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Identification of a commonly amplified 4.3 Mb region with overexpression of *C8FW*, but not *MYC* in *MYC*-containing double minutes in myeloid malignancies

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Double minutes (dmin), the cytogenetic hallmark of genomic amplification, are found in ~1% of karyotypically abnormal acute myeloid leukemias (AML) and myelodysplastic syndromes (MDS). The *MYC* gene at 8q24 has been reported to be amplified in the majority of the cases, and generally it has been assumed that *MYC* is the target gene. However, only a few studies have focused on the extent of the amplicon or on the expression patterns of the amplified genes. We have studied six cases (five AML and one MDS) with *MYC*-containing dmin. Detailed fluorescence *in situ* hybridization analyses identified a common 4.3 Mb amplicon, with clustered proximal and distal breakpoints, harboring eight known genes (*C8FW*, *NSE2*, *POU5FLC20*, *MYC*, *PVT1*, *AK093424*, *MGC27434* and *MLZE*). The corresponding region was deleted in one of the chromosome 8 homologues in five of the six cases, suggesting that the dmin originated through extra replication (or loop-formation)–excision–amplification. Northern blot analysis revealed that *MYC* was not overexpressed. Instead, the *C8FW* gene, encoding a phosphoprotein regulated by mitogenic pathways, displayed increased expression. These results exclude *MYC* as the target gene and indicate that overexpression of *C8FW* may be the functionally important consequence of 8q24 amplicons in AML and MDS.

INTRODUCTION

Among the acquired molecular genetic abnormalities occurring in, and characterizing, hematologic malignancies, most studies have focused on translocation-generated fusion genes, inactivated tumor suppressor genes and activating point mutations in oncogenes (1–3). Less is known about neoplasia-associated genes that may be amplified and overexpressed. Cytogenetic hallmarks of genomic amplification are homogeneously staining regions (hsr) and double minutes (dmin), the latter being small, paired, acentric and usually spherical chromatin bodies (4,5). Despite the quite frequent occurrence of hsr and dmin in human neoplasia, particularly in neuroblastoma, alveolar rhabdomyosarcoma and carcinomas of the breast, head and neck region, ovary and pancreas (6), little is known about the underlying molecular mechanisms involved in the genesis of hsr and dmin, although several possible models have been suggested, such as 'chromosome breakage–interstitial deletion–pulverization' (7) and 'deletion-plus-episome' or 'extra replication (or loop-formation)–excision–amplification' (8). Furthermore, in spite of the fact that several amplified genes have been identified, for example *MYCN*, *PAX7/PAX3-FOXO1A* and *MDM2* (9–13), few studies have focused on the functional outcome of the amplifications—that is, have included expression data (11–13)—or have addressed the size of the amplicons (14–16).

Double minutes are quite rare in hematologic malignancies. In general, they are found in $<1\%$ of cytogenetically abnormal

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acute myeloid leukemias (AML), chronic myeloid leukemias (CML), myeloproliferative disorders, myelodysplastic syndromes (MDS), acute lymphoblastic leukemias, plasma cell dyscrasias and malignant lymphomas, being more common in elderly AML/MDS patients (6,17). Because of the rarity of dmin in malignant hematologic disorders, their clinical and prognostic impact has not been well elucidated. It has been suggested that dmin in AML are associated with reduced responsiveness to chemotherapy and hence a poor prognosis (18), leading to their inclusion in the 'unfavorable' cytogenetic category by some groups (19,20). However, this prognostic impact has been questioned. It may not be the presence of dmin as such, but rather the number of dmin per cell as well as the general cytogenetic pattern that is correlated with survival. In fact, AML patients with complex karyotypes harboring dmin have a dismal outcome, whereas those with an otherwise normal karyotype or with only a single chromosomal aberration in addition to numerous dmin seem to fare better (17,21,22).

