RAPID COMMUNICATION

Alternative, Out-of-Frame runt/MTG8 Transcripts Are Encoded by the Derivative(8) Chromosome in the t(8;21) of Acute Myeloid Leukemia M2

By Jane E. Tighe and Franco Calabi

In the t(8;21) of acute myeloid leukemia (AML) M2, breakpoints are clustered on both chromosomes. The chromosome 21 breakpoint cluster region (bcr) falls within the runt locus, in the intron immediately downstream of the exons encoding an evolutionary conserved domain (the runt box). Transcripts in which the runt box is fused in frame to a novel sequence derived from chromosome 8 (MTG8) have been previously identified and have been assumed to constitute a critical leukemogenic event. Here we show physical linkage of the chromosome 8 bcr to the MTG8 locus. Unexpectedly, not only does the bcr map upstream of the most 5' MTG8 exon found in runt/MTG8 fusion transcripts, but it also maps upstream of a further 5' exon. In addition, we demonstrate the presence of alternative transcripts, originating from the der(8) chromosome, in which runt is out of frame with MTG8. Thus, runt truncation per se, rather than its fusion to MTG8, may be the crucial leukemogenic event.

CONSIDERABLE PROGRESS has been made recently in the molecular study of the t(8;21) of acute myeloid leukemia (AML) M2. On chromosome 21, the locus involved, originally named AML1, has been shown to be a member of the runt family, based on a domain of high homology (the runt box) to Drosophila melanogaster runt, a developmental master gene.1,2 In nearly all patients, breakpoints cluster in the intron immediately 3' of the exons encoding the runt box.3,5 suggesting that the separation of the runt box from its COOH-terminal neighboring domain(s) is a crucial event. As for chromosome 8, cDNA analysis has shown transcripts originating from the der(8) chromosome that encode the runt box fused, in frame, to a chromosome 8 sequence.6,6 The corresponding normal chromosome 8 locus, MTG8 (ETO), has been reported to give rise to at least two transcripts. These have alternative 5' ends (MTG8a and MTG8b), which contain the candidate translational start sites, spliced onto the same sequence (MTG8 common exon) that is found fused to the runt box in transcripts originating from the der(8) chromosome.5 In the fusion runt/MTG8 transcript, the first few amino acids of either normal MTG8a or MTG8b are replaced by the amino-terminus of runt, including the whole runt box.

In parallel studies, we have shown that in most cases breakpoints are tightly clustered at the genomic level not only on chromosome 21, but also on chromosome 8.4 We now show physical linkage of the chromosome 8 breakpoint cluster region (bcr) to the MTG8 locus. Unexpectedly, the bcr is found upstream not only of the MTG8 common exon, but also of a further 5' exon, the MTG8a exon. Furthermore, we demonstrate the presence of alternative transcripts, originating from the der(8) chromosome, in which runt is out of frame with MTG8. Thus, runt truncation per se, rather than its fusion to MTG8, may be the crucial leukemogenic event.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides used as PCR primers and/or as hybridization probes are listed in Table 1.

RNA and DNA preparation. Total cytoplasmic RNA was extracted from patient samples (bone marrow and/or blood) and cell lines concurrently with DNA. After removal of red blood cells by NH4Cl lysis, cells were lysed at 4°C in 0.5% NP40/140 mmol/L NaCl/10 mmol/L Tris pH 7.4/5 mmol/L MgCl2 in the presence of vanadyl ribonucleoside complexes. The postnuclear supernatant was phenol extracted and ethanol precipitated. Concentrations were estimated after gel electrophoresis by reference to a standard. The 25C-G3 YAC strain was grown in YPD medium and DNA plugs were prepared essentially as described, except that the cells were treated with yeast lytic enzyme (ICN Biomedicals, Thame, Oxfordshire, UK) before inclusion in agarose. Plugs were incubated at 37°C in 1% Sarkosyl (Merck, Poole, Dorset, UK)/10 mmol/L EDTA pH 8, with several changes, for 24 to 36 hours, washed in distilled water, and equilibrated in 1X TBE before electrophoresis. For total DNA extraction, the yeast cell pellet after spheroplasting was resuspended in 1% Sarkosyl/15 mmol/L EDTA pH 8/0.1 mg/mL proteinase K, heated at 50°C for 1 to 3 hours, and phenol extracted.

