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Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia

(transcript/gene/leukemogenesis/pediatric)

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ABSTRACT **Ikaros, a zinc finger-containing DNA-binding protein, is required for normal lymphocyte development, and germline mutant mice that express only non-DNA binding dominant-negative "leukemogenic" Ikaros isoforms lacking critical N-terminal zinc fingers develop an aggressive form of lymphoblastic leukemia 3–6 months after birth. Therefore, we sought to determine whether molecular abnormalities involving the *Ikaros* gene could contribute to the development of acute lymphoblastic leukemia (ALL) in infants. Primary leukemic cells were freshly obtained from 12 infants (<1 year of age) with newly diagnosed ALL. In leukemic cells from each of the 12 infants with ALL, we found high level expression of dominant-negative isoforms of Ikaros with abnormal subcellular compartmentalization patterns. PCR cloning and nucleotide sequencing were used to identify the specific Ikaros isoforms and detect *Ikaros* gene mutations in these cells. Leukemic cells from seven of seven infants with ALL, including five of five MLL-AF4^{+} infants, expressed dominant-negative Ikaros isoforms Ik-4, Ik-7, and Ik-8 that lack critical N-terminal zinc fingers. In six of seven patients, we detected a specific mutation leading to an in-frame deletion of 10 amino acids (Δ KSSMPQKFLG) upstream of the transcription activation domain adjacent to the C-terminal zinc fingers of Ik-2, Ik-4, Ik-7, and Ik-8. In contrast, only wild-type Ik-1 and Ik-2 isoforms with normal nuclear localization were found in normal infant bone marrow cells and infant thymocytes. These results implicate the expression of dominant-negative Ikaros isoforms and the disruption of normal *Ikaros* function in the leukemogenesis of ALL in infants.**

Infants with acute lymphoblastic leukemia (ALL), particularly those with a t(4;11)(q21;q23) or *MLL-AF4* fusion transcript, have extremely poor outcomes despite very intensive therapy or bone marrow transplantation (1–6). A major unresolved question in the molecular etiology of t(4;11)$^{+}$ infant ALL is whether expression of the *MLL-AF4* fusion gene is sufficient to lead to a fully transformed phenotype (7, 8). Initial studies on pairs of infant identical twins with concordant leukemia indicated that this molecular rearrangement likely occurred during fetal hematopoiesis (9, 10). In a subsequent report, Greaves *et al.* (11) detected clonotypic genomic *MLL-AF4* fusion sequences in neonatal blood from infants who subsequently were diagnosed with ALL. This finding provided conclusive evidence that the *MLL-AF4* gene fusions arose during fetal hematopoiesis. However, we detected *MLL-AF4* fusion transcripts in a proportion of fetal liver, fetal bone marrow, and normal infant bone marrow specimens (12), which suggests that the presence of *MLL-AF4* fusion transcripts may be necessary but not sufficient for neoplastic transformation of lymphocyte precursors. Thus, altered expression of genes other than *MLL* and *AF4* could be involved in leukemogenesis of t(4;11)$^{+}$ ALL in infants.

Ikaros, a member of the Kruppel family of "zinc finger" DNA-binding proteins, is one of the critical transcriptional regulators of lymphocyte ontogeny and differentiation in amphibian, teleost, avian, and murine species (13–19). Alternatively spliced transcripts of the *Ikaros* gene encode at least eight zinc finger proteins (Ikaros isoforms Ik-1 through Ik-8) with distinct DNA binding capabilities and specificities (14, 17). Ikaros proteins are highly conserved between human and mouse, and all share a common C-terminal domain containing a bipartite transcription activation motif and two zinc finger motifs required for hetero- and homodimerization among the Ikaros isoforms and interactions with other proteins (14, 17). They differ in their N-terminal zinc finger (F1 through F4) composition and in their overall DNA binding and transcriptional activation properties (17). At least three N-terminal zinc fingers are required for high affinity DNA binding to the 4-bp core motif GGGA. Thus, only the isoforms Ik-1, Ik-2, and Ik-3, which contain three or more N-terminal zinc fingers, exhibit high affinity DNA binding (17). These DNA binding isoforms localize to the nucleus whereas isoforms Ik-4 through Ik-8, which have fewer than three N-terminal zinc fingers, localize to the cytoplasm (17). The formation of homo- and heterodimers among the DNA binding isoforms increases their affinity for DNA whereas formation of heterodimers between the DNA binding isoforms and those without an intact DNA binding domain are unable to bind DNA and are therefore transcriptionally inactive (17, 19). Therefore, Ikaros proteins with fewer than three N-terminal zinc fingers can exert a dominant-negative effect by interfering with the activity of Ikaros isoforms that can bind DNA (17).