Molecular genetic studies and fluorescence *in situ* hybridization (FISH) analyses of dmin-carrying hematologic malignancies have revealed that most cases show either *MYC* (8q24) (6,18,23–25) or *MLL* (11q23) amplification (6,26–28), with single cases harboring other amplified genes, such as *ETS1*, *FLI1*, *SRPR*, *NFRKB* and *QCRFS1* (29,30). As the *MYC* gene is involved in normal hematopoiesis and is also strongly implicated in leukemogenesis (31,32), generally it has been assumed that the important outcome of *MYC*-containing dmin is amplification and overexpression of this gene—that is, *MYC* has been considered as the target gene (18,23,24,30,33). However, the expression pattern of this gene in hematologic malignancies with *MYC*-positive dmin has only been investigated in the leukemic cell line HL60 (34) and in one MDS, a chronic myelomonocytic leukemia (CMML) (22). In the HL60 line, overexpression of not only *MYC* but also other transcripts mapping to 8q24 were found, whereas *MYC* was not overexpressed in the primary MDS case. Taken together, the presently available data suggest that *MYC* is not the true target of the amplification but that abundant expression of another gene(s) included in the 8q24 amplicon is the pathogenetically important consequence.

In the present study, we have performed a detailed FISH mapping of six AML/MDS cases with *MYC*-containing dmin as well as expression analyses of possible target genes.

RESULTS

Identification of a 4.3 Mb commonly amplified segment

The initial FISH data showing *MYC* amplification in the six AML/MDS cases with dmin, using the LSI C-MYC probe (Abbott, Stockholm, Sweden), were confirmed using the P1 artificial chromosome (PAC) RP1-80K22, which contains the *MYC* gene (Fig. 1). In all cases except one (case 4, Table 1), this PAC hybridized to only one of the two chromosome 8 homologues (Fig. 2A–C). Thus, the dmin were accompanied by a submicroscopic deletion of the corresponding 8q24 segment, del(8)(q24q24), in five of the six cases. Quantitative fluorescent polymerase chain reaction (QF-PCR) with subsequent fragment analysis of case 4 did not reveal any loss of heterozygosity for five chromosome 8 markers, excluding the possibility of uniparental disomy (UPD) for chromosome 8 in this case, i.e. there was no evidence that a del(8) had occurred in case 4, but that the deleted chromosome 8 had been lost and the remaining non-deleted chromosome 8 duplicated.

To characterize the amplified region on the dmin in detail and to map the proximal breakpoints (pb) and the distal breakpoints (db) on the del(8), a bacterial artificial chromosome (BAC) contig consisting of 34 clones was selected and used in further FISH experiments. The overall results are given in Figure 1. The pb in case 1 mapped to the overlapping region between RP11-344N13, RP11-317L5 and RP11-550A5, and could be localized to RP11-344N13 because this clone was only partially amplified, as seen as a weaker signal on the dmin compared with the one on the normal chromosome 8. In case 3, the pb was included in the region covered by RP11-344N13, RP11-144I22 and RP11-550A5. In cases 2 and 4–6, the pb mapped to the border between RP11-550A5 and RP11-136O12, in the middle of RP11-1018E4, which was partially amplified (Fig. 2A). The db were mapped to the border between RP11-419K12 and RP11-625F17, in the middle of RP11-274M4, in cases 1 and 3, to the region covered by RP11-625F17, RP11-473O4 and RP11-274M4 in cases 2, 4 and 6 and to the overlapping region between RP11-625F17 and RP11-473O4 in case 5. The pb and db in the concomitant del(8)(q24q24) in cases 1, 5 and 6 were identical to the breakpoints observed in the dmin in these cases—that is, all probes deleted in one of the chromosomes 8 were amplified (Fig. 2B). In cases 2 and 3, the proximal RP11-344N13 and the distal RP11-625F17 probes were deleted in the del(8) but not amplified on the dmin (Fig. 2C). Thus, in these two cases, the deleted segment was larger than the amplicons.