Pulsed-field gel electrophoresis (PFGE). PFGE was essentially as described, using 1% agarose/1X TBE, at 150 V and 16°C, for 24 hours, with a 70-second pulse time.

Libraries. A total human genomic library, derived from the 31-13 cell line, has been described previously.4 A partial genomic library of 11 to 15 kbp Sac I fragments from the 25C-G3 strain was prepared in λ 2001, after complete digestion and gel fractionation.

cDNA synthesis and polymerase chain reaction (PCR). cDNA was primed off total RNA using either a (dT)20 primer (oligo 288) and a specific MTG8 oligonucleotide (oligo 383). Similar results were obtained with either primer.

PCRs were performed in 30 μL of 50 mmol/L KCl/10 mmol/L Tris pH 8.3 (at room temperature)/1.5 mmol/L MgCl2/0.1 μmol/μL each primer for 30 cycles of 95°C for 45 seconds, 55°C for 1', and 72°C for 0.5 to 1'.

Sequence analysis software. In general, DNA and protein sequences were analyzed using the Staden package.11 Database searches were conducted with the BLAST and FASTA algorithms.12 Secondary structure analysis of the predicted MTG8 polypeptides was performed according to Rost et al.13 Searches for eukaryotic promoter motifs were performed using the program Signal Scan.14 Oligonucleotides were designed using the program PRIMER (Lincoln S.E., Daly M.J., and Lander E.S., MIT Center for Genome

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Table 1. Oligonucleotides Used in This Study

<table>
<thead>
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<th>No.</th>
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<th>Polarity</th>
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<td>oligo(dT)</td>
<td></td>
<td>CCGCTCTAGAAGCTTACCC</td>
</tr>
<tr>
<td>321</td>
<td>Probe 8</td>
<td>tel → cen</td>
<td>GGTTGCTCTATACGTTGGA</td>
</tr>
<tr>
<td>322</td>
<td>Probe 8</td>
<td>cen → tel</td>
<td>GGCTTCTAGCAGGCTGTG</td>
</tr>
<tr>
<td>379</td>
<td>MTG8a (329-348)</td>
<td>Sense</td>
<td>CATTCCAGAACAGGAGGCAT</td>
</tr>
<tr>
<td>380</td>
<td>MTG8b (67-86)</td>
<td>Sense</td>
<td>CAGAGCGATGGTGGAGAAT</td>
</tr>
<tr>
<td>425</td>
<td>MTG8b (148-172)</td>
<td>Antisense</td>
<td>CTTGACAATATTCAAAGTTCCTCTT</td>
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<tr>
<td>329</td>
<td>MTG8 common exon</td>
<td>Antisense</td>
<td>AGTCAGTCTAGATTGCGTCTCCTCA</td>
</tr>
<tr>
<td>383</td>
<td>MTG8 common exon</td>
<td>Antisense</td>
<td>GTGGTGC~GTGTAAATGAACTG</td>
</tr>
<tr>
<td>382</td>
<td>runt (1196-1216)</td>
<td>Sense</td>
<td>GGAAAA~CTTCACTCTGACCA</td>
</tr>
<tr>
<td>398</td>
<td>46 nt (species 11, Fig 4)</td>
<td>Sense</td>
<td>TGACATACATTAACCGATGAGGlTCTCCTGAGGAAGTACTTAATTG</td>
</tr>
<tr>
<td>436</td>
<td>68 nt (species IV, Fig 4)</td>
<td>Antisense</td>
<td>GAGGATCCCACCAGGAACAT</td>
</tr>
<tr>
<td>382</td>
<td>runt (1196-1216)</td>
<td>Sense</td>
<td>GGAAAGCTTCACTCTGA GCA</td>
</tr>
</tbody>
</table>

Numbers in brackets refer to the following accession numbers in the GenBank/EMBL Databases: D14820 (MTG8a and MTG8 common exon), D14821 (MTG8b), D10570 (runt). Underlined nucleotides represent changes introduced to create restriction sites.

