Acquired interstitial deletions of the long arm of chromosome 5 occur frequently in the myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Recently IRF1, a putative tumor suppressor gene localized to the long arm of chromosome 5, has been shown to be deleted from the 5q- chromosome in a group of patients with MDS and AML. It has been suggested that the loss of IRF1 may be critical to the development of the 5q- syndrome. We have investigated the allelic loss of IRF1 in a group of 12 patients with MDS and a 5q deletion and 2 patients with AML and a 5q deletion. Gene dosage experiments demonstrated that 12 of 14 patients had loss of one allele of the IRF1 gene but no evidence of homozygous loss and that 2 patients with the 5q- syndrome retained both copies of the gene. The retention of IRF1 on the 5q- chromosome in these two cases has been confirmed by fluorescent in situ hybridization localization using an IRF1 cosmids. Pulsed field gel electrophoresis was used to determine whether there was any evidence for structural rearrangement in the region encompassing the IRF1 gene in these two patients. No aberrant bands were detected with a range of rare cutter enzyme digest. We conclude that IRF1 maps outside the commonly deleted segment of the 5q- chromosome and that loss of IRF1 is not solely responsible for the development of the 5q- syndrome.

MATERIALS AND METHODS

Patients. Twelve patients with MDS and two patients with AML and a cytogenetically demonstrable 5q deletion in the bone marrow were included in the study (Table I). Classification was according to the French-American-British criteria. At the time of investigation eight patients had refractory anemia (RA), three had refractory anemia with excess blasts (RAEB), one had RAEB in transformation (RAEBt), and two had AML (Table 1). Chromosome preparations were obtained from bone marrow samples using standard techniques. Cultures were harvested after 24 hours with a 1-hour or overnight exposure to colcemid, and at 48 hours after charge payment. This article must therefore be hereby marked in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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to high specific activity by random hexanucleotide priming (Boeh-
99% granulocytes). T-lymphocyte cell populations (>go%) were
electrophoresis through 1% agarose gels. The DNA was transferred
dard procedures for Southern bl~tting.'~ DNA probes were labeled
with the appropriate restriction enzyme and size fractionated by
isolated from the mononuclear fraction by erythrocyte r~setting.'~
From the mononuclear fraction by erythrocyte rosetting.26 The
DNA samples obtained from the patients with MDS and AML
and healthy individuals were digested with EcoRI, BamHI,
and Southern blotted. The resulting filters were hybridized
to the IRFl cDNA fragment HHCP21 to screen for rearrange-
ments of the IRFl gene.

Pulsed field gel electrophoresis. Pulsed field gel electrophoresis
(PFGE) was used to examine the molecular structure of the IRFl
gene and flanking regions in those MDS patients shown to retain
the gene on the 5q-chromosome. Block formation, restriction en-
zyme digestion, and PFGE were performed according to standard
methods.30,34 Patient granulocyte and T-lymphocyte fractions were
embedded in 1% agarose gels and hybridized to IRFl cDNA frag-
ments of the IRFl gene.

The renin probe and each of the IRFl probes gave single hybrid-
ization fragments. After autoradiography the film was scanned by
an enhanced lazer densiometer (LKB Ultrascan XL, Bromma,
Sweden) to quantitate the relative intensities of the two hybridiza-
tions signals. A comparative densimetric ratio was derived from
the two hybridization signals in 10 normal individuals. An approxi-
mately 50% reduction in this ratio indicates a 50% reduction in the
dosage of the chromosome 5 assigned gene of interest and is consis-
tent with the loss of one allele (hemizygous deletion). A greater
than 50% reduction in this ratio indicates a greater than 50% reduction in the
dosage of the chromosome 5 assigned gene of interest and is
consistent with the loss of both alleles (homozygous deletion) in at
least a subpopulation of cells. All gene dosages experiments were
performed on three separate occasions.

DNA samples obtained from the patients with MDS and AML
and healthy individuals were digested with EcoRI, BamHI,
HindIII, and Southern blotted. The resulting filters were hybridized
to the IRFl cDNA fragment HHCP21 to screen for rearrange-
ments of the IRFl gene.