Based on the FISH mapping (Fig. 1), the amplicons in cases 1–6 were shown to be 4.5, 4.4, 4.5, 4.4, 4.5 and 4.4 Mb, respectively. The commonly amplified region was 4.3 Mb. Chromosome 8 sequences surrounding the pb and db and within the entire amplicons (base pairs 126 000 000–131 000 000; Fig. 1) were analyzed using the GenAlyzer program. No intrachromosomal duplicons were identified within the investigated genomic segment.

Overexpression of the *C8FW* gene

Northern blot analysis of cases 4 and 6 (lack of material precluded analysis of the other cases) revealed that the *C8FW* gene was clearly overexpressed when compared with the expression level in bone marrow (BM) cells from a healthy donor, two hematologic malignancies (CMML and AML, respectively) lacking dmin and +8, and the cell lines K562 and U937 (Fig. 3). In contrast, *MYC* expression in cases 4 and 6 was low, almost undetectable, when compared with, for example, the AML case without dmin and the K562 cells. With one exception, the *NSE2*, *POU5FLC20*, *PVT1* and *MGC27434* genes showed equal expression level throughout the samples investigated; *MGC27434* showed a higher expression in K562 cells (Fig. 3). No signals were obtained upon hybridization with probes derived from

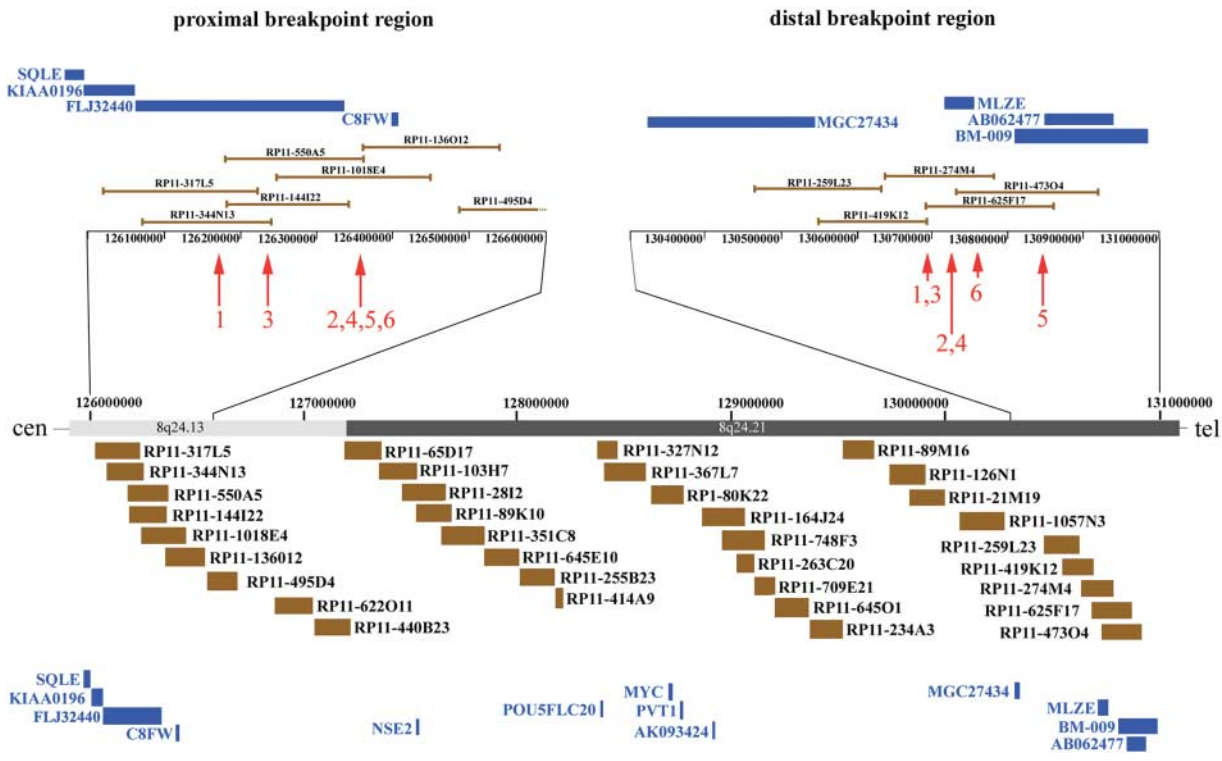

Figure 1. Genomic mapping of the amplified 8q24 segment. The location of the BAC/PAC probes (brown) and genes (blue) is in accordance with the latest update (July 2003) of the UCSC Human Genome Browser (http://www.genome.ucsc.edu/). The pb and db of the dmin in the six AML/MDS patients are denoted by red arrows.

Table 1. Clinical and cytogenetic features of the six myeloid malignancies with *MYC*-containing double minutes

Case no.	Sex/age	Diagnosis	Karyotype	Median no. of dmin/cell (range)[a]
