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Transcription Factor GATA-2 Gene Is Located Near 3q21 Breakpoints in Myeloid Leukemia

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Rearrangements affecting chromosome band 3q21 are observed in a subgroup of patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). However, little is known about the molecular consequences of such aberrations. We therefore established a PAC contig in the 3q21 breakpoint region and identified potential protein coding sequences by exon trapping. One of the exons isolated was from the human GATA-2 gene, which we showed to be transcribed from telomere to centromere. The majority of 3q21 breakpoints are located telomeric to the transcribed portion of this gene in a region that in mice appears to be necessary for proper promoter function. Results of GATA-2 expression analyses in leukemic cell lines as well as primary patient samples are compatible with the hypothesis that 3q21 aberrations contribute to leukemogenesis through deregulation of the hematopoietic transcription factor GATA-2.

Key Words: **acute myeloid leukemia; 3q21; chromosome breakpoint; GATA-2.**

The inversion inv(3) (q21q26) and the translocation t(3;3) (q21;q26) are observed in a subset of patients with myeloid leukemia and are associated with megakaryocytic dysplasia, normal or elevated platelet counts, and a particularly poor clinical outcome (1–4). Even though a candidate oncogene, EVI-1, has been identified in 3q26 (5), other genes located either in 3q26 or in 3q21 might also play a role in the pathogenesis of leukemias bearing the inv(3) or t(3;3) rearrangements. Several transcribed sequences have recently been identified in the relevant region in 3q21 (6, 7), but none of these has been clearly implicated in leukemogenesis. A 3q21 "breakpoint cluster region" (BCR) of approximately 35 kb has been defined (summarized by Rynditch *et al.* (7)); however, more recently, a number of breakpoints have been shown to map centromeric of this region (8–10).

GATA-2 belongs to a family of zinc finger transcription factors that has six known members in mammals. Among these, GATA-1 and GATA-2 show partially overlapping expression patterns in various hematopoietic lineages (11–16). However, while GATA-1 is required for terminal differentiation of erythroid cells (17, 18), expression studies and knockout experiments indicate that GATA-2 is required in the early, proliferative phase of hematopoietic development (15, 16, 18–20). GATA-2 knockout mice could be rescued from embryonic death by YAC transgenes, but properly regulated hematopoietic expression of GATA-2 was dependent on the presence of at least 150kb of upstream sequences (21).

Ectopic expression of GATA-2 has yielded controversial results, promoting proliferation in some experimental systems and differentiation in others (22–25). Nevertheless, since even moderate overexpression had dramatic effects, these experiments clearly demonstrate the importance of precisely regulated expression of this transcription factor in hematopoietic development.

The biological functions of GATA-2 and the importance of its balanced expression have led to the suggestion that it might be involved in leukemogenesis (19). However, despite the fact that GATA-2 seems to be overexpressed in a large percentage of myeloid leukemia samples (26, 27), no correlation has yet been

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The sequence reported in this paper was submitted to GenBank (Accession No. AF 169253).

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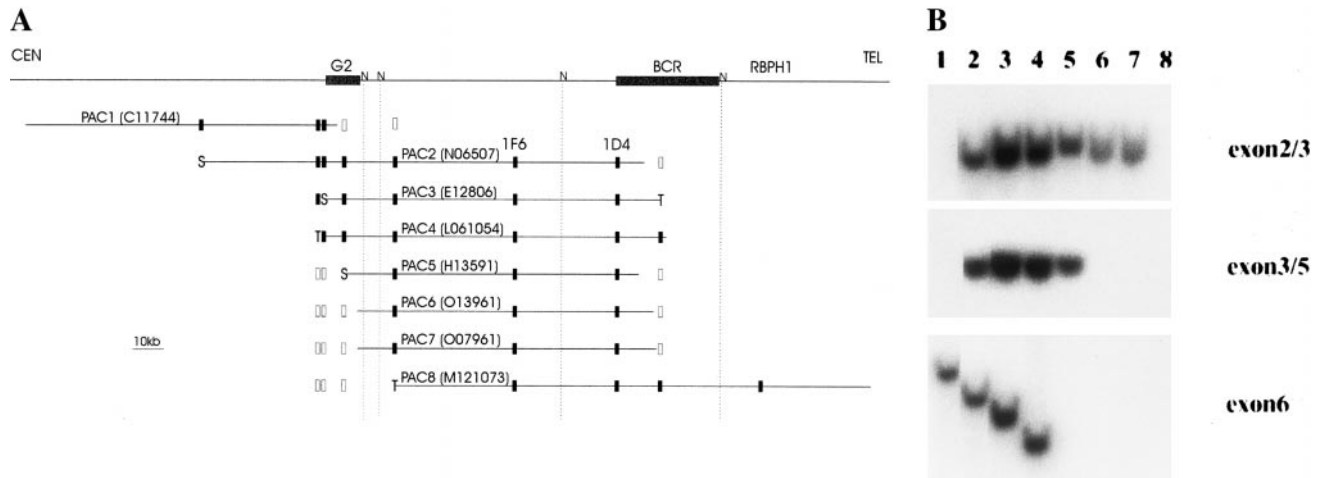

