Differential Regulation of Zfp30 Expression in Murine Airway Epithelia Through Altered Binding of ZFP148 to rs51434084

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ABSTRACT Neutrophil chemotaxis to the airways is a key aspect of host response to microbes and a feature of multiple pulmonary diseases including asthma. Tight regulation of this recruitment is critical to prevent unwanted host tissue damage and inflammation. Using a mouse (Mus musculus) model of asthma applied to the Collaborative Cross population, we previously identified a lung gene expression quantitative trait locus (eQTL) for Zinc finger protein 30 (Zfp30) that was also a QTL for neutrophil recruitment and the hallmark neutrophil chemokine CXCL1. The Zfp30 eQTL is defined by three functionally distinct haplotypes. In this study, we searched for causal genetic variants that underlie the Zfp30 eQTL to gain a better understanding of this candidate repressor’s regulation. First, we identified a putative regulatory region spanning 500 bp upstream of Zfp30, which contains 10 SNPs that form five haplotypes. In reporter gene assays in vitro, these haplotypes recapitulated the three previously identified in vivo expression patterns. Second, using site-directed mutagenesis followed by reporter gene assays, we identified a single variant, rs51434084, which explained the majority of variation in expression between two out of three haplotype groups. Finally, using a combination of in silico predictions and electrophoretic mobility shift assays, we identified ZFP148 as a transcription factor that differentially binds to the Zfp30 promoter region harboring rs51434084. In conclusion, we provide evidence in support of rs51434084 being a causal variant for the Zfp30 eQTL, and have identified a mechanism by which this variant alters Zfp30 expression, namely differential binding of ZFP148.

Recruitment of neutrophils to the lung is a hallmark of innate immune responses to inhaled pathogens and air pollutants, and is associated with decreased lung function and disease susceptibility (Nathan 2006; Borregaard 2010; Mantovani et al. 2011). Neutrophils play an integral role in immune response to external stimuli by migrating toward resulting chemotactic signals and responding to pathogens through phagocytosis and secretion of reactive oxygen species, proteases, and cytokines. These reactive oxygen species and proteases, however, do not distinguish between host and pathogen, therefore a robust recruitment of neutrophils can adversely impact host tissue. This makes tight regulation of neutrophil recruitment and their activity crucial. A neutrophilic infiltrate in the airways is seen in a range of pulmonary diseases, including asthma (Jatakanon et al. 1999; Wenzel et al. 1999), acute respiratory distress syndrome (ARDS) (Chollet-Martin et al. 1992; Grommes and Soehnlein 2011; Williams and Chambers 2014; Kangelaris et al. 2015), chronic obstructive pulmonary disease (COPD) (Qi et al. 2003; Quint and Wedzicha 2007; Hoenderdos and Condliffe 2013; Günay et al. 2014; Duman et al. 2015; Grabcanovic-Musija et al. 2015), and cystic fibrosis (Mackerness et al. 2008; Gifford and Chalmers 2014; Laval et al. 2016). In asthma, neutrophil levels in sputum have been shown to correlate with disease severity and lung function, as well as a lack of responsiveness to glucocorticoids (Stănescu et al. 1996; Green et al. 2002; Uddin et al. 2010).

Previously, we applied a house dust mite model of asthma to incipient lines of the Collaborative Cross (CC) population to identify novel genes and pathways associated with neutrophil recruitment responses in the
context of allergic inflammation. The CC is a new mouse genetics reference population composed of recombinant inbred lines that are derived from eight founder strains (Collaborative Cross Consortium 2012). We measured neutrophil recruitment responses and levels of the hallmark neutrophil chemokine CXCL1 (aka KC) in bronchoalveolar lavage fluid and found that both were regulated by a locus on chromosome 7 (25.6–29.7 Mb) that we called Dpc1 (Rutledge et al. 2014). Likewise, the expression of the gene Zinc finger protein 30 (Zfp30) was also regulated by this locus [i.e., is an expression quantitative trait locus (eQTL)], and Zfp30 expression was strongly correlated with CXCL1 and neutrophil counts (Supplemental Material, Figure S1). We also found that Zfp30 is expressed in airway epithelia and that knockdown of Zfp30 in an epithelial cell line resulted in increased CXCL1 production in response to endotoxin. These results led to development of a model in which ZFP30 negatively regulates CXCL1, and CXCL1 then affects neutrophil recruitment, all under the control of Dpc1.