Homozygous deletion of the *Ikaros* exons 3 and 4 that encode the three N-terminal zinc fingers required for sequence-specific DNA binding of Ikaros results in a complete arrest in the development of all lymphoid lineages in mice (13). Mice heterozygous for this germline mutation express higher than normal levels of Ik-6, Ik-7, and Ik-8 isoforms and develop a highly aggressive form of lymphoblastic leukemia with a concomitant loss of heterozygosity between 3 and 6 months after birth (18). Immediately before the development of leukemia, lymphocyte precursors show both a significant decrease in their Ikaros-

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Abbreviations: ALL, acute lymphoblastic leukemia; RT, reverse transcription; INF, infant.
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Table 1. Patient characteristics

Patient	Age, months	Sex	WBC, $\times 10^3/\mu l$	HSM	Immuno-phenotype	Karyotype	MLL-AF4 fusion transcript
INF-1	2	F	6	−	B	46,XX,t(4;11)(q21;q23)[12]/46,XX[8]	+
INF-2	4	F	346	+	B	46,XX,t(4;11)(q21;q23)[10]	+
INF-3	3	F	285	+	B	Not determined	+
INF-4	1	F	410	+	B	Not determined	+
INF-5	8	M	1,000	+	B	46,XY,t(4;11)(q21;q23),t(14;19)(q11.2;p13)[12]/46,XY[8]	+
INF-6	2	F	670	+	B	46,XX,t(4;11)(q21;q23)[20]	+
INF-7	3	F	684	+	B	46,XX,t(4;11)(q21;q23)[17]/46,XX[3]	+
INF-8	9	F	41	+	B	46,XX[29]	+
INF-9	2	M	187	+	B	46,XY,t(4;11)(q21;q23)[16]/46,XY[6]	+
INF-10	5	M	317	+	B	Not determined	+
INF-11	2	M	270	+	B	46,XX,t(11;19)(q23;p13)[20]	−
INF-12	6	M	83	−	B	Not determined	−

MLL-AF4 fusion transcript expression was determined by RT-PCR. HSM, hepatosplenomegaly; WBC, white blood cells; INF, infant; M, male; F, female; B, B-lineage.

specific DNA binding activity and a change in compartmentalization of Ikaros from nucleus to cytoplasm (17, 18).

Based on the critical role played by *Ikaros* in the development of the normal immune system in mice and the rapid development of leukemia in mice expressing dominant-negative isoforms of Ikaros, we sought to determine whether specific molecular defects involving this candidate leukemia suppressor gene may contribute to the leukemogenesis of infant ALL. Using Western blot analysis, confocal laser scanning microscopy, PCR cloning, and nucleotide sequencing, we have demonstrated that primary leukemic cells from infants with ALL express high levels of dominant-negative Ikaros isoforms that lack critical N-terminal zinc fingers and localize to the cytoplasm, similar to the dominant-negative Ikaros isoforms that lead to leukemia in germline mutant mice. Furthermore, in all but one patient, we detected a specific mutation leading to an in-frame deletion of 10 amino acids (Δ KSSMPQKFLG) upstream of the transcription activation domain and adjacent to the C-terminal zinc fingers of Ik-2, Ik-4, Ik-7, and Ik-8. In contrast, only wild-type Ik-1 and Ik-2 isoforms with normal nuclear localization were found in normal infant bone marrow cells and infant thymocytes. These results uniquely implicate the expression of dominant-negative Ikaros isoforms and/or the disruption of normal *Ikaros* function in the leukemogenesis of ALL in infants.

MATERIALS AND METHODS

Cells and Tissues. Leukemic cells were isolated from diagnostic bone marrow specimens of 12 infants with newly diagnosed ALL who had complete cytogenetic and/or molecular genetic, immunophenotypic, and clinical data and were treated at Children's Cancer Group-affiliated institutions between October, 1996 and February, 1998. Diagnosis of ALL was based on morphological, biochemical, and immunological features of the leukemic cells, as described (20–22). Normal infant bone marrow specimens were obtained from the Hughes Institute Bone Marrow Cell Bank. Normal thymuses were obtained from three infants undergoing thoracic surgery for a cardiac defect. One fetal thymus was obtained from a prostaglandin-induced human abortus of 21 weeks gestational age. These tissues were used according to the guidelines of the Hughes Institute Committee on the Use of Human Subjects.