FIG. 1. GATA-2 is located near 3q21 breakpoints in AML. (A) 3q21 PACs used in this study. For clarity, PACs have been numbered 1 to 8, and actual clone names are indicated in brackets. The centromere is to the left and the telomere to the right. N, *Not*I restriction site. G2, GATA-2 coding region, BCR, breakpoint cluster region (7), RBPH1, Ribophorin1 gene. Relative positions of the PACs were determined by hybridization of various probes to PAC DNA blots. 1F6 and 1D4 are transcribed sequences previously mapped to this region (7). S and T designate probes generated from the Sp6 and T7 ends of PACs, respectively. Closed rectangles indicate hybridization of the respective probe to the PAC; open rectangles indicate absence of hybridization. The centromeric ends of PACs 3 and 4 recognize an aberrantly sized fragment in the respective other PAC, most likely indicating that these end probes overlap. (B) Probes corresponding to the indicated exons of GATA-2 were hybridized to a Nylon membrane containing *Eco*RI digested PAC DNA. Numbering above the lanes corresponds to PAC numbers as indicated in A. The exon3/5 probe hybridized to 2 or 3 bands in each of the PACs; only one of these is shown for reasons of clarity. Fragments detected by the exon 6 probe vary in size, most likely due to the fact that they represent the ends of the respective PAC inserts. All fragment sizes are compatible with the genomic map shown by Nagai *et al.* (12). Hybridization patterns indicate that the GATA-2 gene is transcribed from telomere to centromere.

made between a leukemia associated genetic alteration and the GATA-2 gene.

In this report we show that the hGATA-2 gene is located approximately 100kb centromeric of the proposed 3q21 'breakpoint cluster region'. The t(3;3) breakpoint of the cell line HNT-34, which maps centromeric of the 'BCR', splits the signal of a PAC containing the GATA-2 gene in FISH experiments. In addition, most previously described breakpoints are located in the presumptive GATA-2 regulatory region which might be crucial for proper expression during hematopoiesis. Indeed, we found that GATA-2 is deregulated in primary AML samples as well as in established cell lines with 3q21 aberrations.

METHODS

Cell culture. All cell lines were cultured in RPMI medium supplemented with 10% FCS at 37°C in a 5% CO_2 atmosphere. Culture medium for MUTZ-3 cells contained 100 U/ml GM-CSF.

PAC contig construction and Southern hybridizations. The PACs used in this study were isolated from the RPCI_1, 3, 4, and 5 libraries, which had been constructed at the Roswell Park Cancer Institute by P. Ioannou and P. de Jong from DNA of a normal male blood donor (28). PAC clones were provided by the Resource Centre of the German Human Genome Project (RZPD) in Berlin. Details of contig construction are described elsewhere (10).

For Southern hybridizations, the following GATA-2 cDNA probes were used: exon 2/3 probe, bases 425 to 835, and exon 3/5 probe, bases 1055 to 1327 of the sequence published by Lee *et al.* (29) (GenBank Accession No. M68891); and exon 6 probe, bases 2690 to 3062 of the sequence published by Nagai *et al.* (12). A 1-kb genomic clone containing the newly identified IS exon was also used. Probes were hybridized to DNA blots at 65°C in Church buffer (0.5 M $NaPO_4$, pH 7.0, 7% SDS, 1 mM EDTA) and filters were washed at 65°C with 40 mM $NaPO_4$ pH 7.0, 1% SDS, 1mM EDTA.

