JunD, a member of the Jun family of nuclear transcription proteins, dimerizes with Fos family members or other Jun proteins (c-Jun or JunB) to form the activator protein 1 (AP-1) transcription factor. The junD gene contains no introns and generates a single mRNA. Here we show that two predominant JunD isoforms are generated by alternative initiation of translation, a 39-kDa full-length JunD protein (JunD-FL) by initiation at the first AUG codon downstream of the mRNA 5′-cap and a shorter, 34-kDa JunD protein (ΔJunD) by initiation at a second in-frame AUG codon. The JunD mRNA contains a long, G/C-rich 5′-untranslated region that is predicted to be highly structured and is important for regulating the ratio of JunD-FL and ΔJunD protein expression. A third functional out-of-frame AUG directs translation from a short open reading frame positioned between the JunD-FL and ΔJunD start sites. In addition, three non-AUG codons also support translation, an ACG codon (in-frame with JunD) and a CUG are positioned in the 5′-untranslated region, and a CUG codon (also in-frame with JunD) is located downstream of the short open reading frame. Mutation of these start sites individually had no affect on ΔJunD protein levels, but mutation of multiple upstream start sites led to an increase in ΔJunD protein levels, indicating that these codons can function cumulatively to suppress ΔJunD translation. Finally, we show that the JunD mRNA does not possess an internal ribosome entry site and is translated in a cap-dependent manner.

JunD is a basic region leucine zipper DNA-binding protein and a member of the Jun family of proteins, which also includes c-Jun and JunB (1, 2). Jun proteins can homodimerize or heterodimerize with other Jun family members or heterodimerize with Fos family members (cFos, FosB, Fra-1, and Fra-2) to form the activator protein 1 transcription factor (3–5). Activator protein 1 binds to the tetradecanoyl-13-phorbol acetate response element (TGA(G/C)TCA) located within the promotors of a wide range of genes, many important for regulation of cell growth and transformation (6). Activator protein 1 is also an important signaling component in cell stress responses to UV irradiation, hypoxia, and cytokines (7–9).

Several studies show that JunD has unique properties compared with c-Jun and JunB. Mice lacking c-Jun or JunB are not viable, whereas mice lacking JunD are viable with only mild growth defects and some abnormalities in spermatogenesis (10–12). Overexpression of c-Jun or JunD exerts antagonistic effects on fibroblasts; c-Jun promotes cell proliferation and cooperates with activated ras to transform cells, whereas JunD overexpression slows cell growth and antagonizes transformation by ras (13–15). c-Jun and JunB behave as “early response genes” whose expression, at both the mRNA and protein levels, increases as cells enter the G1 phase of the cell cycle after serum stimulation (16, 17). JunD expression is less responsive to serum levels but is significantly increased as cells enter quiescence (1, 18).

Similar to the other Jun family members, the junD gene has no introns and produces a single messenger RNA (19). The JunD mRNA encodes two ubiquitously expressed protein isoforms, JunD-FL and ΔJunD, generated by translational initiation at two AUG codons that are in-frame with one another (20). A small out-of-frame AUG-initiated open reading frame (μORF) is positioned between these two AUG codons. We show that in addition to these three AUG codons, three non-AUG start codons are functional for initiation of translation, a CUG and an ACG, located within the 5′-untranslated region (5′-UTR), and a CUG that resides between the JunD-FL and ΔJunD start sites. Thus, a total of six translation start sites reside within the rodent JunD mRNA. The JunD mRNA also contains a long, G/C-rich 5′-UTR that is predicted to have a low free energy of folding and to be highly structured (1, 2, 21). Previous studies reveal that long, structurally encumbered UTRs can be inhibitory to cap-dependent translation (22, 23).