1	F/65	AML M2	45,XX,−5,−9,−11,add(17)(p13), + 2mar,dmin[32]	50 (14–125)
2	M/64	AML M2	41–44,X,−Y,del(1)(p13),del(5)(q13q33), + 6,−7, del(9)(p21)x2,−17,−22,−22, + der(?)t(?;1)(?;p13), + mar,dmin,inc[28]	36 (10–60)
3[b]	F/82	AML M2	42,X,−X,?der(5;22)(p10;q10),inv(8)(p?q?),−9,−11, add(12)(q11),−17,−18, + der(?)t(?;12)(?;q2?),dmin,inc[25]	40 (10–100)
4[b]	F/77	CMML	46,XX,8–40dmin[12]/46,XX[13]	28 (9–61)
5	F/61	AML M1	46,XX,del(5)(q15q33),?del(14)(q21q23),add(17)(p11), del(20)(q11–12),dmin[6]/46,idem,der(9)del(9) (p21)add(9)(q34)[13]/46,XX[4]	43 (14–100)
6	M/66	AML M2	46,XY,1–40dmin[22]/46,XY[3]	52 (16–93)

F, female; M, male; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia.
[a]As ascertained by FISH.
[b]The karyotypes of cases 3 and 4 have been published previously (22,35).

AK093424 and *MLZE*, indicating that these genes were not expressed in the investigated samples (data not shown).

DISCUSSION

The salient results of the present genomic mapping and expression analyses of the AML/MDS cases with *MYC*-containing dmin were the identification of a common 4.3 Mb amplicon, with clustered pb and db, the detection of concomitant deletions, corresponding to the amplified segments, in one of the chromosome 8 homologues in all but one of the cases, and the finding that the *C8FW* gene—but not the *MYC* gene—was overexpressed.

We know of only two previous studies which, to some extent, investigated the size of the amplicons in primary hematologic malignancies with *MYC*-containing dmin (14,15). They showed, using Southern blot analysis in two AML cases, that the amplified segment also included the *PVT1* gene and that it was at least 780 kb in one of the cases, concluding that *MYC*-positive dmin may harbor other genes

