH2 (9) and (4) on human genomic DNA or human cDNA from 9-week-old embryos. A control with no DNA was also performed under the same conditions.

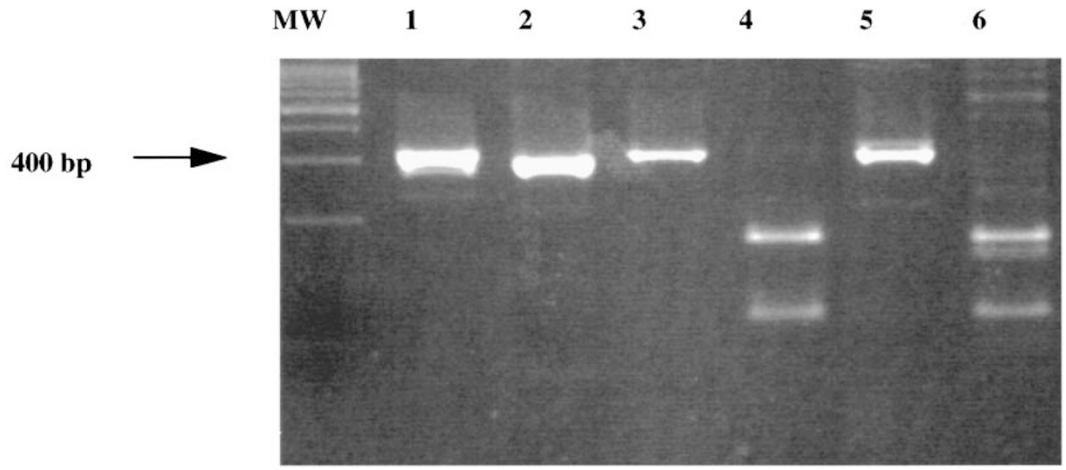


**Figure 4** Expression analysis of the chromosome 7 and chromosome 21 *EZH2* sequences. RT-PCR fragments produced using primers EZH2 (8) and (9) were analysed by AluI restriction. 1: undigested human genomic DNA; 2: digested human genomic DNA; 3: undigested human embryonic cDNAs; 4: digested human embryonic cDNAs; 5: undigested human foetal brain cDNAs; 6: digested human foetal brain cDNAs.

were also tested: foetal liver and HeLa cells and gave the same results (data not shown). These data together provide strong evidence that the chromosome 21 sequence corresponds to an *EZH2* pseudogene.

### Localisation of the *EZH2* gene within a critical region for myeloid disorders

Since previous analysis indicated that 7q31–qter is a critical region for malignant myeloid disorders,[19] patients with either acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS) were tested for deletion of the *EZH2* gene by testing the LOH for markers flanking the *EZH2* gene. Our results (data not shown) indicate that the allele loss is detected in five out of 21 patients (three with *de novo* AML and two with acute transformation of MDS) for the four informative microsatellite markers tested, including marker D7S688 located within intron 2 of the *EZH2* gene. This proportion is in agreement with a previous report[9] and support the finding that the *EZH2* gene is located within a region that is commonly deleted in myeloid disorders.

## Discussion

We have defined the multiexonic structure of the human *EZH2* gene and determined the sequences of the exon–intron boundaries. We have also shown by cytogenetic and molecular analysis that this gene maps to the long arm of chromosome 7 at position 7q35, in a region corresponding to the polymorphic marker D7S688. Interestingly, the murine *Ezh2* gene has been recently mapped to a region of mouse chr 6,[20] which has synteny with human chromosome 7q35. Further, we found that the sequence previously isolated from chromosome 21q22 corresponds to a pseudogene, ruling out the possibility that overexpression of the chromosome 21 copy could be responsible for some of the clinical features of Down syndrome. Finally, since our Southern blot analysis indicates that DNA fragments detected with a *EZH2* probe on human genomic DNA were all accounted for by the bands in YAC 803 g 1 and cosmid AF015726, the presence of another *EZH2*-related sequence in the human genome is very unlikely.

The precise function of *EZH2* is not known. However, its specific *in vitro* and *in vivo* interaction with the Vav proto-oncoprotein suggests involvement in haematopoietic development and signal-dependant T-cell proliferation. In addition, the EZH2 protein shares the SET domain, a domain characteristic of a family of chromatin regulators,[21] with the human trithorax homolog ALL-1/HRX, another proto-oncogene that is frequently disrupted in 11q23 translocations in acute leukaemias.[22,23] Finally, studies of another vertebrate *Pc-G* gene, the mouse *Bmi-1* gene has demonstrated that unregulated activation of this gene generates B- and T-cell lymphomas and its deletion causes severe defects in haematopoietic development, particularly in lymphopoiesis and myelopoiesis,[24] providing further evidence for a specific function of *Pc-G* genes in haematopoiesis. In this regard, the demonstration that *EZH2* maps within a region known to exhibit LOH in myeloid disorders is of great interest and makes it a potential candidate for being involved in these malignancies. Determination of the *EZH2* genomic structure should facilitate further investigations aiming to screen for inactivating mutations in such patients.

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