Low stringency hybridization with the murine IS exon probe (bases 1571 to 1849 of the sequence published by Minegishi *et al.* (30) (Accession No. AB009272) was performed in Church buffer at 55°C or 58°C. Washes were carried out at increasing stringency, with final washes in 0.2× SSC, 0.1% SDS at 65°C.

Exon trapping, cloning procedures, and sequencing. For exon trapping, the exon trapping system from Gibco Life Technologies (Rockville, MD) was employed essentially according to the manufacturer's instructions.

A 3-kb *Hin*dIII fragment of PAC 4 that hybridized to the murine GATA-2 IS exon probe was cloned into pBluescript, and a 1-kb exonuclease III (Promega, Madison, WI) deletion subclone thereof was sequenced. PCR products were cloned into pGEM-T (Promega) before sequencing. Sequence analysis was performed on a LiCor sequencer (MWG Biotech, Ebersberg, Germany) using the thermosequenase sequencing system (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

RNA isolation, RT-PCR, and RACE. RNA was extracted using Trizol reagent (Gibco Life Technologies), and cDNA was synthesized using M-MLV reverse transcriptase (Roche Diagnostics, Basel, Switzerland) according to standard protocols. PCRs were carried out in a buffer containing 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.001% gelatin, pH 8.3, in a Perkin–Elmer 9700 thermal cycler (Perkin–Elmer, Norwalk, Connecticut). For amplification of GC-rich sequences (i.e., the IS exon sequences), Dynazyme (Finnzymes, Espoo, Finland) was used with the buffer supplied by the manufacturer. DMSO was added to a final concentration of 5%.

Transcription of the hGATA-2 IS exon was verified using hGATA2-IS1, 5′-AAGGTAGCGAGGCCAGCGT-3′, and hGATA2-444rev, 5′-TGCGAGTCGAGGTGATTGAAG-3′, the latter of which is located in exon 2. Annealing was at 55°C. For long distance PCR on PAC DNA

