RAPID COMMUNICATION

Functional and Biochemical Analysis of the Cloned Duffy Antigen: Identity With the Red Blood Cell Chemokine Receptor

By Kuldeep Neote, John Y. Mak, Lee F. Kolakowski, Jr, and Thomas J. Schall

The Duffy blood group antigen has been postulated to be a receptor on red blood cells (RBCs) for the malarial parasite *Plasmodium vivax* and a promiscuous receptor for the chemokine superfamily of inflammatory proteins. Recently, the Duffy antigen glycoprotein D cDNA has been cloned (Chaudhuri et al: Proc Natl Acad Sci USA 90:10793, 1993). We have analyzed the binding properties of the cloned Duffy antigen. Duffy-antigen cDNAs expressed in human embryonic kidney cells produced cell-surface proteins that reacted with two known anti-Duffy monoclonal antibodies. Direct ligand binding and displacement experiments using recombinant chemokine proteins also show that the cloned Duffy protein is the RBC chemokine receptor. Radiolabeled chemokines of both the C-C (RANTES and MCP-1) and C-X-C (IL-8 and MIP-1α) subclasses bound reversibly to transfected cells with dissociation constants in the nanomolar range.

Chemokines of either class displaced heterologous chemokines, indicating that they were competing for a single site on the transfected cells. Although the chemokines bound to the transfected cells with high affinity, there was no evidence for signal transduction, as measured by transient increases in intracellular calcium ion concentration, through the Duffy antigen/RBC chemokine receptor in transfected cells. Lastly, we have performed a computer analysis on the amino acid structure of the Duffy antigen/RBC chemokine receptor. Although the cloned Duffy antigen has been postulated to be a nine-transmembrane-spanning receptor, our analysis suggests that the molecule most likely belongs to the seven-transmembrane-spanning receptor superfamily and is therefore similar to other chemokine receptors previously identified.

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MATERIALS AND METHODS

Polymerase chain reaction (PCR), subcloning, and sequencing. Total RNA was isolated by the guanidinium isothiocyanate-cesium chloride procedure or purchased from Clontech (Palo Alto, CA). Total RNA (1 to 2 μg) from various sources was used as substrate for reverse transcription-PCR (RT-PCR) as described previously. Oligonucleotides used in the PCR were as follows: sense, 5'-GAC-TCTTCGACAGGACCTGGTCTC; and antisense, 5'-CGGGAT-CCCGTAGACTTTAATTCAGGTTGAC. PCRs were performed in 100-μL reactions as described with the following conditions: 30 cycles at 94°C for 0.5 minutes, 50°C for 1 minute, 72°C for each cycle. PCR products were subcloned into the EcoRI BamHI site of pRK7 and sequenced as described previously.

DNA transfections and binding analysis. Plasmid DNA was purified using the Qiagen Plasmid Kit as recommended by the supplier (Qiagen, Chatsworth, CA). Transfections were performed using Lipofectamine as recommended by the supplier (BRL, Gaithersburg, MD). Transfectants were analyzed after 12 to 24 hours. Binding analysis was done as described previously.

Flow cytometry. Approximately 10^6 cells were incubated with various antibodies in 100 μL of staining buffer (phosphate-buffered saline [PBS] containing 0.5% fetal calf serum [FCS] and 0.01% sodium azide) for 30 minutes at 4°C. Cells were centrifuged, washed once with 100 μL of staining buffer, and incubated for 30 minutes at 4°C with the secondary antibody (goat-antimouse IgG conjugated with phycoerythrin; CalTag, South San Francisco, CA). Cells were centrifuged, washed once with staining buffer, and analyzed by flow cytometry on a Becton Dickinson FACScan (San Jose, CA). Appropriate negative controls using irrelevant antibodies were also performed.

