Transcriptional Regulation of the Human Reduced Folate Carrier in Childhood Acute Lymphoblastic Leukemia Cells

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Abstract

Purpose: The transcriptional regulation of the human reduced folate carrier (hRFC), involved in cellular uptake of methotrexate and reduced folates, was studied in childhood acute lymphoblastic leukemia (ALL). The hRFC gene is regulated by six noncoding exons (A1/A2 and A to E) and multiple promoters. In ALL, hRFC-A1/A2 and hRFC-B are the major transcript forms.

Experimental Design: RNAs from 18 ALL lymphoblast specimens and 10 nonobese diabetic/severe combined immunodeficient ALL xenografts were assayed by real-time reverse transcription-PCR for hRFC-A1/A2 and hRFC-B transcripts and for transcripts encoding USF1, GATA1, Sp1, and Ikaros transcription factors. For the xenografts, gel shift and chromatin immunoprecipitation assays assessed transcription factor binding to the hRFC-A1/A2 and hRFC-B promoters. CpG methylation density within a 334-bp region, including the core hRFC-B promoter, was established by bisulfite sequencing. hRFC-A1/A2 and hRFC-B promoter polymorphisms were assayed by DNA sequencing.

Results: For the 28 ALLs, hRFC-A1/A2 and hRFC-B transcripts spanned a 546-fold range. By chromatin immunoprecipitation and gel shift assays, binding was confirmed for USF1 and GATA1 for hRFC-A1/A2, and for Sp1, USF1, and Ikaros for hRFC-B. hRFC transcript levels correlated with those for GATA1 and USF1 for hRFC-A1/A2 and with Sp1 and USF1 transcripts for hRFC-B. CpG methylation in ALL did not correlate with hRFC-B transcripts. In 40 ALL and 17 non-ALL specimens, 2 cosegregating high-frequency polymorphisms (T-1309/C-1217 and C-1309/T-1217; allelic frequencies of 36% and 64%, respectively) were detected in the A1/A2 promoter; none were detected in promoter B. The hRFC-A1/A2 polymorphisms only slightly affected promoter activity.

Conclusions: Our results show a complex regulation of hRFC in ALL involving the hRFC-A1/A2 and hRFC-B promoters and noncoding exons. Although Sp1, USF1, and GATA1 levels are critical determinants of hRFC transcription in ALL, neither DNA methylation nor promoter polymorphisms contribute to differences in hRFC expression.

Methotrexate remains an important component of modern treatment protocols for childhood acute lymphoblastic leukemia (ALL) in connection with both intensification and maintenance therapies (1, 2). In addition, methotrexate is administered intrathecally in the prophylaxis and treatment of central nervous system leukemia. Major determinants of methotrexate response and resistance in ALL have been described, including dihydrofolate reductase levels and heterogeneity, levels and activity of the human reduced folate carrier (hRFC), and capacity for synthesis of methotrexate polyglutamates (3, 4). To elucidate mechanisms resulting in differences in methotrexate polyglutamate accumulations between ALL subtypes, oligonucleotide microarrays were used recently to analyze folate pathway gene expression profiles in diagnostic leukemia cells, resulting in identification of specific alterations in expression of hRFC, ABCC1, ABCG2, and folylpolyglutamate synthetase genes between ALL subtypes (5).

hRFC is of particular interest given its central role in the uptake of methotrexate and reduced folates in human cells (6), its relation to methotrexate polyglutamylation (7), and the extraordinarily wide range of hRFC transcripts reported in both diagnostic and relapsed ALL (8–10). The latter may, in part, reflect differences in hRFC gene expression between subgroups of ALL (e.g., T-ALL, B-precursor nonhyperdiploid, B-precursor hyperdiploid, TEL-AML1, and E2A-PBX1; ref. 5).

Studies on hRFC gene structure and regulation have begun to shed light on the molecular bases for these results in primary ALL specimens. Regulation of hRFC gene expression involves
the tissue-specific utilization of up to six alternatively spliced noncoding exons (designated hRFC-A1/A2 and hRFC-A to E) and multiple promoters (10, 11). In both B-precursor and T-ALL lymphoblasts, use of the hRFC-A1/A2 and hRFC-B promoters/5′-untranslated regions (UTR) predominated (>90% of total transcripts; ref. 10). Although critical transcription factors, including Sp1, USF1, GATA1, and Ikaros, have all been implicated in regulating the hRFC-A1/A2 and hRFC-B promoters in cell culture models (10, 12, 13), these results have not yet been corroborated in clinically relevant ALL specimens. Epigenetic controls may also be important, because decreased histone H3 acetylation accompanies loss of transcriptional activity for at least one promoter (i.e., promoter B). Moreover, DNA methylation of a CpG-rich region downstream of promoter B has been reported to regulate levels of hRFC transcripts in MDA-MB-231 human breast cancer cells (14) and is associated with lower complete remissions in primary central nervous system lymphomas treated with methotrexate-based chemotherapy (15). Finally, translation from an upstream AUG in the A1/A2 hRFC noncoding region results in synthesis of a larger (~7 kDa) functionally distinct hRFC protein from the hRFC form translated from transcripts including the B 5′-UTR (10). Thus, both transcriptional and post-transcriptional controls are likely to be important determinants of hRFC levels and function in ALL.

