Lipopolysaccharide (LPS) activation of murine RAW 264.7 macrophages influences the expression of multiple genes through transcriptional and post-transcriptional mechanisms. We observed a 5-fold increase in CstF-64 expression following LPS treatment of RAW macrophages. The increase in CstF-64 protein was specific in that several other factors involved in 3′-end processing were not affected by LPS stimulation. Activation of RAW macrophages with LPS caused an increase in proximal poly(A) site selection within a reporter mini-gene containing two linked poly(A) sites that occurred concomitant with the increase in CstF-64 expression. Furthermore, forced overexpression of the CstF-64 protein also induced alternative poly(A) site selection on the reporter mini-gene. Microarray analysis performed on CstF-64 overexpressing RAW macrophages revealed that elevated levels of CstF-64 altered the expression of 51 genes, 14 of which showed similar changes in expression of 3′-untranslated regions of these 51 genes revealed that over 45% possess multiple putative poly(A) sites. Two of these 51 genes demonstrated alternative polyadenylation under both LPS-stimulating and CstF-64-overexpressing conditions. We concluded that the physiologically increased levels of CstF-64 observed in LPS-stimulated RAW macrophages contribute to the changes in expression and alternative polyadenylation of a number of genes, thus identifying another level of gene regulation that occurs in macrophages activated with LPS.

As a critical component of the innate immune system, macrophages respond to LPS from Gram-negative bacteria by activating multiple signaling pathways that result in the rapid expression of pro-inflammatory cytokines and arachidonic acid metabolites. The induced expression of many of these genes is attributed to the activation of transcription factors linked to Toll-like receptor 4-related signal transduction pathways (reviewed in Refs. 1 and 2). Aside from transcriptional contributions to gene expression in stimulated macrophages, post-transcriptional mechanisms have also been shown to influence gene expression. For example, the half-lives of TNF-α (3) and lysozyme (4) mRNAs are increased following LPS stimulation by a lengthening of the poly(A) tail. Additionally, transcriptional regulatory and mRNA stability elements have been identified in the 3′-untranslated region (3′-UTR) of the LPS-inducible mRNAs encoding COX-2 (5–7) and TNF-α (8, 9).

Post-transcriptional pre-mRNA cleavage and polyadenylation, although essential in creating translationally competent mRNA, is a greatly under-appreciated contributor to the regulation of gene expression. Because it has been estimated that only 30% of primary transcripts are polyadenylated (10), changes in 3′-end processing can have a profound effect on the amount of mature transcript produced. The frequency with which a poly(A) site is selected for the cleavage/polyadenylation reaction is greatly impacted by the strength of that poly(A) site. Relevant to the present study, the frequency of cleavage/polyadenylation at a specific poly(A) site is also sensitive to the abundance and/or activity of trans-acting mRNA processing factors (11–13).

Studies of pre-mRNA processing in vitro have identified soluble trans-acting factors that are required for the cleavage and polyadenylation of gene transcripts. The 160-kDa subunit of the tetrameric complex cleavage polyadenylation specificity factor (CPSF-160) binds to the consensus poly(A) signal AUAAA which is located upstream of most functional poly(A) sites (14). The cleavage stimulatory factor (CstF) trimeric complex binds to the downstream element, a guanosine/uridine (GU)-rich region located downstream of the cleavage site (15), through its 64-kDa subunit (CstF-64) (16). CstF-64 is composed of a conserved RNA recognition motif near its N terminus (17) that binds preferentially to GU-rich regions (16). Additional factors involved in pre-mRNA processing include cleavage factor I w and II w and poly(A) polymerase. We investigated the expression of CstF-64 and other cleavage/polyadenylation factors after stimulation of murine RAW 264.7 and murine bone marrow-derived macrophages (BMDMs) with LPS. We found that LPS stimulation of RAW macrophages and murine BMDMs for 18 h significantly increased CstF-64 protein expression. Because LPS stimulation of macrophages arrests the cell cycle (18, 19), the increase in CstF-64 protein expression occurs in the absence of cellular proliferation. This is in contrast to the increases in CstF-64 expression observed in cells that were induced to proliferate (12, 20). Because exogenous overexpression of CstF-64 in chicken B-lymphoma DT40 cells increased the use of the weak poly(A) site in the IgM heavy chain gene
product (12), we hypothesized that the increased levels of CstF-64 observed in LPS-stimulated macrophages would have a similar effect on poly(A) site choice in many genes. To test this, we first monitored the effect increased levels of CstF-64 in RAW macrophages had on poly(A) site choice by using a stably transfected reporter construct that contained two linked poly(A) sites. We observed an increase in the use of the weaker promoter-proximal poly(A) site in the reporter construct concomitant with an increase in CstF-64 protein levels following LPS stimulation. Because of the pleiotropic effects LPS stimulation has on macrophages, we stably overexpressed CstF-64 in RAW macrophages to ascertain if CstF-64 specifically influences poly(A) site choice. Indeed, RAW macrophages that stably overexpress CstF-64 also demonstrated an increase in weak poly(A) site choice on the reporter construct. We therefore hypothesized that induction of CstF-64 by LPS has a functional consequence for gene expression in activated macrophages. To determine how many genes could be influenced, we performed microarray analysis of gene expression from RAW macrophages stably overexpressing CstF-64. We found that 10-fold constitutive overexpression of CstF-64 in RAW macrophages significantly altered the expression of 51 genes, of which over 25% share common gene expression changes with LPS-stimulated RAW macrophages that had a 5-fold maximal increase in CstF-64 expression. Closer analysis of two of the genes whose expression changed under both LPS-stimulating and CstF-64-overexpressing conditions, Id-2 and Mmp-9, revealed that an alternative polyadenylation event does occur on these gene transcripts and that this change in poly(A) site choice may contribute to the increase of mature transcripts by the removal of mRNA instability elements. From this, we conclude that increases in expression of the pre-mRNA cleavage factor CstF-64 observed in LPS-stimulated macrophages significantly contribute to changes in the expression of a multitude of genes through alternative polyadenylation events that occur in the context of infection by Gram-negative bacteria.