Nucleotide and amino acid alignment, and computer analysis. Amino acid sequence comparisons were performed using the program BLAST. Amino acid sequence analyses were done by the Kyte Doolittle and KKD hydrophathy methods. Alignments were performed using CLUSTALV and manually adjusted to optimize the consensus regions of GCRs. The rates of synonymous (Ks) and nonsynonymous (Kn) codon substitutions for each chemokine gene were determined with the program EXON (kindly provided by William T. Starmer, Syracuse University, Syracuse, NY) using the algorithm of Li et al as follows: the amino acid alignment shown in Fig 4 was used to align the DNA sequence of each of the coding regions, and the regions least similar or containing many gaps were deleted leaving regions of highest overall similarity. Kn and Ks values for each receptor gene were determined from the alignments using EXON.

RESULTS AND DISCUSSION

To investigate the interaction of chemokines with the Duffy antigen, it was cloned by molecular techniques and expressed in the human embryonic kidney cell line 293. A cDNA containing the coding region of the Duffy antigen was obtained by performing RT-PCR, using as substrate total placental RNA and primers flanking the coding region. The PCR product obtained corresponded to the anticipated size of 1.2 kb (data not shown). Subcloning and sequencing of several clones and the PCR product showed the presence of two different sequences, one identical to the published sequence of the Duffy antigen and a second with a base substitution (A → G) at position 479 (nucleotide number corresponds to the sequence deposited in Genbank, accession no. U01839); this nucleotide difference changes Ala102 to Thr. Sequence differences such as these could be polymorphism and therefore represent different allelic forms of the Duffy blood group. Detailed genetic analysis has to be performed to unequivocally correlate amino acid differences with the Duffy blood group alleles. Similar amino acid differences near the N-terminus were also observed by Chaudhuri et al (1993).

To show that the cloned cDNA encodes for the Duffy antigen, 293 cells were transfected with an expression vector construct containing the coding region of the Duffy antigen (pRK-DFA10) and analyzed with Duffy antigen-specific MoAbs anti-Fy3 and anti-Fy6. Both of these antibodies react with Fy^a or the Fy^b antigens but not with RBCs having the Fy (a−b−) phenotype. Positive staining was obtained with both anti-Fy3 and anti-Fy6 antibodies on cells transfected with pRK-DFA10 (Fig 1). Approximately 30% of the transfected cells stained with the antibodies reflecting the transfection efficiency (Fig 1). No staining was observed when the appropriate irrelevant antibody was used (Fig 1) or when cells were transfected with the expression vector alone (data not shown). Thus, the cloned cDNA codes for the Duffy antigen.

Chemokine-binding analysis was next performed on 293 cells transiently expressing the clone containing alanine at position 102. The ability of increasing concentrations of various chemokines to displace radiolabeled interleukin-8 (IL-8), RANTES, and MGSAlgru was assessed. Representative experiments using 125I-MGSAlgru and 125I-RANTES are shown in Fig 2A and 2B, respectively, and Scatchard analysis of the competitive binding data to obtain binding affinities are shown in Table 1. The affinities for MCP-1, IL-8, MGSAlgru, and RANTES on 293 cells transiently expressing the Duffy antigen were approximately 4, 12, 16, and 16 nmol/L, respectively, and are slightly lower than those observed on RBCs. It was observed that homogeneous displacement (ie, displacement of radiolabeled ligand with the same unlabeled ligand) was not as effective as heterogeneous displacement (ie, displacement of the radiolabeled ligand with a different unlabeled ligand). Furthermore, displacement of radiolabeled chemokines with RANTES was not as effective as displacement with other unlabeled chemokines (Fig 2 and Table 1). The reasons for these observations are not readily apparent, but could be related to our previous observation of difficulty with RANTES binding on nucleated cells. It is possible that increasing concentration of the same chemokine causes aggregation and decreases the effective concentration of the chemokine and, consequently, complete heterogeneous displacement does not occur. Also, aggregation on the cell surface can give an apparent increase in the amount of chemokine bound to the cell, and this could be reflected as lack of homogeneous displacement.