In this report, we directly assess the transcriptional regulation of hRFC in primary ALL specimens and ALL xenografts engrafted into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Our results further confirm the importance of hRFC-A1/A2 and hRFC-B as major promoters in ALL and establish that intracellular levels of critical transcription factors, including Sp1, USF1, and GATA1, are key determinants of hRFC transcription. Finally, our results suggest that neither DNA methylation nor promoter polymorphisms significantly contribute to differences in hRFC expression among ALL specimens.

Materials and Methods

Cell culture. HT1080 fibrosarcoma, HepG2 hepatoma, and MDA-MB-231 and MCF7 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). HepG2 and HT1080 cells were cultured as described previously (12, 13, 16). MDA-MB-231 and MCF7 cells were cultured in RPMI 1640, including 2 mmol/L l-glutamine and antibiotics, supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

Patient specimens. The childhood ALL specimens used were obtained at diagnosis from children treated at the Children’s Hospital of Michigan (Detroit, MI). Leukemia blasts were purified by standard Ficoll-Hypaque density centrifugation. Sample handling and data analysis protocols were approved by the Committee on Investigation Involving Human Subjects at Wayne State University (Detroit, MI).

Engraftment of primary ALL specimens into NOD/SCID mice. All procedures involving NOD/SCID mice were approved by the Wayne State University Animal Investigation Committee. Female NOD/SCID mice at 4 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). At 7 weeks, mice were irradiated (2.2 Gy) with a cesium-137 source. After 4 to 6 hours, mice were inoculated by tail vein injection with 2 million to 10 million primary ALL specimens (see above) in RPMI 1640. After ~1 month, mice were monitored for 4-week intervals for human ALL engraftment by collecting ~50 μL from the retro-orbital sinus for flow cytometry analysis. Erythrocytes were removed by osmotic shock and mononuclear cells were incubated in PBS/30% bovine serum albumin and then added to a two-tube four-color antibody panel (CD34-FITC/CD10-PE/CD19-ECD/CD45-PC5 and DR-FITC/CD33-PE/CD3-ECD/CD2-PC5; all from Coulter/Immunootech, Miami, FL). Samples were analyzed with a Coulter XL-MCL flow cytometer equipped with a 488 nm argon laser using the F5/SSLog gate to include all mononuclear cells while excluding dead cells and debris. Results were reported as percent positive cells expressing human antigen based on total mononuclear cells gated and were sufficiently reliable to detect down to 0.2% human leukemia cell engraftment. When CD45 levels exceeded 50% or at the first indication of morbidity, mice were sacrificed by cervical dislocation. Single-cell suspensions of mononuclear cells were then prepared from bone marrows and spleens and purified by Ficoll-Hypaque density centrifugation. Yields of human ALL lymphoblasts routinely exceeded 2 × 10⁶ cells and purities of >90% blast cells.

Real-time PCR quantitation of gene expression. Total RNAs were extracted from primary ALL lymphoblasts using the RNeasy Midiprep kit (Qiagen, Valencia, CA). For cell lines, RNAs were prepared with TriReagent (Molecular Research Center, Inc., Cincinnati, OH). cDNAs were prepared from 1 μg RNAs using random hexamers and a reverse transcription-PCR (RT-PCR) kit (Perkin-Elmer Life Sciences, Boston, MA) and were purified with the QiAquick PCR Purification kit (Qiagen). hRFC-A1/A2 and hRFC-B transcripts and transcripts for assorted promoters/5′-untranslated regions (UTR) predominated in ALL specimens as before (12, 16) with RNA standards prepared from 1 μg total RNA using random hexamer primers as above (10). Primers and PCR conditions used are shown in Table 1. Following each run, the products were analyzed by melting curve analysis from 40°C to 99°C and a final cooling step to 40°C. PCR conditions were designed to ensure linearity and were 95°C for 4 minutes followed by 35 cycles of 95°C for 55 seconds, 60°C for 55 seconds, and 72°C for 60 seconds followed by 72°C for 7 minutes. The amplions were fractionated on a 2% agarose gel in the presence of ethidium bromide.

