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Identification, Cloning, and Functional Characterization of a Murine Lipoxin A4 Receptor Homologue Gene

Michael W. Vaughn, Rita J. Proske, and David L. Haviland

To identify additional members of the murine N-formyl-Met-Leu-Phe peptide receptor family (fMLF-R), a mouse macrophage cDNA library was screened using the open reading frame of murine N-formyl peptide receptor. Four individual hybridizing cDNA clones were maintained through tertiary screening. One cDNA clone was a truncated, polyadenylated version of the previously described murine-fMLF-R. The other three cDNA clones varied in length, but contained identical open reading frame sequences. One clone, 8C10, was selected for further study and shared 75% sequence identity with murine-fMLF-R and 89% sequence identity with murine lipoxin A4 receptor cDNA. When placed into the pcDNA-3 expression vector and cotransfected with Gal16 cDNA into COS-1 cells, 8C10 cDNA induced the production of inositol-1,4,5-triphosphate when concentrations of 1–1600 nM lipoxin A4 (LXA4) were tested as ligands. Northern blot analysis of murine organs indicated that the 8C10 message is present in lung, spleen, and adipose tissue. Moreover, mice treated with LPS demonstrated increased expression of LXA4 message in spleen and adipose tissue, while showing a slight reduction in lung. We have also characterized the 8C10 structural gene from a 129Sv/J genomic library and have determined its size to be >6.1 kb in length and comprised of two exons separated by a 4.8-kb intron. Collectively, these data indicate that this homologue receptor is closely related to the murine LXA4 receptor and functionally responds to LXA4 as a ligand. The Journal of Immunology, 2002, 169: 3363–3369.

The directed migration of polymorphonuclear neutrophils can be mediated by a number of chemotactic factors that are released as a consequence of inflammation. Such factors include platelet-activating factor (1), leukotriene B4 (2), the anaphylatoxin complement fragment C5a (3), IL-8 (NAP-1/IL-8) (4), and N-formylated peptides (5, 6). In addition to chemotaxis, these factors mediate a variety of cellular and biochemical responses in neutrophils and macrophages. Such changes include bacteriocidal superoxide radical production, granule release of proteolytic enzymes, aggregation, and phagocytosis (reviewed by Snyderman and Pike (7)). These inflammatory factors mediate their responses through pertussis toxin-sensitive GTP-binding proteins, and the subsequent signal transduction can be abrogated by pertussis toxin (8, 9).

The high affinity human receptor for N-formyl peptides was cloned as two distinct cDNAs (R62 and R98) from a library prepared using dibutyryl cAMP-differentiated HL-60 cells (10, 11). The two cDNAs were found to be products of alternative polyadenylation of transcripts derived from the same gene (12–14) and mapped to chromosome 19q13.3 (13, 15). Differentiation of either U937 or HL-60 cells using dibutyryl cAMP produces additional, higher m.w. messages that hybridize to human N-formyl-Met-Leu-
Phe peptide receptor (fMLF-R) cDNA by Northern analysis (13, 16). The expression of these additional messages probably represents the expression of structurally homologous genes related to fMLF-R. Two related genes were identified, termed formyl peptide receptor like-1 and -2, using the human fMLF-R cDNA probe under low stringency conditions (15). Currently, no ligand has been identified to bind the formyl peptide receptor like-2 receptor. However, Serhan and colleagues (17) identified the formyl peptide receptor like-1 receptor as the high affinity receptor for the anti-inflammatory ligand lipoxin A4.

Lipoxins are members of the eicosanoid family that can be generated either from single cells or during cell-to-cell interactions. In contrast to fMLF, lipoxin A4 (LXA4) has been shown to inhibit the chemotaxis of neutrophils toward leukotriene B4 as well as toward fMLF. In addition, LXA4 and lipoxin B4 (LXB4) have been found to inhibit fMLF-induced migration of neutrophils across intestinal epithelium (18) as well as reduce neutrophil adherence (by ~70%) to HUVECs (19) and antagonize the mitogenic effects of leukotriene D4 (20) on renal mesangial cells. LXA4 and LXB4 have also been shown to down-regulate leukotriene-induced CD62P (P-selectin) expression on HUVECs (19) and reduce the cytolytic activity of NK cells (21). Additionally, LXA4 has been found to block airway constriction and promote vasodilation in asthmatic patients, suggesting their expression on lung epithelia and/or smooth muscle (22). It is likely that lipoxins may be the effectors of well-established anti-inflammatory therapies such as aspirin, which has been found to trigger the production of a modified form of LXA4 (recently reviewed in Ref. 23). This form, called 15-epimeric lipoxin, may mediate the beneficial effects of aspirin (24–26).

