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The C/ebp-Atf response element (CARE) location reveals two distinct Atf4-dependent, elongation-mediated mechanisms for transcriptional induction of aminoacyl-tRNA synthetase genes in response to amino acid limitation

Jixiu Shan[1], Fan Zhang[1], Jason Sharkey[1], Tiffany A. Tang[1], Tönis Örd[2] and Michael S. Kilberg[1,*]

[1]Department of Biochemistry and Molecular Biology, Shands Cancer Center and Center for Nutritional Sciences, University of Florida College of Medicine, Gainesville, FL 32610, USA and [2]Estonian Biocentre, Riia 23, Tartu, 51010, Estonia

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ABSTRACT

The response to amino acid (AA) limitation of the entire aminoacyl-tRNA synthetase (ARS) gene family revealed that 16/20 of the genes encoding cytoplasmic-localized enzymes are transcriptionally induced by activating transcription factor 4 (Atf4) via C/ebp-Atf-Response-Element (CARE) enhancers. In contrast, only 4/19 of the genes encoding mitochondrial-localized ARSs were weakly induced. Most of the activated genes have a functional CARE near the transcription start site (TSS), but for others the CARE is downstream. Regardless of the location of CARE enhancer, for all ARS genes there was constitutive association of RNA polymerase II (Pol II) and the general transcription machinery near the TSS. However, for those genes with a downstream CARE, Atf4, C/ebp-homology protein (Chop), Pol II and TATA-binding protein exhibited enhanced recruitment to the CARE during AA limitation. Increased Atf4 binding regulated the association of elongation factors at both the promoter and the enhancer regions, and inhibition of cyclin-dependent kinase 9 (CDK9), that regulates these elongation factors, blocked induction of the AA-responsive ARS genes. Protein pull-down assays indicated that Atf4 directly interacts with CDK9 and its associated protein cyclin T1. The results demonstrate that AA availability modulates the ARS gene family through modulation of transcription elongation.

INTRODUCTION

Mammalian cells, *in vivo* or maintained *in vitro* culture, face challenges with regard to fluctuation in nutrient supply and have evolved adaptive strategies. For instance, when the intracellular compartment is depleted of one or more amino acid (AA), a signaling cascade, the amino acid response (AAR), is activated. Depletion of any one of the AA leads to the accumulation of the corresponding uncharged tRNA, which binds to and activates the general control non-derepressible 2 (Gcn2) protein kinase. Gcn2 mediates the phosphorylation of eukaryotic protein initiation factor 2α (eIF2α), which suppresses the eIF2-GDP to -GTP exchange necessary for optimal ribosome assembly (1,2). Thus, there is a suppression of global protein synthesis, but paradoxically the translation of some specific mRNA species that contain short upstream open reading frames in their 5′ untranslated regions is increased. Among those mRNAs is activating transcription factor 4 (Atf4). Mammalian cells express three other eIF2α kinases that are triggered by a wide variety of stimuli (2,3), but all four kinases result in increased p-eIF2α, global suppression of protein synthesis, and up-regulation of Atf4 protein. Collectively, these pathways are often referred to as the Integrated Stress Response (ISR). Increased Atf4 synthesis subsequently activates the transcription of a large number of target genes and thus, directs transcriptional programs that allow cells to correct, adapt, or undergo apoptosis in response to the initial stress. Global gene expression profiling, along with other functional studies, has demonstrated that Atf4-upregulated genes are involved in a broad spectrum of cellular functions and includes many members of AA tRNA synthetase (ARS) gene family (2,4–7). ARSs are a ubiquitously expressed and evolutionally conserved family of en-

*To whom correspondence should be addressed. Tel: +1 352 294 8388; Email: mkilberg@ufl.edu

zymes that link an AA to its cognate tRNA (8,9). This primary function of ARSs is highly conserved for all living organisms from bacteria to humans, but secondary functions of many ARSs are also quite common (9–13). These additional, non-canonical functions of ARSs, include involvement in translational and transcriptional regulation of gene expression, extra- and intra-cellular signaling, angiogenesis, inflammation and tumourigenesis.

Emerging evidence also indicates that ARSs can function as intrinsic sensors for intracellular AA concentrations (10). For example, leucine binding by leucyl-tRNA synthetase (LARS) activates mammalian Target of Rapamycin 1 (mTORC1) and promotes cell growth (14). Binding of glutamine by glutaminyl-tRNA synthetase (QARS) is a prerequisite for QARS-dependent proapoptotic activity of apoptosis signal-regulating kinase 1 (ASK1) (15), consistent with the observation that glutamine deprivation activates ASK1-induced apoptosis (16). Despite these important cellular functions, few reports have focused on the molecular mechnism by which the expression of these genes are transcripitonally regulated. In our previous study (6), genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) and mRNA expression analysis (mRNA-seq) identified a group of ARS genes characterized by increased binding of Atf4 and/or C/ebp homology protein (Chop) transcription factors in response to the ISR. However, the mechanism by which these factors transcripitonally regulate these genes has not been investigated.

In response to the ISR, increased Atf4 translation leads to recruitment of the factors onto an enhancer element within target genes named a C/ebp/Atf response element (CARE). Atf4, as a heterodimer with other transcription factors, can facilitate the assembly of the general transcription machinery to promote increased transcription (4,17–19). However, it is largely unknown if this mechanistic model applies to the Atf4-mediated regulation of the ARS genes.

RNA polymerase II (Pol II)-driven eukaryotic transcription can be regulated at the initiation, elongation or termination steps (20). Genome-wide studies have indicated that Pol II pausing at a gene promoter region is a widespread phenomena, especially for genes involved in development and stress responses (20,21). Consequently, the key regulatory step for these genes is the release of the paused Pol II and the subsequent transition to productive elongation, which is controlled by three major protein complexes (20–22). Negative elongation factor (NELF) complex and 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB)-sensitivity inducing factor (DSIF) interact with the pre-initiation complex (PIC) to stabilize paused Pol II and slow elongation. Release of the paused Pol II is regulated by positive transcription elongation factor-b (P-TEFb), which consists of cyclinT1 and cyclin-dependent kinase 9 (CDK9) (21,23). CDK9 phosphorylates NELF, DSIF and serine 2 in the carboxy-terminal domain of Pol II. After phosphorylation, NELF is evicted from the PIC and DSIF is converted from a negative to a positive elongation factor, which together with phosphorylated Ser2-Pol II, promotes enhanced elongation (21,24).

Given that ARSs mediate a critical step in protein synthesis and are induced by specific stress responses, it is critical to understand how this transcriptional control is regulated. In this study, we measured the change in expression of genes encoding the 20 cytoplasmic and 19 mitochondrial ARSs in mouse embryonic fibroblasts (MEFs) in response to AAR activation. The data show that 16 of the 20 genes for the cytoplasmic localized proteins were induced by the AAR in an Atf4-dependent manner, whereas only four of the 19 genes encoding the mitochondrial localized proteins were up-regulated. Two different classes of the 'cytoplasmic' genes were identified, one class in which the Atf4-binding CARE sites are immediately upstream of the transcription start site (TSS) and a second class in which the CARE is downstream within the body of the gene. After AAR activation, Atf4 was actively recruited to the CARE regardless of its location. Conversely, Pol II was constitutively bound to the TSS region for both classes of genes, but there was an AAR-induced and Atf4-dependent enhanced recruitment of Pol II to the CARE region of those genes that had the enhancer downstream of the TSS. There were also appropriate changes in association of both positive and negative elongation factors in response to Atf4, consistent with increased elongation. Collectively, the data illustrate that whereas most of the ARS genes encoding cytoplasmic enzymes are transcriptionally induced by AA limitation, there are two different mechanisms that mediate that induction.

MATERIALS AND METHODS

Cell culture

Human hepatocellular carcinoma cells (HepG2), MEFs and human embryonic kidney HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), pH 7.4 (#10-013-CV, Mediatech, Herndon, VA) supplemented with 1X non-essential amino acids, 2 mM glutamine, 100 mg/ml streptomycin sulfate, 100 units/ml penicillin G, 0.25 mg/ml amphotericin B and 10% fetal bovine serum (FBS). The medium for the Atf4 wild type and knockout MEFs (kindly provided by Dr Steve Abcouwer, University of Michigan) also contained 55 μM β-mercaptoethanol (25). The FLAG-Atf4–293T cell line was created by transforming HEK293T cells with a tetracycline-inducible construct that contains the Atf4 coding region tagged with the FLAG epitope at its N-terminal (26). These cells were cultured with a medium supplemented with 10% (v/v) tetracycline-free FBS, 25 μg/ml zeocin and 2.5 μg/ml blasticidin in addition to the supplements mentioned above. All cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air, and cultured to about 60–70% confluence so that they were still in a growth phase during the experimental treatment. At 15–16 h before initiation of treatment, all cells were given fresh medium and serum to ensure that no nutrient deprivation took place before the start of the experimental incubations. To assess AAR signaling, cells were incubated in control DMEM or DMEM supplemented with 5 mM histidinol (HisOH) for the period of time indicated. HisOH blocks charging of histidine onto the corresponding tRNA and thus, mimics histidine deprivation and triggers activation of the AAR (27), as shown in Supplementary Figure S1.