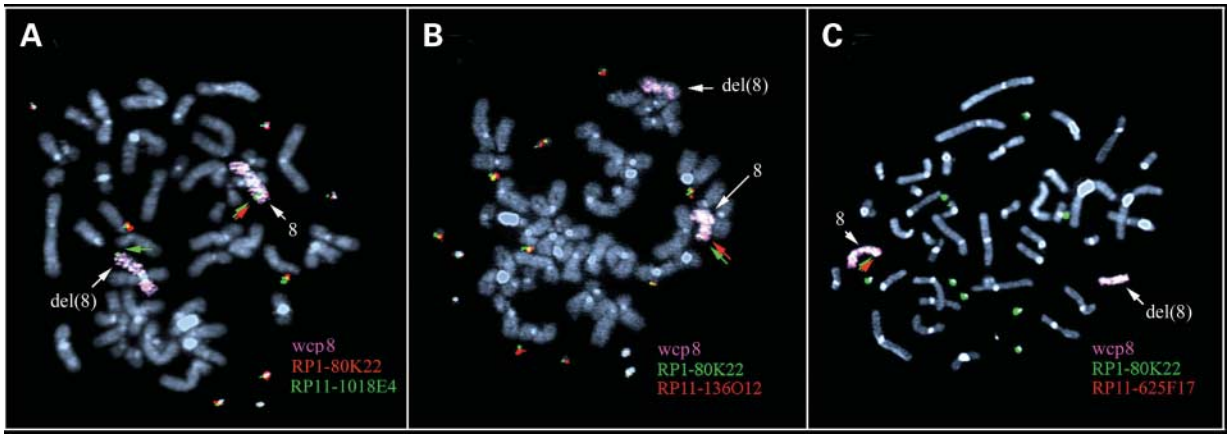

Figure 2. Examples of FISH findings. Co-hybridization experiments using wcp8, RP1-80K22 (*MYC*) and (**A**) the BAC RP11-1018E4 in case 6 [the BAC spans the pb, being deleted partially in the del(8)(q24q24) and amplified partially in the dmin], (**B**) the BAC RP11-136O12 in case 6 [the BAC is deleted in the del(8) and amplified in the dmin] and (**C**) the BAC RP11-625F17 in case 2 [the BAC spans the db, being deleted in the del(8) but not amplified in the dmin].

that contribute to the leukemogenic process. The finding of a common 4.3 Mb amplicon (Fig. 1) in the present six AML/MDS cases clearly agrees with this conclusion, revealing that the amplified region is notably larger than the previous estimates. Further, although indirect, support for amplifications involving several megabases in *MYC*-harboring dmin comes from comparative genomic hybridization (CGH) studies (21,24) revealing CGH-detectable amplifications of 8q24. Considering the resolution limit of CGH, these amplicons must have been at least 2 Mb (36). Furthermore, the dmin in the HL60 cell line were estimated to extend between 8q24.11 and 8q24.2 (34), an ∼10 Mb segment. That dmin-associated amplicons may be several megabases has also been shown in non-*MYC*-containing dmin in AML, in some solid tumors and in several cell lines (7,9,11,13,27,29). Thus, the sizes of the amplified regions far exceed the size of a single gene, making expression analyses mandatory before deciding that the mere presence of a particular gene—for example, *MYC*—is the pathogenetically and functionally important one.

The pb and db of the dmin in all the six AML/MDS cases clustered, at the resolution level of FISH with BAC clones, within genomic regions of 200 and 150 kb, respectively (Fig. 1). This recurrence of both pb and db is indicative of a particular genomic architecture—that is, low copy repeats, known as duplicons—in the vicinity of the breakpoint regions. Such duplicons may play a crucial role in triggering intrachromosomal homologous recombination, as has been described in numerous disease-associated somatic DNA rearrangements (37) as well as in some acquired chromosomal changes, for example t(9;22)(q34;q11) in CML (38) and i(17q) in AML, CML and MDS (39). However, the present *in silico* analyses revealed no duplicons surrounding the breakpoints or within the amplified region. Although this would seem to argue against the involvement of repetitive element in the origin of dmin, it should be stressed that other types of recombination promoting sequences (40–42) cannot be excluded.