In an effort to determine whether additional members of the murine fMLF-R family exist, we screened a thioglycolate-stimulated murine macrophage cDNA library with the open reading...
frame of the murine fMLF-R using high stringency hybridization conditions. In these studies we have 1) identified a homologue of the previously identified murine LXA4 receptor (LXA4-R); 2) demonstrated that the cloned receptor can bind Lipoxin A4, suggesting that it may act as a second murine receptor for LXA4; 3) detected RNA expression of this receptor in adipose, lung, and spleen tissue derived from saline- and LPS-treated mice; and 4) determined that the this receptor is encoded by a two-exon structural gene analogous to other genes of the fMLF-R family.

Materials and Methods

Materials and reagents

Restriction enzymes and other molecular biology reagents were purchased from Roche (Indianapolis, IN) and used according to the manufacturer's recommendations. Hybond-N+ nylon membranes and radionucleotides [γ-32P]dCTP (3000 Ci/mmol) and myo-[3H]inositol (17 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Iodogen was purchased from Pierce (Rockford, IL). Sodium iodine [125I] was purchased from ICN (Costa Mesa, CA). N-formyl-Met-Leu-Phe (fMLF) and N-formyl-nor-Leu-Leu-Phe-nor-Leu-Tyr-Lys were purchased from Sigma (St. Louis, MO). LXA4 and LXB4 were purchased from Sigma and BIORON (Plymouth Meeting, PA).

Murine macrophage cDNA library screening

A Uni Zap XR mouse macrophage cDNA library constructed using mRNA isolated from B10.A thioglycollate-stimulated peritoneal macrophages was obtained from Stratagene (La Jolla, CA). Approximately 100,000 plaques were plated, and duplicate filters were screened with a random-primed (27) 32P-labeled cDNA probe that was generated by PCR using oligonucleotides corresponding to the complete murine fMLF-R open reading frame (28). Duplicate filters were prehybridized and hybridized for 16 h at 65 °C in a solution containing 5X SSC, 10X Denhardt's solution, and 2.0% SDS, pH 7.4, washed at room temperature for 20 min and then at 65 °C in 0.2X SSC containing 1.0% SDS, and exposed to autoradiography film (Amersham Hyperfilm MP) overnight at −70°C with intensifying screens. After identification of fMLF-R-hybridizing clones, a second and a subsequent third set of filters were screened under identical conditions with the murine fMLF-R probe. Four of the original 10 clones carried in duplicate through tertiary screening were plaque-purified, converted to p-Bluescript, and analyzed by restriction digestion and agarose gel electrophoresis.

Oligonucleotide synthesis and DNA sequence analysis

All oligonucleotides were synthesized using an Oligo 1000 M DNA synthesizer (Beckman, Fullerton, CA). The oligonucleotides used as primers in the sequencing reactions were 20 mers. All cDNA and genomic sequencing reactions were 20 mers. All cDNA and genomic sequencing reactions were analyzed using double-stranded templates and a model 377A automated sequencer. All oligonucleotides were synthesized using an Oligo 1000 M DNA Synthesizer (Beckman, Fullerton, CA). The oligonucleotides used as primers in the sequencing reactions were 20 mers. All cDNA and genomic sequencing reactions were analyzed using double-stranded templates and a model 377A automated sequencer. All cDNA and genomic sequencing reactions were analyzed and plotted using PRISM 3.0 (GraphPad, Berkeley, CA).