In this study, we have investigated the role of the JunD 5′-UTR and the unusually large number of functional initiation start codons in controlling translation of the JunD-FL and ΔJunD isoforms. Our results demonstrate that the JunD 5′-UTR is important in regulating the ratio of JunD-FL and ΔJunD. Furthermore, we show that the JunD mRNA is translated in a cap-dependent manner and that upstream codons act cumulatively to repress expression of the ΔJunD isoform.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—A human keratinocyte cell line (HaCat), human lung epithelial cell line (WI-38), simian kidney epithelial cell line (COS-1), rat osteosarcoma cell lines (UMR-106 and UMR-106), and human osteosarcoma cell lines (U2-OS and SAOS) were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal calf serum (HyClone), 50 units/ml penicillin, and 50 μg/ml streptomycin (BioWhittaker). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone), 50 units/ml penicillin, and 50 μg/ml streptomycin. For protein extraction or RNA isolation, HeLa cells were plated onto 6-well dishes
at a density of 5 \times 10^4 cells/well. For luciferase assays, HeLa, CHO, or COS-1 cells were plated onto 24-well dishes at a density of 8 \times 10^4 cells/well. DNA (0.4 or 2 \mu g of total DNA/well) was transfected into 24-well dishes or 6-well dishes, respectively, using the Cytofectene transfection reagent according to the manufacturer's protocol (Bio-Rad). Lysates for protein analysis or luciferase assays were prepared 24 h after transfection.

**Luciferase Assays**—After transfection, cells were lysed in 1× passive lysis buffer, and luciferase activity was determined using the dual luciferase assay system according to the manufacturer's instructions (Promega) with a TD-20/20 luminoimeter (Turner Designs). Firefly luciferase activities were normalized using Renilla luciferase.

**Plasmid Construction and Site-directed Mutagenesis**—The full-length JunD ORF used to generate Dciferase activities were normalized using luciferase assay system according to the manufacturer. The JunD open reading frame was amplified by PCR using 5'-GGAAATTCGAGGATCCACAAGGCGCAGC-3′ as the forward primer, a portion of the JunD ORF used to generate D-Δ1 was amplified using 5′-CGGAATTTCGGGACCCCGCAGTGCTGG-3′ as forward primer for full-length mouse JunD ORF. The human 5′-GGAGTCCAGCAGCATGCGTCTAGGAGG-3′ as forward primer, and a portion of the JunD ORF used to generate D-Δ2 was amplified using the 5′-GGAAATTCGAGGATCCACAAGGCGCAGC-3′ as forward primer. The rat JunD ORF was amplified by PCR using the mouse forward primer for full-length mouse JunD ORF. The human JunD open reading frame was amplified by PCR using 5′-GGAAATTCGAGGATCCACAAGGCGCAGC-3′ as forward primer, a portion of the JunD ORF used to generate D-Δ1 was amplified using 5′-CGGAATTTCGGGACCCCGCAGTGCTGG-3′ as forward primer, respectively. The human CDNA from the TATAA region of the 5′-3′ end of the ORF was amplified by PCR using 5′-CCGCGCCGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. The view D-luciferase ORF was amplified by PCR using 5′-CCGCGCCGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. PCR products were subcloned into the pcDNA 3.1(+) expression vector (Invitrogen) after digestion with EcoRI and HindIII restriction enzymes. Rat JunD or human JunD sequences containing the wild-type 5′-untranslated region were amplified by PCR as follows. A BsiWI restriction site was generated upstream of the pcDNA3.1-MHB TATAA region using the QuikChange site-directed mutagenesis protocol according to the manufacturer’s instructions (Stratagene). The rat JunD cDNA from the TATAA region through the 3′-end of the ORF was amplified by PCR using 5′-CCCGTCCGGGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. The truncated junD-Δ1 region of the JunD ORF was amplified by PCR using 5′-CCGCGCCGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. The PCR products were subcloned into the pcDNA3.1MB vector after digestion with BstI and HindIII.