Relatively little is known about Zfp30 except that it encodes an as yet uncharacterized C2H2 zinc finger protein that contains a Krüppel-associated box domain, signifying a role in gene repression via heterochromatin formation (Friedman et al. 1996). However, two features of the Zfp30 eQTL provided important insights about Zfp30 regulation. First, allele-specific expression data provided convincing evidence that the eQTL acts in cis. Second, haplotypes spanning the 5′ region of the gene, but not the 3′ region, were strongly correlated with Zfp30 expression. More specifically, we found that expression levels in MLE12 mouse lung epithelium cells were strongly correlated with the strain distribution pattern of 5′ region haplotypes: mice with haplotypes from NOD/ShiLtJ, NZO/H1LtJ, 129S1/SvImJ, or A/J founder strains had high expression; mice with haplotypes from C57BL/6J or WSB/EiJ founder strains had moderate expression; and mice with haplotypes from CAST/EiJ or PWK/PhJ founder strains had low gene expression. These findings prompted us to seek to identify the specific variant(s) that regulate Zfp30 expression. In this study, we utilized sequence data, epigenomic data, reporter gene assays, and electrophoretic mobility shift assays (EMSA) to interrogate the effect that specific variants have on Zfp30 expression. In aggregate, our data point to a causal variant responsible for the modulation of Zfp30 expression, and show that this variant alters transcription factor binding.

MATERIALS AND METHODS

Cell culture

The MLE12 mouse lung epithelial cell line (generated from FVB/N mouse strain) was cultured in a 1:1 mix of Dulbecco’s Modified Eagle Medium and Ham’s F12 supplemented with 5% fetal bovine serum (FBS). The LA4 mouse lung airway cell line (generated from the A/He mouse strain) was cultured in Ham’s F12K medium supplemented with 15% FBS. Both cell lines were grown at 37°C with 5% CO2. Twenty-four-well plates were seeded at a density of 120,000 cells per well for MLE12 or 125,000 cells per well for LA4 24 h prior to transfection for luciferase assays.

Dual luciferase reporter assay

A 500 bp genomic region of the Zfp30 promoter surrounding a eukromatic region containing a variant of interest was amplified from five of the eight CC founder strains. Amplified candidate promoters were subcloned into the multiple cloning site upstream of the firefly luciferase gene in the promoterless pGL4.10 vector (Promega, Madison, WI) using a Gateway subcloning approach, and then verified by sequencing. MLE12 or LA4 cell lines were cotransfected in 24-well cell culture plates with 500 ng of candidate promoter constructs and 28 ng of Renilla control vector pRL-SV40 (Promega), using Lipofectamine 3000 (Life Technologies, Carlsbad, CA), according to manufacturer’s protocol. Transfected cells were cultured 48 hr before cell lysates were collected. Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity as a control for transfection efficiency. Data are reported as the ratio of firefly to Renilla luciferase activity, and these data are provided in Table S1. Assays include four replicates for each candidate promoter and two technical replicates for each lysate collected. Luciferase assays were performed in each cell line at least two times, and results were consistent across experiments. Welch’s two-sided t-tests were performed to compare luciferase activity between promoter constructs.

qPCR of luc2 and Renilla luciferase

mRNA expression of firefly and Renilla luciferase was assayed in MLE12 RNA samples collected 48 hr post-transfection with candidate promoters and pRL-SV40. iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) was used in conjunction with luc2 primers (Fwd: 5'-GTGGTGTCAGCGAAGATG-3'; Rev: 5'-CGCTCGTTGTGATATTGTTAG-3'). We validated the luc2 primers for efficiency by performing assays with serial dilutions of template and verifying the expected changes in Cq values. In subsequent assays, Renilla luciferase levels were used to normalize luc2 Cq values, and fold change in expression of A/J and 129S1/SvImJ over C57BL/6J was calculated.