RNA isolation and real-time quantitative PCR

Total RNA was isolated with the TRIzol reagent (#15596018, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. A 1-μg aliquot of total RNA was used to synthesize first-strand cDNA with the qScript cDNA Synthesis Kit (#95047-100, Quanta BioSciences Inc., Gaithersburg, MD, USA). For real-time quantitative PCR (RT-qPCR), synthesized cDNA was diluted 10X with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and then 2 μl was mixed with 10 μl of SYBR Green master mixture (#4309159, Applied Biosystems, Warrington, UK) and 5 pmol of forward and reverse primers in a total volume of 20 μl. The mixture was subjected to 40 cycles at 95°C for 15 s, 60°C for 60 s after an initiation at 95°C for 10 min and RT-qPCR was performed with a CFX Connect real time system (Bio-Rad, Hercules, CA, USA). The primers used are listed in Supplementary Table S1. After RT-qPCR, melting curves were acquired by a stepwise increase of the temperature from 55 to 95°C to ensure that a single product was amplified in the reaction. The data were processed with the software Bio-Rad CFX Manager 3.0 (Version:3.0.1224.1015). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA content was used as an internal control. It has been well established in our laboratory that the expression of this housekeeping gene is not regulated by amino acid limitation. All calculations were based on the difference of Ct (the threshold cycle) of the analyzed gene relative to the internal control mRNA content in the same sample.

Chromatin immunoprecipitation (ChIP)

HepG2 cells were seeded at 1.5×10^7 per 150 mm dish and ChIP analysis was performed according to our previously published protocol (18). For the ChIP assay with MEFs, the protocol was essentially the same as with HepG2 cells, except that the cells were seeded at 1.5×10^6 per 150 mm dish and three dishes were used for each treatment. The Atf4 rabbit polyclonal antibody was described previously (28). Commercial antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) as follows: rabbit anti-total RNA Polymerase II Subunit B1 (RPB1) polyclonal antibody (#sc-899); rabbit anti-CHOP polyclonal antibody (#sc-575), rabbit anti-TBP polyclonal antibody (#sc-204), rabbit anti-NELF-A polyclonal antibody (#sc-32911), rabbit anti-SPT5 polyclonal antibody (#sc-28678) and normal rabbit IgG (#sc-2027). The antibody against tri-methylated histone 3 lysine 4 (H3K4me3) (#ab8580) was from Abcam (Cambridge, MA, USA). DNA enrichment was analyzed with RT-qPCR and the reaction mixtures were incubated at 95°C for 15 min, followed by amplification at 95°C for 15 s and 60°C for 60 s for 35 cycles. All experiments were performed in triplicate and were repeated to ensure reproducibility. The results are presented as the ratio to input DNA and the primers used for analysis are listed in Supplementary Table S1.

Co-IP, FLAG pull-down and Immunoblotting

Whole cell protein was extracted by lysing cells with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 0.5% sodium deoxycholate, 1.0% Triton X-100 and 0.1% SDS) supplemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (#88668, ThermoScientific, Waltham, MA, USA). For the nuclear extract, cells were resuspended in hypotonic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing the protease inhibitor mixture and incubated on ice for 15 min. To the swollen cells, 10% (W/V) Nonidet P-40 was added to a final concentration of 0.3%, vortexed vigorously for 10 s and then centrifuged for 5 min at 2500 *xg* at 4°C. After discarding the supernatant, the nuclear pellet was washed two more times with the same solution. The nuclear pellet was resuspended in 200 μl high salt nuclear extraction buffer (50 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 450 mM NaCl) containing the protease inhibitor mixture. After vortexing vigorously for 30 s, the pellets were collected at 20,000 *xg* for 30 min at 4°C. This nuclear protein was diluted to a final concentration of 1 mg/ml with an IP buffer (50 mM Tris-HCl, pH8.0, 1% NP-40, 150 mM NaCl, 1 mM EDTA) containing protease inhibitor mixture, then pre-cleared by incubating with non-specific IgG (2 μg/ml) and protein G-conjugated Sepharose 4B beads (5% of the total volume) (#101243, Invitrogen, Frederic, MD, USA) for 2 h at 4°C. For immunoprecipitation, 2 μg primary antibody was added in 1 mg pre-cleared total nuclear protein solution and incubated overnight at 4°C. Protein-antibody complex was then captured by adding protein G-conjugated Sepharose 4B beads (6% of the total volume) and rotating for 2 h at 4°C. After washing with the IP buffer for 5 times, the protein-antibody complex was eluted by boiling in Laemmli buffer (#1610737, Bio-Rad Laboratories, Hercules, CA, USA) and then processed for immunoblot analysis to assess co-IP.

For FLAG-tagged Atf4 pull-down analysis, expression of FLAG-Atf4 was induced by incubating FLAG-Atf4-293T cells with medium containing 0.1 μg/ml tetracycline hydrochloride (#T7660, Sigma, St. Louis, MO, USA) for 6 h. Nuclear protein extraction and dilution were performed as described above for the co-IP studies. The diluted nuclear protein was pre-cleared by incubating with mouse IgG-conjugated agarose beads (5% of the total volume) (#A0919, Sigma, St. Louis, MO, USA) and rotating for 2 h at 4°C. For immunoprecipitation and antibody–antigen complex capture, the pre-cleared nuclear protein solution was incubated with mouse anti-FLAG conjugated agarose beads (6% of the total volume) (#A2220, Sigma, St. Louis, MO, USA) and rotated overnight at 4°C. Washing the beads and the protein complex elution were performed as above described for co-IP. The protein complex was separated with a 10.5–14% Tris-HCl polyacrylamide gel (#345-9949, Bio-Rad Laboratories, Hercules, CA, USA) and after separation, electro-transferred to an Immun-Blot PVDF membrane (0.2 μm) (#162-0177, Bio-Rad Laboratories, Hercules, CA, USA). The membrane was stained with Fast Green to check for equal loading and then incubated with rotating in blocking solution consisting of 5% (w/v) Carnation non-fat dry milk in TBST (30 mM Tris-base pH 7.5, 200 mM NaCl and 0.1% (v/v) Tween-20) for 1 h at room temperature. The blots were washed 5 × 5 min in TBST and then incubated with goat anti-rabbit IgG (H+L)-HRP-conjugated secondary antibody (#170-6515, Bio-Rad Lab-

oratories, Hercules, CA, USA), goat anti-mouse IgG-HRP-conjugated secondary antibody (#sc-2005, Santa Cruz, CA) or HRP-conjugated protein A (#1706522, Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature. The blots were then washed for 5 × 5 min in TBST. The bound secondary antibody was detected using an enhanced chemiluminescence kit (#32106, Pierce/ThermoFisher Scientific, Waltham, MA, USA) and exposing the blot to Classic Blue Autoradiography film (#EBNY2, MIDSCI, St. Louis, MO, USA). The antibodies used were rabbit anti-Atf3 (#sc-188) and anti-cyclin T1 (#sc-10750) from Santa Cruz, rabbit anti-CDK9 (#ab6544) from Abcam, rabbit anti-FLAG (#F7425) from Sigma, rabbit anti-phospho-eIF2α (#9721s) and anti-total eIF2α (#9722s) from Cell Signaling, and mouse anti-Chop (#PK208449) from Pierce.