In some experiments, MIP-1α seemed to marginally displace radiolabeled chemokines on transfected cells (Fig 2A). However, Scatchard analysis of the displacement showed an affinity greater than 300 nmol/L, suggesting that MIP-1α has a very weak interaction with the Duffy antigen. This is consistent with our previous observation of lack of binding
with RBCs. Taken together, the chemokine binding analyses on the cloned Duffy antigen provide evidence that the Duffy antigen is indeed the chemokine receptor on RBCs and strongly suggests that no additional components are needed for chemokine binding, though we cannot exclude the possibility that other components might be necessary to impart higher affinity binding as seen on native erythrocytes. Further biochemical characterization is needed to define precisely the chemokine binding and parasite-binding domains on the Duffy antigen/RBC chemokine receptor.

To confirm that the binding affinities obtained in transfected cells are indeed lower than those observed on RBCs, parallel binding analysis was performed using RBCs and 293 cells transiently expressing the Duffy antigen. In each case, the affinity on RBCs was consistently between 1 and 5 nmol/L, whereas the affinity on the transfected cells was slightly higher, between 4 and 18 nmol/L (data not shown). Reasons for the apparent differences between the affinities on RBCs and on transfected cells are not clear. It is possible that there are other accessory molecules or subunits that associate with the Duffy antigen and their presence increases the affinity for chemokines. The presence of additional proteins associated with the Duffy antigen has been shown by Chaudhuri et al (1989). However, the most likely explanation is that the lipid environment of the Duffy antigen determines the conformation and therefore the affinity of chemokine binding. In 293 cells, the cell-membrane lipid composition and therefore the lipid environment could be
different than that of RBCs, and these differences could contribute to the different chemokine affinities observed.

Chemokine receptors belong to the superfamily of G-protein coupled receptors (GCRs) that span the membrane seven times. They transduce intracellular signals that result in the mobilization of intracellular calcium stores. In general, these increases in intracellular calcium have been observed in monocytes and neutrophils, although chemokine receptors expressed in 293 cells also respond by mobilizing calcium stores. Because the Duffy antigen/RBC chemokine receptor binds chemokines with high affinity, we determined whether it has the capacity to mobilize intracellular stores of calcium. 293 cells transiently expressing the Duffy antigen/RBC chemokine receptor were loaded with the calcium probe INDO-1-AM and challenged with various chemokines. Changes in intracellular calcium were followed in a spectrofluorometer as described. Regardless of the chemokine used, no increases in intracellular calcium was observed (Fig 3, A and 3B, and data not shown). To show that intracellular calcium stores could be mobilized, cells were subsequently challenged with thapsagargin (Fig 3). As a positive control, cell transfected with the IL-8 receptor type A could mobilize intracellular calcium when challenged by IL-8 (Fig 3C). Finally, all chemokines used could increase intracellular calcium in the premonocytic cell line THP-1 (Fig 3D, and data not shown). These observations suggest that the Duffy antigen/RBC chemokine receptor most likely does not transduce an intracellular signal in the transfected cells tested here. However, one cannot rule out the possibility that other second messengers distinct from those that mobilize intracellular calcium are activated and that the Duffy antigen/RBC chemokine receptor does indeed signal.