**MATERIALS AND METHODS**

**Cell Culture and Treatment**—Murine BMDM were derived from C57BL/6 bone marrow based on adherence. In brief, the bone marrow from euthanized mice was flushed out of the femur and tibia bones with Dulbecco’s modified Eagle’s medium (Invitrogen) as described previously (21). The bone marrow suspension was then washed twice with 2% fetal bovine serum (Atlanta Biologicals, Norcross, GA) in PBS. Nontissue culture-treated Petri dishes (Labtek) were seeded at 2 \( \times 10^6 \) cells per dish in 25 ml of macrophage media (25% L-cell supernatant, 20% fetal bovine serum, 1% L-glutamine, 1% pyruvate, and 1% nonessential amino acids). After 4 days in culture the cells were fed with 10 ml of fresh macrophage media. BMDM were generated until 80% confluent and then trypsinized. Cells were collected, spun down, and resuspended in 1 ml of PBS; cell number was determined from an aliquot. Triplicate 200-μl samples were removed. Nucleic acids were precipitated by 10% trichloroacetic acid and collected on glass fiber filters. Radioactivity was determined using scintillation counting, and the \([^{3}H]dT\) normalized was normalized for cell counts.

**RNA Isolation and Poly(A)\(^+\) RNA Purification**—Total RNA was extracted from RAW macrophages using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. Poly(A)\(^+\) RNA was purified from total RNA using oligo(dT)-cellulose (Stratagene) according to manufacturer’s instructions. Purified mRNA was suspended in diethyl pyrocarbonate (Sigma)-treated water and 2 volumes 95% ethanol and stored at \(-20^\circ\)C until needed.

**T2 RNase Protection Assay**—RAW macrophages were stably transfected with a guanine phosphotransferase (gpt)-containing plasmid with linked poly(A) sites and cultured in the presence of MPA, as described previously (23). The gpt gene has a weak poly(A) site from the \(\alpha_2\)-globin gene upstream of the strong SV40 late poly(A) site that is contained in the pSV2gpt vector (24). The RAW macrophages that contain the gpt-encoding reporter gene are subsequently referred to as RAW-a2 macrophages. RNase protection assays were performed using an antisense radiolabeled riboprobe that hybridizes to the 3′-UTR of the gpt mRNA to detect poly(A) site usage (see Fig. 3A). The riboprobe was synthesized with a specific activity of \(~7.3 \times 10^8\) cpm/\(\mu\)g as described previously (23). Radioactivity contained in the proximal and distal poly(A) site-protected fragments was measured with a PhosphorImager (MD 860, Amersham Biosciences), and quantification was performed using ImageQuant software (Amersham Biosciences). The proximal/distal ratio (P/D) was computed using Office XP Excel (Microsoft), and all ratios from experimental samples were normalized to the untreated control within each experiment. Statistical significance of the data was calculated using S.E. and the paired Student’s t test from two to three trials performed from at least two separate RNA preparations, for a total of five trials from each condition.

**Overexpression of CstF-64 in RAW 264.7 Murine Macrophages**—The complete open reading frame of human CstF-64 [CstF-2] was cloned Imaging Station 2000R and associated one-dimensional analysis software. CstF-64 expression was normalized to the GAPDH signal on the same blot. Rabbit antiserum recognizing murine CstF-64 was generated for peptide CIALPMPEPQQRQSLILKEQIQKSTGAP, corresponding to the 26 C-terminal amino acids (a cysteine residue was added to the N terminus to aid keyhole limpet hemocyanin coupling). Injections and collections of serum were performed at Charles River PharmServices. The anti-CstF-50 and anti-CstF-77 rabbit anti-peptide antibodies were made in our laboratory as described previously (20) as well as the anti-human RNAP F and anti-human RNAP H/H′ rabbit polyclonal antibodies (11). Anti-CPSF-30 (rabbit polyclonal) and anti-CPSF-100 (rabbit polyclonal) were generously provided by Walter Keller (University of Basel). We purchased the mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Chemicon. Horseradish peroxidase-conjugated secondary antibodies for mouse IgG (Sigma A7282) and rabbit IgG (Sigma A0545) were used according to the manufacturer’s instructions.

**Measurement of Cell Proliferation by \([^{3}H]Thymidine Incorporation**—RAW macrophages were plated in 35-mm culture dishes and allowed to grow to \(~80%\) confluency (3 \( \times 10^5\) cells/plate) with 5 ml of medium and were left untreated or stimulated with LPS. To measure DNA synthesis (22), 10 μCi of \([^{3}H]dT\) (6.7 Ci/mmol) in aqueous solution was added to the cells covered with 3 ml of medium. After 6, 14.5, and 18 h at 37°C, the medium was removed, and the cells were washed twice with 1× PBS and then trypsinized. Cells were collected, spun down, and resuspended in 1 ml of PBS; cell number was determined from an aliquot. Triplicate 200-μl samples were removed. Nucleic acids were precipitated by 10% trichloroacetic acid and collected on glass fiber filters. Radioactivity was determined using scintillation counting, and the \([^{3}H]dT\) normalized was normalized for cell counts.

**Western Blot Analysis and Antibodies**—Ten micrograms of whole cell lysates were loaded per lane unless otherwise indicated. Proteins were visualized after treatment of the appropriate secondary antibody with the Renaissance chemiluminescence system (PerkinElmer Life Sciences) according to manufacturer’s instructions. All Western blots were re-probed with GAPDH to demonstrate equal loading between lanes. Quantification of signal intensities was performed on the Kodak Digital Science Imaging System (2000R) according to manufacturer’s instructions.
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into the EcoRI sites of pBluescript II SK(+)(pBSSK-64), using EcoRI linkers as described (17), and was a gift from Jim Manley (Columbia University). To avoid difficulties with cytomegalovirus promoter activity in RAW 264.7 macrophages, we used pEF1/Myc-His B (Invitrogen), a plasmid driven by the human EF-1α promoter that has been shown to achieve high levels of exogenous gene expression in RAW macrophages (25). A double digest of both pBSSK-64 and pEF1/Myc-His B with KpnI (Roche Applied Science) and XbaI (Roche Applied Science) was performed followed by gel purification of an ~2.0-kb fragment from pBSSK-64 and an ~6.5-kb fragment from pEF1/Myc-His B. Ligation of the purified plasmid fragments with T4 DNA ligase (New England Bio-labs) was done in a 16°C water bath overnight followed by direct trans- formation of the ligation reactions into One Shot MAX Efficiency DH5α-1 competent cells (Invitrogen) resulting in a sufficient number of colonies on penicillin G (Sigma) LB plates for miniprep screenings. The resulting CstF-64 expression plasmid is called pEF1-64.