Molecular Studies. High molecular weight DNA was obtained
from peripheral blood leucocytes by phenol/chloroform extraction using standard methods.29 From fractionated peripheral blood leucocytes and blast samples. The mononuclear cells and granulocytes were separated by 40 mL
of EDTA peripheral blood by Ficoll gradient centrifugation.26 The
granulocyte fractions showed a high level of purity in all cases (95% to
99% granulocytes). T-lymphocyte cell populations (>90%) were
isolated from the mononuclear fraction by erythrocyte rosetting.27
Blast samples were obtained from the two patients with AML.

Gene dosage and Southern analysis. The DNA was digested
with the appropriate restriction enzyme and size fractionated by
electrophoresis through 1% agarose gels. The DNA was transferred
by Hybond N (Amersham Int, Amersham, UK) according to stan-
dard procedures for Southern blotting.25 DNA probes were labeled
to high specific activity by random hexanucleotide priming (Boeh-
ringer Mannheim Int, Lewes, UK) and hybridization was per-
duced at 65°C for 16 hours. Filters were washed for 30 minutes in
standard saline citrate (SSC), and 0.1% sodium dodecyl sulfate
at 65°C and autoradiographed between intensifying screens at
-70°C.

Gene dosage experiments28-30 designed to allow for a quantita-
tive assessment of IRFl loss were performed. DNA samples from the
fractionated peripheral blood leukocytes of 10 healthy individuals
were used as controls. DNA samples obtained from the patients
with MDS and AML and healthy controls were digested with
EcoRI, Southern blotted, and the resulting filters simultaneously
hybridized to two probes; a 1.9-kb genomic EcoRI-Sst I fragment
from the renin gene (the probe pHRnES 1.9 was obtained from the
American Type Culture Collection [ATCC], Rockville, MD) and a
probe for IRFl. Two IRFl probes were used; one generated by
polymerase chain reaction amplification using primer sets designed
from published sequence data by Wamngton et al (the primer se-
dences, product size, and annealing temperatures are as described
in Warington et al)14 and an IRFl cDNA fragment (clone
HHCP21 obtained from the ATCC). The renin gene is localized to
chromosomes 1q32-42,31,32 a region karyotypically normal in the
MDS patients included in this study, and acts as an internal hybrid-
ization standard.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age</th>
<th>Diagnosis</th>
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<td>RA</td>
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* Patient with the 5q- syndrome.
ALLELIC LOSS OF IRF1 IN MDS AND AML

0.5 mg/mL proteinase K (Boehringer Mannheim) and incubated at 50°C for 48 hours. The blocks were washed twice with TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8), twice with TE containing 40 μg/mL phenyl methylsulfonyl fluoride (Sigma), and finally, three times with TE alone. Blocks were incubated with the appropriate restriction enzyme buffer containing 5 mmol/L spermidine and incubated at the appropriate temperature overnight in fresh buffer with 20 U of the restriction enzyme. The samples were electrophoresed in 1% or 0.8% agarose gels on a CHEF II PFGE system (Bio-rad, Hemel Hempstead, UK). Electrophoresis was performed in 0.5× TBE buffer (45 mmol/L Tris-HCl, 45 mmol/L boric acid, 0.5 mmol/L EDTA, pH 8.3) at 14°C. Ramp pulse times from 50 to 90 seconds over 24 hours at 200 V were used to separate DNA fragments in the 200- to 2,200-kb size range. Sacccharomyces cerevisiae and Schizosaccharomyces pombe, and oligomers of lambda phage were used as size markers. Southern blotting was performed as for conventional gels except that transfer times were for at least 24 hours. Hybridization with the IRF1 cDNA probe was performed as for conventional blots.