Results

Isolation of cDNA clones related to the murine fMLF-R

Using synthetic oligonucleotides, the open reading frame of the murine fMLF-R was amplified by PCR using DBA/l genomic DNA as template. The PCR product encoding the murine fMLF-R was subcloned into pcDNA-3, and the identity was confirmed by sequencing. It was subsequently used as a probe to screen a B10.A thioglycollate-stimulated peritoneal macrophage cDNA library. Ten primary isolates from 100,000 original clones were picked and rescreened under identical conditions. Four of the original 10 clones were taken to single colony isolates. These four phage clones were turned into plasmids, sequenced with vector-based oligonucleotides, and analyzed. A single clone partially encoded the cDNA for the murine fMLF-R described by Gao and Murphy (28). This cDNA clone was truncated at position 748 of the open reading frame (at Arg261) and polyadenylated.

In addition, we obtained three clones that were different from the murine fMLF-R. Although these three clones varied in overall length, all had putative open reading frames that were identical and shared 70% sequence identity with the murine fMLF-R. One clone, called 8C10, was chosen for further study, and its complete sequence was determined (Fig. 1). The 8C10 cDNA consists of 1179 bp (not including the poly(A)+ tail) and encodes 351 aa.

Initially, it was thought that 8C10 may be a new member of the recently identified murine fMLF-R family. To better understand the relationship of 8C10 to other known members of the family, the sequence of the 8C10 cDNA and derived protein were compared with those of other known members of the human and murine fMLF-R family that have been identified as well as to those of the recently cloned human and murine LCA4 receptors. A BLAST search was conducted, and the relationships are shown in Table I. Comparisons above the diagonal are the percent nucleotide sequence identity with the length of the ORFs (in parentheses). Below the diagonal are comparisons of peptide sequence identity and similarity over the length of the peptides. As shown in Table I, the...
The murine 8C10 clone shared the highest identity with the murine LXA4-R (24), at both the nucleotide and peptide levels (89 and 83% respectively). Due to the structural similarity between the 8C10 clone and the murine LXA4-R, it was hypothesized that the 8C10 clone may be an LXA4-R homologue.

**Structural organization of the LXA4-R homologue (8C10) gene**

A 129Sv/j/H9261 phage genomic library was screened for LXA4-R homologue (8C10) genomic clones under high stringency using the 8C10 ORF cDNA as a probe. One hybridizing phage clone was identified, and the colony was purified. The DNA that was isolated from this clone was digested with the enzymes BamHI, EcoRI, HindIII, SacI, XhoI, and XhoII. A Southern blot analysis revealed that two hybridizing EcoRI bands probably contain the 8C10 gene. These two bands (1Eco6, at 6.3 kb and 1Eco4 at 4.1 kb) were isolated and subcloned into p-Bluescript KS-II°.

**FIGURE 1.** The nucleotide and derived protein sequences of the murine LXA4-R homologue (8C10). The 8C10 cDNA consists of 1179 bp (not including the poly(A)' tail) and encodes an ORF (1056 bp) for 351 aa with an estimated unglycosylated Mᵣ of 39,421 Da. The selection of amino acids was made using mammalian codons, and the stop codon (TGA) is indicated by the asterisk.
### Table I. **Comparison of murine and human N-formyl peptide and lipoxin receptor families**

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<th></th>
<th>MuFMLF-R</th>
<th>HuFMLF-R</th>
<th>HuFMLF-RL1</th>
<th>HuFMLF-RL2</th>
<th>8C10</th>
<th>MuLXA4</th>
<th>HuLXA4</th>
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<td>57/65 (353)</td>
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*Comparisons of the nucleotide and peptide sequences were performed using GCG Bestfit. The following sequences and accession numbers were used in the analysis: MuFMLF-R (M84562) (28), HuFMLF-R (M60627, M33538) (16), HuFMLF-RL1 (M76673) (15), HuFMLF-RL2 (M76672) (15), MuLXA4 (U78299) (24), and HuLXA4 (AF054013) (45).*

end of exon 1 was determined from the 1Eco6 genomic clone and examined for the presence of transcription-related motifs. Fig. 3 summarizes the location and type of *cis* regulatory elements found in the 5′-flanking region of the 8C10 structural gene. A classical TATA box was found at −49 as well as two CCAAT boxes at positions −154 and −637 that are potentially recognized by CCAAT-binding transcription factors (33, 34). Unique motifs included a GATA box at −423, which may be involved with expression in myeloid cell lineages (35). In addition to the TATA box found at −49, a second TATA motif was located at −183 which may also serve as a transcription initiation factor IID initiation point (36). Finally, an IL-6 response element was located at −128 (37), which overlapped a consensus NF-κB motif at position −126 (38). Assessment of any actual role these sequence motifs play in regulating the expression of this LXA4-R homologue gene must await functional analysis performed with deletion and mutant reporter fusion gene constructs prepared from the 5′-flanking sequence.