RAW-α2 macrophages were stably transected using FuGENE 6 (Roche Applied Science) with pEF1-64 or pEF1-lacZ, as a control, as instructed by the manufacturer. In brief, RAW-α2 macrophages were seeded in 6-well culture dishes and grown until ~75% confluent. Macrophages were transected with a FuGENE 6 (μl):DNA ratio (μg) of 8:1 in the presence of serum. At day 3 post-transfection, the cells were split and placed under selective pressure of 400 μg/ml G418 sulfate (Cellgro) and MPA. Media were changed as needed. After 18 days of selective pressure, polyclonal populations of RAW-α2-64 or RAW-α2-LacZ cells were stored in liquid nitrogen. Raw-α2-64 cultures were assessed for constitutive CstF-64 expression by Western blot.

Affymetrix Gene Chip Analysis—Three unique samples of RNA from each treatment (untreated, 100 ng/ml LPS-treated for 18 h and constitutively overexpressed CstF-64) were used to create biotinylated cRNA at the PittArray DNA microarray facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Fifteen micro- grams of fragmented RNA were hybridized to the murine 430A Affymetrix gene chip according to manufacturer’s instructions. Affymetrix gene chips were scanned using MAS 5.0 (Affymetrix) to obtain raw expression values and signal calls (i.e. present, absent, or marginal) for each probe set on the array chip (GEA accession number GSE2002). Data sets of intensities for 22,690 probe sets per expression array were analyzed by BRB-ArrayTools version 3.02 (linus.nci.nih.gov/BRB-ArrayTools.html). Data were filtered through the software by using the following parameters: all signals with an intensity value below 10 were given a threshold value of 10, and each array was normalized to the 64 Affymetrix control probe sets intrinsic to the murine 430A chip. Furthermore, probe sets were excluded from all arrays under the following conditions: when less than 30% (i.e. 0, 1, or 2 of 9) of expression data values have a 2-fold change in either direction from the probe set’s median value or when 50% or more of the data for a probe set is missing or absent. Using these filtering parameters, 1145 probe sets passed the criteria. To identify genes that demonstrated statistically significant changes in expression between control and treatment (LPS or CstF-64 overexpressing) groups, the probe sets that had passed the initial filtering process also had to pass the following criteria. 1) The mean of the treatment group must have demonstrated a 2-fold increase or decrease in signal over the mean of the control group. 2) The signal values for each probe set must have passed a statistical univariate significance test with p value<0.05 with 1000 permutations and specifying a random variance for each calculation (class comparison feature of BRB Array Tools). 3) A probe set was excluded if it scored an absent call in at least two of three measurements for the treatment group (for up-regulated genes) or scored an absent call in at least two of three measurements for the control group (for down-regulated genes). Marginal calls were considered absent for this criterion.

A statistical algorithm was designed to test if the genetic overlap between LPS-stimulated and CstF-64 overexpressing conditions was beyond the random chance of occurrence. Briefly, the test was designed with one set consisting of a draw of 515 random numbers from a pool of 22,690 and another set consisting of a draw of 51 random numbers again from a pool of 22,690 numbers. The test was run 30,000 times looking for the number of random chance overlap from the two sets of randomly selected numbers. A graph depicting the frequency of zero numbers of overlaps, one number, two numbers, etc. up to 14 numbers from 30,000 trials was constructed using Microsoft Excel and appears in supplemental Fig. 1.

Semi-quantitative RT-PCR—Single-stranded cDNA was synthesized for each condition from 10 μg of total cellular RNA using the Super- script first-strand synthesis system for RT-PCR with oligo(dT) primers (Invitrogen). PCR reaction mixtures were assembled in quadruplicate for each primer pair using either 0.5 or 1.0 μl of cDNA, 50 pmol of each primer, 800 nm dNTP mix, 5 μl of 10× Taq buffer, and 2.5 units of Taq (Genechoice) in 50-μl volumes. At the conclusion of 20, 25, and 30 cycles, reaction tubes were removed from the PCR machine and placed on a heat block for final extension. The last tube of each set of four reactions was allowed to complete the PCR program to 35 cycles on the thermocycler. Ten microlitrer aliquots of the PCRs representing 20, 25, 30, and 35 cycles were loaded on 1.5% agarose gels, stained with ethidium bromide, and visualized using the Kodak Imaging Station 2000R. The hypoxanthine-guanine phosphoribosyltransferase gene, which is shown to be equally expressed between the RAW macrophage culture conditions on the microarray data (GSE2002) and has been shown previously to be a good candidate for RT-PCR loading control (26), was used to demonstrate cDNA potency between preparations. The nucleotide sequences for the primer pairs used are listed in TABLE ONE. All primer were designed using Primer3 (frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) unless otherwise indicated (27).

Multiple-Reverse Primer PCR (MRP-PCR)—Single-stranded cDNA was synthesized, and PCR mixtures were assembled as described above. A common forward primer (F) was designed for all five genes under study (Fig. 7A and TABLE ONE). An upstream reverse primer (R1) was designed 5’ to the most proximal putative poly(A) site and amplifies all gene products regardless of poly(A) site usage. A second downstream reverse primer (R2) was designed to the region on the gene that lies between putative poly(A) sites and amplifies only gene products that are cleaved at the distal poly(A) site(s). PCR products were loaded on agarose gels and analyzed on the Kodak Imaging Station 2000R.