Research and Whitehead Institute for Biomedical Research, Cambridge, MA).

DNA subcloning and sequencing, restriction mapping, Southern blotting, and hybridization were performed according to standard protocols.15

RESULTS

Physical linkage of the chromosome 8 bcr to the MTG8 locus. We have previously described the derivation of a chromosome 8 probe (probe 8) that detects rearranged BamHI or EcoRI fragments on Southern blots of 14 out of 18 patients with t(8;21) AML M2.4 An 800-nucleotide (nt) AvaII internal fragment containing sequences highly conserved in the mouse, as judged by Southern blotting analysis, was subcloned and sequenced (J.E.T. and F.C., in preparation). The sequence was not significantly related either to any entry in the available databases, or to the subsequently published chromosome 8 sequence (MTG8) shown to be fused to the runt box in transcripts from t(8;21) AML patients.6,8

Because the MTG8 common exons were predicted to lie centromeric to the chromosome 8 bcr, chromosome walking was undertaken, initially using probe 8 on a random human genomic library in A 2001. However, the clones isolated extended only a few kbp in a centromeric direction (Fig 1).

A genomic library in a YAC vector16 was then screened. PCR primers (oligos 321 and 322) were designed from within the probe 8 sequence and used on primary YAC pools provided by the UK HGMP Resource Centre (Harrow, Middlesex, UK). From the two pools that scored positive on the first screening, one clone (25C-G3) was isolated following two further rounds of screening. By reference to Saccharomyces cerevisiae chromosomes on PFGE, the YAC was sized at ~1 megabasepair (Mbp) (Fig 2). Southern blotting of the same gel showed the whole YAC to be clearly positive both with a probe mapping 1.8 kbp centromeric to probe 8 (probe 8C, Fig 1) and with an oligonucleotide derived from the first MTG8 common exon sequence (oligo 329, data not shown). Thus, the chromosome 8 bcr and MTG8 are physically linked, being within ~1 Mbp of each other.

To achieve finer mapping, advantage was taken of the observation that probe 8 maps to the telomeric end of a 12 kbp Sall fragment, whereas oligo 329 maps immediately 5’ to an Sall site of a 10.4-kbp Sall fragment. A size-selected Sall library was established in A 2001 from total DNA of 25C-G3 and screened in parallel with both probes. Two sets of non-overlapping positive clones were isolated (λG3S1 and λG3S7) (Fig 1). On Southern blots of 25C-G3 DNA, unique sequence probes made from the near ends of the two Sac I inserts may detect the same 27-kbp Xba I

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Fig 1. Partial restriction map of the chromosome 8 bcr and its relationship to MTG8 exons. Restriction sites are: B, BamHI; R, EcoRI; S, SacI; X, XbaI. The position of probes 8 and 8C is indicated. Also represented are the inserts of clones isolated from genomic libraries: λ31.3 and λ31.11 from the 31-13 library; λG3S1 and λG3S7 from a library made from 25C-G3.
ALTERNATIVE runt/MTG8 TRANSCRIPTS

Fig 2. (A) Ethidium bromide-stained PFGE gel of chromosomes from YAC strain 25C-G3 (left) and host AB1380 (right). The position of the YAC is indicated by an arrow. The approximate sizes of AB1380 chromosomes are indicated in megabases. (B) Southern hybridization of the left lane in (A) to probe 8C.