Gel mobility shift assays. Nuclear extracts were prepared from cell lines and ALL xenografts by standard methods (17), and 10 μg nuclear extracts were used in each binding reaction. The hRFC-A1/A2 and hRFC-B oligonucleotide probes (Table 2) were end labeled with [γ-32P]ATP (Perkin-Elmer Life Sciences). Gel shift assays were done exactly as described previously (12, 16, 18). The gels were dried and visualized by autoradiography.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done in ALL specimens as before (12, 16) with antibodies to Sp1 (Active Motif, Carlsbad, CA), USF1 (Santa Cruz Biotechnology, Santa Cruz, CA), GATA1 (Santa Cruz Biotechnology), and Ikaros (Santa Cruz Biotechnology) and normal IgG (Santa Cruz Biotechnology). A fragment of the hRFC-A1/A2 promoter (positions −1,311 to −987) was amplified from the precipitated chromatin with the sense and antisense primers used to create the hRFC-A1/A2(1311-987) reporter construct (see below) and Taq polymerase (Promega, Madison, WI). Secondary amplifications used sense (5′-AGCTGCTTC-GAGCCGCAATGGCTTGGCCTACAGGTTGCGTTG-3′) and antisense (5′-CTACGTTAACGTTGGAGCTTGGCCTAT-3′) primers. Standard PCR for the hRFC-A1/A2 promoter region was done with forward (5′-CGTCCCACCGGTACCTGCGACT-3′) and reverse (5′-CGTCCCACCGGTACCTGCGACT-3′) primers spanning positions −4,560 to −4,353 using a GC-rich reagent and polymerase (Roche).
For all immunoprecipitations, an unrelated gene sequence not including transcription factor binding sites [bone marrow stromal antigen 2 (BST2)] was also amplified with forward 5'-CTGCTCCGGCATTTCCCTGGAACAT03' and reverse 5'-CCGGACGGCGCGCTGAGGAGC-3' primers to confirm the specificity of the chromatin immunoprecipitation assays. PCR conditions were 95°C for 5 minutes followed by 32 cycles of 95°C for 30 seconds, 62°C for 45 seconds, and 72°C for 45 seconds. 

**Methylation analysis by bisulfite sequencing.** Methylation analysis involved an adaptation of the method of Rein et al. (19). Briefly, genomic DNAs from MDA-MB-231 and MCF7 breast cancer cells and from all specimens were isolated using the PureGene Genomic DNA purification kit (Gentra, Minneapolis, MN). DNAs (2 mcg) were denatured in 0.3 mol/L NaOH for 15 minutes at 37°C and freshly prepared 3.6 mol/L sodium bisulfite (120 A) purifying solution (120 A) was added. The reactions were cycled in a thermal cycler at 99°C for 3 minutes and 50°C for 15 minutes for 20 cycles. Following treatment, DNAs were desalted with the Wizard DNA Purification kit (Promega). The DNAs were eluted in 50 mcL water and then desulfonated in 0.3 mol/L NaOH for 15 minutes at 37°C. The DNAs were precipitated in ethanol and resuspended in 30 mcL water.

For the upper DNA strand, a 384-bp stretch (334 bp minus primers) of the Cpg island from positions +4,626 to +4,293 (in the hRFC upstream sequence spanning the hRFC noncoding exon) was analyzed. The primary (forward 5'-GGGAGGGATGGCGAGCTG-3' and reverse 5'-CCAACCCCACTCTCCTGAAAC-3') and secondary (forward 5'-GGTCAGGAGCGGCTGTTTGGTGGAGG-3', same reverse as the primary reaction) amplifications were done with either Taq or Easy A (Stratagene, La Jolla, CA) polymerases. PCR conditions were 93°C for 3 minutes followed by 32 cycles of 93°C for 55 seconds, 58°C for 55 seconds, and 72°C for 1 minute with 1 cycle of 72°C for 7 minutes. For four samples, the corresponding bisulfite-treated lower strand, including a Cpg-rich stretch implicated as functionally important (14), was analyzed using both primary (forward 5'-GTAGTTTTATATATATATATATATATAGG-3' and reverse 5'-AATAACCCCAAAAAATATACACAC-3') and secondary (same forward as primary reaction; reverse 5'-AATAAAATATATTCCCTGGAACAC-3') amplifications. Primary PCR conditions were 93°C for 3 minutes, 35 cycles of 93°C for 55 cycles, 54°C for 55 seconds, and 72°C for 1 minute followed by 1 cycle of 72°C for 7 minutes. Secondary PCR conditions were 95°C for 3 minutes, 5 cycles of 95°C for 1 minute, 35°C for 1.5 minutes, and 72°C for 1.5 minutes followed by 1 cycle of 72°C for 7 minutes.

The amplicons were resolved on a 2% agarose gel, gel purified, and cloned into PCRII-TOPO vector (Invitrogen, Carlsbad, CA). Plasmid DNAs from eight or nine bacterial clones were prepared for automated DNA sequencing.

**Identification of hRFC-A1/A2 promoter polymorphisms.** Genomic DNAs were isolated from 40 primary ALL samples from patients, and 40 normal control samples. Reverse PCR conditions were 93°C for 55 seconds, and 72°C for 45 seconds.