Identification of genetic variants among CC founder strains

We utilized the Sanger Mouse Genomes Project variant database (Keane et al. 2011) (http://www.sanger.ac.uk/sanger/Mouse_SnpViewer/snp-1505) to identify variants within our cloned promoter region, which spans 29783622–29784093 bp on chromosome 7. These variants are shown in Table 1.

Site-directed mutagenesis: Site-directed mutagenesis to change the rs5143408 allele in pGL4.10 constructs was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) according to manufacturer’s protocol. Primers (Fwd: 5'- CCTCTCA ACCCCTTCCCCGTGACCAAGCTGTTAAAGCCC-3'; Rev: 5'- GG CCCCTAGCCTTTTGTCAGCGGAAGGGGGTTGGAGG-3') were designed to mutate the position corresponding to rs5143408 from C to G.

EMSA: Complementary 17 bp oligonucleotide probes centered on rs5143408 allele in pGL4.10 constructs were purchased from Integrated DNA Technologies, Coralville, IA) for use in EMSA experiments. Sequences for the moderate (C57BL/6J) and high (A/J) expression haplotypes, respectively, were as follows: Fwd: 5'-CCCTCCCTCCGTGACCAAGCTGTTAAAGCCC-3'; Rev: 5'-GG CCCCTAGCCTTTTGTCAGCGGAAGGGGGTTGGAGG-3'. Sets of complementary probes were ordered with and without biotin end-labeling (Integrated DNA Technologies). Complementary oligos were annealed into double-stranded DNA probes at a concentration of 2 pmol/µl by heating complementary oligos to 95°C in an annealing buffer (10 mM Tris, 50 mM NaCl; 1 mM EDTA pH 8.0) and cooling them to 25°C in a thermocycler at a rate of 1°C per minute. NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA) were used to collect nuclear protein lysates from MLE12 cells. Protein concentrations were quantified using the Pierce BCA Protein Assay (Thermo Scientific).
EMSA experiments were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer’s protocol. DNA–protein binding reactions contained 1× binding buffer, 1 μg poly(dIdC), 4 μg nuclear protein lysate, 200 fmol biotinylated DNA probe, and water, for a total reaction volume of 20 μl. Reactions were incubated at room temperature for 30 min prior to gel loading. Reactions including nonbiotinylated probes for DNA competition were incubated with 45-fold excess unlabeled probe for 25 min prior to addition of biotinylated probe. ZFP148 supershift reactions contained either 10 μg (Figure 4) or 20 μg (Figure S3) antibody incubated for 30 min prior to addition of biotinylated probe. Antibodies for ZFP148 (Fisher Scientific, Hampton, NH) and KLF4 (Millipore, Burlington, MA) were ordered for use in EMSA supershifts. DNA–protein complexes were separated on 6% DNA retardation gels (Life Technologies) using 0.5× TBE buffer (Life Technologies). Complexes were transferred to Biodyne B Pre-Cut Modified Nylon Membranes (Thermo Scientific) and UV cross-linked before chemiluminescent detection.

**Transcription factor binding site prediction:** To identify transcription factor binding sites potentially affected by rs51434084, we used TRANSFAC (http://gene-regulation.com/pub/programs.html#match) and HOCOMOCO (http://opera.autosome.ru/perfectosape/scan/new) prediction tools. Based on EMSA results indicating enhanced binding of nuclear proteins to the C57BL/6J allele (vs. 129S1/SvImJ), we limited our search to putative transcription factor binding sites in which the C57BL/6J allele is predicted to bind the transcription factor better than the 129S1/SvImJ allele. We then performed literature searches on the initial list of candidates to prioritize any transcription factors with a known connection to regulation of neutrophil recruitment.

**Data availability**
Luciferase reporter data are provided in Table S1. Original EMSA images for Figures 3 and 4 are provided as Figure S4 and Figure S5.