Site-directed mutagenesis of JunD constructs was performed using the QuikChange protocol, and all mutations were verified by ABI sequence analysis (Texas Tech University Biotechnology Institute). The sequence of specific mutagenesis primer pairs can be obtained upon request. Dicistronic constructs were generated as follows. An XhoI restriction site was generated in the pRL-CMV expression vector (Promega) downstream of the Renilla luciferase open reading frame. The 5′-CCCGTCCGGGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ primer served as primer for PCR of the JunD fragments described above. PCR products were subcloned into the pcDNA3.1-MHB expression vector (Invitrogen) after digestion with EcoRI and HindIII restriction enzymes. Rat JunD or human JunD sequences containing the wild-type 5′-untranslated region were amplified by PCR as follows. A BsiWI restriction site was generated upstream of the pcDNA3.1-MHB TATAA region using the QuikChange site-directed mutagenesis protocol according to the manufacturer’s instructions (Stratagene). The rat JunD cDNA from the TATAA region through the 3′-end of the ORF was amplified by PCR using 5′-CCCGTCCGGGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. The truncated junD-Δ1 region of the JunD ORF was amplified by PCR using 5′-CCGCGCCGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′ as forward and reverse primers, respectively. The PCR products were subcloned into the pcDNA3.1MB vector after digestion with BstI and HindIII.

**RNA Isolation and Northern Blotting**—Total cellular RNA was isolated using TRIzol reagent (Invitrogen). RNA (10 μg) was separated by electrophoresis on 1% agarose gels containing formaldehyde and transferred to nylon membranes (Schleicher & Schuell). Membranes were hybridized overnight with 2.5 × 10^6 cpn of DNA probe in modified Church's buffer (0.25 mM Na2HPO4, pH 7.2, containing 7% SDS) (25). After incubation, membranes were washed with 1× SSC and 0.1% SDS at 65 °C and then exposed to a PhosphorImager screen for 6–15 h. Signal was detected using a PhosphorImager instrument (Model 445SI, Molecular Dynamics). A DNA probe corresponding to the myc-his region was generated as follows. The myc-his region of pcDNA3.1-MHB was amplified by PCR using 5′-AAGCTTTCTAGAAGCCAAAACGTC3′ as the forward primer and 5′-TCAAGTTGAATGCGTCTGG-3′ as reverse primers. The PCR product was subcloned into the pcDNA3.1-MHB expression vector (Promega) after digestion with XhoI and XhoI. The insert was then subcloned into the modified pRL-CMV vector after digestion with XhoI and BamHI. The dicistronic construct containing the encephalomyocarditis virus insert was a kind gift from Dr. Kristen Boris-Lawrie and was described previously (24). A hairpin structure was inserted into the dicistronic constructs by first generating an EcoRI site within the pRL-CMV promoter region upstream of the Renilla luciferase ORF. Next, a primer (5′-CCCGTCCGGGCTGATTGCCTGG-3′ and 5′-CCGCGCCGCTGATTGCCTGG-3′) containing a modified synthetic stem-loop structure was denatured at 95 °C for 5 min and placed on ice for 20 min (21). The primer was extended using Klenow polymerase I at 16 °C for 1 h and was then digested with EcoRI, gel-purified, and subcloned into the dicistronic-EcoRI plasmids.

**Cell Extracts and Western Blotting**—Whole cell extracts were prepared using 0.5% SDS lysis buffer (50 mM Tris-Cl, pH 8.0, 0.5% SDS, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, all reagents from Sigma) as described previously (18). Mouse tissue extracts were a gift from the lab of Dr. C. McDonald. Total protein concentration was normalized using the Bio-Rad protein assay reagent. Equal amounts of cellular protein were denatured in sample buffer at 95 °C, separated by electrophoresis on 10% SDS-polyacrylamide gels, and blotted to nitrocellulose membrane (Schleicher & Schuell). Blots were preincubated for 1 h at room temperature using phosphate-buffered saline containing 0.1% Tween 20 (PBST) supplemented with 5% nonfat dry milk. Primary antibodies were diluted in PBST with 1% bovine serum albumin and incubated at room temperature for 1 h. After washing with PBST, an appropriate peroxidase-conjugated secondary antibody (Jackson Laboratories) was diluted in PBST with 1% bovine serum albumin and incubated in 1 h at room temperature. Peroxidase-conjugated antibody complexes were visualized with SuperSignal chemiluminescent substrate (Pierce). The Myc epitope was detected using a mouse anti-Myc antibody (Invitrogen) diluted 1:5000. JunD proteins were detected using either a rabbit anti-JunD antibody (Santa Cruz #329, 0.2 μg/ml) or a rabbit anti-JunD antibody described previously (15).