RESULTS

We treated RAW macrophages with increasing amounts of LPS for 18 h and measured CstF-64 protein expression by Western blot. We observed that CstF-64 protein levels increased with LPS stimulation of RAW macrophages and that this increase is LPS dose-dependent (Fig. 1A). The induced expression of CstF-64 plateaued at 100 ng/ml LPS (Fig. 1A). A semi-quantitative Western blot revealed that 100 ng/ml LPS stimulation of RAW macrophages for 18 h resulted in a 4–5-fold increase in CstF-64 expression (Fig. 1B). Quantification of the luminescence from the Western blot using the Kodak Imaging Station 2000R corroborated the semi-quantitative Western data by measuring the induction of CstF-64 with 100 ng/ml LPS at 5-fold that of unstimulated RAW 264.7 macrophages (see “Materials and Methods”). Therefore, we concluded that LPS treatment of RAW macrophages increases CstF-64
protein expression ~5-fold. From these observations, 100 ng/ml LPS treatment for 18 h was used for all subsequent experiments.

LPS stimulation of B-lymphocytes not only increases CstF-64 expression (12) but also induces cell cycle progression resulting in rapid clonal expansion (28). We have shown previously that CstF-64 protein levels increase in the presence of cell cycle progression. Notably, LPS stimulation of B-lymphocytes not only increases CstF-64 expression (12) but also induces cell cycle progression resulting in rapid clonal expansion (28). We have shown previously that CstF-64 protein levels increase when serum-starved, resting mouse 3T3 fibroblasts were induced into the S-phase of the cell cycle by the addition of serum-containing media (20). Both of these examples demonstrate that CstF-64 induction by Western blot (1) and by immunofluorescence (29) is not obligatorily coupled to the cell cycle.

The protein levels of several other cleavage/polyadenylation factors were also assessed under LPS-stimulating conditions. Western blots showed no measurable changes for CstF-77, CPSF-100, CPSF-30, and CF-1m in LPS-stimulated RAW macrophages (Fig. 2, left panel). Furthermore, the expression of hnRNP H/H' and hnRNP F, RNA processing factors we have found to have effects on cleavage/polyadenylation in B-lymphocytes and plasma cells (11), also remained unchanged (Fig. 2, left panel).

The protein levels of several other cleavage/polyadenylation factors were also assessed under LPS-stimulating conditions. Western blots showed no measurable changes for CstF-77, CPSF-100, CPSF-30, and CF-1m in LPS-stimulated RAW macrophages (Fig. 2, left panel). Furthermore, the expression of hnRNP H/H' and hnRNP F, RNA processing factors we have found to have effects on cleavage/polyadenylation in B-lymphocytes and plasma cells (11), also remained unchanged (Fig. 2, left panel). To determine whether the specific increase in CstF-64 protein expression observed in the transformed RAW cell line also occurred in primary macrophages, we stimulated murine BMDMs with 100 ng/ml LPS for 18 h. CstF-64 protein was increased ~10-fold in murine BMDMs stimulated with 100 ng/ml LPS (Fig. 2, right panel). As with the RAW macrophage cell line, no other mRNA processing factors assayed showed changed expression upon LPS stimulation (Fig. 2, right panel). Studies have shown that CstF-64 is the limiting component for CstF trimer formation, the active form of CstF (12, 20). Therefore, we hypothesized that the specific increase in CstF-64 observed in LPS-stimulated RAW macrophages and BMDMs has a significant impact on 3'-mRNA processing and would thus alter the expression of specific genes.

### TABLE ONE

| Gene name | Sequence (5'–3') |
|-----------|-----------------|
| TNF-α     | CAC GTC GTA GCA AAC CAC CAA GTG GA* |
| Antisense | TGG GAG TAG ACA AGG TAC AAC CC |
| Nos2      | CAC CTT GGA GTT CAC CCA GT |
| Antisense | ACC ACT CGT ACT TGG GAT GC |
| Cox-2     | AAA CCG TGG GGA ATG TAT GAG C |
| Antisense | TCG CAG GAA GGG GAT GTT GTT C |
| Chap      | GCA TGA AGG AGA AGG AGC AG |
| Antisense | CTT CCG GAG AGA CAG ACA GG |
| Id-2      | ATC AGC CAT TTC ACC AGG AG |
| Antisense | TCC CCA TGG TGG GAA TAG TA |
| Tyki      | AGC TCT GCA ATC CCG TTT TA |
| Antisense | CCA GAA TTC ACG GTG TTT AT |
| Mnp-9     | TGA ATC AGC TGG TTT TTT TT |
| Antisense | GTG GAT AGC TGG GTG GTG TT |
| P4ha2     | GTC AAG CAG AAG CCA GGA AC |
| Antisense | CCA CAA TCT TCC ATG ACG TG |
| Mnp-12    | TTT CTT CCA TAT GGG CAA GC |
| Antisense | GGT CAA AGA CAG CTA CTA |
| RARβ      | ACA AGT CAT CGG GCT ACC AC |
| Antisense | CTG TGG ATT CCT GCT TGG AA |
| Cte-1     | GAT GGC TTC AAG GAT GTT GG |
| Antisense | TAA GGG GGC TCG ATG TAA TG |
| Cd48      | CGA TCT CAT CGT CAC ACC AC |
| Antisense | TGC TTC CAA GAT GCC TTC TT |
| Hprt      | CGT CGT GAT TAG CGA TGA TG |
| Antisense | AGA GGT CCT TTT CAC CAG GA |
| Id-2      | F CCA GCA TCC CCC AGA ACA AG |
| R1        | GGG AAT TCA GAT GCC TGC AA |
| R2        | GCT TGG TTT ATT TCA GAC AAC CAG TG |
| Mnp-9     | F CTG GAC TCC GCC TTC TTT GAG GA |
| R1        | GAA AGG ACG GTG GGC AGA GA |
| R2        | CAG CTA GGA ACC ACC GTA |