Patient characteristics are listed in Table 1: Nine of 12 infants were diagnosed at <6 months of age. There were seven female and five male infants. Ten infants had high white blood cell counts at diagnosis (range: 83,000–1,000,000 cells/μl), and ten presented with massive hepatosplenomegaly. Immunophenotypically, all cases were identified as $CD19^+$ B-lineage ALL by standard immunophenotyping criteria (20–22). Cytogenetic analyses (see below) were performed on samples from 8 of the 12 infants, and reverse transcription (RT)–PCR assays for MLL-AF4 fusion transcript expression (see below) were performed in all 12 cases. A total of 10 cases had PCR-detectable MLL-AF4 fusion transcripts: Six of these cases had a cytogenetically (24) detected t(4;11)(q21;q23); one case had a normal diploid karyotype. Of the two MLL-AF4-negative patients, one had a cytogenetically detected t(11;19)(q23;p13).

RT-PCR and Nucleotide Sequencing. All RT-PCR assays for *MLL-AF4* fusion transcript expression as well as *Ikaros* mRNA expression were performed centrally in the Children's Cancer Group ALL Biology Reference Laboratory with all due precautions to avoid false positive results, as described (12). Primers for *Ikaros* cDNA amplification were F1:5′ATGGATGCTGACGAGGGTCAAGAC3′ and R1:5′TTAGCTCATGTGGAAGCGGTGCTC3′. For enhanced sensitivity, the *Ikaros* PCR products were amplified further by nested PCR. Primers for nested PCR were F2:5′CTCATCAGGGAAGGAAAGCC3′ and R2: 5′GGTGTACATGACGTGATCCAGG3′. RNA integrity was confirmed by PCR amplification of the cABL mRNA (12). Negative controls included PCR products from an RNA-free cDNA synthesis and amplification reaction and a DNA polymerase-free reaction. Purified RT-PCR products (QIAquickTM PCR purification kit; Qiagen, Valencia, CA) were cloned into the pCR II vector by using the TA Cloning kit (Invitrogen). The cloned PCR products were purified with a Qiagen plasmid isolation kit and were sequenced automatically with the Thermosequenase sequencing kit (Amersham Pharmacia) and the ALF Sequencer (Amersham Pharmacia) (12). Manual sequencing by the dideoxynucleotide chain termination method was performed by using the T7 Sequenase Quick-denature Plasmid Sequencing kit (Amersham Pharmacia) according to the manufacturer's instructions. The sequences were compared with the published human *Ikaros* cDNA sequence obtained through the GenBank database (accession nos. S80876 and U40462).

Western Blot Analysis of Ikaros Protein Expression. Whole cell lysates were prepared by using a 1% Nonidet-P40 lysis buffer as described (25). Western blot analysis of whole cell lysates for Ikaros expression was performed by immunoblotting using a polyclonal anti-Ikaros antibody (17) reactive with all eight Ikaros isoforms and enhanced chemiluminescence detection system

Table 2. Expression of dominant negative Ikaros isoforms in leukemic cells from infants with ALL

	Frequency
Cytoplasmic localization, confocal microscopy	12/12
Apparent mass <45 kDa, Western blot analysis	11/11
Dominant negative isoforms, RT-PCR	7/7
Ik-4 or Ik4(del)	3/7 (INF-1, INF-2, INF-3)
Ik-7 or Ik7(del)	2/7 (INF-5, INF-12)
Ik-8(del)	2/7 (INF-4, INF-11)

Confocal microscopy, Western blot analysis, and RT-PCR analysis, including PCR cloning and sequencing, were performed as described in *Materials and Methods*. del, deletion mutant.