Examination of 8C10 cDNA indicated that a polyadenylation signal (AATAAA) was not present upstream from the poly(A) tail of the cDNA. It was evident that the poly(A) sequence in the 3′-untranslated sequence of the 8C10 cDNA was present in the gene and served as an oligo(dT) priming site in construction of the cDNA library. In the genomic sequence there was a polyadenylation signal 35 bp downstream from the A-rich region that would potentially lengthen the true size of the mRNA to ~1227 bp.

**FIGURE 2.** Structural organization of the murine LXA4 receptor homologue (8C10) gene. The isolated phage clone containing the 8C10 gene was digested with EcoRI and the two bands identified by Southern analysis that hybridized to the 8C10 cDNA were subcloned into p-Bluescript SK−/H18554 and sequenced using synthetic oligonucleotides as primers. The 1Eco6 fragment was completely sequenced using PCR-based cycle sequencing as described in Materials and Methods. ■ Coding region is shown as a shaded box; □ 5′- and 3′-untranslated regions.

**Tissue expression of the LXA4-R (8C10) homologue**

To determine the tissue distribution of the murine LXA4-R homologue, an RNA hybridization analysis was conducted using tissues from both saline- and LPS-treated mice, with the radiolabeled 8C10 cDNA ORF as a probe. A variety of LPS- and saline-treated tissues were examined, including adipose, brain, heart, kidney, lung, large intestine, small intestine, liver, and spleen. Of these tissues, only RNA derived from spleen, lung, and adipose tissue hybridized to the murine LXA4-R homologue ORF, as shown in Fig. 4. Basal expression was found in murine lung, spleen, and adipose tissue; however, with LPS treatment, a marked increase in the LXA4-R homologue expression was noted, particularly in lung and adipose tissue. In tissues expressing the LXA4-R homologue RNA, two hybridizing bands were detected. This is possibly due to 1) alternative processing of the 8C10 mRNA in these tissues, or 2) cross-hybridization to the murine LXA4-R or some other unidentified homologue. For the second case to be true, this would indicate that the two genes are regulated identically, which, although possible, is unlikely. The most reasonable explanation is that the two bands are derived from alternative processing of the 8C10 message.

**Functional studies of the 8C10 cDNA clone**

The 8C10 cDNA was excised from p-Bluescript and then placed into the mammalian expression vector pCDNA-3. HEK-293 cells were transfected and cultured under neomycin (G418) selection. When [125I]fMLF radiolabeled ligand (N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys) displacement assays were conducted, it was determined that the protein translated from the 8C10 message did not bind labeled fMLF, whereas cells transfected with human fMLF-R demonstrated binding in a displacement assay shown in Fig. 5. [125I]N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (5 nM) was applied to HEK cells or to HEK cells that were stably transfected with 8C10 cDNA or cDNA encoding human fMLF-R. Neither HEK nor the HEK 8C10 transfectants demonstrated binding of the radiolabeled ligand. Only HEK cells expressing human fMLF-R cDNA demonstrated binding of the labeled ligand and subsequent displacement using unlabeled fMLF. Given the marked primary DNA and amino acid sequence identity that exists between 8C10 protein sequence and that of the murine LXA4-R, we tested the expressed...
8C10 for biological activity with LXA4 as ligand using a phospholipase C assay. COS cells were cotransfected with cDNAs alone or in combination. As shown in Fig. 6, the protein encoded by the 8C10 cDNA was capable of responding to LXA4 ligand, as shown by the increased incorporation of [3H]inositol over the tested range of 1–1600 nM (groups A and B). In addition, the response to LXA4 was specific, as no incorporation of inositol-1,4,5-triphosphate was observed with 8C10 cDNA in the absence of the cotransfected G/H9251 expression vector (group A). LXA4 also induced the incorporation of inositol when the expression vector containing cDNA encoding murine LXA4-R (a gift from Dr. C. Serhan) was cotransfected with the G/H9251 expression vector (group C). Two irrelevant ligands, the complement anaphylatoxins C3a and C5a, were tested in the assay at a single dose of 400 nM and did not elicit a response in COS cells transfected with the 8C10 cDNA (group D). These functional data along with the sequence data have lead us to conclude that the 8C10 clone encodes a murine LXA4-R homologue that responds to LXA4.