**RESULTS**

**Candidate regulatory region for Zfp30**
We sought to identify the causal variant (or variants) responsible for the Zfp30 cis-eQTL on chromosome 7 we previously identified in whole lung tissue from incipient CC lines, which we showed was associated

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Table 1 Zfp30 expression levels among CC founder strains and strain distribution patterns (SDPs) for 5' region SNPs

| Strain | Zfp30 Expression | Relative Expression | Expression Group |
|--------|------------------|---------------------|------------------|
| A/J    | 7.72             | 1.86                | High             |
| NOD/ShiLtJ | 7.65           | 1.77                | High             |
| NZO/H1LtJ  | 7.56           | 1.67                | High             |
| 129S1/SvImJ | 7.57           | 1.68                | High             |
| C57BL/6J | 6.82            | 1.00 (Ref.)         | Moderate         |
| WSB/EJ   | 6.69            | 0.91                | Moderate         |
| CAST/EJ  | 6.48            | 0.79                | Low              |
| PWK/PhJ  | 6.42            | 0.76                | Low              |

The Sanger Mouse Genomes Project variant database was utilized for SNP data.

Expression data (log2 units) based on data from Rutledge et al. (2014).

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Figure 1 Zfp30 promoter region haplotypes recapitulate in vivo patterns of gene expression in reporter assays. Dual luciferase reporter assays were performed, and firefly/Renilla ratios are shown. Reporter clones are grouped by in vivo Zfp30 expression level: high (H), moderate (M), and low (L). (A) Results from assays in MLE12 cells recapitulate in vivo differences between moderate-expressing (C57BL/6J) and high-expressing (A/J and 129S1/SvImJ) strains, but not low-expressing (CAST/EJ and PWK/PhJ) strains. (B) Results from assays in LA4 cells recapitulate in vivo differences between all three expression groups. * P < 0.05.
with variation in CXCL1 and neutrophil counts in response to allergen challenge (Rutledge et al. 2014). In that study, we found that incipient CC lines with chromosome 7 QTL region haplotypes from NOD/ShiLtJ, NZO/H1LtJ, 129S1/SvImJ, or A/J strains had high Zfp30 expression, mice with haplotypes from C57BL/6J or WSB/EiJ founder strains had moderate expression, and mice with haplotypes from CAST/EiJ or PWK/PhJ founder strains had low gene expression. This same pattern of expression was observed among CC founder lines (Table 1), indicating that the cis-eQTL is the major determinant of Zfp30 expression.

To identify regulatory regions that may contribute to variation in Zfp30 expression, we examined ENCODE data for euchromatic regions marked by DNase I hypersensitivity near the 5′ region of Zfp30 in mouse lung tissue, because our previous data indicated a strong correlation between 5′ region haplotypes and gene expression (Rutledge et al. 2014). We examined a region spanning 10 kb upstream through intron 1 and identified a ~500 bp region of open chromatin near the putative Zfp30 promoter and transcription start site (Figure S2), which contains 10 SNPs. An examination of the strain distribution patterns within the identified region of open chromatin at the Zfp30 promoter revealed a single shared SNP (rs51434084) among the high expression strains and six shared SNPs among the low-expressing strains (Table 1). These variants were thus considered priority candidates that might affect regulation of Zfp30 in a manner consistent with the three expression groups.

**Candidate regulatory region significantly modulates reporter gene activity in mouse airway epithelia cell lines**

To interrogate the effect of this candidate regulatory region on Zfp30 expression, we cloned the 500 bp putative promoter region from representative strains of each Zfp30 expression group into a promoterless luciferase vector for use in luciferase reporter assays. More than one strain was chosen from the high and low expression groups to account for potential differences in gene expression due to variants in the region. Specifically, promoter regions were cloned from A/J and 129S1/SvImJ to represent the high expression group, from C57BL/6J to represent the intermediate expression group, and from CAST/EiJ and PWK/PhJ to represent the low expression group.