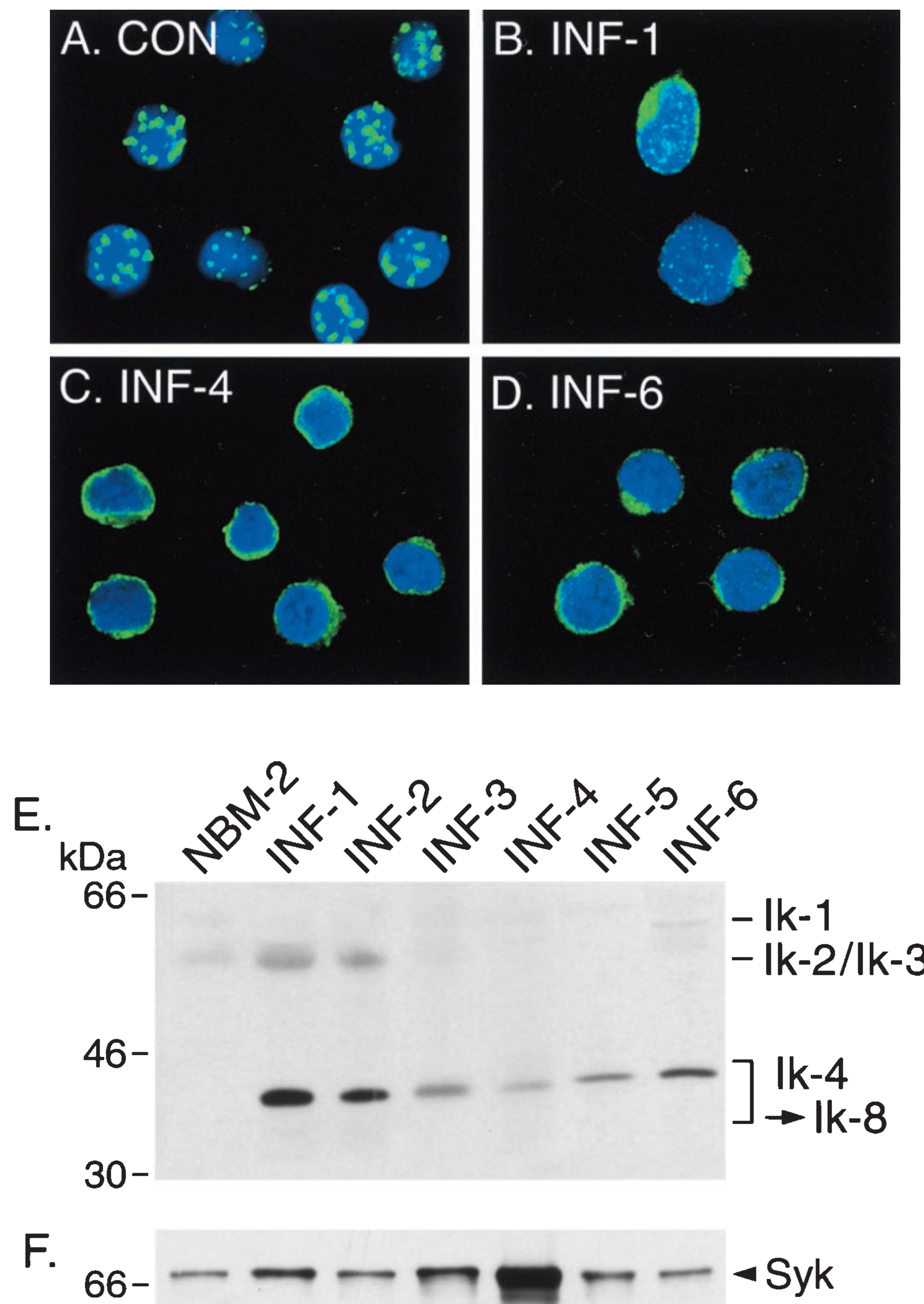


FIG. 1. Expression and subcellular localization of Ikaros in leukemic cells from infants with newly diagnosed ALL. Cells were stained with a polyclonal anti-Ikaros antibody (1:300 dilution), were labeled with a fluorescein-labeled secondary antibody (1:40 dilution) (green fluorescence) as well as the DNA specific dye toto-3 (blue fluorescence), and were visualized by using confocal laser scanning microscopy, as described in *Materials and Methods*. (*A*) Confocal images of normal infant thymocytes showing the characteristic multifocal nuclear localization pattern of Ikaros (i.e., bright punctate green fluorescent staining in toto-3-labeled blue nuclei). (*B–D*) Confocal images of leukemic cells from seven infants with ALL, showing cytoplasmic expression of Ikaros (i.e., bright green fluorescent rim surrounding the toto-3-labeled blue nuclei). (Bar = 10 μm.) (*E*) Anti-Ikaros Western blots of whole cell lysates from normal infant bone marrow mononuclear cells (NBM-2) and leukemic bone marrow blasts from six representative infants with newly diagnosed ALL. The positions corresponding to the migration patterns of Ik-1 ($\approx$57 kDa), Ik-2/Ik-3 ($\approx$47 kDa), and Ik-4 through Ik-8 ($\approx$37–40 kDa) proteins are indicated. (*F*) Anti-SYK Western blots of the whole cell lysates shown in *E*.

(Amersham Pharmacia) as described (25, 26). In brief, 30-μg samples of whole cell lysates were loaded on a 12% mini-tall SDS/PAGE gel, and the size-fractionated proteins were transferred onto a poly(vinylidene difluoride) membrane (Millipore). The membrane was blocked in 5% milk for at least 1 hr at room temperature and then was immunoblotted with a polyclonal anti-Ikaros antibody (1:1000 dilution)[12] in PBS with 5% milk overnight at 4°C. Control membranes were immunoblotted with a polyclonal anti-SYK antibody instead of the anti-Ikaros antibody, as reported (26). The membrane was washed three times with PBST (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4/0.1% Tween 20, pH 7.3) at room temperature and was incubated with a peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution) as the secondary antibody (The Jackson Laboratory) for 2 hr at room temperature before enhanced chemiluminescence.