The chromosome 8 bcr maps downstream of MTG8b, but upstream of MTG8a. The MTG8b exon was mapped to an ~2 kbp BamHI/XbaI fragment immediately telomeric to the insert of clone λ31-11 (Fig 1), using a combination of PCR with two primers mapping within MTG8b (oligos 380 and 425) and hybridization of the 3' primer (oligo 425) to Southern blots of YAC and λ clones spanning the chromosome 8 bcr. Its identification was confirmed by subcloning the 2-kbp BamHI/XbaI fragment and sequencing it using oligos 380 and 425 as primers. Analysis of the genomic sequence upstream of position 1 in MTG8b does not show a pattern of motifs typical of eukaryotic promoters. The 3' boundary of MTG8b corresponds to a canonical donor splice site.

In a similar way, using oligonucleotide 379, MTG8a was mapped to a 3.1-kbp BamHI fragment 1.8 kbp centromeric to probe 8 (Fig 1). A 1-kbp fragment from this region, extending from the telomeric BamHI site to the centromeric end of the λ clone λ31-11 (probe 8C, Fig 1), was subcloned and sequenced. It was found to contain the whole of the MTG8a sequence bound at its 3' end by a canonical donor splice site. Thus, in the MTG8 transcriptional orientation, the chromosome 8 bcr is downstream of the MTG8b exon, but upstream of the MTG8a exon. Analysis of the genomic sequence upstream of position 1 in MTG8a does not show a pattern of motifs typical of eukaryotic promoters. Nucleotide sequence protection experiments were performed on RNA from a number of cell lines with a probe spanning 625 nt upstream of position 252 in MTG8a. No protected fragment could be detected in JMI, NH17,18 WT52, Daudi, Raji, K562, HL60, and U937 cell lines. The Raji findings contrast with the previously reported results8 and, if the two Raji samples are indeed identical, may be caused by the low abundance of the MTG8a species in this cell line. In contrast, full-length probe protection was observed in the t(8;21) cell line Kasumi-119 (data not shown). Because in the latter line the breakpoint on the der(8) chromosome does not fall between MTG8a and MTG8b (F.C. and J.E.T., unpublished results), this result suggests that the MTG8a exon extends for at least an extra ~370 nt in a 5' direction.

Alternative fusion transcripts. A reverse transcription/PCR strategy was used to investigate the occurrence of fusion runt/MTG8 transcripts in the series of patients with t(8;21) AML M2 previously tested for rearrangement at the runt locus and at the chromosome 8 bcr.4 cDNA was primed off total RNA either with oligo(dT) (oligo 288) or with a specific oligonucleotide mapping to the first MTG8 common exon (oligo 383), and subjected to PCR amplification using oligo 383 in combination with an oligonucleotide mapping to the runt box (oligo 382).

Using ~1 μg of total RNA and 1 round (30 cycles) of PCR, an ethidium bromide-stained band of a size corresponding to the previously described runt/MTG8 fusion product was readily detected in most patients (data not shown). However, additional ethidium bromide-stained bands were visible in some patients. Southern blotting analysis using a runt box probe at high stringency showed that all of the ethidium bromide-stained bands were strongly positive, whereas further bands were shown in some cases (Fig 3). To determine the nature of these extra bands, total PCR products and/or size-selected bands were cloned and sequenced from unique patient numbers (UPN) 1, 12, 13, 14, and 16. Four different species of fusion transcripts were identified (I through IV, Fig 4). The first (species I) was 241 nt long, and corresponded to the previously described in-frame runt/MTG8 fusion. In the other three species, an extra 46, 82, and 68 nt were found to be inserted between the two sequences. These insertions cause the runt and MTG8 reading frames to be out of register. Moreover, all three contain stop codons that terminate the runt frame 17, 11, and 20 nt, respectively, 3' of the junction.
Comparison with chromosome 8 sequences showed the origin of the insertions in every case (Fig 5). The 46 extra nucleotides of species II derive from the region spanned by probe 8; the extra nucleotides of species III correspond to the last 82 nt of the \textit{MTG8}a exon, correctly spliced onto the first \textit{MTG8} common exon; the extra nucleotides of species IV correspond to the last 68 nt of the intron immediately upstream of the first \textit{MTG8} common exon. The genomic sequences surrounding the inserted nucleotides were compared with the consensus splice sites. Using the Mount weight matrix, both sites flanking the 46-nt insertion and the acceptor splice site at the 5' end of the 82-nt insertion score midway between the best and the worst scores. However, the acceptor splice site at the 5' end of the 68-nt insertion scores below the worst score.