CARS and SARS promoter cloning, Site-directed mutagenesis and firefly luciferase assay

Human genomic DNA isolated from HepG2 cells was used as the template for cloning of promoter- and CARE-containing fragments of the *CARS* and *SARS* genes. A DNA fragment of 489 bp upstream and 1380 bp downstream of the annotated TSS of the human *SARS* gene (ENSG00000031698, Transcript ENST00000234677) was amplified and then cloned into the pGL3.1 luciferase reporter vector. Similarly, a DNA fragment of 518 bp upstream and 959 bp downstream of the human *CARS* TSS (ENSG00000110619, Transcript ENST00000397111) was cloned likewise. The Q5 site-directed mutagenesis kit (#E0554, New England Biolabs, Ipswich, MA, USA) was used for the deletion and single site mutation of the CARE element of *the CARS* (5′-GTTGCATCA-3′) and *SARS* (5′-TGATGCAAT-3′) genes. Primers for CARE deletion of the *CARS* gene are: Forward, 5′-GATTCTAGGAAGTGTCTGTAGCCGCAGC-3′ and Reverse, 5′-CGCCGCCCCGGAAGTCGC-3′. Primers for CARE mutation of *CAR*S gene are: Forward, 5′-ATTGGATTCTAGGAAGTGTCTGTAGCCGCAGC-3′ and Reverse, 5′-ACGGACGCCGCCCCGGAAGTCGC-3′. Primers for CARE deletion of the *SARS* gene are: Forward, 5′-AGGTCTGCAACCATCTGG-3′ and Reverse, 5′-GACAAATCAAATCACAAGTACTTG-3′. Primers for CARE mutation of *SAR*S gene are: Forward, 5′-TATTAGGTCTGCAACCATCTGG-3′ and Reverse, 5′-CATCTGACAAATCAAATCACAAGTACTTG-3′.
The deletion and mutation constructs were confirmed by DNA sequencing. For Firefly luciferase reporter assays, HepG2 cells were seeded on 24-well plates at a density of 0.05–0.1 × 10^6 cells per well 18–24 h before transfection. Cells were then transfected with plasmid DNA (0.5 μg per well), which was mixed with Superfect reagent (#301307, Qiagen, Valencia, CA, USA) at a ratio of 6 μl per μg plasmid DNA. At 24–36 h after transfection, cells were treated with 2 mM HisOH for 16 h. Whole cell extracts were then prepared by incubating cells with 200 μl of lysis buffer (#E367A, Promega, Madison, WI, USA) and shaking vigorously for 20 min. The cell lysates were stored at −80°C until luciferase assays were performed. The expression activities of the genomic fragments were then analyzed with a Luciferase Assay System (#E4530, Promega, Madison, WI, USA) using a Synergy 2 microplate reader (Biotek, Winooski, VT). All values were normalized to the amount of total protein per well.

RLM-5′-RACE analysis

To detect the transcription start site of the *Sars* gene, the FirstChoice RLM-5′-RACE kit (AM1700, Ambion/ThermoFisher Scientific, Waltham, MA, USA) was used. This approach uses RNA ligase-mediated rapid amplification of cDNA ends (RACE) to selectively amplify 5′-capped RNA. For each reaction, 5–10 μg of total RNA was used to generate 5′ adaptor-ligated mRNA as the template for synthesizing first strand cDNA with a random-primed reverse transcription reaction. *Sars* gene-specific cDNA molecules were amplified by nested PCR with primers corresponding to the DNA sequences located in Exon 2 and 3 of *Sars* gene and the manufacture's 5′ RACE primers corresponding to the 5′ adapter sequence. The sequences of the *Sars* gene-specific primers were: 5′ outer, 5′-TCGTCGTCTCCCACTGCTTCCTTT-3′ and 5′ inner, 5′-CTTCCTTTTTCTTCATTTTCTCCCCAA-3′. PCR products were cloned into pCRII-TOPO vector with the TOPO TA Cloning kit (K4520-01, Invitrogen/ThermoFisher Scientific, Waltham, MA, USA) and then sequenced.

Statistical analysis

Each experiment contained three or more individual samples to detect experimental variation, and each experiment was repeated one or more times with separate batches of cells to ensure reproducibility between experiments. The data are expressed as the averages ± standard deviations within an individual experiment and the results, analyzed using Student's *t*-test, with $P \leq 0.05$ were considered statistically significant.

RESULTS

AA responsive tRNA synthetase genes

A number of genome-wide screening analyses have indicated that at least a few of the ARS genes are regulated in an AA-dependent manner (7,29,30). To confirm and extend these reports, the mRNA content for each of the ARS genes, both cytoplasmic (Figure 1A) and mitochondrial (Figure 1B) localized proteins, was analyzed in MEFs following activation of the AAR by incubation in 5 mM HisOH for 8 h. The data reveal a striking contrast between the genes encoding cytoplasmic and mitochondrial localized enzymes in that 16 of the 20 genes encoding cytoplasmic proteins were transcriptionally increased by the AAR (Figure 1A), whereas only four genes encoding mitochondrial proteins were significantly enhanced and only by 2-fold or less (Figure 1B). One of the four was *Gars*, which encodes both the cytoplasmic and mitochondrial enzymes. For the reminder of the studies, only the cytoplasmic localized genes were investigated.

Depending on the cell type, the AAR can be comprised of several signaling pathways, but the one that appears to

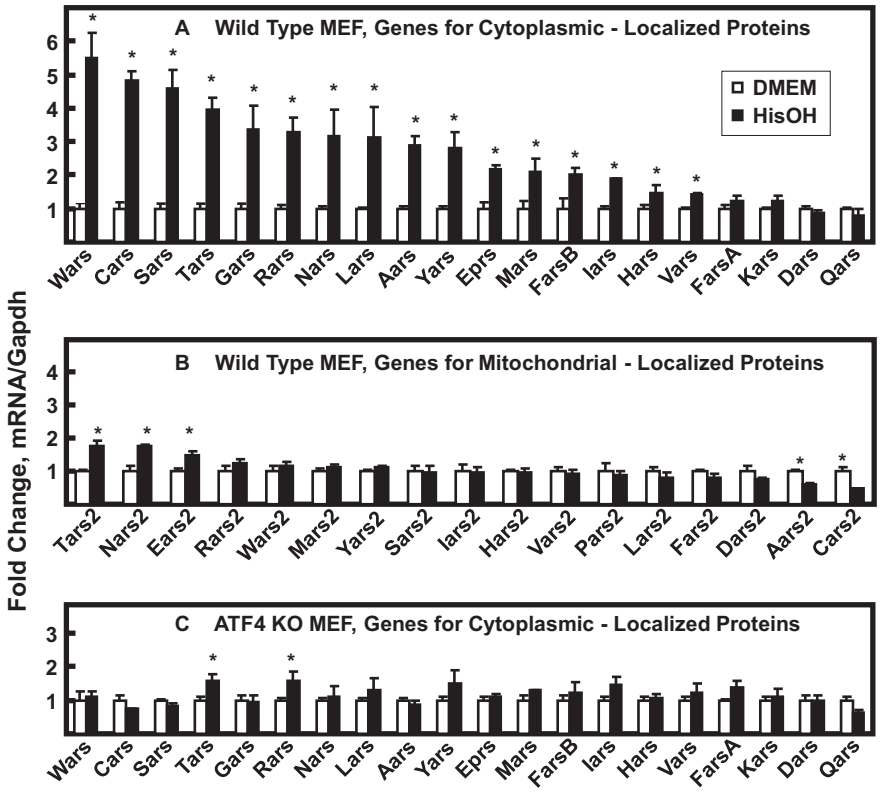

Figure 1. Differential induction of ARS genes in response to AA deprivation. Mouse embryonic fibroblasts (MEFs) were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. Total RNA was isolated and the steady state mRNA of genes encoding cytoplasmic (Panel A) or mitochondrial (Panel B) localized ARS proteins were analyzed. The data were plotted as the ratio of ARS/Gapdh mRNA and normalized relative to the DMEM value to yield the fold induction. The results are shown as the averages ± standard deviations of three individual samples per experiment and are representative of multiple independent experiments. An asterisk (*) indicates a statistically significant change at $P \leq 0.05$. (Panel C) MEFs from Atf4 knock-out (Atf4 KO) mice were treated as described above and then mRNA of genes encoding cytoplasmic localized ARS proteins were analyzed. The primer sequences used for RT-qPCR are listed in Supplementary Table S1.

be universal across all tissues and species, is the Gcn2-eIF2-Atf4 cascade (1,4,31). To determine if the induction of the ARS genes was Atf4-dependent, their AA sensitivity was monitored in Atf4-wild type (Figure 1A) and Atf4-knockout MEFs (Figure 1C). In the cells lacking Atf4 activity, the induction of each of the regulated genes for the cytoplasmic proteins was strongly reduced with most showing little or no increase. To establish that increased transcription contributed to this increase in ARS mRNA, analysis of the short-lived hnRNA was performed for two representative genes, *Cars* and *Sars* (32). A time course of induction showed that for both genes, the abundance of hnRNA increased during the initial 4 h after activation of the AAR and then declined, returning to the basal level by 24 h (Figure 2). These results show that a significant component of the increased mRNA for the ARS genes is due to transcriptional mechanisms.