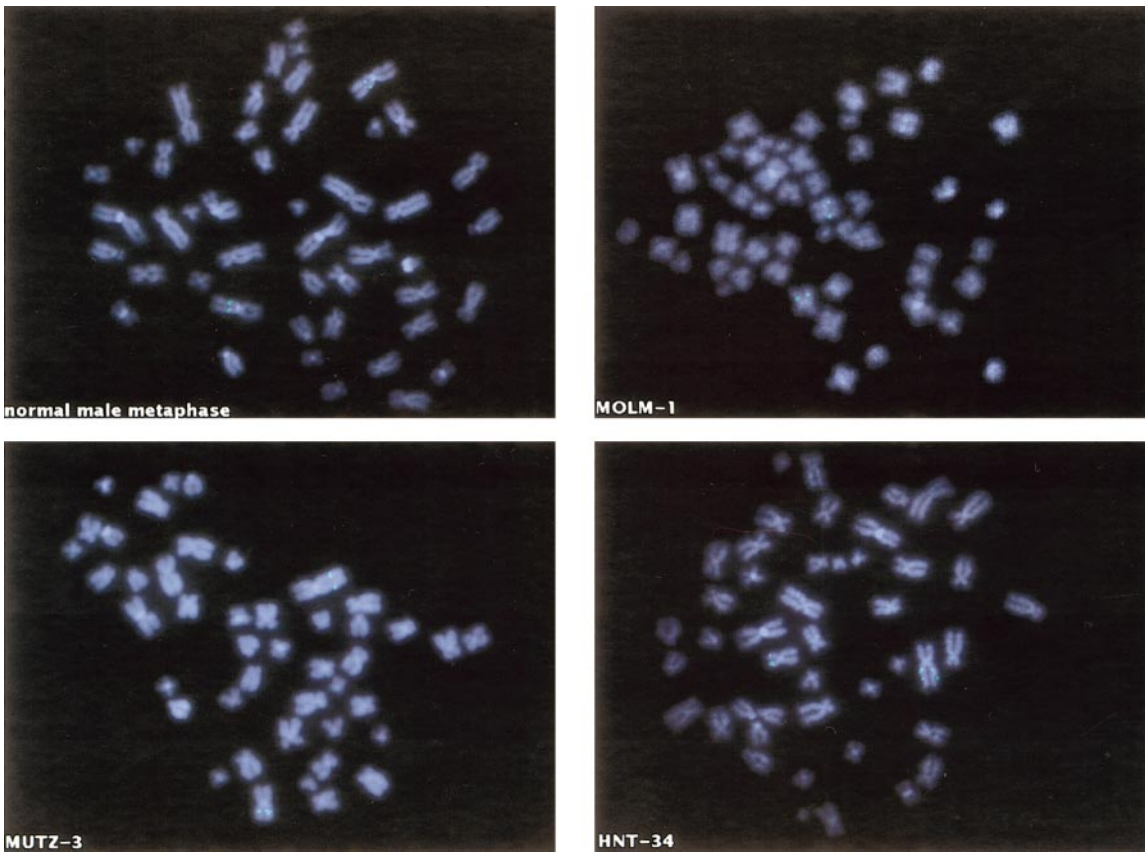

FIG. 2. FISH analysis of cell lines with 3q21 aberrations. Biotin-dUTP-labeled PAC 4 DNA was hybridized to metaphases from the sources indicated in the figure. MUTZ-3 cells (34, 35) contain a t(1;3) (q43;q13.12)inv3(q21.2q26.3), MOLM-1 cells (36), an inv(3) (q21q26), and HNT-34 cells (8) a t(3;3) (q21;q26). The inv(3) breakpoints in MUTZ-3 and MOLM-1 cells are telomeric of PAC 4, the t(3;3) breakpoint in HNT-34 cells splits this PAC. The presence of the indicated 3q aberrations was verified in each case by classical cytogenetic analysis as well as by FISH analyses using additional probes.

as a template, the same primers and conditions were used, except that extension was for 6 min at 72°C.

RT-PCR primers for GATA-2 were as follows: hGATA2-1055fwd, 5′-GACTACAGCAGCGGACTCTTC-3′; and hGATA2-1327rev, 5′-ACAACAGGTGCCGGCTCTTC-3′; and annealing was at 62°C. Primers for GAPDH were: hGAPDHfwd, 5′-AAGGTGAAGGTCG-GAGTCAACG-3′; and hGAPDHrev, 5′-CAGCCTTCTCCATGGTG-GTGAA-3′; and annealing was at 58°C. For cell lines, GATA-2 and GAPDH were amplified in 21 and 16 cycles, respectively. For patient samples, the number of PCR cycles was raised to 25 (GATA-2) and 17 (GAPDH). To compare the relative use of the alternative exon 1 sequences, the following primers were used: for the IG exon, hGATA2-192fwd (5′-TCACTCTCAGAGGCCGAGTC-3′) in conjunction with hGATA2-444rev, with annealing at 53°C; and for the IS exon, hGATA2-IS3fwd (5′-GTCCGCTGAACACCATGCG-3′) in conjunction with hGATA2-444rev, with annealing at 54°C. 26 and 30 PCR cycles were carried out, respectively. Aliquots from PCRs were electrophoresed and transferred to Nylon membranes. These blots were hybridized to an excess of ^{32}P-labeled specific probe. Detection as well as quantification was performed using a Storm 840 Phosphoimager (Amersham Pharmacia). This method yielded semiquantitative data, as shown by PCR on serial dilutions of a cDNA sample.

For RACE, the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) and Dynazyme DNA polymerase (Finnzymes) were used. The human placenta cDNA supplied as a positive control with the Marathon kit served as a template for primers AP-1 and hGATA2-444rev. 35 PCR cycles were carried out with annealing at 66°C. The resultant product was further amplified in 25 cycles with the AP-2 primer and hGATA2-ISrev (5′-CCTGGAGTAGAGCTGGGAGCA-3′). The 300-bp PCR product was cloned into pGEM-T (Promega) and 7 clones were subjected to sequence analysis.