### Table 1. Summary of primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Size (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S RNA</td>
<td>Forward GATGCGGAGGGCCGGTAT and reverse TGAGGTTTCCCGGTGTTGCA</td>
<td>167</td>
<td>59</td>
</tr>
<tr>
<td>Ikaros</td>
<td>Forward AGCGGGAGCAGGATGAAGTGTA and reverse CTGACCCTGGTCGTTG</td>
<td>148</td>
<td>61</td>
</tr>
<tr>
<td>USF1</td>
<td>Forward CCAGGGGTCTCAGAGGCGACTG and reverse GCGTTTCTCATCCAGGTGTC</td>
<td>176</td>
<td>61</td>
</tr>
<tr>
<td>Sp1</td>
<td>Forward GGGGCCCCAATGGAGCAGTGCAG and reverse TGAGGCAATGGTGAGATG</td>
<td>174</td>
<td>63</td>
</tr>
<tr>
<td>GATA1</td>
<td>Forward TGAGACTTTTGAAGACAGGCTGAG and reverse GAAGGTTGGAGAGGAGAATGCGTA</td>
<td>156</td>
<td>64</td>
</tr>
<tr>
<td>hRFC-A1/A2</td>
<td>Forward GGCCTGCAAGCACCCTTCCAGGTG and reverse GGTACGAGGCCAGGTCAAGG</td>
<td>170</td>
<td>66</td>
</tr>
<tr>
<td>hRFC-B</td>
<td>Forward GGGAGCCCCAGGGCCCGGC and reverse AGCAAGGCCGCCAGGACCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** hRFC promoter A1/A2 and B oligonucleotides used for gel shift assays

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRFC-A1/A2/-1145</td>
<td>-1,114 to -1,113</td>
<td>AAACACAGGGGGCCAGGCGTGGGACATGAG</td>
</tr>
<tr>
<td>hRFC-A1/A2/-1118</td>
<td>-1,118 to -1,090</td>
<td>ACATGATTCTGATAAGGGAGACGGCTTTT</td>
</tr>
<tr>
<td>hRFC-B/-4534</td>
<td>-4,534 to -4,506</td>
<td>CGGGCAGGGGCGGAGCCGCCCAGGTCAG</td>
</tr>
<tr>
<td>hRFC-B/-4434</td>
<td>-4,434 to -4,414</td>
<td>TTCCCGAGTCGGCGGGCGG</td>
</tr>
<tr>
<td>hRFC-B/-4573</td>
<td>-4,573 to -4,554</td>
<td>GTGGGAGGGTGCCCGGTCG</td>
</tr>
</tbody>
</table>

**NOTE:** Double-stranded oligonucleotide sequences in the sense direction for hRFC-A1/A2 and hRFC-B promoter regions are shown.
including 11 from the group in Fig. 1 using the PureGene kit. Seventeen DNAs from peripheral blood or umbilical blood samples were provided by Drs. Murray Norris and Michelle Haber (Children’s Cancer Institute Australia, Randwick, New South Wales, Australia). The A1/A2 promoter region was amplified with sense (5’-TGGCGAGGCACAGATGGTCCAATCGT-3’) and antisense (5’-CAGCTGAGCAACGACCTCG-TGGGCGC-3’) primers using Easy A high-fidelity polymerase. The promoter B region was amplified with primary (sense 5’-GAGGGCG-3’) and antisense 5’-CCCACACTCACCTCACAG-3’) primers using Easy A high-fidelity polymerase. The PCR conditions were 95°C for 30 seconds, 60°C for 45 seconds, and 72°C for 60 seconds (35 cycles). Amplicons were purified with a PCR purification kit and sequenced with an automated DNA sequencing machine.

Preparation of hRFC-A1/A2 luciferase reporter constructs and reporter gene assays. A wild-type hRFC-A1/A2(1311-987) construct (positions −1,311 to −987) was prepared with sense (5’-ACTGCGCTGACCAGTGGCGG-3’) and antisense (5’-CTAGCCTAGCTAGCAGATGGTCCAATCGT-3’) primers. The promoters included restriction sites (XhoI and HindIII, respectively, are italicized) for cloning. A hRFC-A1/A2 construct, corresponding to wild-type hRFC-A1/A2(1311-987) (10, 13) but including polymorphisms at positions −1,309 and −1,217 (T-1309/C-1217 as opposed to the wild-type C-1309/T-1217 forms), was prepared by primary amplification from ALL patient genomic DNA using upstream sense (5’-ACTGCGCTGACCAGTGGCGG-3’) and downstream antisense (5’-CTAGCCTAGCTAGCAGATGGTCCAATCGT-3’) primers (which they were derived (median, 8.93 × 10^−3 and 4.57 × 10^−3 relative units, respectively; and the corresponding primary specimens from which they were derived (median, 5.90 × 10^−3 and 2.14 × 10^−3 relative units, respectively; P = 0.36 and 0.67, respectively) specimens. These results further document the remarkably broad range of hRFC transcript levels for both hRFC-A1/A2 and hRFC-B transcripts and promoter activities in pediatric ALL specimens.

**Results**

**ALL xenografts exhibit identical patterns and expression of surface markers to primary ALL specimens.** As a backdrop for our studies of hRFC in pediatric ALL and to provide sufficient numbers of ALL lymphoblasts for our experiments, we engraved 10 primary ALL specimens (9 B-precursor and 1 T-ALL) into NOD/SCID mice. Lymphoblasts were harvested from spleens, purified, and analyzed by flow cytometry for surface markers. Results confirmed that the xenografts exhibit virtually identical patterns of surface marker expression to the original patient specimens (data not shown). Similar results have been reported elsewhere (21, 22).