**RESULTS**

**JunD-FL and ΔJunD Are Generated by Alternative Translational Initiation**—To examine endogenous JunD protein levels, whole cell extracts were generated from mouse tissues and a variety of mammalian cell lines. Tissue extracts were probed with a commercially available rabbit polyclonal antibody specific for a C-terminal peptide of JunD (Santa Cruz #329) (Fig. 1A), and cell line extracts were probed with a rabbit polyclonal antibody directed against amino acids 1–114 of JunD (Fig. 1B). Both of the antibodies were highly specific, with no detectable cross-reaction to the other Jun proteins, and both antibodies yielded similar results (data not shown). Each of the extracts contained two isoforms of JunD. The larger isoform, full-length JunD (JunD-FL), was detected at 39 kDa, and a smaller isoform, ΔJunD, was detected at 34 kDa (20). All tissue extracts expressed JunD-FL and ΔJunD at approximately 32668

**Fig. 1. Two isoforms of JunD are expressed in mouse tissues and mammalian cell lines.** A, Western blot analysis of whole cell extracts prepared from mouse tissues. Two dominant JunD-reactive bands were observed in each JunD isoform (JunD-FL) and a shorter 34-kDa isoform (ΔJunD). B, Western blot analysis of SAOS (human osteosarcoma), HaCaT (human keratinocyte), UMR-106 and UMR-108 (rat osteosarcomas), U2-OS (human osteosarcoma), WI-38 (human lung epithelial), and HeLa (human cervical epithelial) cell lines.
equal stochiometries; however, the ratio of JunD-FL to ΔJunD varied somewhat between cell lines. HaCat, U2-OS, and HeLa cells expressed JunD-FL and ΔJunD at approximately a 1:1 ratio, whereas SAOS, UMR-106, UMR-108, and WI-38 cells expressed JunD-FL at slightly higher levels than ΔJunD (Fig. 1B). Sequence analysis of the JunD mRNA revealed three potential AUG initiation codons positioned within good Kozak contexts to support translational initiation. These three start codons are conserved between chicken, rodents, and human (Fig. 2B). The first AUG (S1) in the JunD mRNA downstream of the 5’ cap gives rise to JunD-FL (1). The third AUG downstream of the 5’ cap (S3) is in-frame with JunD-FL, and the predicted protein size generated from the S3 start site is comparable with the ΔJunD isoform seen in Fig. 1 (Fig. 2A). The second AUG downstream of the 5’ cap (S2) is out-of-frame with JunD-FL (Fig. 2B). If translated, the S2 ORF (referred to here as the μORF) would encode a 3–5 amino acid product depending upon species (Fig. 2A).