Contribution of the transcription factor Chop

Genome-wide screening indicated that some of the ARS genes are controlled by the combined action of Atf4 and C/ebpβ homology protein (Chop) (6). To establish the breadth of this mechanism, each of the ARS genes were analyzed in Chop-wild type and Chop-deficient MEF cells. The basal expression of several genes was suppressed in the absence of Chop, but the degree of decrease varied widely (Figure 3). After AAR induction of expression, the greatest reduction of the induced activity was observed for *Sars, Aars, Eprs, Mars, Iars* and *Vars*. Although most of the AA-responsive genes exhibited some degree of Chop-dependence, the difference in relative contribution between Atf4 and Chop was striking. At this time, we cannot rule out the possibility that another transcription factor can partially substitute for Chop in the knockout MEF cells.

Association of Atf4 and Chop at CARE enhancer sites

To study the molecular role for Atf4 and Chop, ChIP analysis was performed to determine association with each of the regulated genes (Figure 4). For each gene, the genomic sequence 3 kb upstream and downstream from the TSS was analyzed *in silico* for Atf4-responsive CARE (Supplementary Table S2). In most cases, only one likely CARE sequence (5′-TGATGxAAx-3′) was identified, but if there were multiple potential sites each was checked for Atf4 binding. With the exception of *Rars,* each of the Atf4-dependent genes exhibited significant Atf4 association at the identified CARE site (Figure 4). These data show that Atf4-dependence is associated with factor binding at the gene locus. For the *Rars* gene only one potential CARE site was present within the scanned 6 kb genomic region (Sup-

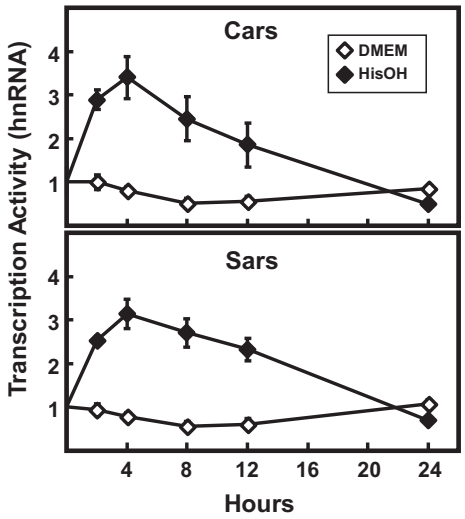

Figure 2. Transcription activities of the *Cars* and *Sars* genes in response to AA deprivation. MEFs were incubated in DMEM or DMEM + 5 mM HisOH to activate the AAR for the period of time indicated. The hnRNA abundance, which represents an indirect measure of transcription activity, was analyzed with primers spanning an exon-intron or intron-exon junction. The data were plotted as the fold induction using the ratio of ARS/Gapdh mRNA and normalized relative to the DMEM value of time zero. The results are shown as the averages ± standard deviations of three individual samples per experiment and are representative of multiple independent experiments. The primer sequences used for RT-qPCR are listed in Supplementary Table S1.

plementary Figure S2). When this site did not show significant AA-responsive Atf4 binding, two more distal CARE sequences were also tested, both of which yielded negative results as well. There may be an even more distal CARE that regulates the *Rars* gene, but it is interesting to note that *Rars* was only one of two genes that exhibited significant Atf4-independent induction (Figure 1C). ChIP analysis was also performed to determine if Chop was recruited to the Atf4-binding ARS CARE sites in an AA-responsive manner (Figure 4). The results revealed that, in general, the genes that exhibited the strongest Chop-dependence (Figure 3) were among those with the greatest degree of Chop recruitment. Negative controls included a non-specific IgG antibody and PCR of a region known not have a CARE sequence (negative control example data are shown for two genes in Figure 5).

To investigate the molecular mechanism of Atf4- and Chop-dependent control of the ARS genes, two genes were chosen for further study based on three criteria. *Cars* was chosen as a representative gene that showed: (i) Chop independence, (ii) little or no AAR-inducible Chop recruitment; and (iii) a functional CARE site near the TSS. Conversely, *Sars* was chosen as an example gene for which: (i) the AAR induction is at least partially dependent on Chop, (ii) Chop was recruited to the CARE in response to the AAR and (iii) the functional CARE site is downstream of the TSS by at least 500 bp (Supplementary Table S2). ChIP analysis for these two genes was used to analyze the effect of the AAR on association of Atf4, Chop, RNA polymerase II (Pol II), and TATA binding protein (TBP). For *Cars*, activation of the AAR led to enhanced Atf4 binding, but minimal Chop association with the CARE site (Figure 5A), consistent with the lack of Chop-dependence (Figure 3). Binding of both factors was negligible when a non-CARE containing region was screened and these results, along with non-specific IgG binding, serve as negative controls. The association of Pol II near the *Cars* TSS was readily detectable in cells incubated in DMEM, but the amount was not altered after activation of the AAR (Figure 5A). The Pol II enrichment near the promoter region in the basal condition with no further induction during the AAR appears to be common to almost all of the ARS genes analyzed (Supplementary Figure S3). This result is interesting because previously identified AAR-induced genes exhibit increased recruitment of Pol II to the TSS region, as illustrated in Supplementary Figure S4 for *Asns* and *Atf3*. The functional CARE site for *Sars* is located in Intron 1, about 1 kb downstream from the TSS (Supplementary Table S2). Atf4 association was increased at the *Sars* CARE site after activation of the AAR, with little or no increased binding at either the TSS or a distal downstream site chosen to serve as a negative control (Figure 5B).

Activation of *Sars* showed partial dependence on Chop activity (Figure 3) and consistent with those results, Chop recruitment to the intronic CARE site was enhanced in response to the AAR (Figure 5B). Similar results were obtained with other genes having a TSS-localized CARE (*Nars*) or a downstream CARE (*Wars*) (data not shown). Although these four examples might suggest that increased Chop recruitment only occurs at downstream CARE sequences, other genes, such as *Aars* and *Iars*, contain CARE sites near the TSS and yet exhibit enhanced Chop recruitment in response to the AAR (Figure 4). As with *Cars* and *Nars*, the AAR did not lead to a large increase in RNA Pol II recruitment to the TSS region of *Sars* or *Wars*, but in striking contrast, an increased Pol II association at the CARE region was observed (Figure 5B). Similarly, TBP association at the distal *Sars* CARE was also increased by AAR. These data are consistent with the current view that enhancer elements recruit transcription pre-initiation complexes (PIC) and function much like promoters (33). Previously, CARE sequences for other AA-responsive genes have been documented to exhibit all of the properties that define an enhancer element (34). Given the similarity between this intronic CARE element and the gene promoter in terms of Pol II and general transcription machinery assembly, it could be proposed that this CARE element may function as an alternative promoter. To test this possibility, we did 5′ RNA Ligase Mediated Rapid Amplification of cDNA Ends (5′ RLM-RACE) analysis, which selectively detects the 5′ end of the 7mG-capped mature mRNA. PCR with a gene-specific 3′ primer showed discreet bands at about 300 bp, a size consistent with a predicted size of mRNA transcribed from the annotated canonical TSS. Further sequencing of independent clones confirmed this result (Figure 6). Therefore, the mRNA encoding for Sars protein is transcribed from the canonical TSS, not the CARE. However, we can't exclude the possibility of local transcription at the CARE region including the production of an enhancer RNA (eRNA), and its function in regulating transcription from the proximal promoter. Indeed, preliminary experi-

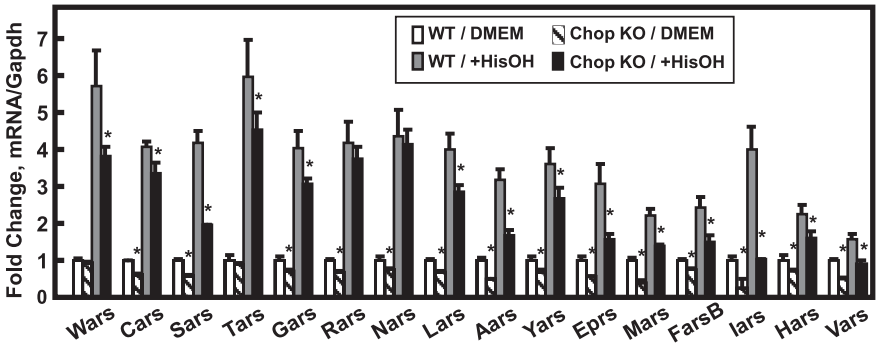

Figure 3. Effect of Chop on the expression of ARS genes. Wild type (WT) and Chop knock out (Chop KO) MEFs were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. Total RNA was isolated, and the steady state mRNA of genes encoding cytoplasmic localized ARS proteins were analyzed. The data were plotted as the fold induction using the ratio of ARS/Gapdh mRNA and normalized relative to the DMEM value of WT MEF. The results are shown as the averages ± standard deviations of three individual samples per experiment and are representative of multiple independent experiments. An asterisk (*) indicates a statistically significant change at $P \leq 0.05$. The primer sequences used for RT-qPCR are listed in Supplementary Table S1.