Although Chaudhuri et al (1993) report that the deduced amino acid sequence of the Duffy antigen/RBC chemokine receptor is a nine-transmembrane–spanning protein, our analyses using Kyte Doolittle and KKD hydropathy methods show six regions that are clearly membrane spanning, and an additional region that is more weakly hydrophobic and most likely corresponds to the seventh transmembrane-spanning domain. Detailed comparisons of the deduced amino acid sequence of Duffy antigen/RBC chemokine receptor using BLAST show that it is weakly similar to members of the chemokine receptor subgroup of the GCRs superfamily. The highest score is a match to the mouse IL-8/gro receptor homologue. Further comparisons against a complete database of GCRs using portions of the translated amino acid sequence show that transmembrane segments 1 through 4 are significantly similar to the mouse IL-8/gro receptor homologue. However, the best regions of similarity between the Duffy antigen/RBC chemokine receptor and the mouse IL-8/gro receptor homologue are atypical for GCRs. When comparing the sequences of most GCRs, common motifs are present in the transmembrane segments 1, 2, 3, 6, and 7, with potential disulfide forming cysteines in the extracellular loops 1 and 2. Within the chemokine receptor subgroup, an additional pair of cysteines are conserved in the N-terminus and the third extracellular loop (Fig). The regions of strongest similarity between the Duffy antigen/RBC chemokine receptor and the mouse IL-8/gro receptor homologue are in the extracellular regions.

### Table 1. Chemokine Cross-Competition on Duffy Antigen/Chemokine Receptor

<table>
<thead>
<tr>
<th>Cold Competitor Kd (mmol/L)</th>
<th>IL-8</th>
<th>MGSA/gro</th>
<th>RANTES</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>No Disp*</td>
<td>12.5</td>
<td>No Disp</td>
<td>3.67</td>
</tr>
<tr>
<td>MGSA/gro</td>
<td>12.5</td>
<td>16.1</td>
<td>16.21</td>
<td>3.97</td>
</tr>
<tr>
<td>RANTES</td>
<td>11.6</td>
<td>18.6</td>
<td>16.91</td>
<td>4.86</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* No Disp: No appreciable displacement of radiolabeled ligand was observed.

† Complete displaceable binding was not observed (see Fig 1).
Intracellular calcium measurements. (A) 293 cells transiently expressing the Duffy antigen/RBC chemokine receptor were harvested, loaded with calcium probe INDO-1-AM, and assayed by spectrofluorometric methods as previously described. Cells were first challenged with 500 nmol/L IL-8 followed by 10 nmol/L thapsagargine (THAPS) as shown. (B) Details as in (A) except cells were challenged with 500 nmol/L RANTES. Similar results were obtained when the cells were challenged with MCP-1 or MGSA/gro (data not shown). (C) 293 cells transiently expressing the IL-8 receptor A were assayed as in (A), and challenged with 500 nmol/L IL-8 followed by thapsagargine as shown. (D) THP-1 cells were assayed as indicated in (A) and challenged with 500 nmol/L RANTES as shown. Similar results were obtained when THP-1 cells were challenged with IL-8, MCP-1, or MGSA/gro (data not shown).

The striking features of the Duffy antigen/RBC chemokine receptor are: (1) the very low similarity of this GCR with regions of GCRs typically conserved in others; and (2) the high affinity and nonselective binding despite the lack of intracellular signaling. These observations suggest that the evolution of this receptor may have unique features that might distinguish it from other chemokine receptors. To investigate this, the rates of synonymous (silent, $K_s$) and non-synonymous (missence, $K_a$) codon substitutions were determined as outlined in Materials and Methods. For many gene
Fig 4. Alignment of the coding sequence of the Duffy antigen/RBC chemokine receptor with chemoattractant receptors. Shown are the Duffy antigen/RBC chemokine receptor (DFNCR), mouse homologue of the human IL-8 receptor (mlL-8rH), human IL-8 receptor type B (hlL-8rB), and the C-C chemokine receptor type 1 (C-C CKR) and the rat GlOd orphan receptor (rGlOd). Shaded amino acid positions are identical in at least three of the sequences compared and the ones highlighted in black are identical in all five. Cysteine residues implicated in disulfide bonding are indicated by asterisks. The seven putative transmembrane domains are indicated by black bars above the aligned sequences.