Subcellular Localization Studies Using Confocal Laser Scanning Microscopy. The subcellular localization of Ikaros protein(s) was examined by immunofluorescence and confocal laser scanning microscopy as described (27). Cells (200 $\times$ 10^3) were attached to poly-L-lysine-coated glass coverslips by a 30-min incubation at room temperature, were washed twice with PBS, and were fixed in ice cold (−20°C) methanol for 15 min. To permeabilize the cells and block the nonspecific antibody binding sites, cells were treated with 0.1% Triton X-100 and 10% goat serum in PBS for 30 min. To detect the Ikaros protein, cells were incubated with an affinity-purified polyclonal rabbit anti-Ikaros antibody (17) of 1:300 dilution for 1 hr at room temperature. Cells were washed with PBS and were incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Amersham Pharmacia) at a 1:40 final dilution for 1 hr. Cells were washed with PBS, were counterstained with the DNA-specific nuclear dye toto-3 (Molecular Probes) at a 1:1,000 dilution for 10 min at room temperature, and were washed again with PBS. The coverslips were inverted, were mounted onto slides in Vectashield (Vector Laboratories) to prevent photobleaching and were sealed with nail varnish (27). Slides were examined by using a Bio-Rad MRC

1024 Laser Scanning Confocal Microscope mounted on an Nikon Eclipse E-800 upright microscope equipped for epifluorescence with high numerical aperture objectives (27). Optical sections were obtained and turned into stereomicrographs by using LASERSHARP software (Bio-Rad).

RESULTS

Abnormal Subcellular Localization of Ikaros Proteins in Leukemic Cells from Infants with ALL. We compared the subcellular compartmentalization of Ikaros proteins in normal and fetal tissues to that in primary leukemic cells from infants by confocal laser scanning microscopy. The nuclei, but not the cytoplasm, of fetal thymocytes, normal infant thymocytes, and normal infant bone marrow mononuclear cells were stained brightly by the anti-Ikaros antibody, as evidenced by a distinct punctate green fluorescent staining pattern in toto-3-labeled blue nuclei (Fig. 1*A*). These findings are consistent with previous reports regarding the exclusively nuclear localization of Ikaros proteins in normal mouse thymocytes and in transfected fibroblast cells ectopically expressing the DNA binding Ikaros isoforms Ik1, Ik2, or Ik3 (17). In contrast to this exclusively nuclear localization with discrete foci of high level expression in normal cells, Ikaros proteins were expressed predominantly in the cytoplasm of leukemic cells in freshly obtained primary bone marrow specimens from all 10 infants with MLL-AF4 fusion transcript-positive ALL as well as both infants with MLL-AF4 fusion transcript-negative ALL, as evidenced by a bright green fluorescent rim surrounding the toto-3-labeled blue nuclei (Table 2 and Fig. 1 *B–D*).

Western Blot Analysis of Ikaros Proteins in Leukemic Cells from Infants with ALL. Ikaros expression was studied in normal tissues and primary leukemic cells from infants with ALL by Western blot analysis. Normal infant bone marrow cells and thymocytes expressed an ≈57-kDa immunoreactive protein corresponding in size to Ik-1 and an ≈47-kDa immunoreactive protein corresponding to either Ik-2 or Ik-3 (Fig. 1*E*, first lane). Similarly, fetal thymocytes expressed a single, ≈57-kDa protein corresponding to Ik-1. In contrast, primary leukemic cells from all infants with ALL primarily expressed smaller immunoreactive proteins of ≈37–40 kDa corresponding in size and electrophoretic mobility to the dominant-negative Ikaros isoforms Ik-4, Ik-5, Ik-6, Ik-7, and Ik-8 that lack critical N-terminal zinc fingers involved in DNA binding (Table 2 and Fig. 1*E*, lanes INF-1 to INF-6). The absence of an abundant Ik-1, Ik-2, or Ik-3 was not caused by a generalized proteolytic degradation because a 72-kDa SYK protein was detected by Western blot analysis in the same whole cell lysates from each infant with ALL (Fig. 1*F*).