To determine whether the S3 codon can direct translation of the ΔJunD protein, synthetic mouse JunD constructs, D-WT, D-Δ1, and D-Δ2, were created. The synthetic constructs contained a Myc epitope in-frame with the C terminus of JunD, allowing direct detection of exogenous JunD proteins by Western blot analysis. The D-WT construct possessed each of the three AUG codons shown in Fig. 2B. D-Δ1 contained the S2 and S3 codons, and D-Δ2 contained only the S3 codon (Fig. 3A). The D-WT, D-Δ1, and D-Δ2 constructs were transfected into HeLa cells, and expression was analyzed by Western blots probed with mouse monoclonal antibody specific for the Myc epitope. The D-WT plasmid produced both JunD-FL and ΔJunD isoforms. The D-Δ1 and D-Δ2 constructs did not produce JunD-FL, but both generated the ΔJunD isoform (Fig. 3A, lanes 1–3). To directly test the involvement of the S3 AUG in ΔJunD expression, this codon was mutated to UUA. Independently, the −3 and −2 positions of the S3 Kozak sequence were mutated to pyrimidine residues to decrease the efficiency of any translational initiation from the S3 start codon (Fig. 3B) (26). The S3 AUG mutant construct was incapable of generating ΔJunD, and the S3 Kozak mutation nearly abolished ΔJunD protein expression (Fig. 3C, lanes 4 and 5). Taken together, these data indicate that JunD-FL translation is initiated at the S1 AUG and ΔJunD translation is initiated at the S3 codon.

The JunD μORF Is Capable of Supporting Translation Initiation—To determine whether the μORF affected translation of ΔJunD, the S2 AUG was mutated to an ACG codon to prohibit translational initiation. Independently, the μORF stop codon was mutated to a UGG codon to prevent termination of ribosomes that had translated the μORF (Fig. 4A). Wild-type and mutant constructs were transfected into HeLa cells, and cell lysates were analyzed by Western blotting. Neither the μORF start codon mutant nor stop codon mutant significantly altered the expression of ΔJunD or JunD-FL (Fig. 4B).

To determine whether the S2 AUG supported ribosomal initiation, mouse, rat, and human constructs that correspond to D-WT shown in Fig. 3A were used to generate frameshift mutations. A single nucleotide was inserted into the μORF stop codon, placing the μORF in-frame with ΔJunD and placing the S1 AUG out-of-frame with ΔJunD. This frameshift mutation allowed ribosomes initiating translation at the S2 codon to elongate and generate a μORF-JunD hybrid protein (S2 product) in-frame with the C-terminal Myc epitope that was predicted to be slightly smaller than JunD-FL. Subsequently, the frameshift mutant constructs were used to generate a double mutant that also contained the S2 AUG mutation (AUG to ACG) (Fig. 5A). Mouse, rat, and human frameshift mutants...
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A schematic diagram of the μORF sequence. The D-WT construct, shown in Fig. 3A, was used to generate mutations within the μORF start codon (S2*) or the μORF stop codon. B, the wild-type and mutated plasmids were transfected into HeLa cells, and equal amounts of whole cell extracts were analyzed for expression of Myc-tagged JunD isoforms by Western blotting.

The JunD 5′-UTR Alters the Ratio of JunD-FL and ΔJunD Expression—The 5′-UTR of the JunD mRNA is a long (120 and 138 nucleotides in rodent and human, respectively) G+C-rich sequence that is predicted to have a low free energy of folding (21). Both the rat and human 5′-UTRs from the mRNA 5′ cap to the S1 AUG codon were analyzed using mfold software (27, 28). The rat and human UTR structures had comparably low ΔG* values (within 11% of each other). The predicted structures of lowest free energy for the rat and human 5′-UTRs both possess three dominant stem-loop structures, with the first stem-loop positioned only 2-3 nucleotides from the JunD mRNA 5′ cap (Fig. 6). To more accurately assess the role of the 5′-UTR in JunD mRNA translation, plasmid constructs were generated that possessed the complete rat or human wild-type 5′-UTR (JunD 5′-UTR) including the JunD TATAA box (Fig. 7A). Constructs containing the JunD wild-type 5′-UTR and D-WT constructs that contained a 5′-untranslated region from the pcDNA3.1 plasmid were transfected into HeLa cells. RNA samples were isolated and analyzed by Northern blots hybridized with a DNA probe specific for the myc-his region of the mRNA. Comparable levels of mRNA were expressed by the D-WT and JunD-5′-UTR constructs (Fig. 7B, bottom panel). Western blotting indicated that JunD-FL protein levels were reduced in cells transfected with the JunD 5′-UTR constructs, accompanied by a corresponding change in the ratio of JunD-FL to ΔJunD (Fig. 7B, top panel). The expression ratio of JunD-FL/ΔJunD was ~1:1 from JunD-5′-UTR constructs, a ratio similar to that found for the endogenous JunD isoforms (Fig. 1). These data indicate that the JunD 5′-UTR has a role in determining the ratio of JunD-FL and ΔJunD in cells that the JunD-5′-UTR constructs are a better model with which to study JunD translation.