* Primer sequences were obtained by Grolleau et al. (27).
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Previous studies have shown that increases in CstF-64 expression in LPS-stimulated splenic B-lymphocytes are associated with an increase in weak poly(A) site selection on the IgM heavy chain gene (12). With the specific increase in CstF-64 protein observed in LPS-stimulated macrophages (Fig. 2), we hypothesized that the nuclear mRNA processing machinery has a heightened level of mRNA 3’-end processing in activated macrophages that results in an increased selection of weak poly(A) sites. To test this, we stably transfected into the RAW macrophage cell line a reporter mini-gene that possesses two contiguous poly(A) sites of differing strengths in its 3’-UTR (Fig. 3A). The reporter mini-gene codes for the bacterial gene guanosyl phosphotransferase and is under the control of the SV40 early promoter (24). Stable incorporation of the mini-gene in the RAW macrophages is thus conferred through MPA resistance. Contained in the 3’-UTR of the mini-gene is the weak α2-globin poly(A) site upstream of the strong SV40 early poly(A) site (Fig. 3A). The RAW macrophage cell line that stably expresses the α2-globin poly(A) site containing gpt mini-gene is referred to as RAW-α2. LPS stimulation of RAW-α2 macrophages exhibited the same protein expression levels for CstF-64 and the other cleavage/polyadenylation factors measured in untransfected RAW macrophages (Fig. 2 and data not shown). Previous studies using this same reporter mini-gene in different B-cell lines demonstrated that the upstream, weaker poly(A) site was selected more often in the plasma cell environment, which exhibits heightened 3’-end processing activity than in memory B-cell lines (23). Here we wished to test the hypothesis that the LPS-stimulation of RAW macrophages that exhibit elevated levels of CstF-64 expression induces an increase in poly(A) site selection of the weaker promoter-proximal poly(A) site.

We performed T2 RNase protection assays on mRNA isolated from unstimulated or LPS-stimulated RAW-α2 macrophages to measure poly(A) site selection within the 3’-UTR of the gpt reporter mini-gene, as described previously (23). The full-length probe is 649 nucleotides in length. A protected fragment 487 nucleotides in length indicates that the promoter-distal poly(A) site was chosen for mRNA cleavage and polyadenylation (Fig. 3A). Likewise, a protected fragment 185 nucleotides in length indicates the choice of the promoter-proximal poly(A) site for mRNA processing. More importantly, the poly(A)-containing RNA for the T2 RNase protection assay was harvested at 18 h after LPS stimulation. This allowed the cells to achieve the 5-fold increase in CstF-64 protein expression we had observed previously (see Fig. 1B). A representative result of the RNase protection experiment displays the protected, radiolabeled riboprobe fragments separated on a denaturing urea gel (Fig. 3B). Quantification of the amount of transcripts corresponding to each protected fragment was performed using a Phosphorimager (Amersham Biosciences) and the ImageQuant software package (see “Materials and Methods”). By calculating the P/D of the protected fragments, we were able to obtain a relative measurement of poly(A) site usage that can be compared between experiments and treatments. An increase in P/D for a particular treatment indicates that there is an increase in selection of the weaker promoter-proximal poly(A) site of the gpt mini-gene transcripts relative to the stronger promoter-distal poly(A) site. Likewise, a decrease in P/D would indicate increased selection of the stronger promoter-distal poly(A) site. Although the absolute values of transcripts processed at the upstream

FIGURE 2. LPS stimulation of RAW 264.7 macrophages and BMDM does not alter the expression levels of other factors involved in the cleavage/polyadenylation reaction. Protein extracts from macrophages stimulated for 18 h with 100 ng/ml of LPS were probed by Western blot for the indicated cleavage/polyadenylation factors. Cont, control.
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We have demonstrated that both LPS stimulation and CstF-64 overexpression induced an increase in the selection of weak poly(A) sites in RAW macrophages on a reporter mini-gene that possesses two contiguous poly(A) sites of varying strengths. Because of the impact that post-transcriptional processing has on gene expression, we compared global gene expression profiles in unstimulated, LPS-stimulated, and CstF-64-overexpressing RAW macrophages using the microarray technique. RNA from untreated and CstF-64-overexpressing RAW macrophages and RAW macrophages stimulated with 100 ng/ml LPS for 18 h was used for microarray analysis. By using Affymetrix murine gene chip 430A, 22,690 genes were examined from three replicates of each treatment group (see "Materials and Methods"). Statistical analysis was performed as described under "Materials and Methods" using BRB Array Tools developed by the National Cancer Institute (linus.nci.nih.gov/BRB-ArrayTools.html).

Treatment of RAW macrophages with 100 ng/ml LPS for 18 h resulted in a statistically significant 2-fold or greater increase in the expression of 245 known genes and 45 expressed sequence tags (ESTs) and a 2-fold or greater decrease in the expression of 162 known genes and 63 ESTs (supplemental Tables S1 and S2). To ensure that our data sets from LPS-stimulated macrophages accurately represent an activated macrophage gene expression profile, we performed semi-quantitative RT-PCR using the same RNA sample used for the microarray experiment to measure TNF-α, Nos2, and Cox-2 transcript levels, all of which are identifiers of macrophage activation. As with the microarray data, we have shown that TNF-α, Nos2, and Cox-2 transcript levels all significantly increased in the presence of LPS stimulation (Fig. 5). We also confirmed the change in expression of several other genes by RT-PCR (Fig. 5). None of the CPSF subunits, poly(A) polymerase, or poly(A) tract-binding protein changed expression upon LPS stimulation (supplemental Tables S1 and S2). Probes for CstF-77, CstF-50, hnRNP F, hnRNP H/H\(^\text{t}\), or CF-Im-68 were not included on the Affymetrix 430A murine gene chip.

Microarray analysis of RAW-α2 macrophages overexpressing CstF-64 protein ~10-fold showed a statistically significant 2-fold or greater increase in the expression of 21 known genes and 1 EST, of which 11 of the genes demonstrated a significant increase in LPS stimulation (TABLE TWO). Likewise, 29 known genes showed a 2-fold or
TABLE TWO

Genes that exhibit increased expression in CstF-64 overexpressing RAW macrophages

Data are from three independent experiments and were analyzed as described under “Materials and Methods.” Genes shown were induced an average of 2-fold or greater.