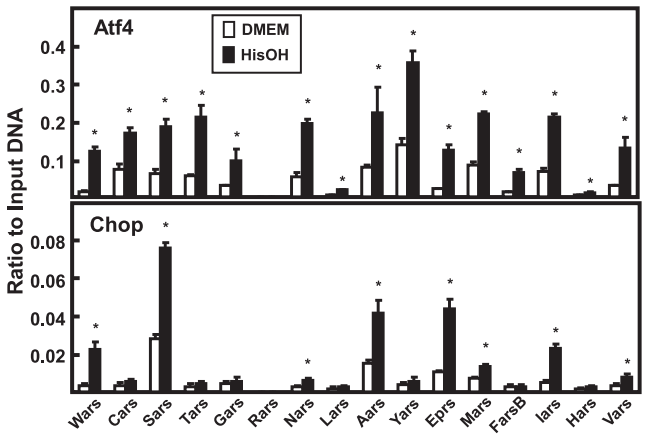

Figure 4. Enrichment of Atf4 and Chop at CARE sites of ARS genes. MEFs were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. The cells were subjected to ChIP analysis with antibodies specific for Atf4 or Chop. The data are plotted as the ratio to input DNA and are the averages ± standard deviations for at least three samples. An asterisk (*) denotes a significant difference of $P \leq 0.05$ relative to the corresponding DMEM control value for that particular primer set. The primer sequences are listed in Supplementary Table S1.

ments suggest this possibility, as described in the Discussion section.

Mechanism of Atf4 action

To determine the role of Atf4 and Chop on the recruitment of Pol II and the PIC, ChIP analysis was performed on the *Cars* and *Sars* genes in wild type (WT) and Atf4-deficient MEFs (Figure 5). For both *Cars* and *Sars*, the association at the TSS of Pol II and a second member of the PIC, TBP, was significantly decreased by about 3- to 4-fold in the absence of Atf4. This result documents the importance of Atf4 in the establishment and initiation of transcription machinery assembly at the TSS for both genes. Surprisingly, regardless of the increased recruitment of Atf4 in WT cells, the enrichment of Pol II and TBP at the TSS of both genes was not increased further by the AAR. Thus, for these ARS genes, in contrast to *Atf3* and *Asns* (Supplementary Figure S4), AAR-induced Atf4 plays a distinct role other than increasing Pol II recruitment and transcription machinery assembly at TSS (Figure 5). However, for the *Sars* gene, the AAR significantly enhanced the recruitment of Chop, Pol II and TBP at the distal intronic CARE, but the absence of Atf4 completely abolished the recruitment of these factors (Figure 5B). These results demonstrate the requirement for Atf4 in Pol II recruitment and the transcription machinery assembly at the distal CARE enhancer. Consequently, there are two important observations for Atf4-mediated transcriptional control of AA-responsive ARS genes. (i) Regardless of the location of the CARE, Atf4-mediated transcriptional up-regulation of gene expression does not require an increase in Pol II recruitment and PIC assembly at the proximal promoter. (ii) For those genes that have a distal enhancer, a key function of Atf4 is to facilitate the increased recruitment of Pol II and the PIC formation at the CARE. The precise function of this Pol II/PIC assembly remains unknown at present. For both sets of ARS genes, Atf4 appears to function differently than for a third class of AA-responsive genes represented by *Asns* and *Atf3* that have a CARE immediately upstream of the TSS and an Atf4-dependent, AAR-enhanced recruitment of Pol II and the PIC to the proximal promoter region (Supplementary Figure S4).

To determine if Chop contributed to the association or AA-dependent recruitment of Atf4 or Pol II to the *Cars* and *Sars* genes, ChIP analysis was performed in Chop knockout MEF cells (Figure 5). Consistent with no binding of Chop to the *Cars* gene, Atf4 and Pol II association was unaffected by the absence of Chop (Figure 5A). Interestingly, for the *Sars* gene, which does exhibit AAR-induced Chop association at the CARE, the enhanced recruitment of Atf4 and Pol II at the CARE and the constitutive binding of Pol II at the TSS was also unaffected by the absence of Chop (Figure 5B). Collectively, the results indicate that AA-responsive Atf4 recruitment to the *Sars* gene is critical for subsequent recruitment of Chop and the PIC complex to the intronic CARE, but that despite PIC assembly at this location, mRNA-producing transcription starts from the canonical TSS.

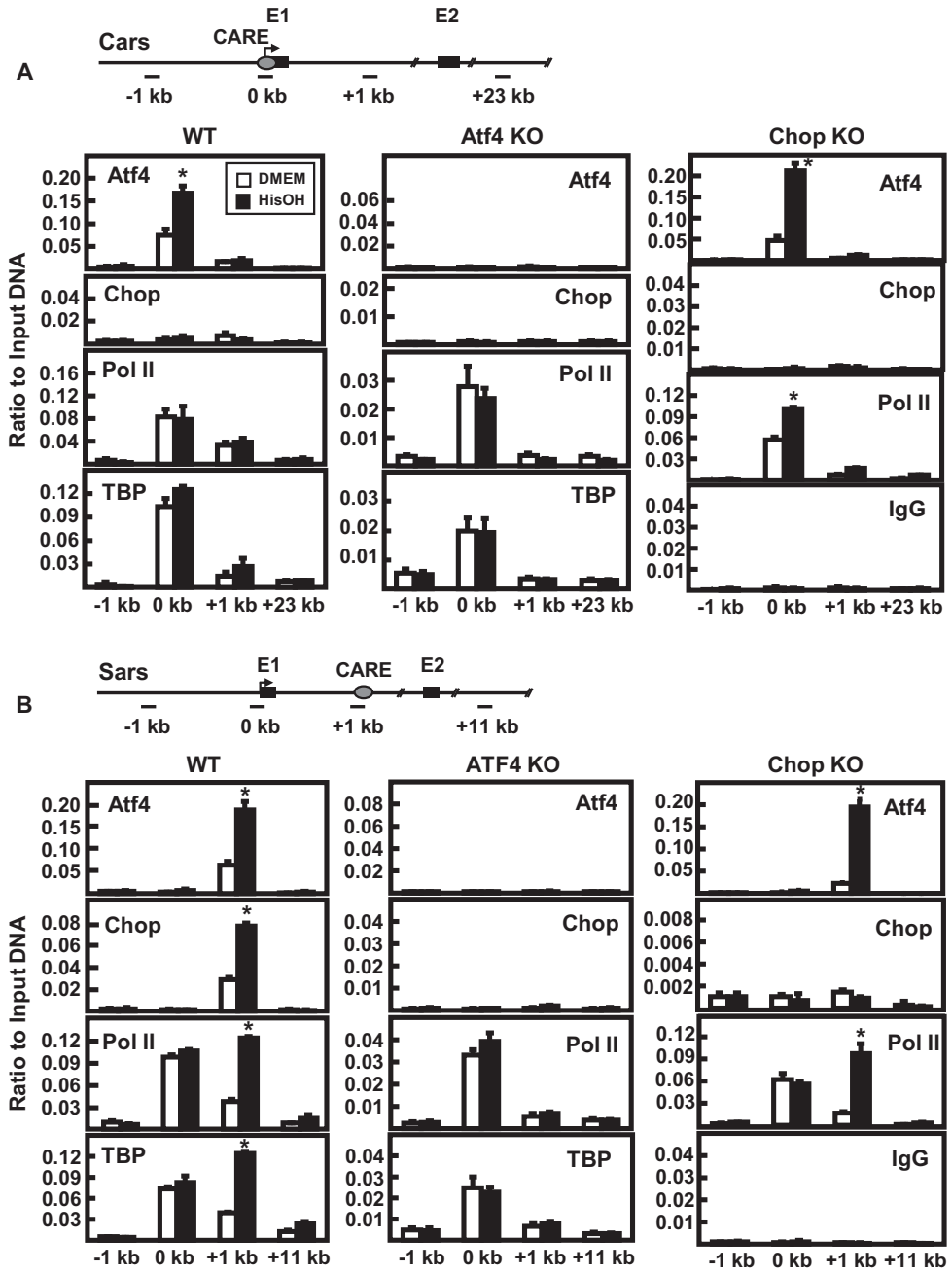

Figure 5. Enrichment of Atf4, Chop, Pol II and TBP within the *Cars* and *Sars* genes. MEFs from WT, Atf4 KO or Chop KO mice were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. The cells were subjected to ChIP analysis with antibodies specific for Atf4, Chop, the subunit 1 of Pol II (RPB1) or the TATA binding protein (TBP). Immunoprecipitation with a non-specific goat-anti rabbit antibody (IgG) and PCR at a distal site (+23 or + 11 kb) were used as negative controls. The data are plotted as the ratio to input DNA and are the averages ± standard deviations for at least three samples per experiment. An asterisk (*) denotes a significant difference of $P \leq 0.05$ relative to the corresponding DMEM control value for that particular primer set. The arrows denote the annotated TSS of each gene, the boxes represent exons 1 and 2, and the grey ovals represent the approximate locations of the CARE sequence. The primer sequences used for RT-qPCR are listed in Supplementary Table S1.