We then tested whether these promoter constructs recapitulate relative patterns of expression seen in vivo, using luciferase reporter assays in two mouse lung epithelia cell lines, MLE12 and LA4. Mouse lung epithelia cell lines were chosen because Zfp30 expression was previously reported in this tissue. Additionally, we previously reported that knockdown of Zfp30 using siRNAs in MLE12 cells led to increased CXCL1 secretion in response to LPS exposure, which is consistent with predictions based on in vivo data (Rutledge et al. 2014). Concordant with previously measured in vivo expression data (see Table 1), initial luciferase experiments in the MLE12 mouse lung epithelia cell line (Figure 1A) revealed a significant twofold difference in expression driven by promoters from high-expressing strains (A/J and 129S1/SvImJ) compared with lower-expressing strains (C57BL/6J, CAST/EiJ, and PWK/PhJ). No difference in luciferase activity was observed between the moderate-expressing (C57BL/6J) and low-expressing (CAST/EiJ and PWK/PhJ) promoter constructs in this cell line. This assay was also performed in the LA4 mouse airway cell line to compare results across differing genetic backgrounds. In LA4 cells, the expression difference between high and moderate expression groups was also evident, and at a similar magnitude. Additionally, CAST/EiJ and PWK/PhJ promoters produced significantly lower expression than C57BL/6J promoter (Figure 1B), at a magnitude roughly equivalent to the in vivo differences shown in Table 1. To verify that the differences we observed exist at both the protein and RNA levels, we measured luc2 and Renilla

![Figure 2](image-url)
luciferase mRNA levels in transfected MLE12 cells using a custom qPCR assay. The results, shown in Table S2, confirmed that the difference we observed in protein activity was also present at the RNA level.

We then focused our attention on the identification of SNPs that could explain the observed twofold expression difference between moderate and high Zfp30 expressing promoters, for two reasons. First, the difference between moderate and high expression strains is much larger than the expression difference between moderate and low expression strains (Table 1). Second, in addition to our previous finding regarding the link between Zfp30 expression and immune response in the lung (Rutledge et al. 2014), a previous report from a different research group indicates that the aforementioned expression difference is likely also the cause of contrasting response to Streptococcus pneumoniae infection (Denny et al. 2003).

Within the cloned regulatory region, A/J and 129S1/SvImJ share only one variant, rs51434084. To test the regulatory effect of this SNP, we used a site-directed mutagenesis approach to modify a C57BL/6J promoter at one variant, rs51434084. To test the regulatory effect of this SNP, we used a site-directed mutagenesis approach to modify a C57BL/6J promoter at one variant, rs51434084.

**Variation at rs51434084 results in differential binding of ZFP148**

We then performed EMSA experiments to detect differential binding of transcription factors from MLE12 nuclear lysates to 17 bp probes mimicking either the C57BL/6J or 129S1/SvImJ allele at rs51434084 (Figure 3). Multiple DNA–protein complexes were observed for each probe, and each complex showed higher affinity for the C57BL/6J probe. Competition reactions with unlabeled probes revealed that the unlabeled C57BL/6J probe competed these interactions away with higher affinity than the unlabeled 129S1/SvImJ probe. Given that in vivo data and in vitro reporter assays both indicated that the C57BL/6J haplotype is associated with lower gene expression than the 129S1/SvImJ haplotype, we reasoned that the observed nuclear binding to the probe sequence containing rs51434084 is repressive.

**DISCUSSION**

Neutrophil levels in human airways correlate with asthma disease severity (Wenzel et al. 2003; Moore et al. 2014), poor response to inhaled corticosteroids (Green et al. 2002), and sudden-onset fatal asthma
attacks (Sur et al. 1993). In COPD, neutrophil-to-lymphocyte ratio (NLR) is associated with disease state, and increased NLR is associated with longer hospital stays and higher readmission rates (Günay et al. 2014; Duman et al. 2015). Additionally, neutrophil extracellular traps are associated with increased airflow limitation among patients with COPD, and neutrophilic inflammation leads to decreased lung function (Quint and Wedzicha 2007; Hoenderdos and Condiffe 2013; Grabcanovic-Musija et al. 2015). In cystic fibrosis, neutrophil secretory proteins drive disease states and increase mucus production (Mackerness et al. 2008; Gifford and Chalmers 2014; Laval et al. 2016). In acute lung injury, neutrophils are a key component to lung damage, and their recruitment is controlled by complex chemokine networks (Grommes and Soehnlein 2011; Williams and Chambers 2014). Genes related to neutrophils and their recruitment are also upregulated in early sepsis-induced ARDS (Kangelaris et al. 2015). These findings indicate that increased understanding of neutrophil recruitment to the lung would have broad importance.