Molecular Characterization of Ikaros Transcripts in Leukemic Cells from Infants with ALL. RT-PCR was used to amplify *Ikaros* transcripts in normal fetal thymocytes, infant thymocytes, and bone marrow cells as well as leukemic cells from seven infants with ALL, and the purified PCR products were characterized by electrophoresis and nucleotide sequencing (Fig. 2). A single, ≈1.4-kilobase PCR product was observed in normal fetal thymocytes, and 10 of 10 different PCR clones had the coding sequence of wild-type *Ik-1* (Fig. 2*B*). Similarly, a single, ≈1.2-kilobase PCR amplification product was detected in normal bone marrow cells from a healthy child and three of three different PCR clones had the coding sequence of wild-type *Ik-2* (Fig. 2*B*). By comparison, multiple PCR products were obtained from the patient leukemia samples, and many of these PCR products were smaller than *Ik-2* (Fig. 2*B*). Sequence analysis by PCR cloning was successful in seven of seven infants with ALL (Table 2 and Figs. 2*C* and 3). Half of the PCR clones from patients INF (infant)-1, INF-2, and INF-3 had a mutant coding sequence of the dominant-negative Ikaros isoform Ik-4, with a 30-bp deletion at the 3′ end of exon 6 resulting in deletion of 10 amino acids (Δ KSSMPQKFLG). The PCR clones from INF-4 as well as INF-11 were either Ik-2 or non-DNA binding dominant-negative isoform Ik-8 with the same deletion mutation. All three PCR clones from INF-5 had the wild-type coding sequence of the dominant-negative Ikaros isoform Ik-7. Half of the PCR clones from INF-12 were mutant Ik-7, with the same 30-bp deletion at the 3′ end of exon 6 identical to the deletion mutation in INF-1, INF-2, and INF-3. The observed C-terminal deletions did not cause frame shift and therefore did not change the downstream amino acid sequences.

Thus, extending the confocal microscopy and Western blot analyses, RT-PCR and sequencing confirmed that each infant ALL case expressed dominant-negative and/or mutant isoforms of Ikaros: Leukemic cells from seven of seven infants with ALL expressed dominant-negative Ikaros isoforms, including Ik-4 (three of seven patients: INF-1, -2, and -3), Ik-7 (two of seven patients: INF-5 and -12), and Ik-8 (two of seven patients: INF-4 and -11). Furthermore, in six of seven infant ALL cases, the PCR

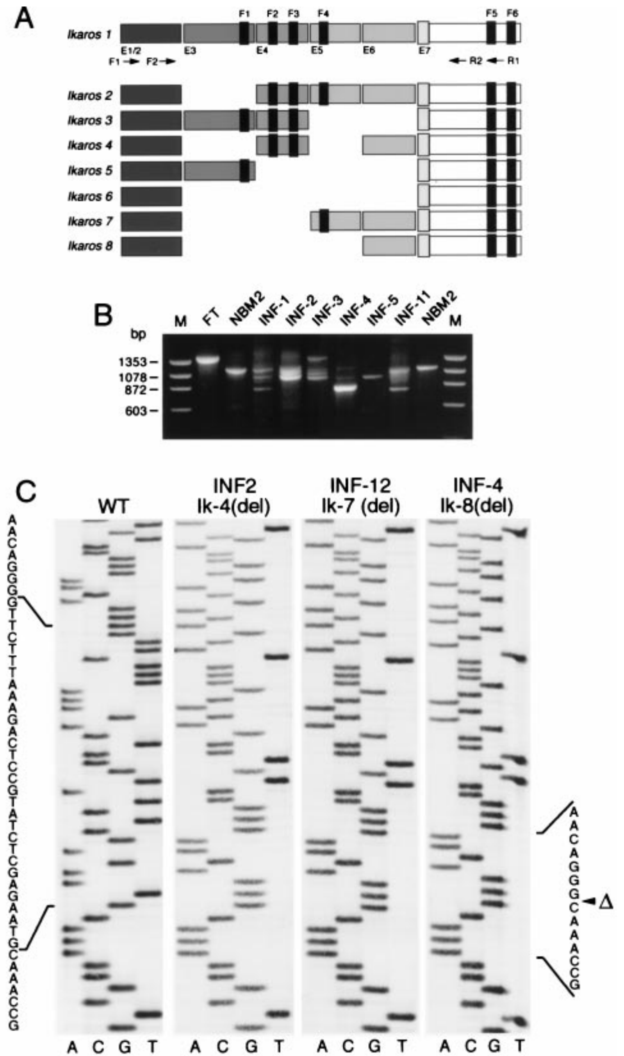
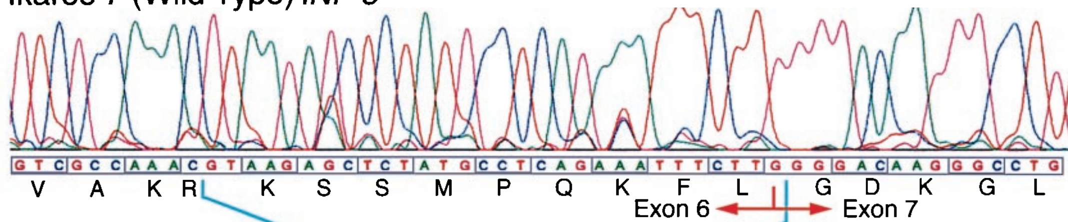
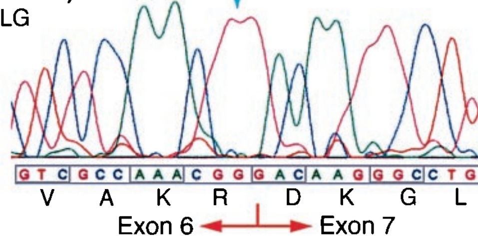
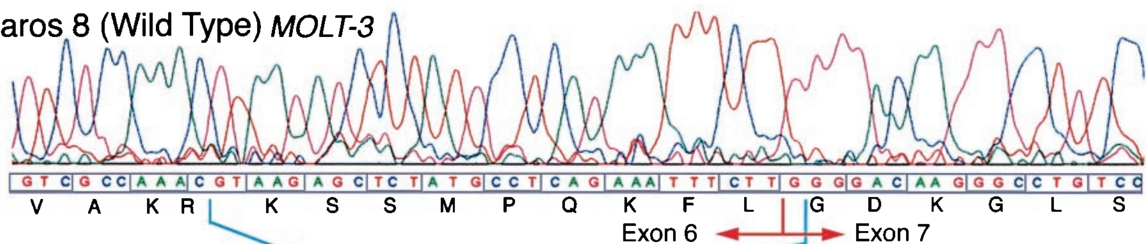