Functional activity of the CARE sequences

To determine if the *Cars* and *Sars* CARE sequences exhibit functional activity, DNA fragments of both human genes were cloned in front of a luciferase reporter and transfected into HepG2 cells (Figure 7). Fragments that contained the proximal promoter region and the WT CARE sequence for either gene showed basal transcription activity that was induced 3- to 5-fold in response to AAR activation. For both genes, deletion of the CARE resulted in a reduction of basal transcription activity as well as a complete inhibition of the induction by the AAR (Figure 7). Mutation of several nucleotides within each CARE also caused a loss of inducibility with no effect on basal transcription activity (Figure 7). These functional data show that the CARE sequences me-

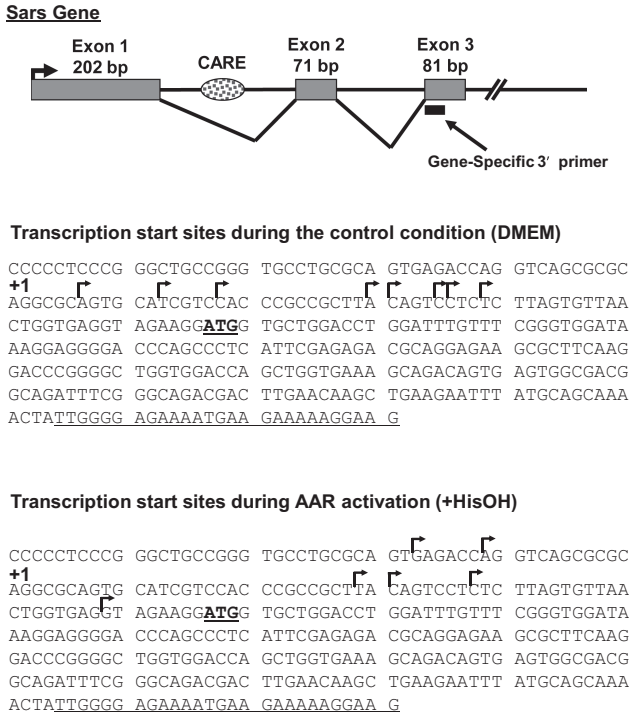

Figure 6. Transcription of *SARS* mRNA starts at the annotated proximal promoter. Human hepatocellular carcinoma (HepG2) cells were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. The total RNA was subjected to RLM-5′ RACE analysis, as described in the Materials and Methods section. PCR products amplified with a 5′ RACE primer and a gene-specific primer were cloned into the pCRII-TOPO TA vector and the resulting clones were randomly selected for sequencing analysis. The top panel is the SARS gene structure; the grey boxes are the first three exons and the dotted oval is the approximate location of the CARE element. The arrow indicates the annotated transcription start site. The gene-specific primer used for PCR is labeled with a thick line under Exon 3. In the lower panels showing sequence, the annotated transcription start site is indicated with a '+1' and the bold ATG represents the translation start codon. The underlined sequence is the location of the gene-specific primer. The multiple arrows inside the sequences are the actual transcription start sites established by the analysis of the clones obtained by the RLM-5′ RACE experiments.

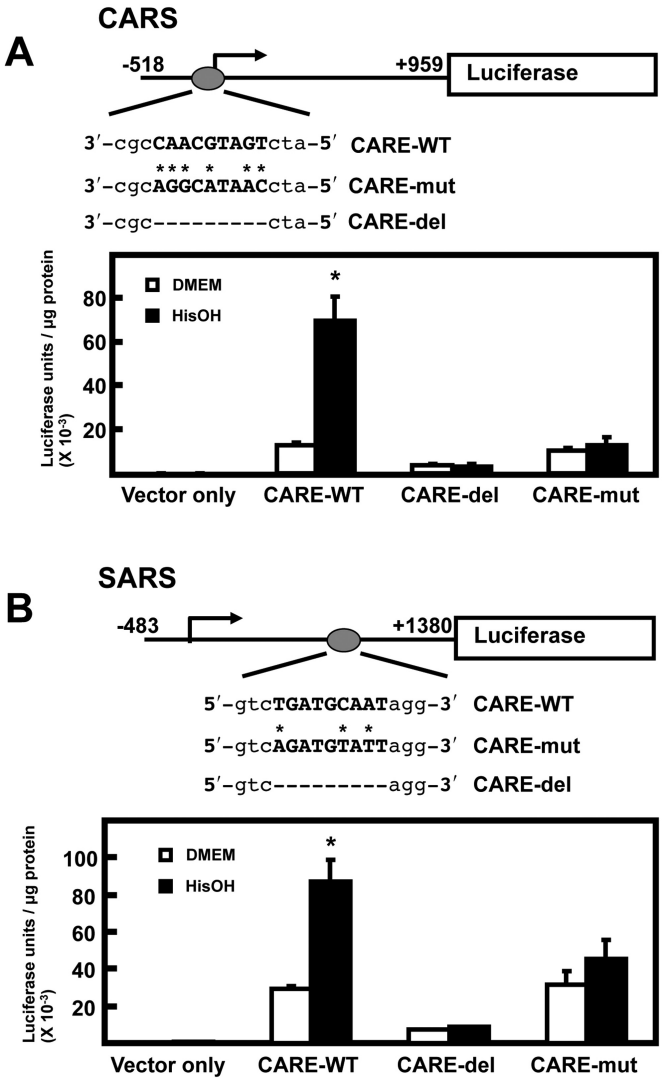

Figure 7. Functional assay of the CARE-containing enhancer regions of the *CARS* and *SARS* genes. DNA fragments of the human (**A**) *CARS* (nt −518 to +959) and (**B**) *SARS* (nt −483 to +1380) genes were cloned in front of a luciferase reporter. As indicated, CARE deletion (del) or mutagenized (mut) constructs were also tested. The plasmid DNA was transfected into HepG2 cells, which were cultured for 16 h in DMEM or DMEM + 2 mM HisOH to activate the AAR. The luciferase intensities were normalized to the total protein per individual sample and are the averages ± standard deviations for at least three samples per experiment. An asterisk (*) denotes a significant difference of $P \leq 0.05$ relative to the corresponding DMEM control value.

diate the ability of these genes to respond to the AAR, regardless of location within the gene structure.

AAR activation alters association of elongation factors

In mammalian cells, Pol II pausing is induced and stabilized by NELF and DSIF complexes. When subunits within these two complexes are phosphorylated, NELF is evicted from the transcription machinery, but DSIF is converted to a positive factor leading to release of paused Pol II and increased elongation (21,24). Mammalian NELF complex consists of NELF/A-E subunits, and DSIF contains subunits Spt4 and Spt5 (24). NELF and DSIF are regulated by the positive transcription elongation factor b (P-TEFb) protein complex, which facilitates the transition of paused Pol II into the elongating form. P-TEFb consists of cyclin T1 and cyclin-dependent protein kinase 9 (CDK9), the latter of which phosphorylates the subunits of NELF and DSIF (21,23). An inhibitor of CDK9, flavopiridol (35), totally blocked the AAR-driven induction of both *Sars* and *Cars* (Figure 8A), indicating that CDK9 is involved in AAR-induced transcriptional up-regulation. Pull-down assays in HEK293T cells that stably express a tetracycline-inducible FLAG-tagged Atf4 were used to test for interaction between Atf4 and the P-TEFb components cyclin T1 and CDK9 (Figure 8B). The results show that both proteins are present in Atf4-containing complexes and the abundance of these complexes is increased after induction of Atf4 expression.