To gain insight into the regulation of neutrophil recruitment, we exploited a mouse model of asthma featuring neutrophilic inflammation. In the context of this model system, we identified Zfp30 as a candidate regulator of CXCL1 levels and neutrophil recruitment (Rutledge et al. 2014). Here, we follow up this finding by identifying a causal variant for the variation in Zfp30 expression, namely rs5143408.

Our reporter assays with Zfp30 promoter constructs provided two main findings. First, this 500 bp region successfully recapitulated the twofold range of variation in Zfp30 expression we previously characterized in vivo (Rutledge et al. 2014). Second, allele-swapping of rs5143408 using site-directed mutagenesis shows that this variant is the primary driver of the twofold expression difference between these strain groups. We also note that in our in vitro reporter assays, the expression pattern for CAST/EiJ and PWK/PhJ differed between the two cell lines we used (MLE12 and LA4). The expression pattern seen in the LA4 cell line (generated from the A/He mouse strain) matched mRNA expression results seen in vivo, but the pattern seen in MLE12 (generated from FVB/N mouse strain) did not. This suggests that these cell lines differentially express a repressor protein that downregulates expression of Zfp30 in CAST/EiJ and PWK/PhJ. Future experiments will be directed toward identifying the causal variant(s) and protein(s) responsible for this modulation of Zfp30 expression.

Our EMSA results indicate that ZFP148 is among the transcription factors that bind to the rs5143408 locus in the Zfp30 promoter region and thereby regulate Zfp30 expression. Thus, we propose that our initial model, in which Zfp30 expression regulates CXCL1 and neutrophil recruitment (Rutledge et al. 2014), be expanded to include ZFP148. ZFP148 has a known role in the negative regulation of Cxcl5, a CXCR2 ligand similar to CXCL1, in human colonic epithelial cells (Keates et al. 2001). In combination with previous findings, our results suggest that ZFP148 downregulates both Cxcl5 and Zfp30. These functions may seem contradictory at first, as Zfp30 is proposed to downregulate Cxcl1, but decreased expression of Cxcl5 has actually been linked to increased neutrophil recruitment into the lungs in an Escherichia coli pneumonia mouse model (Mei et al. 2010) through a mechanism involving Duffy antigen receptor for chemokines (DARC)–mediated sequestration of CXC chemokines. This mechanism results in a steeper gradient of CXCL1 and CXCL2 chemokines between vasculature and lungs. Thus, the function of ZFP148 proposed here could be cooperative with its known effect on CXCL5 levels.

ZFP30 is a C2H2 zinc finger protein with domains that suggest DNA-binding and KAP-1–binding functions. Wherever ZFP30 binds in the genome, a complex of proteins recruited by KAP-1 are predicted to induce heterochromatin domains and silence nearby genes (Friedman et al. 1996; Ryan et al. 1999; Schultz et al. 2002; Medugno et al. 2005). Specific binding sites for ZFP30 have not yet been identified, and additional work is needed to characterize the mechanism of ZFP30-mediated control of neutrophil recruitment. Cxcl1 is a priority locus of interest; however, KAP-1/zinc finger protein complexes could have multiple binding sites and can act over tens of kilobases (Groner et al. 2010), so multiple genes may be affected.

This work contributes to an increased understanding of how Zfp30 is regulated as a function of an allelic variant, which we previously showed was linked to neutrophils in the airways in a mouse model. Future work examining the relevance of the genes/proteins studied here, namely ZFP148 and ZFP30, in neutrophil chemotaxis in other model systems and in human patients is a critical next step to evaluating the biomedical importance of our findings. Additionally, identification and characterization of additional functional variants that impact neutrophil recruitment will further develop our understanding of the complex regulatory networks that drive disease severity phenotypes in both model systems and human patients with asthma, acute lung injury, cystic fibrosis, or COPD.

ACKNOWLEDGMENTS

We thank the following associates at University of North Carolina at Chapel Hill: Karen Mohlke, Mark Heise, Mauro Calabrese, and Jason Mock for support and advice throughout this project; Karen Mohlke and James Davis for research support in luciferase and EMSA assays; and Max Lowman and Autumn Sanson for technical support in qPCR. Funding for this work was provided by National Institutes of Health grants R01ES024965 and R01HL122711.

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Communicating editor: D. Threadgill