Identification of Multiple Translational Initiation Sites within the Rodent JunD mRNA—The rat JunD 5′-UTR construct was used to determine whether the S2 codon supported translation initiation in the context of the JunD 5′-UTR. Mutations corresponding to those shown in Fig. 5A were introduced into the rat JunD 5′-UTR construct, and expression from these plasmids was tested in HeLa cells. A Myc-reactive S2 product resulted from the frameshift mutation that placed the S2 AUG in-frame with the S3 (ΔJunD) AUG. Similar to the D-WT constructs, the S2 product was abolished by mutation of the μORF start codon (Fig. 8D, lane 4). Mutation of the S2 AUG codon alone or the μORF stop codon in the JunD-5′-UTR construct had no effect on JunD-FL or ΔJunD protein levels (data not shown).
The JunD-5′-UTR construct containing a frameshift mutation also generated a Myc-reactive band that migrated to a higher molecular weight than JunD-FL (A1 product) (Fig. 8D, lane 2). This was surprising since there are no other AUG codons upstream of the S1 AUG codon. To determine the origin of the higher molecular weight product, the rat JunD mRNA sequence upstream of the S1 AUG was analyzed for potential non-AUG start codons. A putative alternative initiation codon, CUG (A1), within the JunD 5′-UTR that was in-frame with the 5′ cap region of the A1 start site and a CUG codon (A3) just downstream of the μORF stop codon were found at positions that could explain these additional in-frame JunD proteins. Mutations were introduced that disrupted these two putative alternative start sites. Mutation of the A2 start codon abolished expression of the high molecular weight product (Fig. 8C, lane 2), and mutation of the A3 codon abolished expression of the smaller protein (Fig. 8C, lane 4). One additional potential alternative start codon, a CUG located 25 nucleotides from the mRNA 5′ cap, was also mutated, but this had no effect on the expression of the A2 product (data not shown). Taken together, these data demonstrate that the rat JunD mRNA has multiple sites of translation initiation that occur at both AUG and non-AUG start codons.

**Mutation of Multiple Upstream Start Codons Increase ΔJunD Expression—**Each of the start codons within the rat JunD-5′-UTR construct was mutated to a non-start codon (Fig. 8B). Mutation of the A1 codon led to increased A2 protein levels, and the S2 mutation led to increased A3 protein levels. However, the single mutation of any upstream start codon, including S2 (Jun-D-FL start codon), had no detectable affect on ΔJunD protein levels (data not shown). The mutation of upstream start codons in combination did lead to detectably increased ΔJunD protein expression. Two separate double mutants (A1 + A2 and A2 + S1) led to a slight increase in ΔJunD protein levels (Fig. 9, lanes 2 and 3). A triple mutant targeting the S1, S2, and A3 codons led to a larger increase in ΔJunD protein levels, and a quadruple mutation of the A1, A2, S1, and A3 codons led to a yet larger increase in ΔJunD protein levels (Fig. 9, lanes 4 and 5). Mutation of all upstream start sites together led to the greatest increase in ΔJunD protein expression (Fig. 9, lane 6). There was no observable difference in JunD mRNA expression between the different mutant constructs as determined by Northern blotting (data not shown).