The finding that five of the six AML/MDS cases harbored a deletion, corresponding to the segment included in the amplicons, in one of the chromosome 8 homologues strongly suggests a likely underlying mechanism for dmin generation—that is, the 8q24 segment was deleted, retained, circularized and then amplified. Similar concomitant interstitial deletions have been reported previously in leukemias and solid tumors with dmin harboring *MYC*, *MLL*, *HMGIC* and *MDM2* (11,18,25,28,33). Thus, dmin formation seems to be coupled to chromosomal deletions, as would be expected based on the 'deletion-plus-episome' or 'extra replication (or loop-formation)–excision–amplification' model (8). However, the lack of a deletion in one of the cases (case 4) and the finding that the deletions were larger than the amplified segments in two of the cases (cases 2 and 3) could indicate that dmin formation may be more complex. That a deletion nevertheless had occurred also in case 4 but that the deletion-harboring chromosome 8 had been lost and the remaining non-deleted homologue duplicated was excluded based on the result of the UPD analysis, showing that the two chromosomes 8 were not identical. However, if trisomy 8 occurred prior to the dmin formation, an interstitial 8q24 deletion in one of the trisomic chromosomes followed by loss of the del(8) remains a possibility. The finding that the deleted segments in cases 2 and 3 were larger than the amplicons may not be surprising considering the quite common occurrence of concomitant deletions flanking the breakpoint regions of neoplasia-associated translocations and inversions (43,44).

The results of the present northern blot analyses, including all the eight known genes in the amplified segment, clearly support our previous conclusion (22) that *MYC* may not be the target gene in *MYC*-positive dmin. The expression of *MYC* was not increased compared with the control samples; in fact, there were higher transcript levels of *MYC* in some of the control samples, including the AML and CMML cases without dmin (Fig. 3). Of the eight investigated genes, only *C8FW* was overexpressed in the dmin-harboring cases, showing notably lower expression in all other samples (Fig. 3). Unfortunately, little is known about this gene. According to LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/), *C8FW* encodes a phosphoprotein, which is regulated by mitogenic pathways. Interestingly, C8FW is homologous to the protein kinase SKIP3 that recently has been reported to be

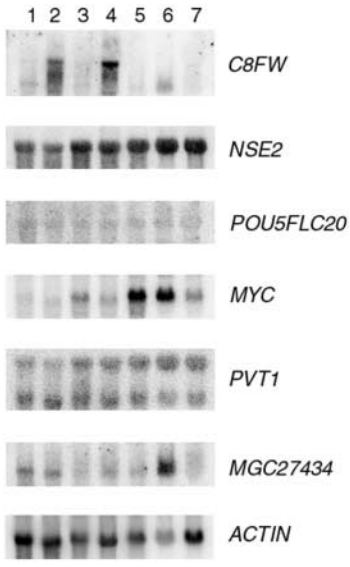

Figure 3. Expression patterns of the genes included in the amplified 8q24 segment. Northern blot results of the six genes expressed in the investigated samples. The genes analyzed are listed to the right; the order (top to bottom) is in accordance with their position (cen to ter). Lane 1, normal BM; lane 2, dmin-positive CMML (case 4 in Table 1); lane 3, CMML case without dmin and trisomy 8; lane 4, dmin-positive AML (case 6 in Table 1); lane 5, AML case without dmin and trisomy 8; lane 6, cell line K562 and lane 7, cell line U937.

overexpressed in several tumor types and suggested to play a crucial role in tumor progression (45). Furthermore, C8FW has been shown to bind to the arachidonic acid metabolizing enzyme 12-LOX, which is also overexpressed in human cancer tissue (46). These data, together with the present results, indicate that overexpression of the *C8FW* gene may be the functionally important outcome of 8q24-amplicons in AML and MDS.

MATERIALS AND METHODS

Patients

Among the 1000 AML and 734 MDS cytogenetically investigated since 1972 at the Department of Clinical Genetics, Lund, Sweden, eight (0.8%) AML and four (0.5%) MDS have displayed dmin. Of these, cells in fixative were available in 11 cases. Initial FISH analyses using the LSI C-MYC probe (Abbott), mapping to 8q24, revealed that *MYC* was amplified in six AML/MDS cases. The dmin in these were further characterized by a detailed FISH mapping. The clinical and cytogenetic features of the six patients are listed in Table 1. The investigation was approved by the Research Ethics Committee of Lund University.