FIG. 2. Detection of PCR-amplifiable Ikaros transcripts in leukemic cells from infants with ALL. (*A*) Schematic representation of Ikaros isoforms 1–8 with specific composition of domains encoded by exons (E) 1–7 and the PCR primers. (*B*) A representative ethidium bromide stained gel of the PCR products from six infants with ALL. The first and last lanes contain molecular size markers. PCR products from normal fetal thymocytes (FT) and normal infant bone marrow mononuclear cells (NBM2) were included as controls. (*C*) Cloned PCR products were subjected to manual sequencing as described in *Materials and Methods*. The nucleotide sequences spanning the junction between exon 6 and exon 7 are shown for representative PCR clones from NBM2 (WT, wild-type) and INF-2 with mutant Ik-4, INF-12 with a mutant Ik-7, and INF-4 with a mutant Ik-8 coding sequence. The PCR clones from infants with ALL had the same 30-bp deletion at the 3′ end of exon 6 (deletion mutation Δ KSSMPQKFLG), which does not cause a frameshift.







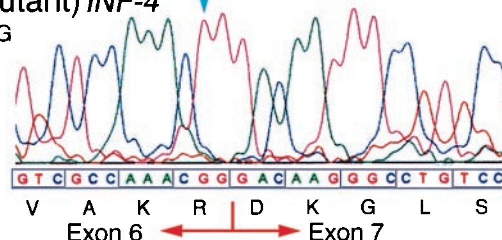


FIG. 3. Sequence analysis of Ikaros PCR clones from infant leukemia cells. Cloned PCR products were subjected to automated sequencing as described in *Materials and Methods*. The exon 6 coding sequence was normal in three of three Ik-7 clones from INF-5 and three of three Ik-8 clones from the MOLT-3 cell line. In contrast, a 30-bp deletion at the 3′ end of exon 6 (deletion mutation Δ KSSMPQKFLG), which does not cause a frameshift, was detected in Ik-7 clones from INF-12, Ik-8 clones as well as Ik-2 clones from INF-4. Sequence traces spanning the junction between exon 6 and exon 7 are shown for some of the individual clones from the MOLT-3 cell line and leukemic cells of three infants with ALL. (*A*) Patients INF-5 (wild-type Ik-7) and INF-12 (Ik-7, Δ KSSMPQKFLG) deletion mutant. (*B*) MOLT-3 leukemia cell line (wild-type Ik-8) and patient INF-4 (Ik-8, Δ KSSMPQKFLG deletion mutant).

clones with coding sequences of *Ik-2*, *Ik-4*, *Ik-7*, and *Ik-8* had the same specific 30-bp deletion at the 3′ end of exon 6 (Table 2 and Figs. 2*C* and 3).

DISCUSSION

The *Ikaros* gene encodes, by means of alternative pre-mRNA splicing, multiple zinc finger proteins with distinct DNA binding capabilities and specificities (14, 17). Furthermore, other DNA binding proteins, such as Aiolos (28) and Helios (29), which can dimerize with all Ikaros isoforms via their shared C-terminal zinc finger domains to form stable multimeric complexes, act in concert with Ikaros and may partially complement its function. These different multimeric complexes are thought to control the transcription of developmentally important genes during lymphocyte ontogeny and thereby play pivotal roles for the orderly maturation of lymphocyte precursors (14). Non-DNA binding Ikaros proteins with fewer than three N-terminal zinc fingers can act as "dominant-negative" regulators by interfering with the ability of DNA binding Ikaros isoforms to form homo- and heterodimers or complexes with Aiolos and Helios (17). It is therefore conceivable that inappropriate expression of non-DNA binding Ikaros isoforms during early lymphopoiesis may dysregulate normal lymphocyte development. Such a developmental error could lead to a maturational arrest at discrete stages of lymphocyte ontogeny and predispose lymphocyte precursors to second hits and leukemic transformation. This hypothesis is supported by the fact that a deletion of three N-terminal zinc fingers of Ikaros results in a dominant-negative mutation and

leads to development of lymphoblastic leukemia in germline mutant mice between the third and sixth months after birth (18).