Cytogenetics and fluorescence in situ hybridization (FISH). The identity of all cell lines used in this study was verified by G-band analysis.

Metaphases for FISH experiments were prepared as described previously (31, 32). PAC DNA was labeled with Biotin-16-dUTP by nick translation (Roche Diagnostics) according to the manufacturer's instructions. 100 ng of labeled PAC DNA were hybridized to one half of a microscopic slide; and specific signals were detected with FITC-labeled avidin (Oncor, Gaithersburg, MD). Chromosomes were visualized by counterstaining with DAPI.

RESULTS AND DISCUSSION

The hGATA-2 Gene Is Located in Close Proximity to the Breakpoint Cluster Region and Is Transcribed from Telomere to Centromere

As part of our effort to understand the molecular basis for the clinical features associated with chromosome aberrations affecting band 3q21, a PAC contig covering the 3q21 breakpoint region was established

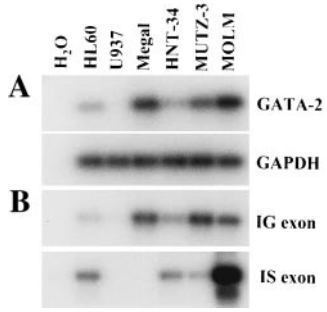

FIG. 3. Expression of hGATA-2 in leukemic cell lines with and without 3q21 aberrations. (A) Primers specific for the hGATA-2 gene were used for RT-PCR analysis of the indicated cell lines. HL-60, U937, and Megal cells do not harbor 3q21 aberrations, whereas HNT-34, MUTZ-3, and MOLM-1 cells do (see the legend to Fig. 2). GAPDH served as a control for the amounts of cDNA used. PCR products were blotted onto nylon membranes, hybridized to ^{32}P-labeled specific internal probes, and detected using a Phosphoimager. (B) Primers that specifically amplify hGATA-2 cDNA containing either the IS or the IG exon were employed to compare their relative use in the cell lines. The same cDNAs as in A were used, and PCR products were detected by blotting and hybridization to ^{32}P-labeled probes. The experimental conditions employed in A and B allow a semiquantitative estimate of expression, as determined by PCR analysis of serial dilutions of a cDNA sample (Fig. 4, and data not shown).

(10). A subset of these PACs that is relevant to the studies described here is shown in Fig. 1A. PAC 8 essentially covers the region corresponding to the two P1 clones described by Rynditch *et al.* (7), and contains the 3q21 breakpoint cluster region as well as Ribophorin 1 gene sequences. Exon trapping was used in order to identify coding sequences within the PAC contig. One of the exons isolated corresponded to the published sequence of exon 4 of the human GATA-2 gene.

To map the GATA-2 gene more precisely within the contig, Southern analysis was carried out. Three probes corresponding to exons 2 to 3, exons 3 to 5, and to exon 6 of hGATA-2 were hybridized to membranes containing *Eco*RI digested PAC DNA (Fig. 1B). None of these probes hybridized to the most telomeric PAC 8. Whereas the exon2/3 probe recognized PACs 2 to 7, the exon3/5 probe hybridized only to the more centromeric PACs 2 to 5. The exon 6 probe recognized the most centromeric PAC 1, in addition to PACs 2 to 4. The genomic size of the hGATA-2 gene has been reported to be approximately 10kb (12). Together with our mapping data, this indicates that the GATA-2 gene is located approximately 100kb centromeric of the 'breakpoint cluster region' (7). In addition, the hybridization pattern indicates that the gene is transcribed from the telomere toward the centromere.

Identification of an Alternative Exon 1 of the hGATA-2 Gene

In the mouse, transcription of GATA-2 can be initiated either from the proximal IG exon or, alternatively, from the more distal IS exon (30). The exon 1 of the human GATA-2 gene that was published originally corresponds to the murine IG exon.