Table 2. Rate of Synonymous (Ks) Substitutions Within the Transmembrane Domains of Chemoattractant and Related Receptors

<table>
<thead>
<tr>
<th></th>
<th>DFA/CR</th>
<th>mlL-8rH</th>
<th>hlL-8rB</th>
<th>C-C CKR1</th>
<th>rGlOd</th>
<th>hSST1R</th>
<th>US28</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA/CR</td>
<td>1.6845</td>
<td>0.6908</td>
<td>0.0205</td>
<td>1.6460</td>
<td>1.3608</td>
<td>3.2078</td>
<td>0.7637</td>
</tr>
<tr>
<td>mlL-8rH</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
<td>1.4357</td>
</tr>
<tr>
<td>hlL-8rB</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
<td>1.4357</td>
</tr>
<tr>
<td>C-C CKR1</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
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<tr>
<td>rGlOd</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
<td>1.4357</td>
</tr>
<tr>
<td>hSST1R</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
<td>1.4357</td>
</tr>
<tr>
<td>US28</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.6373</td>
<td>1.4357</td>
<td>1.4357</td>
<td>1.4357</td>
</tr>
</tbody>
</table>

Rate values are rate of substitutions per codon.

Abbreviations: DFA/CR, Duffy antigen/RBC chemokine receptor; mlL-8rH, mouse IL-8 receptor homolog; hlL-8rB, human IL-8 receptor type B; C-C CKR1, C-C chemokine receptor type 1; rGlOd, rat GlOd orphan receptor; hSST1R, human somatostatin receptor 1; hCMV-US28, viral gene US28 from human cytomegalovirus.

* Denotes pairs that are too distant for comparison.
families, it has been shown that functionally conserved genes tend to have higher $K_\sigma$ values than $K_\alpha$ values. As can be seen in Table 2, the Duffy antigen/RBC chemokine receptor has $K_\sigma$ values twofold higher than other chemokine receptors, whereas $K_\alpha$ values between the Duffy antigen/RBC chemokine receptor and a nonchemokine GCR (hSST1R) are highly comparable. This indicates that as a group, chemokine receptors are distinct from other GCRs. The Duffy antigen/RBC chemokine receptor $K_\sigma$ rates behave in a similar fashion (Table 3), with higher rates than all other comparisons. Because there is an inverse relationship between evolutionary pressures on an invading foreign organism and its host target in some disease states, a similar phenomena could be reflected here for the Duffy antigen/RBC chemokine receptor and its potential ligand, the malaria parasite erythrocyte-binding protein. Thus, it is possible that the inverse parasite/host relationship is reflected by a $K_\sigma > K_\alpha$ value for the erythrocyte-binding protein (as has been shown for the malaria parasite surface binding protein, MSA-1) as opposed to a $K_\sigma > K_\alpha$ value for the Duffy antigen/chemokine receptor (Tables 2 and 3). Finally, it is also possible that the positive selection for the erythrocyte binding protein on the malaria parasite and the enhanced rate of $K_\sigma$ and $K_\alpha$ in the Duffy antigen/RBC chemokine receptor may be related. The Duffy antigen/RBC chemokine receptor may be under negative selection against the binding of the parasite, and this pressure leads to mutations that cause resistance to infection. Another possibility is that the enhanced $K_\sigma$ and $K_\alpha$ values in the Duffy antigen/RBC chemokine receptor are caused by replication in a viral genome for some period of time, or retrotransposition events as rates of $K_\alpha$ and $K_\sigma$ are much higher in RNA genomes.