In the present study, we sought to determine whether molecular abnormalities involving the *ikaros* gene could contribute to the development of infant ALL. Our findings provide unprecedented evidence that, in contrast to normal infant thymocytes and infant bone marrow cells, which express the DNA binding Ikaros isoforms Ik-1 and Ik-2, leukemic cells from infants with ALL preferentially express the alternatively spliced *Ikaros* mRNA species lacking two or more of the exons 3, 4, and 5 that encode crucial DNA binding zinc finger domains. Consequently, these leukemic cells primarily express the non-DNA binding dominant-negative isoforms of Ikaros Ik-4, Ik-7, and Ik-8. Moreover, these non-DNA binding isoforms are localized in the cytoplasm, in contrast to the exclusively nuclear localization of DNA-binding Ikaros isoforms. Consistent with our findings, previous studies in mice with leukemogenic germline Ikaros mutations also demonstrated that dominant-negative Ikaros isoforms lacking exon 3 and exon 4 localized to the cytoplasm (17). These results indicate that the posttranscriptional regulation of alternative splicing of *Ikaros* RNA is defective in leukemic cells from infants with ALL.

A recurring in-frame mutation leading to deletion of a 10-aa peptide was detected in leukemic cells from six of seven infants with ALL and involved the Ik-2, Ik-4, Ik-7, and Ik-8 coding sequences. We speculate that this deletion might have resulted from the selection of an alternative 5′ splice site because it starts with a GU sequence (i.e., GT in cDNA) at the 5′ junction, which could very well serve as a donor-site recognition sequence. The deleted peptide is located in close proximity to the conserved bipartite transcription activation domain (17) within the first 81 amino acids of exon 7 adjacent to the C-terminal zinc finger dimerization motifs. The structural changes caused by deletion of this peptide could affect the accessibility of the Ikaros activation domain for interactions with members of the basal transcription machinery and stability of such interactions. These Ikaros deletion mutants also could be impaired in their ability to form dimers or other higher order complexes with other Ikaros isoforms or other proteins. Such impairments could lead to altered DNA-binding or altered subcellular localization of Ikaros, as we observed in the experiments described above. These possibilities should be examined further in future experiments.

Taken together, our findings provide direct evidence that infants with ALL have mutations in *Ikaros* and express high levels of dominant-negative Ikaros isoforms. Our study extends previous studies in mice (14, 18) that demonstrated that germline mutant mice expressing dominant-negative isoforms of Ikaros develop ALL and implicates the *Ikaros* gene in the leukemogenesis of infant ALL. We hypothesize that, besides the MLL gene rearrangement and expression of the MLL-AF4 fusion gene, the expression of dominant-negative Ikaros isoforms and/or the disruption of normal *Ikaros* function may contribute to leukemogenesis of $t(4;11)^+$ ALL in infants. Because MLL-AF4-negative leukemic cells from one infant who had a t(11;19) translocation and one infant who had a normal diploid karyotype also expressed mutant Ikaros isoforms Ik-7 and Ik-8 with the C-terminal deletion, neither the dysregulation of alternative splicing of *Ikaros* RNA nor the recurrent mutation involving exon 6 can be attributed to t(4;11) translocation or expression of MLL-AF4 fusion transcripts as the primary triggering events. Alternatively, leukemic cells from the infant with the normal diploid karyotype could have a partial duplication of MLL or another MLL fusion that is not apparent cytogenetically. In that case, a molecular abnormality of MLL might well be a triggering event for the Ikaros abnormalities. We are planning to examine, in future studies, leukemic cells from a larger number of non-t(4;11) infants as well as children with B-lineage ALL with and without MLL rearrangements for Ikaros abnormalities. Recent studies have shown that immature lymphocyte precursors express DNA-binding isoforms of Ikaros that localize to centromeric heterochromatin, which may lead to gene silencing (15, 16). Thus, Ikaros might play an important role in recruitment and centromere-associated silencing of growth regulatory genes (15, 16). An abundance of dominant-negative or mutant Ikaros isoforms that no longer bind DNA would interfere with centromeric recruitment and repression of specific genes during lymphocyte development. Expression of Ikaros isoforms incapable of lineage-specific gene silencing might also contribute to the lineage infidelity frequently observed in infant ALL (1, 30).

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