To determine if Atf4 modulates these elongation-controlling complexes, we measured the association of

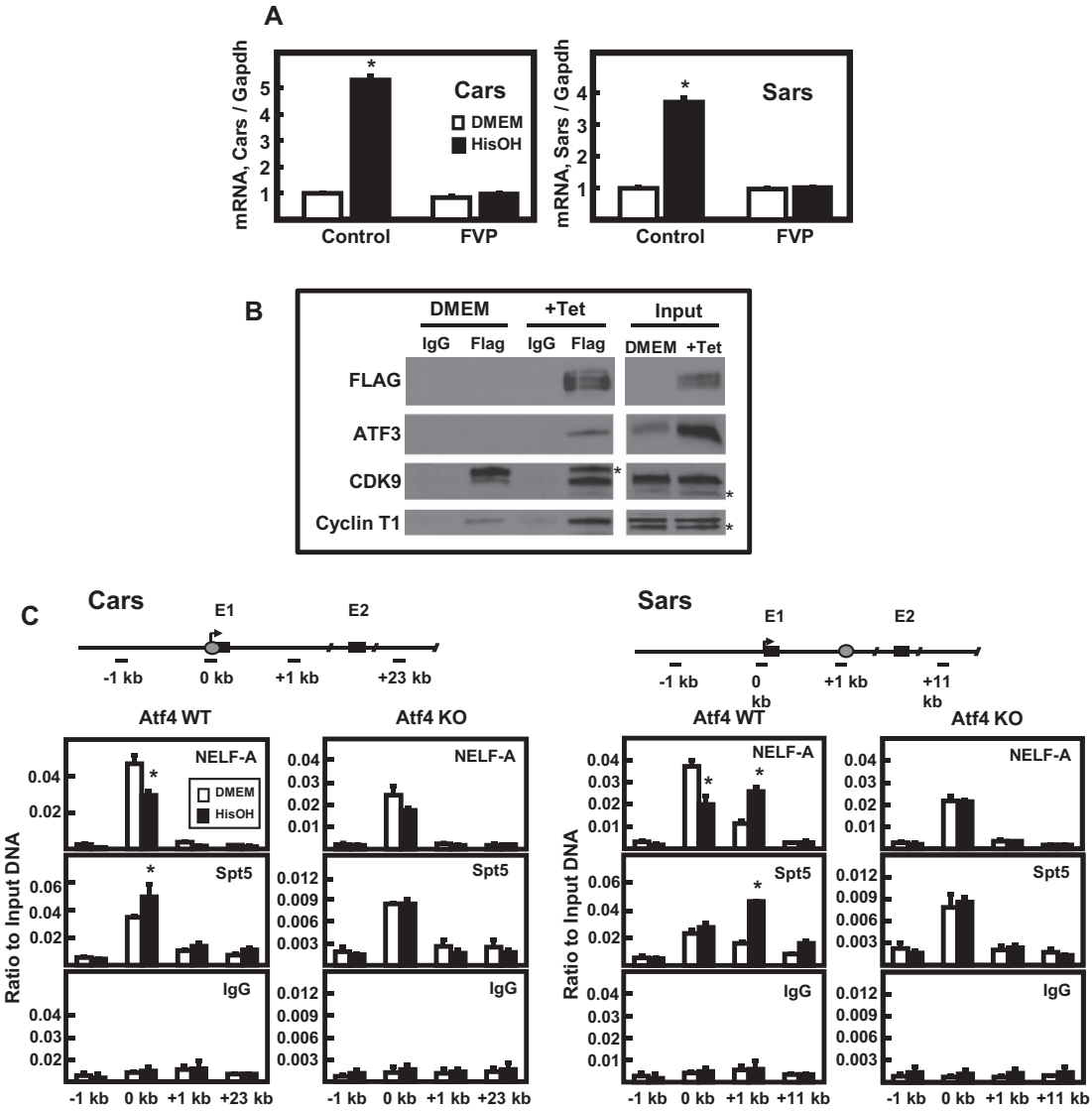

Figure 8. ATF4 modulates association of positive and negative elongation factors within AA-responsive ARS genes. (**A**) Inhibition of CDK9 abolishes the induction of *CARS* and *SARS* during the AAR in HepG2 cells. HepG2 cells were pre-treated with or without flavopiridol (FVP) for 30 min, then incubated for 8 h in DMEM or DMEM + 5 mM HisOH, with (FVP) or without FVP (control). The steady state mRNA content for *CARS* and *SARS* was analyzed and the data plotted as the ratio to *GAPDH*. The results shown are the fold induction normalized relative to the DMEM value of control samples and presented as the averages ± standard deviations of three individual samples per experiment. The data are representative of multiple independent experiments. An asterisk (*) indicates a statistically significant change at $P \leq 0.05$. (**B**) ATF4 interacts with both CDK9 and cyclin T1. FLAG-tagged ATF4 was expressed in HEK293T cells that bear a tetracycline-inducible construct encoding FLAG-ATF4 by incubating the cells in DMEM containing 0.1 μg/ml tetracycline for 6 h. Nuclear protein was isolated and subjected to immunoprecipitation with an antibody specific for the FLAG epitope. The immunoprecipitated protein was subjected to immunoblot analysis with antibodies against CDK9 or cyclinT1. Probing with anti-Atf3, a known Atf4 interacting protein, was used as a positive control. (**C**) ATF4 modulates changes in the gene association of NELF-A, and Spt5 during the AAR in MEFs. MEFs from WT or Atf4 KO mice were incubated for 8 h in DMEM or DMEM + 5 mM HisOH to activate the AAR. The *Cars* and *Sars* genes were subjected to ChIP analysis with antibodies specific for NELF-A or Spt5. Immunoprecipitation with a non-specific goat-anti rabbit antibody (IgG) or PCR at a distal site (+23 or +11 kb) were used as negative controls. The data are plotted as the ratio to input DNA and are the averages ± standard deviations for at least three samples per experiment. An asterisk (*) denotes a statistically significant difference of $P \leq 0.05$ relative to the corresponding DMEM control value for that particular primer set. The arrows denote the annotated TSS of each gene and the boxes represent exons 1 and 2. The grey ovals represent the approximate location of CARE sites. The primer sequences used for RT-qPCR are listed in Supplementary Table S1.

NELF-A and Spt5 at the *Cars* or *Sars* promoter and CARE element during AAR activation. For the *Cars* gene, the data show that after AAR activation NELF-A declines and Spt5 increases in abundance near the TSS (Figure 8C). Both of these changes were suppressed in Atf4-deficient cells, suggesting that these transcription factors are necessary for the transition to elongating Pol II. For the *Sars* gene, the expected Atf4-dependent eviction of NELF-A from the TSS region was observed. However, an Atf4-dependent increase in both NELF-A and Spt5 occurred at the intronic CARE (Figure 8C). The elevated association of the Spt5 at this downstream region of the gene is consistent with its proposed role in elongation, whereas the functional role of the increased association of NELF-A near the CARE is not as obvious. As mentioned above, it has been proposed that eRNAs produced at distal enhancers may serve as a decoy by directly binding NELF and thereby, promoting NELF eviction from the promoter-localized PIC (21,36). One possibility is that an eRNA generated from the *Sars* CARE functions in this way. Alternatively, NELF, which can bind directly to Pol II (20), may be associated with the PIC complex that is recruited to the CARE and serve as a feedback mechanism to suppress further transcription from the CARE. More extensive investigation will be required to establish this mechanism. None-the-less, the results indicate that at least one of the mechanisms by which Atf4 induces the ARS genes is by controlling the NELF and DSIF complexes leading to increased transcriptional elongation through the release of paused Pol II. This is the first demonstration of this mechanism for AA-responsive genes.