To investigate whether an IS-like exon 1 also exists in the human genome, we hybridized a PCR product corresponding to the murine IS exon to PAC DNA blots under low stringency conditions. A 3-kb *Hin*dIII fragment of PAC 4 that was specifically recognized by this probe was subcloned into pBluescript, and a 1-kb region thereof was sequenced (GenBank Accession No. AF 169253). Using the GCG program 'Bestfit', clear similarity with the published mGATA-2 IS promoter and exon sequence was detected. Through RT-PCR, RACE, and sequence analysis it was shown that the new sequence is in fact transcribed as part of the human GATA-2 gene, and that its 5′ and 3′ ends correspond to those of the murine IS exon. Long distance PCR as well as Southern analysis showed that the human IS exon, like its murine counterpart (30), is located approximately 6 kb upstream of the IG exon (data not shown). While this work was in progress, other authors reported similar findings on the IS exon of the human GATA-2 gene (33). Their results are entirely consistent with ours.

FISH Analysis of Cell Lines

PACs 3 and 4 contain the hGATA-2 coding region near their centromeric ends, as well as approximately 90kb of 5′ sequences. PAC 8, on the other hand, overlaps the 'breakpoint cluster region', but ends sharply before the transcribed portion of GATA-2. To determine whether the GATA-2 gene might be affected by chromosome breakpoints in any of several cell lines with 3q21 aberrations, fluorescence *in situ* hybridiza-

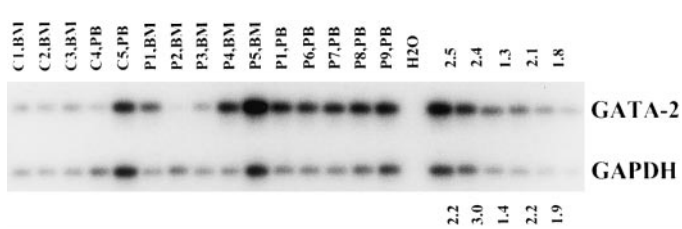

FIG. 4. Expression of hGATA-2 in samples from AML patients with 3q21 aberrations. cDNA prepared from bone marrow (BM) or peripheral blood (PB) samples from AML patients (P1 to P9) or healthy controls (C1 to C5) was amplified with primers specific for hGATA-2 (upper panel) or hGAPDH (lower panel). PCR products were blotted onto nylon membranes, hybridized to ^{32}P-labeled specific internal probes, and detected and quantified using a Phosphoimager. To ensure that the procedure allows semiquantitative evaluation of the results, serial 1:2 dilutions of a cDNA sample were amplified in the same experiment (last 6 lanes in each panel). The difference in intensity between the resulting PCR products is indicated by the numbers above the GATA-2 panel and underneath the GAPDH panel, respectively. Relative expression of GATA-2 and GAPDH in samples from patients and healthy controls is indicated in Table 1. The experiment shown is representative of three repetitions.

TABLE 1

Characteristics of Primary Samples Analyzed for GATA-2 Expression

Sample No.	Age/Sex	FAB[a]	chrom 3	Disease status	PB/BM[b]	GATA2/GAPDH
C1	40/M	—	—	—	BM	0.8
C2	41/F	—	—	—	BM	0.9
C3	48/M	—	—	—	BM	1.0
C4	38/F	—	—	—	PB	0.3
C5	34/F	—	—	—	PB	1.0
P1	25/F	M6	t(3;3)	Therapy	BM	3.9
					PB	5.7
P2	62/F	M2	inv(3)	Therapy	BM	0.1
P3	59/M	M5b	inv(3)	Diagnosis	BM	0.9
P4	29/M	M1	t(3;3)	Relapse	BM	7.0
P5	20/F	AML	inv(3)	Diagnosis	BM	4.0
P6	56/F	M1	inv(3)	Diagnosis	PB	4.3
P7	31/F	M0	inv(3)	Diagnosis	PB	5.2
P8	53/F	M5a	inv(3)	Therapy	PB	3.5
P9	68/F	M7	t(3;3)	Therapy	PB	2.5

[a] FAB, French–American–British classification of acute myeloid leukemia.