To investigate the occurrence of each species in individual patients, Southern blots of PCR products were reanalyzed with probes specific for each of the inserted sequences. In addition, PCR products from some patients were subjected to a second round of amplification using a primer specific for species IV (oligo 436) in combination with a runt box primer (oligo 382). The results are summarized in Table 2.

**Fig 4.** Sequences of the insertions found in the alternative runt/\textit{MTG8} fusion transcripts. For each one, the top line shows the join to runt (5') and \textit{MTG8} (3'), whereas the bottom line shows the flanking genomic sequences (small letters).
Species IV was detected in all patients, except for UPN 11. However, in the latter only a very weak signal was detected on Southern analysis of PCR products using a runt box probe (Fig 3). Species III was clearly detected in 20 of 25 patients (80%). However, by hybridization to oligo 398, species II was detected only in eight patients (32%), all of whom also scored positive for both species III and species IV.

Sequence homology of the MTG8 gene. The MTG8 nucleotide sequences were compared with both the GenBank and EMBL databases. A highly significant score was observed to EST04880, which corresponds to a human fetal brain cDNA clone (clone HFBCB51). Positions 1 to 294 in this clone match positions 1328 to 1627 in the MTG8a cDNA, with 66% identity. Homology is even higher in the first 200 nt (73.5%). Over this stretch, comparison of the predicted translation products shows 76% identity, with similarity increasing to 86% if conservative changes are taken into account (Fig 6). The boundaries of the homology domain correspond nearly exactly to a region that is most likely to fold in an α-helical conformation, according to secondary-structure predictions. The homology domain is not related to any other known motif in the database. MTG8 has been reported to be expressed most highly in mouse brain.

DISCUSSION

Up until now, three main findings have emerged from the molecular analysis of the t(8;21). First, translocation breakpoints involve the runt locus on chromosome 21 and the MTG8 locus on chromosome 8. Second, at the genomic level, breakpoints are clustered within both loci. Third, at the mRNA level, transcripts originating from the der(8) chromosome have been identified, which have an in-frame fusion between the runt box and the whole MTG8 coding sequence, but for the first few codons. It has been assumed that synthesis of the chimeric runt/MTG8 polypeptide represents a critical leukemogenic event. However, the fusion transcripts have been detected not only in the leukemic phase, but also in remission. Thus, they may not be sufficient, per se, for leukemogenesis.

The present work contributes two novel, provocative, findings. First, the majority of chromosome 8 breakpoints map not only upstream of the most 5' MTG8 exon present in the previously identified runt/MTG8 fusion transcripts, but also upstream of a further 5', largely noncoding, MTG8 exon (MTG8a). This is the case in at least 11 of the 18 patients with t(8;21) AML M2 that we have analyzed (J.E.T. and F.C., in preparation). Second, alternative fusion transcripts, in which the runt coding frame is blocked a short distance 3' of the runt box, have been identified in virtually all patients.

The position of the chromosome 8 bcr cannot be explained simply by a leukemogenic requirement for an in-frame runt/MTG8 common exon fusion. If this was the case, breakpoints would be expected to fall anywhere upstream of the first MTG8 common exon, rather than to cluster upstream of the MTG8a exon. We propose, instead, two possible explanations. The chromosome 8 bcr may represent a recombination hotspot. Alternatively, its position may reflect positive, or

### Table 2. Occurrence of Individual Out-of-Frame runt/MTG8 Transcripts in the Panel of t(8;21) Patients Analyzed in This Study

<table>
<thead>
<tr>
<th>UPN</th>
<th>Species II (46 nt)</th>
<th>Species III (82 nt)</th>
<th>Species IV (68 nt)</th>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
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</tr>
</tbody>
</table>