**Analysis of hRFC-A1/A2 and hRFC-B transcripts in primary ALL specimens and engrafted ALLs by real-time RT-PCR.** In childhood ALL, transcripts including 5’-UTRs from exons A1/A2 and B predominate, although these are distinguishable by their different translation start sites and encoded hRFC proteins differing in mass and transport function. We used real-time RT-PCR methods to measure hRFC transcripts with the hRFC-A1/A2 and hRFC-B 5’-UTRs reported previously (10) to account for >90% of total hRFC transcript forms. 5’-UTR utilization is a direct reflection of endogenous promoter activities. Samples included 18 primary ALL specimens (9 B-precursor and 9 T-ALL) in addition to the 10 ALL xenografts. The data on patterns of quantitative hRFC gene expression (Fig. 1) were similar to those reported previously (10). For this cohort, we measured a 190-fold range of hRFC-B transcripts and a 546-fold range of hRFC-A1/A2 transcripts. There were only slight and statistically insignificant differences between the median or the distributions of hRFC-B and hRFC-A1/A2 transcripts for the xenograft specimens (median, 13.0 × 10^−3 and 2.31 × 10^−3 relative units, respectively) and the corresponding primary specimens from which they were derived (median, 8.93 × 10^−3 and 4.57 × 10^−3 relative units, respectively; Mann-Whitney test) or for the B-precursor (median, 3.29 × 10^−3 and 1.81 × 10^−3 relative units, respectively) and T-ALL (median, 5.90 × 10^−3 and 2.14 × 10^−3 relative units, respectively; P = 0.36 and 0.67, respectively) specimens. These results further document the remarkably broad range of hRFC transcript levels for both hRFC-A1/A2 and hRFC-B 5’-UTRs and promoter activities in pediatric ALL specimens.

**Analysis of transcription factor binding to the hRFC-A1/A2 and hRFC-B promoters in xenograft ALL specimens.** A major advantage of using the NOD/SCID xenograft model for ALL lies in its ability to circumvent limitations of cell numbers for assays of gene expression commonly associated with use of primary ALL specimens directly from patients. Accordingly, we used ALL xenografts to prepare chromatin and nuclear extracts to study transcription factor binding in *vivo* by chromatin immunoprecipitation and *in vitro* by gel shifts.

We characterized previously a 123-bp region in the hRFC-B promoter, including Ikarsos(c), Ikarsos(b), GC-box(b), Ikarsos(a), GC-box(a), and E-box(a) elements (ref. 12; Fig. 2A). For hRFC-A1/A2, a 270-bp promoter was identified, including E-box(a), E-box(b), GATA1, and E-box(c) elements (ref. 13; Fig. 2B). For cloning site of pGL3-Basic vector. All constructs were verified by automated DNA sequencing. Reporter gene assays with HepG2 cells were done exactly as in our prior reports (10, 16, 18). For all transfections, three or more experiments were done in duplicate.

**Fig. 1.** Real-time RT-PCR analysis of patient samples. hRFC transcripts, including the individual hRFC-A1/A2 and hRFC-B 5’-UTRs for 10 NOD/SCID xenograft and 18 primary ALL specimens (10 T-ALL and 18 B-precursor ALL), were measured by real-time PCR. Results are expressed in the same relative units and are normalized to 18SRNA. The xenograft samples are B10087, B10089, B10056, B10054, B10067, B10093, B10085, B10069, B10063, and T20079.
hRFC promoter B, in vivo binding of Sp1, USF1, and Ikaros was localized to within 208 bp of 5’ flanking sequence. For hRFC-A1/A2, in vivo binding of USF1 and GATA1 was confirmed by chromatin immunoprecipitation in ALL specimens, involving a 330-bp fragment flanking the A1/A2 transcriptional start sites and including the 270-bp transcriptionally important region (13). Representative chromatin immunoprecipitation results are shown in Fig. 3A. Neither sample showed any transcription factor binding within a control sequence (BST2) that did not include DNA-binding elements.

On gel shifts with oligonucleotides, including major cis elements (USF1 and GATA1 for hRFC-A1/A2 and Sp1, USF1, and Ikaros for hRFC-B; Table 2), in vitro binding of all these transcription factors was confirmed, analogous to results in HepG2 and HT1080 cells (Fig. 3B and C; refs. 12, 13). Binding specificity was confirmed by competition with unlabeled hRFC oligonucleotides (lanes 3 and 18 show results with cell lines); complex identities were confirmed with specific competitor oligonucleotides (GATA, USF, and Sp1; lanes 4, 5, and 19). In some cases, there were substantial variations in the levels of bound factors among the 10 xenograft specimens. However, any correlation between in vitro protein binding and hRFC transcript levels was, at best, inexact. Nonetheless, for hRFC-A1/A2 transcripts, there was a trend toward higher transcript levels with increased binding of USF and, to some degree, GATA1 (data not shown). This likely reflects the largely qualitative character of these in vitro binding assays as well as the small number of samples with sufficient cells for our gel shift analyses. Importantly, these chromatin immunoprecipitation and gel shift results validate our previous results on hRFC transcription in cell line models (12, 13) in ALL.