DISCUSSION

The data presented in this report illustrate the following novel observations. (i) A survey of the entire set of mammalian ARS genes reveals that most of those encoding cytoplasmic localized enzymes are activated by the AAR pathway in response to AA limitation, whereas nearly all of those encoding mitochondrial localized enzymes are not. (ii) There are isolated reports of Atf4-dependent regulation of a few individual ARS genes following insulin treatment (37) or activation of the UPR pathway (25,38,39). The complete gene family screening in the present work illustrates that the induction of all AAR-responsive ARS genes show strong or complete Atf4-dependence. (iii) The induction of some, but not all, AAR-responsive ARS genes depends partially on the transcription factor Chop. (iv) Interestingly, almost all AAR-responsive ARS gene are highly enriched with Pol II and the PIC at their promoters even under basal conditions. However, the AAR-induced, Atf4-mediated transcriptional induction of the ARS genes does not result from a further increase in Pol II and PIC assembly at the promoter. (v) Most AAR-responsive ARS genes have a functional CARE enhancer that is near the TSS, but a few have the CARE enhancer located at least 500 bp downstream from the TSS. (vi) Regardless of the CARE location, increased Atf4 recruitment initiates a mechanism that increases transcriptional elongation controlled by NELF and DSIF, maybe Atf4 recruitment of P-TEFb. (vii) For those ARS genes that have a distal CARE element, Atf4, Chop and the general transcription factors, including Pol II, exhibited AAR-induced, Atf4-dependent recruitment to the downstream CARE rather than to the promoter region. (viii) This distal CARE sequence, as exemplified by the *Sars* CARE, functions as an enhancer. Despite the assembly of the general transcription machinery at this distal enhancer, transcription of the mRNA encoding for the ARS enzyme still begins from the annotated TSS. Collectively, these results document that the ARS genes are coordinately regulated by AA availability in an Atf4-dependent manner and that one of the functions of Atf4 for this group of genes is to regulate transcription through the association of elongation factors.

RNA Pol II was highly enriched at the promoters of almost all ARS genes and was not further enhanced by AA limitation, even for those genes for which there was significant up-regulation of transcription. The present and published data illustrate that there are at least three different mechanisms associated with Atf4-dependent gene activation. The first of these mechanisms is illustrated by genes such as *Asns* and *Atf3* in which there is a CARE sequence located within the proximal promoter. For these genes, the AAR results in enhanced Atf4 recruitment to the CARE and then enhanced PIC recruitment to the TSS region in an Atf4-dependent manner (18,19). The second and third mechanisms are illustrated by the two sets of ARS genes described in this report. One set having the CARE at the TSS (e.g. *Cars*). For these ARS genes, Atf4 binding is increased in response to the AAR, whereas the level of Pol II and TBP association is constitutive. In contrast, the third mechanism is revealed by those AAR-induced ARS genes (e.g. *Sars*) that possess CARE elements downstream of the TSS. In the case of those genes, Atf4, Chop, TBP and Pol II recruitment to the CARE region is enhanced by the AAR, whereas the Pol II and TBP association at the TSS is constitutive. The enrichment of TBP, the major component of the transcription factor TFIID, indicates increased assembly of the general transcripiton machinery at this distal enhancer. Furthermore, knockout of Atf4 completely abolished the recruitment of Chop, Pol II and TBP, demonstrating the mechanistic function of Atf4 in the process of recruiting and assembly of co-activators and the general transcripiton machinery. The role of Chop remains unclear. That some ARS genes require Chop, whereas others do not, may reflect an evolutionary change caught midstream. Further analysis of Chop function will be required to fully understand its mechanistic role. Despite the assembly of the PIC at the distal enhancer, a 5′-RACE study demonstrated that *Sars* mRNAs have capped 5′ ends aligned with the annotated TSS only, indicating that mRNA production from the *Sars* gene does not start from the downstream enhancer.

Genome-wide studies have demonstrated that promoter proximal Pol II pausing is common and that increased transcription from these genes results from release of the paused Pol II and increased elongation (40,41). It is proposed that promoter paused Pol II favors a synchronized response to environmental stimuli (42). Two protein complexes, NELF and DSIF, act to control Pol II pausing by directly interacting with transcribing Pol II (41). Our ChIP assays demonstrated that the NELF-A subunit and the Spt5 DSIF subunit are enriched at the promoter region of the *Cars* and *Sars* genes. The AAR significantly reduced the enrichment

of NELF-A, but not Spt5, suggesting AA limitation induces paused Pol II release. P-TEFb, consisting of CDK9 kinase and cyclin T1, promotes elongation by phosphorylating NELF and DSIF subunits. Upon phosphorylation, NELF is released from the Pol II complex, whereas phosphorylated DSIF stays associated with Pol II and functions as a positive elongation factor (21,40). The CDK9 inhibitor flavopiridol completely blocked the induction of both *Cars* and *Sars*, while the basal expression was barely affected, illustrating a critical role for P-TEFb in the transcriptional induction of these genes. P-TEFb can be recruited by DNA-bound transcription factors (43–45) and knockout of Atf4 abolished the reduction of the NELF-A enrichment at the promoters of both *Cars* and *Sars* genes, indicating that one of the functions of Atf4 is the recruitment of P-TEFb. Indeed, FLAG-tagged Atf4 associated with a protein complex containing both CDK9 and CyclinT1. Future studies are needed to further detail Pol II release mechanisms.

Several models have been proposed for how distal enhancers increase transcription (46,47). In general, enhancers regulate transcription either by increasing the recruitment of the general transcription machinery to the promoter or by facilitating paused Pol II release to promote increased elongation (48). The enrichment of Pol II and TBP at the *Sars* promoter was not significantly increased by the AAR despite their increase at the distal enhancer, suggesting that it is unlikely that the CARE complex upregulates transcription by increasing the general transcription machinery abundance at the promoter. In contrast, the enrichment of NELF-A at the promoter was significantly decreased during the AAR and this decrease was completely attenuated by Atf4 knockout, indicating that Atf4-mediated molecular events occur at the distal enhancer that control release of the promoter-associated NELF complex and thereby, Pol II release and increased elongation.

The AAR-induced recruitment of Pol II and the general transcription machinery may reflect local transcription that generates a non-mRNA species of RNA, such as an eRNA. Accumulating data have shown that most enhancers, if not all, can transcribe the DNA template bidirectionally from sites to which the specific transcription factors bind and trigger assembly of the general transcription machinery to produce eRNA (33,49). Although the exact mechanisms by which eRNAs act to regulate transcription from the promoter are still under debate, an increasing number of studies have illustrated their ability to do so (50). While in some cases eRNA has been proposed to facilitate the formation or stabilization of an enhancer-promoter loop (51–53), other studies have suggested that eRNAs promote paused Pol II release and productive elongation by acting as a decoy to directly bind NELF and thus, remove this negative elongation factor from the promoter (36,54). Interestingly, preliminary experiments show that a luciferase reporter construct containing the CARE enhancer region (nt +747/+1380) of the *Sars* gene exhibits transcription activity, albeit at significantly lower rates than constructs containing the proximal promoter. The AAR-induced induction of transcription from these CARE-only constructs is almost the same as constructs containing both the promoter and enhancer (data not shown). Furthermore, in response to the AAR, the Atf4-dependent abundance of H3K4me3, an indicator of active transcription (33), is increased at both the promoter and the CARE distal enhancer region of *Sars* (Supplementary Figure S5). If confirmed, these results suggest that Atf4-mediated Pol II and PIC assembly at the distal CARE lead to local transcription. Additonal experimentation will be required to test the hypothesis that there is production of a functional eRNA that regulates mRNA transcripiton from the promoter.

It may first appear to be a paradox as to why tRNA synthetase genes would be induced during a cellular stress such as AA limitation or ER stress, in which protein synthesis is expected to be decreased. As we have documented for ER stress (6), initially after the onset of a cellular stress, eIF2α is phosphorylated which does indeed cause a transient suppression of protein synthesis. Concurrently, there is an increase in transcription and translation of Atf4 and Chop that subsequently serves to restore protein synthesis for either recovery or apoptosis. One aspect of cellular stress that is not well understood is the role of the specific compartments within the cell. The ARS genes that encode the mitochondrial enzymes were largely unaffected by the AAR, despite the fact that they are encoded by nuclear genes. To our knowledge, there have been no published reports describing the impact of AA limitation on mitochondrial protein synthesis. Further analysis will be required to determine if the genes for the mitochondrial enzymes respond to other cellular stresses. For the cytoplasmic enzymes, there appears to be no obvious correlation between the AA-responsive ARS genes and: (i) essential versus non-essential AA; (ii) Class 1 or Class 2 ARS protein structure; (iii) members of the multisynthetase complex; or (iv) ARS proteins that are known to have additional, non-canonical activities (9,10,13). Related to gene specificity, there are additional questions raised by our results. (i) Why are there a few ARS genes that are not subject to AA control? (ii) Given the likelihood that coordination of ARS expression is required to maintain protein synthesis balance, why is there such a disparity in the degree of induction among those genes that are subject to AA control? These and many other questions provide interesting opportunities for future investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: J.Shan, F.Z., T.A.T. and J.Sharkley conducted the experiments. J.Shan analyzed the data, prepared the figures and tables, and wrote most of the manuscript. M.S.K. devised the study, analyzed the data, planned new experimental avenues, prepared the figures and tables, and co-wrote the manuscript with J.Shan.

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Conflict of interest statement. The authors declare that they have no conflicts of interest with the contents of this article.

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