26 t(8;21)∗

The presence of each species was assessed on the Southern blots shown in Fig 4 by sequential hybridization to oligo 398 (species II), probe 8C (Fig 1, species III), and oligo 436 (species IV), except for the cases marked with an asterisk (*), where detection was by PCR with oligo 362 and 436 on the same PCR material as that used for the blots.
The S' boundaries of both the MTG8a and MTG8b exons have not been defined. The genomic sequences upward of position 1 in either of the cloned cDNAs do not show patterns of motifs (cap sites, TFIID, and Sp1 binding sites) characteristic of eukaryotic promoters. Furthermore, nuclearase protection experiments suggest that at least 370 nt upstream of the reported position 1 of MTG8a are transcribed into RNA. Consistent with these findings, the corresponding region is well conserved in the mouse, as judged by Southern blotting analysis. A similar degree of conservation, typical of transcribed regions, is found also in other stretches of DNA extending telomeric to MTG8a. The MTG8 species identified on Northern blotting analy-

Fig 6. Alignment of MTG8 and cDNA clone HFBCB51 (EST04880). Vertical lines indicate nucleotide identity; dashes, amino acid identity; italics, conservative amino acid replacements.

products. We cannot at present give an accurate estimate of the relative amounts of the different fusion transcripts.

Although the in-frame runt/MTG8 species appears to be the most abundant, a differential efficiency of amplification and, in particular, a bias against the longer species cannot be excluded. Thus, the relative intensities resulting from hybridization to the common runt box probe (Fig 3) may be misleading. Given the complexity of the pattern of hybridization, further variants may well exist, at least in some patients. In addition, the experimental approach does not exclude the presence of other alternative transcripts involving runt upstream exons or MTG8 downstream exons.

Alternative fusion transcripts have been described in other reciprocal chromosome translocations, of which the closest parallel may be the t(15;17) of AML M3. In this, some such transcripts would encode truncated forms of the PML gene product, analogous to the truncated runt predicted by our findings. However, the significance of these has not been investigated further.

If runt truncation, rather than its fusion to MTG8, is the leukemogenic mutation, it may be expected to exert its effects through a dominant negative mechanism. Truncation occurs at the COOH-end of the runt box, which has been reported to be a DNA binding domain. Its separation from the COOH-terminal domain(s) may produce a polypeptide capable of competing for DNA binding sites, but not capable of interacting with other factors. Thus, it may behave as a competitive repressor/activator. Alternatively, the normal runt locus may be functionally haploid, perhaps because of imprinting.
sions have been sized at greater than 5.7 kb, of which only ~2.6 kb have been cloned.\(^8\) It is possible that the additional sequences are at the 5' end. \(MTG8a\) and \(MTG8b\) may represent either two alternatively spliced internal \(MTG8\) exons or two alternative first exons with separate promoters. Further mapping data, by way of nucleoside protection/primer extension, complemented by functional promoter tests, will be required to address these questions. The finding that the \(MTG8b\) exon is consistently 5' of the bcr implies that at least one \(MTG8\) promoter is potentially active on the der(21) chromosome. However, using PCR on patients' samples, we have been unable to find any evidence of transcripts containing both \(MTG8b\) and sequences 3' to the runt box in the published cDNA.\(^{25}\)

ACKNOWLEDGMENT

We are particularly indebted to the UK HGMP Resource Centre for providing yeast chromosome plugs from the ICI human YAC library and for excellent computing facilities, and to Prof N. Kamada for providing the Kasumi-1 cell line. We are very grateful to Prof L. Luzzatto for encouragement and for critically reading the manuscript, and to Prof Luzzatto and the Department of Haematology, Royal Postgraduate Medical School, for generous hospitality.

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Alternative, out-of-frame runt/MTG8 transcripts are encoded by the derivative (8) chromosome in the t(8;21) of acute myeloid leukemia M2

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