| Accession no. | Gene name | -Fold increase<sup>a</sup> | No. putative poly(A) sites<sup>b</sup> | Accession no. | Gene name | -Fold increase<sup>a</sup> | No. putative poly(A) sites<sup>b</sup> |
|---------------|-----------|-----------------------------|---------------------------------|---------------|-----------|-----------------------------|---------------------------------|
|               |           | CstF-64 | LPS |                  |            | CstF-64 | LPS |                   |            | CstF-64 | LPS |                   |            |
| Biological regulation/metabolism |           |        |    |                  |            |        |    |                   |            |        |    |                   |            |
| NM_020557  | Tyki     | 2.64   | 35.12 | 3          | AK089198  | Gpcr-35  | 4.90 | 5          |            |        |    |                   |            |
| NM_013599  | Mmp-9    | 2.05   | 7.11  | 2          | NM_022881 | Rgs-18   | 3.59 | 2          |            |        |    |                   |            |
| NM_011961  | Plod-2   | 17.62  | 1    | 1          | AF220524  | Dnmt3L   | 2.84 | ND |            |        |    |                   |            |
| BC018411   | Phh2a    | 13.01  | 1    | 1          | AF220524  | Dnmt3L   | 2.84 | ND |            |        |    |                   |            |
| BC019135   | Mmp-12   | 5.97   | 1    | 1          |            |         |     | ND |            |        |    |                   |            |
| NM_080555  | Ppap2b   | 4.88   | ND   | 1          | AK041118  | Rhpms    | 14.81 | 3.22 | 1          |        |    |                   |            |
| Transcriptional regulation |           |        |    |                  |            |        |    |                   |            |        |    |                   |            |
| NM_007837  | Chop     | 2.81   | 7.22  | ND          | BC022751  | IFN-stimulated protein | 2.29 | 22.78 | ND |            |        |    |                   |            |
| NM_010496  | Id-2     | 2.64   | 3.45  | 4          | BC057864  | Digr2    | 2.15 | 7.31 | 1          |        |    |                   |            |
| NM_011243  | RARβ     | 4.93   | 2    | 1          | BC010564  | Histone H2a | 2.09 | 7.83 | ND |            |        |    |                   |            |
| Transport   |           |        |    |                  |            |        |    |                   |            |        |    |                   |            |
| NM_023044  | Stc15α3  | 2.40   | 20.09 | ND          | BC009118  | cDNA sequence BC009118 | 2.51 | 1          |        |    |                   |            |
| NM_007752  | Ceruloplasmin (Cp) | 2.40 | 17.98 | 1 | | BC009118  | cDNA sequence BC009118 | 2.51 | 1 | | |
| NM_007443  | Bikunin  | 5.29   | 1    | 1          |            |         |     | ND |            |        |    |                   |            |

<sup>a</sup>- Fold increase in average signal value observed in CstF-64-overexpressing or LPS-stimulated RAW macrophages compared with untreated control is shown.

<sup>b</sup>- Number of AAUAAA and AUUAAA poly(A) signals with associated GU-rich regions identified in the 3'-UTR of the listed GenBank™ sequence is shown.

*ND indicates no identifiable AAUAAA or AUUAAA poly(A) site in the 3'-UTR of the listed GenBank™ sequence.

FIGURE 5. Validation of differential gene expression observed in LPS-stimulated and CstF-64-overexpressing RAW 264.7 macrophages by semi-quantitative RT-PCR. Ethidium bromide-stained agarose gels show increases in product formation from 20, 25, 30, or 35 PCR cycles, which ensure that the reactions lie within the linear range and therefore can be accurately compared between RAW macrophage culture conditions.
greater decrease in the expression with CstF-64 overexpression, of which 3 of the genes also demonstrated a significant decrease with LPS stimulation (TABLE THREE). Analysis of the 3'-UTRs of all 51 genes that change expression under CstF-64 overexpression revealed that over 45% of them (24 of 51) possess two or more putative poly(A) sites (TABLES TWO and THREE). Putative poly(A) sites were chosen by their consensus AAUAAA or nonconsensus AUUAAA poly(A) signal (shown to be greater than 58 and 14%, respectively, of the functional poly(A) signals determined from human data base analysis) (29) and an associated GU-rich region that possesses at least two contiguous uracils and lies within 20–60 nucleotides downstream of the poly(A) signal. Many of these genes that may support an alternative polyadenylation event also possess adenine/uridine (AU)-rich elements (AREs) that may, themselves, dictate mRNA stability or translatability (reviewed in Refs. 9 and 30).

Notably, we did not detect increases in CstF-64 gene expression in LPS-stimulated RAW macrophages by microarray analysis. This result was not unexpected because repeated attempts to measure changes in CstF-64 gene expression in LPS-stimulated RAW macrophages by Northern blot or RT-PCR demonstrated no change in CstF-64 message (data not shown). This result led us to believe that the observed increase in CstF-64 protein is the result of a post-transcriptional/translational control mechanism, a characteristic of other genes in LPS-stimulated macrophages (6, 9). Furthermore, we were unable to measure increases in CstF-64 gene expression in RAW-a2-64 macrophages by microarray because 9 of the 11 probe pairs in the CstF-64 probe set for the murine 430A gene chip target 3'-UTR genomic sequences (Affymetrix NETAFFX<sup>TM</sup> Analysis Center, www.affymetrix.com/analysis/index.affx), which are not contained in the CstF-64 expression vector that is stably incorporated into the RAW macrophage genome. Nevertheless, the Western blot data for both LPS-stimulated (Fig. 1, A and B) and CstF-64-overexpressing (Fig. 4A) RAW macrophages firmly established that protein levels are indeed increased over basal levels under both conditions. Therefore, we concluded that increased expression of CstF-64 contributes, in part, to the gene expression changes that accompany the late macrophage response to LPS stimulation.

Because we were interested in whether elevated levels of CstF-64 in the RAW macrophage influence the poly(A) site choice of LPS-responsive genes, we set out to detect by PCR a possible poly(A) site switch on genes that possess multiple putative poly(A) sites, have an ARE, and whose expression changes in both LPS-stimulated and CstF-64-overexpressing RAW macrophages. Fig. 7A describes our MRP-PCR approach to detect the use of alternative poly(A) sites under LPS-stimulating and CstF-64-overexpressing conditions. By pairing the common forward primer (F) with the upstream reverse primer (R1), we were able to detect all of the mature transcripts regardless of which poly(A) site was chosen. Furthermore, by pairing the F primer with the downstream reverse primer (R2), we were able to detect the portion of the mature gene transcript that was cleaved and polyadenylated at a more downstream poly(A) site (FR2).