[b] PB, peripheral blood, BM, bone marrow. C denotes control samples from healthy donors, P denotes patient samples. The GATA2/GAPDH ratio was determined by Phosphoimager quantification of the results shown in Fig. 4.

tion (FISH) employing PACs 4 and 8 as probes was performed. The inversion breakpoints in MUTZ-3 (34, 35) and MOLM-1 (36) cells split the signal of PAC 8, but appeared to be located telomeric of PAC 4, and therefore 5′ of the GATA-2 coding region. The t(3;3) breakpoint in HNT-34 (8) cells, on the other hand, split the signal of PAC4, indicating that it maps closer to the transcribed region of GATA-2 (Fig. 2, and data not shown). However, Southern analyses using various probes from the GATA-2 gene did not give any indication for a structural alteration of the GATA-2 coding region in any of the cell lines (data not shown). Since, more recently, several 3q21 breakpoints in patient samples have been mapped centromeric of the 'breakpoint cluster region', and in close vicinity to the GATA-2 gene (9, 10), it is nevertheless possible that GATA-2 rearrangements may be found in some cases.

Expression of the hGATA-2 Gene in Cell Lines and Patient Samples Harboring 3q21 Aberrations

Using YAC transgenic mice, Zhou *et al.* have shown that 150 kb of the murine GATA-2 promoter are required for adequately regulated expression of mGATA-2 in the hematopoietic compartment (21). The structure and sequence of the mGATA-2 coding and proximal promoter regions exhibit a striking degree of conservation with their human counterparts ((12, 30, 33), and this study), suggesting that more distal regulatory elements might also be conserved between the two species.

Since hGATA-2 is transcribed from telomere to centromere (Fig. 1B), most previously mapped breakpoints are located in a region that is likely to contain sequences important for transcriptional control in hematopoietic cells. We therefore asked whether 3q21 breakpoints could affect the expression of hGATA-2, and compared the mRNA levels of this gene in the above mentioned cell lines with 3q21 aberrations with those in several control cell lines without such rearrangements. Since some of the cell lines used grow only poorly, we employed a semiquantitative RT-PCR method, which was validated by analyzing serial dilutions of a cDNA sample (Fig. 4). All cell lines with 3q21 rearrangements—HNT-34 (myeloid), MUTZ-3 (monocytic), and MOLM-1 (megakaryocytic)—expressed considerable amounts of GATA-2 transcript. GATA-2 mRNA was also abundant in the control cell lines HL60 (promyelocytic) and Megal (megakaryocytic) (Fig. 3A). In contrast, only minute amounts of GATA-2 mRNA were detected in the monocytic U937 cells using a higher number of PCR cycles. Other monocytic cell lines are also devoid of GATA-2 mRNA (12, 27, 33). MUTZ-3 cells, on the other hand, which display a number of characteristics of monocytic cells, and have a 3q21 breakpoint (34), do transcribe GATA-2. This could be a consequence of a more immature state of these cells compared to other established monocytic cell lines. However, at least in certain experimental settings, GATA-2 overexpression can actually cause a block in hematopoietic maturation (22, 24), raising the possibility that GATA-2 expression in MUTZ-3 cells leads to rather than reflects a less differentiated state.

We further asked whether 3q21 rearrangements might lead to a different use of the alternative exons 1. Primers were designed that specifically amplify hGATA-2 cDNA initiating from either the IS or the IG exon. The relative use of these exons varied considerably between the cell lines (Fig. 3B), however, no clear

correlation with either hematopoietic lineage or the presence of 3q21 aberrations became apparent.

GATA-2 expression was also analyzed in primary samples from leukemia patients with 3q21 rearrangements and from healthy donors. GATA-2 was reproducibly overexpressed in seven of nine patient samples with 3q21 aberrations compared to healthy controls (Fig. 4 and Table 1). Since GATA-2 overexpression has been found in many AML samples irrespective of cytogenetically detectable 3q21 aberrations (26, 27), mechanisms other than gross chromosomal rearrangements might cause such deregulation in the majority of acute myeloid leukemias. Similar situations have been described for the oncogenes scl/tal-1 (37) and c-myc (38).

In summary, GATA-2 would be an attractive candidate for a 3q21 oncogene because of its well established involvement in the proliferation and differentiation of hematopoietic cells. Additionally, its large promoter might provide a basis to explain why 3q21 breakpoints are dispersed over a comparatively large genomic region. Clearly, additional experiments are required in order to further investigate the potential involvement of GATA-2 in leukemia.

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