Correlations between levels of major transcription factors and hRFC-A1/A2 and hRFC-B transcripts in ALL specimens. As an extension of our chromatin immunoprecipitation and gel shift experiments, we measured transcript levels for the major transcription factors implicated in regulating the hRFC-A1/A2 and hRFC-B promoters by amplifying cDNAs prepared from the 10 xenograft and 18 primary ALL specimens in real time. Levels of the individual hRFC transcript forms from Fig. 1 were plotted against GATA1 and USF1 transcript levels for hRFC-A1/A2, and against Sp1 and USF1 transcript levels for hRFC-B. As shown in Fig. 4, there were highly significant (r = 0.89; P < 0.001) correlations between levels of hRFC-A1/A2 transcripts and GATA1 over a 359-fold range of GATA1 transcripts and with USF1 over a 269-fold range of USF1 transcripts (r = 0.74; P < 0.001, Spearman nonparametric analysis). For hRFC-B, increasing transcripts, likewise, paralleled changes in Sp1 over a 394-fold range (r = 0.87; P < 0.001) and in levels of USF1 (r = 0.82; P < 0.0001).

Our previous results (refs. 12, 13; Fig. 3) also suggested that the B promoter is regulated by the family of Ikaros proteins. However, total levels of Ikaros transcripts in the 28 ALL specimens correlated poorly with hRFC-B transcripts (data not shown), a result that was not particularly surprising because Ikaros includes up to eight isoforms generated by alternate splicing of exons 3 to 7 (23, 24). Because at least three of the four internal zinc fingers (i.e., exons 3-5) are needed for high-affinity DNA binding, only Ikaros 1 to 3 significantly bind to DNA, whereupon they can either activate or repress transcription. Ikaros 4 to 8 are dominant-negative forms that bind poorly to DNA and interfere with cellular responses to the DNA-binding Ikaros proteins via the formation of heterodimers. In previous studies of hRFC transcription from the hRFC-B promoter, Ikaros 2 was a potent repressor of transcription (12). However, the effects of Ikaros 2 could be at least partly reversed by Ikaros 8. By amplification of cDNAs prepared from the 28 ALL specimens using primers located in exons 1/2 and 7, only Ikaros 2/3 and 4 were consistently detected (data not shown). There seemed to be a range of overall Ikaros expression, consistent with the real-time PCR results. However, there were only minor differences in the distributions of the dominant-negative (Ikaros 4) to DNA-binding (Ikaros 2/3) isoforms among our cohort.

Methylation of the hRFC-B promoter and flanking sequences. Previous studies have implicated DNA methylation in the regulation of hRFC expression in MDA-MB-231 breast cancer cells (14); however, this was not observed for other methotrexate-resistant cell lines with decreased hRFC expression (25). The upstream region of the hRFC gene includes a
598-bp CpG island from positions −4,802 to −4,204. This encompasses promoter B and a stretch upstream of promoter A that has been reported to be methylated in MDA-MB-231 cells (ref. 14; Fig. 5). To explore the possibility that methylation within this CpG island may contribute to differences in hRFC levels between low and high hRFC-expressing ALL specimens, genomic DNAs from 12 ALL samples were treated with bisulfite to convert unmethylated cytosines to uracils, conditions under which methylated cytosines are unaffected (19). We amplified a 334-bp stretch of the positive DNA strand for sequencing of eight or nine individual subclones. For four ALL samples (samples B1441, B2486, B10056, and B10085) with the lowest and highest hRFC-B levels in our cohort (Fig. 1), we also amplified and sequenced the corresponding negative DNA sequence that included the CpG-rich region implicated previously in regulating hRFC gene expression (14). Results were compared with those for MDA-MB-231 and MCF7 breast cancer cells.

Extensive methylation was detected for CpGs in both positive (Fig. 5) and negative (data not shown) DNA strands from MDA-MB-231 cells, accompanying almost undetectable levels of hRFC-B transcripts (0.05 × 10^{-3} relative units). Conversely, substantially higher levels of hRFC transcripts in MCF7 cells (2.12 × 10^{-3} relative units) were accompanied by almost no CpG methylation. For the 12 ALL specimens, CpG methylation was sporadic. Although somewhat higher levels of methylation were detected in the samples with the lowest hRFC-B transcripts (e.g., samples B1411 and B2486), there was no consistent association between patterns or densities of methylated CpGs and levels of hRFC-B transcripts.

Identification of hRFC-A1/A2 promoter polymorphisms. Based on our earlier finding of a high-frequency polymorphism in the hRFC-A promoter (26), we considered this possibility for the hRFC-A1/A2 and hRFC-B promoters. Accordingly, DNAs were amplified from ALL and non-ALL specimens, including the previously documented transcriptionally important regions (refs. 12, 13; positions −1,463 to −964 for promoter A1/A2 and positions −4,817 to −4,293 for promoter B), for sequencing. For promoter B, there were no sequence alterations in any of the 38 ALL specimens that were tested. In 40 primary ALL samples, including 11 from the group in Fig. 1, two high-frequency polymorphisms were identified at positions −1,309 and −1,217 in the hRFC-A1/A2 promoter (C-to-T transition at position −1,309 and T-to-C transition at position −1,217; noted in Fig. 2B). Identical results were obtained with 17 DNAs from normal (nondisease) patients. Among these 57 DNAs, the sequence changes at positions −1,309 and −1,217 seemed to cosegregate (there was no C-1309/C-2117 or T-1209/T-1217). Frequencies of these hRFC-A1/A2 promoter polymorphisms in ALL and non-ALL specimens are summarized in Table 3. For all 57 DNAs, allelic frequencies were calculated as 36% and 64% for T-1309/C-1217 and C-1309/T-1217, respectively. By Fisher's exact test, there was no statistically significant difference (P = 1.0) between the allelic frequencies for the ALL and non-ALL specimens.