Discussion

In an effort to determine whether additional members of the murine fMLF-R family existed, a thioglycolate-stimulated murine macrophage cDNA library was screened with the ORF of the murine fMLF-R using high stringency hybridization conditions. We identified a receptor that was initially thought to be a member of the murine fMLF-R family. In this study we have examined the gene structure and possible function of this receptor and have determined that it is more closely related to the murine LXA4-R than it is to the murine fMLF-R. As a result, we refer to this gene product as a murine LXA4-R homologue.

LXA4 and LXB4 are members of the eicosanoid family. Lipoxins are formed by the metabolism of arachidonic acid by lipoxigenases during cell-cell interactions and are rapidly metabolized in vivo. Lipoxin biosynthesis has also been shown to occur in many human diseases (reviewed in Ref. 23) and serve as braking or stop signals for the proinflammatory actions of neutrophils. Peripheral blood monocytes have been found to metabolize lipoxins during inflammation, thrombosis, and atherosclerosis (39, 40). In contrast to neutrophils, lipoxins activate monocytes, inducing their

FIGURE 3. Nucleotide sequence of the putative 5′-flanking region of the murine LXA4-R homologue (8C10) structural gene. The sequence shown is derived from an EcoRI (1Eco6)-subcloned fragment derived from the genomic phage clone. Exon 1 is shown in uppercase letters, and the 5′-flanking sequence is shown in lowercase letters. The beginning of the cDNA is denoted +1. Sequences that correspond to identified consensus sequences are boxed and identified as shown above.
C3a and C5a (\textsuperscript{[44x437]}) as described in Materials and Methods triplicate determinations from a representative experiment. Significantly, concentrations of LXA4 (\textsuperscript{[44x447]} and A regions. Initially, we thought that the 8C10 clone was an allelic variant identities among the extracellular, intercellular, and transmembrane domains. The sequence between these two genes. This identity is summarized in comparing the ORFs, the LXA4-R and the LXA4-R homologue is striking (89%). In comparison, their receptors share numerous structural characteristics. Both are members of the rhodopsin superfamily, traverse the membrane seven times, and are coupled to pertussis toxin-sensitive, GTP binding proteins (8, 17). Another characteristic of this superfamily is a very intriguing, but they are limited until immunohistochemistry and/or in situ hybridization studies are conducted to ascertain whether the tissue itself is expressing the LXA4-R homologue or if the signal is due to resident or infiltrating myeloid cells, as may be the case with lung and splenic tissues.

Although interaction of FMLF and lipoxin with their respective receptors have functionally opposite roles in the inflammatory process, their receptors share numerous structural characteristics. Both are members of the rhodopsin superfamily, traverse the membrane seven times, and are coupled to pertussis toxin-sensitive, GTP binding proteins (8, 17). Another characteristic of this superfamily is a very simple gene structure comprised of only two exons. Moreover, human and murine FMLF-R and LX A4-R share surprising 76 and 70% identities at the nucleotide level, respectively.

Although not exhaustive, the screening of the murine thioglycolate-elicited macrophage cDNA library did not produce any clones identical with the murine LXA4-R homologue (8C10) cDNA. Cells were transfected with eukaryotic expression vectors encoding the cDNAs for β-galactosidase (pSV-βGal) and the murine LXA4-R homologue (8C10) either alone or with Ga16, labeled, washed, and stimulated with various concentrations of LXAX (A and B) and with complement anaphylatoxins C3a and C5a (C) as described in Materials and Methods. Levels of inositol phosphates were determined as described. Shown are the mean and SE of triplicate determinations from a representative experiment. Significant differences between the Ga16 control and those with Ga16 and the murine LXA4-R homologue (8C10) are indicated (\textit{p} < 0.05, determined by unpaired Student’s \textit{t} test).