Most interestingly, for Id-2 and Mmp-9, we were able to detect a reduction in FR2 PCR product compared with FR1 PCR product, which represents total gene expression, under LPS-stimulating conditions compared with the untreated control (Fig. 7B). Likewise, we also detected a reduction in FR2 PCR product for Id-2 and Mmp-9 under CstF-64-overexpressing conditions compared with FR1 PCR product (Fig. 7B). Because we demonstrated an increase in the total gene transcript using the FR1 primer pair for Id-2 and Mmp-9 under LPS-stimulating and CstF-64 overexpressing conditions, this proves that the decrease in FR2 PCR product observed is the result of an alternative polyadenylation event similar to what was observed on our reporter mini-gene (Fig. 3).

By identifying a poly(A) site switch in both Id-2 and Mmp-9 under LPS-stimulating conditions, we focused our attention on the region between the proximal and distal poly(A) sites of these gene transcripts in the hope of gaining some insight on the biological effect a change in

### TABLE THREE

| Accession no. | Gene name  | -Fold decrease<sup>a</sup> | No. putative poly(A) sites<sup>a</sup> | Accession no. | Gene name  | -Fold decrease<sup>a</sup> | No. putative poly(A) sites<sup>a</sup> |
|---------------|------------|---------------------------|--------------------------------------|---------------|------------|---------------------------|--------------------------------------|
| AK053680      | Cte-1      | 6.71                      | 3.13                                 | NM_007643     | Cd36       | 16.16                     | ND                                   |
| NM_134188     | Mye-1      | 5.19                      | 2.27                                 | AF057367      | Ufli       | 5.92                      | 4                                    |
| NM_012011     | elf-3a Y-linked | 14.74                  | 5                                     | BC010200      | Fgfr1      | 4.45                      | 1                                    |
| NM_020026     | B3gal3t    | 7.59                      | 1                                     | NM_013036     | Edg2       | 2                         |                                      |
| NM_153153     | Supravinil  | 4.08                      | 2                                     | AB122023      | Pilr1      | 3.37                      | 1                                    |
| NM_007945     | Eps8       | 3.04                      | 2                                     | AK028875      | Cxcl16     | 2.71                      | 3                                    |
| NM_011890     | sarcoglycan,β | 2.10                    | 4                                     | NM_016846     | ralGDS1-1  | 2.67                      | 2                                    |
| BC048929      | Dystonin   | 2.01                      | 3                                     | U66888        | Emr-1      | 2.29                      | 1                                    |
| NM_012495     | Nectin 3   | 2.21                      | 2.38                                 | NM_008967     | Ptgr1      | 2.11                      | 1                                    |
| NM_012008     | Ddx3 Y-linked | 16.06                  | 7                                     | BC016887      | Doc-2      | 2.42                      | 4                                    |
| NM_011419     | Zmusat1 1D | 5.48                      | 3                                     | NM_153098     | Cd109      | 5.40                      | 2                                    |
| NM_029413     | Zwcc2      | 4.19                      | ND                                    | BC060977      | Cd48       | 4.30                      | 2                                    |
| NM_007965     | Evl        | 4.16                      | 1                                     | ND            |            |                           |                                      |
| AJ116823      | Trip6      | 3.39                      | ND                                    | AK030971      | coronin 2A | 2.03                      | 1                                    |

<sup>a</sup> Fold increase in average signal value observed in CstF-64 overexpressing or LPS-stimulated RAW macrophages compared with untreated control is shown.

<sup>b</sup> Number of AAUAAA and AUUAAA poly(A) signals with associated GU-rich regions identified in the 3'-UTR of the listed GenBank<sup>TM</sup> sequence.

<sup>c</sup> ND indicates no identifiable AAUAAA or AUUAAA poly(A) site in the 3'-UTR of the listed GenBank<sup>TM</sup> sequence.
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FIGURE 6. Model of CstF-64 influences on gene expression. LPS treatment of RAW macrophages increases CstF-64 protein levels. Depicted are three possible ways elevated levels of CstF-64 protein influence gene expression. (i) The cleavage activity of the cell is increased allowing a larger number of pre-mRNA transcripts to be 3′-end cleaved and polyadenylated, especially for transcripts that have GU-rich sequences in the 3′-UTR that are sub-optimal for CstF-64 binding. This would create more mature mRNA transcripts of a given gene to be used for protein translation. (ii) An alternative polyadenylation event occurs on a gene transcript that has multiple poly(A) sites surrounding mRNA instability elements. Increased selection of an upstream poly(A) site removes the instability elements from the mature transcript, thus increasing mRNA half-life and allowing the accumulation of more protein product. (iii) The increased expression of transcriptional regulators (e.g. Chop, Id-2, and RARβ) by either of the two aforementioned mechanisms has effects on the expression on their targeted genes. The Venn diagram depicts 515 genes that change expression with LPS stimulation, 51 genes that change expression with CstF-64 overexpression, and 14 genes that change under both conditions. + indicates induction of gene expression; — indicates repression of gene expression.

poly(A) site usage would have on the expression of these genes. In Fig. 8, we show the nucleotide sequence of Id-2 and Mmp-9 that lies between the proximal and distal poly(A) sites. Most interestingly, both of the regions of these genes possess AU-rich elements that resemble biologically functional AREs that have been described previously to influence mRNA half-life and/or translation (30).

From this, we concluded that the physiological increase in CstF-64 protein through LPS stimulation causes, in part, a shift in poly(A) site usage on LPS-inducible genes and that this shift in poly(A) site usage may contribute to the final amount of the gene transcript and influence the translatability of that transcript, thus profoundly influencing the amount of protein produced.

DISCUSSION

We have shown that LPS stimulation of murine RAW macrophages stimulated with LPS for 18 h exhibited CstF-64 expression levels ~5-fold over basal expression and occurred during the cessation of the cell cycle (Fig. 1). This finding is noteworthy because of studies from our laboratory and others that have shown that CstF-64 expression levels increase with cell cycle progression (12, 20). We further showed that LPS stimulation of both RAW macrophages and murine BMDM increased CstF-64 specifically, in that several other proteins associated with pre-mRNA cleavage/polyadenylation did not change expression (Fig. 2).