Although positions −1,309 and −1,217 were outside the major cis elements in promoter A1/A2 (Fig. 2B), it was, nonetheless, important to assess the possible functional significance of these sequence changes. The hRFC-A1/A2 promoter, including C-1309/T-1217 and T-1309/C-1217, was amplified from two ALL DNAs and subcloned into pGL3-Basic to generate constructs analogous to the hRFC-A1/A2(1311-987) construct. Constructs were transiently transfected into HepG2 cells and assayed for luciferase as a measure of promoter activity. In this series of experiments, the presence of the substitutions at both positions −1,309 and −1,217 caused, at most, a minor (~10%) decrease in hRFC-A1/A2 promoter activity from the wild-type construct. Consistent with this result, for the 11 ALL samples for which hRFC transcripts were measured (5 with C-1309/T-1217 and 6 with T-1309/C-1217), there were no significant differences in the levels of hRFC-A1/A2 transcripts between the groups (median, 2.44 × 10^{-3} and 2.79 × 10^{-3} relative units, respectively; P = 0.79, Mann-Whitney test).
Discussion

The present study significantly expands on our prior reports of hRFC expression and function in pediatric ALL (8, 10) and studies of the complex transcriptional and post-transcriptional regulation of hRFC in ALL and non-ALL specimens (10–13, 16, 18, 26). Our current results also further validate NOD/SCID ALL xenografts as relevant models of clinical disease and establish that hRFC transcripts, including 5′-UTRs derived form hRFC-A1/A2 and hRFC-B noncoding exons, are major transcript forms for both primary and xenograft ALLs. Using nuclear extracts and chromatin from ALL xenografts in gel shift and

Fig. 4. Correlations between transcript levels for individual transcription factors and hRFC transcript forms. Results for 28 ALL specimens (10 xenografts and 18 primary ALLs), illustrating the relationships between transcript levels for GATA1, Sp1, and USF1 and hRFC-B and hRFC-A1/A2 as measured by real-time RT-PCR. The Spearman correlations were all highly significant.

Fig. 5. Methylation analysis of CpG island flanking hRFC promoters A and B. Top, CpG island prediction by MethPrimer program (http://www.urogene.org/methprimer/) and its location in the hRFC gene. The sequence flanking hRFC promoters A and B was input into the MethPrimer program. One CpG island spanning parts of promoters A and B was predicted, which stretched 598 bp upstream of promoter A and with a guanine/cytosine content above 0.5 and an observed or expected CpG frequency above 0.6. A 334-bp region, including part of promoter A, exon B, and part of promoter B, was targeted for bisulfite sequencing PCR. Bottom, genomic DNAs from 12 ALL patient samples and 2 breast cancer cell lines (MDA-MB-231 and MCF7) were treated with bisulfite and PCR amplified. The amplicons were cloned into PCR8-TOPo vector. Plasmid DNAs from eight or nine clones were sequenced and aligned to bisulfite converted sequence. Forty-five CpGs within the 334-bp sequence are depicted and methylated CpGs are marked with closed circles.
Table 3. Sequence polymorphisms in the hRFC-A1/A2 promoter region

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>Homozygous T-1309/C-1217 (%)</th>
<th>Heterozygous T-1309C/C-1217T (%)</th>
<th>Homozygous C-1309/T-1217 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>40</td>
<td>7 (17.5)</td>
<td>16 (37.5)</td>
<td>18 (45)</td>
</tr>
<tr>
<td>Non-ALL</td>
<td>17</td>
<td>3 (17.65)</td>
<td>6 (35.29)</td>
<td>8 (47.06)</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>10 (17.54)</td>
<td>21 (36.84)</td>
<td>26 (45.61)</td>
</tr>
</tbody>
</table>

NOTE: Forty genomic DNAs from primary ALL samples and 17 DNAs from normal (nondiseased) samples were isolated, PCR was amplified, and the amplicons were sequenced for the presence of sequence polymorphisms.

chromatin immunoprecipitation assays, we were able to show in vitro and in vivo binding of Sp1, USF, and Ikaros to the hRFC-B promoter and of USF1 and GATA1 to the hRFC-A1/A2 promoter. These findings are consistent with our published studies in cell line models (12, 13). For the expanded cohort, including both primary and engrafted ALLs, levels of the individual hRFC transcripts closely paralleled levels of Sp1 or USF1 transcripts for hRFC-B, and of GATA1 or USF1 transcripts for hRFC-A1/A2. Although conclusions based on these results could be affected by the extent to which these transcript forms are efficiently translated into proteins and/or by the extent to which their transcriptional effects may be influenced by their post-translational modifications, they, nonetheless, strongly imply that intracellular levels of these critical transcription factors are major determinants of transcriptional activities of these hRFC promoters in ALL lymphoblasts.