Fluorescent in situ hybridization (FISH) analysis. FISH of cosmid DNA was performed essentially as described. An IRF1 cosmid (IRF1SL) was isolated from a 412 total human cosmid library in vector eHC2 (donated by Integrated Genetics, Framingham, MA). The IRF1 cosmid was labeled by nick translation with biotin-dUTP and hybridized in situ to chromosome spreads obtained from normal lymphocytes and from the bone marrow of patients 11 and 12. The hybridization mix contained 100 ng of cosmid DNA and 3 μg of COT-1 DNA, which were preannealed at 37°C for 15 minutes before hybridization. The site of hybridization was detected with successive layers of fluorescein-conjugated avidin (Vector, Betton, UK) and biotinylated anti-avidin (Vector). Slides were mounted in antifade (Vector) with 1 μg/mL of propidium iodide, and also 1 μg/mL of DAPI, which permits concurrent G-band analysis when the spreads are viewed under ultraviolet light.

RESULTS

Gene dosage and Southern analysis. Ten patients (no. 1, 2, 4, 5, 6, 7, 9, 10, 13, 14) with MDS and two patients with AML (no. 3 and 8) showed an approximately 50% reduction in IRF1 gene dosage in the granulocyte fraction (Table 2, Fig I). These data are consistent with the deletion of IRF1 from the 5q- chromosome. Two patients (no. 11 and 12) showed no reduction in IRF1 gene dosage in the granulocyte fraction (Table 2, Fig I). These data are consistent with the retention of the IRF1 gene on the 5q- chromosome. No reduction in the gene dosage of IRF1 was shown in the T-lymphocyte fractions obtained from all 12 patients with MDS (not shown). No rearrangements of the IRF1 gene

Fig 1. Gene dosage analysis of IRF1. DNA from peripheral blood samples obtained from healthy individuals (tracks labeled C) and the granulocyte fractions (tracks no. 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14) and blast samples (tracks no. 3 and 8) obtained from the patients with MDS or AML, respectively, were digested with EcoRI and simultaneously hybridized to a renin probe and an IRF1 cDNA probe. Optical densitometric readings were obtained and were used to obtain comparative ratios between the two signals, + + indicates the presence of two gene copies, + - indicates the loss of one gene copy.

Fig 2. PFGE analysis of IRF1. Granulocyte DNA obtained from patient 11 (track 1) and patient 12 (track 2) and granulocyte DNA obtained from a healthy individual (track 3) are shown. (A) The DNA was digested with BssHII and hybridized to an IRF1 cDNA probe. A 250-kb fragment was detected in the patient samples and the normal control. (B) The DNA was digested with BbrI and hybridized to an IRF1 cDNA probe. A 285-kb fragment was detected in the patient samples and the normal control.
were observed with *EcoRI*, *HindIII*, and *BamHI* digests of patient DNA samples.

**PFGE.** The granulocyte and T-lymphocyte fractions obtained from patients 11 and 12 and the granulocyte fractions obtained from four healthy individuals were digested with four restriction enzymes: *Mlu I*, *BssHII*, *EclXI*, and *BbrPI*. Patient and control DNA samples gave identical fragments with the four restriction digests following hybridization with the IRFl cDNA probe. Digestion with *Mlu I* gave fragments of 285 kb and <225 kb, *BssHII* 250 kb, *EclXI* 565 kb, and 365 kb, and *BbrPI* 285 kb (Fig 2). No additional fragments were observed in the granulocyte fractions obtained from patients 11 and 12.

**FISH analysis.** The IRFl cosmid, which contains the IRFl gene, hybridized only to the chromosomes 5 of normal lymphocytes, at 5q23-5q31 (data not shown). When this cosmid was hybridized to bone marrow chromosomes from patient 11, signals were observed on both the normal and the deleted chromosome 5 in all 20 cells examined (Fig 3), in patient 12 signals were observed from both chromosomes 5 in all of 50 cells examined. The small deletion in patient 12 was difficult to identify under FISH conditions but in the three cells in which the deleted chromosome 5 could be unambiguously identified there were signals from both the normal and abnormal chromosome.