Through RNase protection assays, we measured a 2.4-fold increase in the selection of the weaker promoter-proximal poly(A) site of the reporter mini-gene in LPS-stimulated RAW macrophages that exhibit increases in CstF-64 protein expression (Fig. 3). Constitutive overexpression of CstF-64 ~10-fold higher than basal level further demonstrated that specific increases in CstF-64 protein expression were sufficient to induce a 2.0-fold increase in selection of the weaker promoter-proximal poly(A) site on the reporter mini-gene (Fig. 4).

Microarray analysis revealed that LPS stimulation of RAW macrophages for 18 h significantly increased the expression of 245 known genes and 45 unique ESTs (supplemental Table S1). We also identified a significant decrease in the expression of 162 known genes and 63 unique ESTs in LPS-stimulated RAW macrophages (supplemental Table S2). These data demonstrate that the effects of LPS stimulation on macrophage gene expression are sustained beyond the initial induction of gene transcription and thus could be contributed to by a variety of secondary effects. Furthermore, we measured a significant increase in 21 known genes and 1 EST and a significant decrease in 29 known genes in RAW macrophages that overexpress CstF-64. Because the only identified function of CstF-64 to date is its required role in the 3′-end cleavage step of pre-mRNA processing, we propose that genes that exhibit expression increases in the context of CstF-64 overexpression do so because of the following: (i) more gene transcript is cleaved and polyadenylated from the nascent pool of pre-mRNA of that gene, especially in the context of a single, weak poly(A) site; (ii) an alternative polyadenylation event occurs that removes mRNA instability elements from the mature mRNA transcript; or (iii) there are secondary effects from genes that regulate transcription which are themselves directly effected by elevated levels of CstF-64 (Fig. 6). Notably, there are at least three members in our list of CstF-64 inducible genes that have known transcriptional regulatory functions: Chop (31, 32), Id-2 (33, 34), and RARβ (35). Likewise, we believe the genes that exhibit a decrease in expression with CstF-64 overexpression are influenced by an alternative polyadenylation event that removes an mRNA stability element from the mature mRNA transcript, or the transcriptional regulators that increase in expression under elevated levels of CstF-64 repress the expression of these genes (Fig. 6).

Most interestingly, 11 of the 23 genes that increased and 3 of the 28 genes that decreased in the context of CstF-64 overexpression also demonstrated similar gene expression changes with LPS stimulation (TABLES TWO and THREE). By designing a statistical algorithm that tests the random chance of the same 14 genes of 22,690 on the array being selected from two separate pools at random, we found that this overlap between the LPS stimulation and CstF-64 overexpression would occur far less than once in 30,000 trials (supplemental Fig. S1). We therefore believe that the overlap is highly unlikely to have occurred at random and thus has biological significance. From this we conclude that some of the gene expression changes observed with LPS stimulation of RAW macrophages can be attributed, at least in part, to the elevated pre-mRNA cleavage induced by increases in CstF-64 protein expression. A possible explanation of why every gene that demonstrated expression changes in RAW macrophages overexpressing CstF-64 was not identified in LPS-stimulated RAW macrophages is that the stable 10-fold overexpression of CstF-64 is able to influence the expression of...
genes that are unaffected by the LPS-induced 5-fold increase. Examples of genes that changed expression with CstF-64 and not with LPS stimulation are *Plod-2*, *Ph4a2*, *RAR/H9252*, and *Cd48* (TABLE TWO and Fig. 5). Furthermore, stable overexpression of CstF-64 indefinitely elevates the pre-mRNA cleavage of the cell. This sustained elevation of mRNA 3'-end cleavage acts on all newly synthesized RNA. In the context of LPS stimulation, CstF-64 levels increase to 5-fold over basal expression 18 h post-treatment, and therefore heightened levels of pre-mRNA cleavage due to elevated levels of CstF-64 primarily act on gene transcripts produced late in macrophage activation. Transcripts produced before increases in CstF-64 expression, especially those with lengthy mRNA half-lives, can dampen the measurable effect elevated levels of CstF-64 have on newly transcribed genes.

Our studies have shown that the mechanism of alternative polyadenylation is strongly influenced by the physiological increase in the expression of CstF-64, resulting in an increase in the selection of a weaker promoter-proximal poly(A) site over a stronger promoter-distal poly(A) site on a reporter mini-gene. Analyses of EST databases have estimated that as much as 22% of human genes possess more than one poly(A) site, often of varying strengths (29). Indeed, a multitude of studies have demonstrated how the inclusion or exclusion of regions of the 3'-UTR to the final gene product through alternative polyadenylation can have a profound effect on mRNA half-life (36–39), the translational efficacy of the gene transcript (9, 40), and the final structure of the protein (see Ref. 41 and reviewed in Ref. 42). Analysis of the longest 3'-UTRs available through the GenBank® data base of the genes that show changes in abundance with CstF-64 overexpression revealed that 24 of 51 of these genes possess more than one putative poly(A) site.
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Furthermore, many of these genes also possess ARE-containing cis-elements that may regulate mRNA stability. Therefore, increases in CstF-64 protein levels can, in theory, dictate the presence or absence of these mRNA regulatory elements in the mature transcript by inducing an alternative polyadenylation event. To ascertain whether increases in CstF-64 protein cause a poly(A) site switch in LPS-induced genes as demonstrated for our reporter mini-gene, we found, through a PCR-based approach, that the abundance of transcripts that result from distal poly(A) site usage for Id-2 and Mmp-9 are reduced under LPS-stimulating and CstF-64-overexpressing conditions (Fig. 7). Reducing the expression levels of a more mature mRNA to be a template for protein production. To ascertain whether increases in CstF-64 protein cause a poly(A) site switch in LPS-induced genes as demonstrated for our reporter mini-gene, we found, through a PCR-based approach, that the abundance of transcripts that result from distal poly(A) site usage for Id-2 and Mmp-9 are reduced under LPS-stimulating and CstF-64-overexpressing conditions (Fig. 7). Reducing the expression levels of a more mature mRNA to be a template for protein production.

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