Disproportionate levels of dominant-negative forms of Ikaros were reported previously in ALL (27–29); however, this remains controversial (30). In our patient cohort, only DNA-binding Ikaros 2/3 and dominant-negative Ikaros 4 transcript forms were consistently detected. Although Ikaros proteins were implicated previously in the regulation of the hRFC-B promoter (12), in the present study, neither overall expression of Ikaros nor the distributions between these individual DNA-binding and dominant-negative Ikaros isoforms significantly correlated with levels of hRFC-B transcripts in individual ALL specimens. Thus, it would seem that the effects of Ikaros proteins on transcription from promoter B are significantly modulated by the Sp1 and USF families of transcription factors (12).

Our previous findings of both hRFC coding (31) and promoter (26) sequence variants raised the possibility that the extreme variability in hRFC transcript levels in primary ALL specimens may reflect the presence of additional unrecognized sequence alterations in the hRFC-A1/A2 and/or hRFC-B promoters. Although two high-frequency cosegregating (C/T-1309 and T/C-1217) sequence changes were identified in the hRFC-A1/A2 promoter in ALL and non-ALL specimens, these fell outside the critical cis elements and caused only a minor overall effect on promoter activity in reporter gene assays. For promoter B, no sequence alterations were detected over 253 bp spanning the major transcriptional start sites.

Another regulatory consideration involves epigenetic effects because hRFC-B promoter activity is sensitive to changes in histone deacetylation (12), and total hRFC transcripts in MDA-MB-231 breast cancer cells and primary lymphomas are decreased accompanying methylation of a downstream promoter region proximal to exon B (14, 15). We used DNA sequencing of amplified DNAs from both DNA strands isolated from ALL specimens spanning the entire range of hRFC-B transcripts to directly assess possible contributions of DNA methylation within a 598-bp CpG island, including the entirety of promoter B and a stretch of promoter A reported to be methylated (14). Extensive CpG methylation within this stretch was confirmed for the MDA-MB-231 breast cancer subline. For the 12 ALL specimens, CpG methylation was substantially reduced and there were some differences between samples with the highest and lowest transcript levels. However, there was no consistent association between patterns or density of methylated CpGs and levels of hRFC-B transcripts among the ALL cohort. Of course, we cannot exclude the possibility that there might be other regions in the hRFC gene whose methylation results in changes in hRFC expression.

In combination with our prior reports (8, 10, 12, 13), the present results shed light on the possible basis for the wide range of hRFC transcripts in both diagnostic and relapsed ALL. In ALL, >90% of hRFC transcripts are transcribed from the hRFC-B and hRFC-A1/A2 promoters (10) regulated by distinct transcription factor families, including Sp1, USF1, GATA1, and Ikaros. As reported herein, for 28 ALL lymphoblast specimens, there was a close relationship between levels of Sp1 or USF1 and hRFC-B transcripts and between levels of GATA1 or USF1 and hRFC-A1/A2 transcripts. However, the ramifications of separate promoter usage can vary depending on sites of transcription initiation, the extent and manner of splicing of the alternate noncoding exons, and/or the presence of coding frame (i.e., CATG) insertions that result in early translation termination (8, 11, 31). When combined with possible differences in translation efficiencies and transcript stabilities for the resulting hRFC transcripts, let alone the synthesis of a modified, functionally distinct hRFC protein from an upstream AUG in the A1/A2 noncoding exon, this could result in wide-ranging hRFC protein and transport activity with prognostic importance. Indeed, our previous study (8) established, at best, a partial correlation between total hRFC transcripts and methotrexate uptake in ALL lymphoblasts, most likely due to these post-transcriptional regulatory effects.

The use of other hRFC promoters and noncoding exons in nontumor tissues in response to tissue-specific transcription factors may confer a selective pattern of net hRFC expression that manifests as therapeutic selectivity. Thus, the optimal result from these assorted transcriptional and post-transcriptional controls is to provide sufficient hRFC transport and intracellular methotrexate in ALL lymphoblasts for maximal dihydrofolate reductase inhibition and methotrexate polyglutamate synthesis during chemotherapy. Accordingly, any
change that impedes overall transport by hRFC in ALL cells would adversely affect drug activity, an effect that would likely be exacerbated in primary clinical specimens with lower levels of dihydrofolate reductase and hRFC proteins and methotrexate transport than those reported in cultured cells (8, 32). Future studies will focus on the prognostic significance of patterns of total hRFC levels and hRFC promoter usage in a larger retrospective study of childhood ALL. A better understanding of the molecular determinants of hRFC expression and function in ALL and normal susceptible host tissues should foster new approaches for modulating promoter activity to increase the effectiveness of methotrexate chemotherapy in treating this disease.

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References

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