The biologic role of the Duffy antigen is not yet clear. Given that there are ~5,000 receptors per RBC, blood has a capacity of binding ~1.5 mg of monomeric chemokines and, thus, the Duffy antigen/RBC chemokine receptor has been proposed to act as a sink. This notion is further supported by the recent observation that erythrocyte-bound IL-8 is present in patients undergoing IL-2 immunotherapy. Although it could function as a clearing receptor, its absence in Duffy-negative individuals questions its role as a clearance receptor. To obtain an insight into the biologic role of

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**Table 3. Rate of Nonsynonymous ($K_\sigma$) Substitutions Within the Transmembrane Domains of Chemoattractant and Related Receptors**

<table>
<thead>
<tr>
<th></th>
<th>DFA/CR</th>
<th>mlL-BrH</th>
<th>hIL-BrB</th>
<th>C-C CKR</th>
<th>rG10d</th>
<th>hSST1R</th>
<th>US28</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFNCR</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>mlL-BrH</td>
<td>0.9743</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>hIL-BrB</td>
<td>0.9982</td>
<td>0.1415</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C CKR</td>
<td>1.2115</td>
<td>0.5707</td>
<td>0.5097</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rG10d</td>
<td>1.2819</td>
<td>0.7134</td>
<td>0.7134</td>
<td>0.8147</td>
<td></td>
<td></td>
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<tr>
<td>hSST1R</td>
<td>1.0982</td>
<td>0.5691</td>
<td>0.5464</td>
<td>0.6665</td>
<td>0.7621</td>
<td></td>
<td></td>
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<tr>
<td>US28</td>
<td>(−1)*</td>
<td>(−1)</td>
<td>0.6239</td>
<td>0.6356</td>
<td>0.7714</td>
<td>0.7405</td>
<td></td>
</tr>
</tbody>
</table>

*Rate values (boldface) are rate of substitutions per codon. Abbreviations are as in Table 2.

*Denotes pairs that are too distant for comparison.

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**Fig 5.** Northern blot analysis using as probe the Duffy antigen/RBC chemokine receptor cDNA. Blots were obtained from Clontech or Biosis and hybridized with radiolabeled Duffy antigen/RBC chemokine receptor cDNA. The filters were washed at 50°C with 0.5× SSC, 0.1% SDS and the autoradiograph was developed after 4 to 24 hours of exposure at −70°C with intensifying screens.
the Duffy antigen/RBC chemokine receptor, Northern blot analysis of RNA from different tissue was performed. The highest levels of Duffy antigen/RBC chemokine receptor were found in lung and muscle with lower levels in spleen, heart, and pancreas, and barely detectable levels in kidney (Fig 5). Interestingly, a transcript corresponding to ~8.5 kb is found in fetal brain (present at week 20, Fig 5B), which changes into the ~1.35-kb size in adult brain. These observations are consistent with those previously reported and strongly suggest that the Duffy antigen/RBC chemokine receptor might have a biologic role other than functioning solely as a clearance receptor. The availability of the cDNA encoding the Duffy antigen will be useful in assessing its biologic function.

In summary, the Duffy antigen has been functionally characterized and shown to bind chemokines with affinities very similar to those observed previously on RBCs. Furthermore, it failed to transmit an intracellular signal when transiently expressed in human kidney cells. Detailed sequence analysis of the coding region of the Duffy antigen/RBC chemokine receptor suggests that it belongs to the superfamily of G-protein-coupled receptors, with significant differences at the carboxyl-end. These carboxyl terminal differences may account for the lack of signal transduction via this receptor in transfected cells. The precise biologic role of the Duffy antigen/RBC chemokine receptor is not known because its expression is widely distributed and a different size transcript is present in the developing brain. However, evidence presented here is consistent with the idea that this protein may act as a clearance receptor for chemokines in blood and other tissues.

NOTE ADDED IN PROOF

Since the submission of this paper, Chaudhuri et al (J Biol Chem 269:7835, 1994) have also reported the binding of chemokines to K562 cells expressing the cloned Duffy antigen.

ACKNOWLEDGMENT

We thank Drs Ken Soo and David Ansted for providing the anti-Fy3 antibody and Dr Terry Hadley for providing the anti-Fy6 antibody. We also thank the Genentech flow cytometry and oligosynthesis groups. Finally, we thank Dr William T. Starner for providing the computer program EXON.

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