FIGURE 6. Accumulation of \textsuperscript{[3H]}inositol phosphates in COS-1 cells transfected with the murine LXA4-R homologue (8C10) cDNA. Cells were transfected with eukaryotic expression vectors encoding the cDNAs for β-galactosidase (pSV-βGal) and the murine LXA4-R homologue (8C10) either alone or with Ga16, labeled, washed, and stimulated with various concentrations of LXAX (A and B) and with complement anaphylatoxins C3a and C5a (C) as described in Materials and Methods. Levels of inositol phosphates were determined as described. Shown are the mean and SE of triplicate determinations from a representative experiment. Significant differences between the Ga16 control and those with Ga16 and the murine LXA4-R homologue (8C10) are indicated (\textit{p} < 0.05, determined by unpaired Student’s \textit{t} test).

Migration to sites of inflammation where they play a role in wound healing (41). Finally, Godsen et al. (42) recently demonstrated that lipoxins are capable of stimulating human macrophages to phagocytose apoptotic neutrophils.

Although interaction of FMLF and lipoxin with their respective receptors have functionally opposite roles in the inflammatory process, their receptors share numerous structural characteristics. Both are members of the rhodopsin superfamily, traverse the membrane seven times, and are coupled to pertussis toxin-sensitive, GTP binding proteins (8, 17). Another characteristic of this superfamily is a very simple gene structure comprised of only two exons. Moreover, human and murine FMLF-R and LX A4-R share surprising 76 and 70% identities at the nucleotide level, respectively.

Although not exhaustive, the screening of the murine thioglycolate-elicited macrophage cDNA library did not produce any clones identical with the murine LXA4-R (Fpr-rs1) as described by Takano et al. (24) and Gao et al. (43). From the four phage clones identified, three clones varied in overall length, but had identical nucleotide and peptide sequences, we concluded that the murine LXA4-R and the murine LXA4-R homologue (8C10) are indicated (\textit{p} < 0.05, determined by unpaired Student’s \textit{t} test).

Of particular interest was the tissue expression of the LXA4-R homologue on both saline- and LPS-treated murine tissues (Fig. 4). Lung and spleen tissues obtained from saline-treated mice demonstrated a detectable, but low level, expression of the LXA4-R homologue message, which was markedly increased in mice treated with LPS. This finding was not unexpected given the inflammatory response induced by LPS and that receptors for lipoxins have been found primarily on neutrophils and macrophages. The surprising result was finding the LXA4-R homologue message in adipose tissue in both saline- and LPS-treated mice. It was noted that two bands were observed in each of the tissues that hybridized with the 8C10 cDNA. Collectively, these results are intriguing, but they are limited until immunohistochemistry and/or in situ hybridization studies are conducted to ascertain whether the tissue itself is expressing the LXA4-R homologue or if the signal is due to resident or infiltrating myeloid cells, as may be the case with lung and splenic tissues.

During our studies of the 8C10 gene, Gao et al. (43) expanded the murine FMLF-R family to include the murine FMLF-R (FPR1) and five homologues, termed Fpr-rs1 through Fpr-rs5. The murine LXA4-R identified by Takano et al. (24) is identical with the Fpr-rs1 identified by Gao et al. (43). The LXA4-R homologue (8C10) described in this report is identical with Fpr-rs2. In their report Fpr-rs2 (8C10) was described as a possible low affinity FMLF receptor homologue, and at that time the response of this receptor to LXA4 was not considered. Based on our current data it appears more likely that 8C10 is in fact an LXA4-R homologue.

In conclusion, the study of both pro- and anti-inflammatory receptors provides a unique opportunity to examine how the inflammatory response is mediated and modulated. Understanding the biology and biochemistry of both types of receptors will provide insights into processes involved in switching the cellular response from pro- to anti-inflammatory. In addition, understanding the similarities and differences in the biochemistry of pro- and anti-
inflammatory signal transduction may provide venues for future therapeutic approaches.

Acknowledgments
A debt of thanks and appreciation is extended to Drs. Irma Gigi, Rick A. Wetsel, and Scott M. Drouin for critically reading the manuscript, and to Travis J. Hollman and Joie C. Haviland for discussions and technical assistance, respectively.

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