FISH probes and analyses

One PAC clone and 34 BAC probes, mapping to 8q24, were obtained from the RPCI-1 and the RPCI-11 P. de Jong libraries, respectively (http://www.chori.org/bacpac). The locations of the clones were based on the latest update (July 2003) of the UCSC Human Genome Browser (http://www.genome.ucsc.edu/). In addition, a whole chromosome painting (wcp) probe for chromosome 8, used in all hybridizations, was obtained from Abbott. The BAC/PAC probes were labeled directly with Cy3–dUTP, FluorX–dCTP or Cy5–dUTP, or indirectly with Biotin–dUTP using Amersham's Megaprime DNA Labelling System (Amersham Place, UK) and detected with streptavidin–diethylaminocoumarin. FISH was performed essentially as described previously (47), and the signals were analyzed with the Chromofluor System (Applied Imaging, Newcastle, UK).

Bioinformatics and sequence analyses

Segmental duplications surrounding the pb and db regions or occurring within the entire 8q24 amplicon were searched using the GenAlyzer program (http://www.genomes.de/), which is an improved version of the Reputer software (48), on a masked sequence, downloaded from the UCSC site (ftp://genome.ucsc.edu/goldenPath/hg16/chromosomes/).

Analysis of uniparental disomy

In all cases except case 4 (Table 1), the region in 8q24 corresponding to the amplicons was deleted in one of the two chromosome 8 homologues, indicating a possible mechanism for dmin formation. One reason for the lack of such a deletion in case 4 could be that a deletion had occurred, but that the deletion-harboring chromosome 8 had been lost and the remaining non-deleted chromosome 8 duplicated. If so, this would be detectable as a UPD for chromosome 8—that is, both copies would be derived from the same parent—resulting in widespread loss of heterozygosity on this chromosome. This possibility was investigated by QF-PCR with subsequent fragment analysis, using the microsatellite markers *D8S639* (8p22), *D8S381* (8q13), *D8S1804* and *D8S1799* (8q24, centromeric to the amplicon) and *D8S284* (8q24, telomeric to the amplicon) (http://www.genome.ucsc.edu/). The markers were selected based on their location as well as on high maximum heterozygosity (no constitutional DNA was available from case 4). DNA was extracted according to standard methods from cells stored at -80°C for 3 years, and QF-PCR and fragment analyses were performed as described previously (49).

Northern blot analysis

Total RNA from BM cells of cases 4 and 6 (Table 1) (RNA was not available from cases 1–3 and 5), one AML and one CMML without dmin and trisomy 8, a healthy donor and the control cell lines K562 and U937 was extracted using the Trizol reagent as described by the manufacturer (Invitrogen Corporation Inc. Stockholm, Sweden). The total RNA (5 μg) was electrophoresed through 1% formaldehyde/formamide gels, blotted as described (50) and subsequently hybridized with ^{32}P-labeled probes generated by PCR amplification of individual genes (primer sequences available upon request). The identity of all probes was verified by sequencing, using the BigDye mix (Applied Biosystems, Warrington, UK). After hybridization and washing, the filters were analyzed by

phosphorimaging. β-Actin was used as a probe to verify equal loading and transfer of RNA.

The expression patterns of the following eight genes were investigated: cen-*C8FW*, *NSE2*, *POU5FLC20*, *MYC*, *PVT1*, *AK093424*, *MGC27434* and *MLZE*-tel. These genes were selected because they all map within the commonly amplified segment and because all, except *PVT1*, are considered presently as 'known genes', based on SWISS-PROT, TrEMBL, mRNA and RefSeq, as reported in the UCSC Human Genome Browser (http://www.genome.ucsc.edu/). The *PVT1* gene, which is not listed in this database, was included in the expression analyses because of its previously reported involvement in hematologic malignancies and close proximity to the *MYC* gene (51,52). To verify the location of the *PVT1* gene, we mapped the *PVT1* mRNA (accession number M34428), using the BLAT tool (53) in the UCSC Human Genome Browser, and showed that *PVT1* is located 53 kb 3′ of *MYC*, within the commonly amplified segment.

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