**DISCUSSION**

Allelic loss of the IRFl gene has been demonstrated in 13 patients with AML or MDS and deletions of the long arm of chromosome 5 by Willman et al.18 All thirteen patients showed deletion of one allele of the IRFl gene by gene dosage analysis. Furthermore, six of these patients showed the deletion of both alleles in a subpopulation of cells (≥10%) by gene dosage analysis. This finding was also supported by FISH experiments. It has consequently been suggested that the deletion of IRFl may be a critical event in the development of malignant myeloid disorders with a 5q deletion.18 To investigate this hypothesis we have performed a molecular examination of the IRFl gene in 12 patients with MDS and a 5q deletion and two patients with AML and a 5q deletion. Using gene dosage analysis we have demonstrated loss of one allele of the IRFl gene in 10 patients with MDS and in 2 patients with AML. This finding is consistent with the cytogenetic assessment of the 5q deletion breakpoints in these cases; each had 5q deletions encompassing most of the long arm of chromosome 5 and including the region 5q23-5q31. We did not find any evidence for homozygous loss of IRFl in our group of patients. No rearrangements of the IRFl gene were detected in the 12 patients by conventional gel electrophoresis.

Two patients with MDS were shown to retain the IRFl gene on the 5q- chromosome by gene dosage and FISH analysis. The two patients both had the 5q- syndrome with the same karyotypic abnormality del (5)q31-q33. The loss of CSFIR, assigned 5q1-33,13,14,20 has been previously demonstrated in these two cases.20 FISH experiments were used to confirm the retention of IRFl in these two cases. FISH analysis, using an IRFl cosmid, also demonstrated the presence of the IRFl gene on the 5q- chromosome in both patients. No rearrangements of the IRFl gene were detected in these two patients by both conventional gel electrophoresis and PFGE.

Willman et al.18 reported the deletion of one IRFl allele and the rearrangement of the second allele (presumably from the apparently normal homologous chromosome 5) in a patient with acute lymphocytic leukemia (ALL) and a 5q- chromosome. Abnormalities involving the long arm of chromosome 5 are characteristic of AML but not ALL and therefore the relevance of this finding to myeloid malignancies is unclear. In this study we examined two patients with AML and a 5q- chromosome. Loss of one IRFl allele was demonstrated but we were unable to detect any structural rearrangements of the remaining IRFl allele in the two AML patients by conventional electrophoresis.

The 5q deletion typically encompasses most of the long arm and many known genes (and presumably many more yet to be identified) are lost as a result. It has been postulated that the long arm of chromosome 5 contains a myeloid tumor suppressor gene and that this gene is located within the smallest region of common genetic loss, termed the "critical region" of gene loss.37 The precise localization of the critical region of the 5q- chromosome is variably reported.37-41 presumably reflecting differences in the cytogenetic assessment of deletion breakpoints between laboratories. The region most frequently cited as the critical region of the 5q- chromosome is 5q31 to 5q33,30,37,39,41 although it has been speculated that there may be more than one critically deleted segment.42 The IRFl gene is localized to the long arm of chromosome 5 and has recently been shown to possess...
growth inhibitory and antioncogenic activities.\textsuperscript{20,21} It therefore represents an interesting candidate for the myeloid tumor suppressor gene presumed to reside on chromosome 5. The IRF1 gene has been assigned to 5q31.1.\textsuperscript{7,18} This might suggest the localization of IRF1 to a critical region of gene loss of the 5q chromosome. However, we have shown the retention of the IRF1 gene on the 5q-chromosome in two patients with the 5q-syndrome. This finding is consistent with data arising from a detailed examination of the 5q-chromosome in MDS and AML by Le Beau, who demonstrated the retention of IL-3, IL-4, IL-5, and CSF2 on the 5q-chromosome in some patients.\textsuperscript{13} IRF1 maps proximal to IL-3 and CSF2.\textsuperscript{14,18,19} It is therefore most improbable that deletion of the IRF1 gene is solely responsible for the development of the 5q-syndrome. However, it remains possible that multiple genes localized to different regions on the long arm of chromosome 5 are involved in the pathogenesis or progression of these myeloid disorders.

REFERENCES

10. Shows TB, Byers MG, Fiddes J: Assignment of FGFB (basic fibroblast growth factor) to chromosome 4q25 and FGFA (acidic fibroblast growth factor) to chromosome 5q31-33. Cytogenet Cell Genet 51:1079, 1989
Allelic loss of IRF1 in myelodysplasia and acute myeloid leukemia: retention of IRF1 on the 5q- chromosome in some patients with the 5q-syndrome

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