**The JunD mRNA Does Not Contain an Internal Ribosome Entry Site—**To determine whether the 5′ region of the JunD mRNA possessed an internal ribosome entry site (IRES), dicistronic reporter plasmids were prepared as described under “Experimental Procedures.” A portion of the JunD mRNA from the 5′ cap to the S3 start site was placed within the intercistronic space of a dicistronic vector containing firefly and Renilla luciferase coding regions (Fig. 10A). As a positive con-
...was detected. Western blotting. The presence of two additional JunD isoforms (extracts were analyzed for expression of Myc-tagged JunD isoforms by...

...plasmids were transfected into HeLa cells, and whole cell extracts were analyzed by Western blotting using an antibody that recognizes the myc epitope. The nomenclature for the translation start site mutations correspond to the schematic in Fig. 8B.

FIG. 9. Mutation of multiple upstream translation initiation sites increases expression of ΔJunD. Wild-type and the indicated mutated expression plasmids were transfected into HeLa cells, and whole cell extracts were analyzed by Western blotting using an antibody that recognizes the myc epitope. The nomenclature for the translation start site mutations correspond to the schematic in Fig. 8B.

In this report, we have analyzed the role that structural features within the JunD mRNA have in regulating expression of the two dominant JunD protein isoforms, JunD-FL and ΔJunD. We have found that the JunD 5′-UTR alters the ratio of JunD-FL to ΔJunD and that a small ORF (μORF) that resides between the JunD-FL and ΔJunD start sites, out-of-frame with JunD, is readily translated by ribosomes in vitro. We have also determined that the JunD mRNA contains three additional non-AUG codons that can initiate translation, although not as efficiently as the JunD AUG codons. Two of these non-AUG codons, a CUG upstream of JunD-FL and a CUG downstream of the μORF stop codon, produce products that are in-frame with JunD-FL and ΔJunD. Therefore, there are po-

FIG. 8. Identification of multiple translation initiation codons within the rodent JunD mRNA. A, the sequence of the rat JunD mRNA 5′-untranslated region. Two potential alternative initiation codons are indicated by brackets (A1 and A2). B, schematic diagram of rat JunD construct containing the wild-type 5′-UTR. The three conserved AUG initiation start codons are shown (S1, S2, and S3). Potential alternative initiation codons are represented by A1, A2, and A3. Mutations tested to establish whether each of the start codons was capable of supporting translation initiation are indicated. C, wild type and mutant plasmids were transfected into HeLa cells, and whole cell extracts were analyzed for expression of Myc-tagged JunD isoforms by Western blotting. The presence of two additional JunD isoforms (A2 and A3 products) was detected. D, mutations corresponding to Fig. 5A (μORF frameshift) were created in the rat JunD construct containing the wild-type 5′-UTR. Translation initiated at the A1 CUG and the S2 AUG were detected and specifically abolished by the respective additional A1* and S2* mutations.

As an additional experimental test for JunD IRES activity, the entire JunD cDNA from the 5′ cap through the coding region was placed downstream of a firefly luciferase encoding cassette. This construct allowed scanning ribosomes to translate the firefly luciferase ORF but prevented translation of the JunD ORF unless an IRES was present. JunD expression was not detectable even when firefly luciferase was expressed at high levels (data not shown). Taken together, these results indicate that the JunD mRNA does not possess an IRES but is translated in a cap-dependent manner.

DISCUSSION

In this report, we have analyzed the role that structural features within the JunD mRNA have in regulating expression of the two dominant JunD protein isoforms, JunD-FL and ΔJunD. We have found that the JunD 5′-UTR alters the ratio of JunD-FL to ΔJunD and that a small ORF (μORF) that resides between the JunD-FL and ΔJunD start sites, out-of-frame with JunD, is readily translated by ribosomes in vitro. We have also determined that the JunD mRNA contains three additional non-AUG codons that can initiate translation, although not as efficiently as the JunD AUG codons. Two of these non-AUG codons, a CUG upstream of JunD-FL and a CUG downstream of the μORF stop codon, produce products that are in-frame with JunD-FL and ΔJunD. Therefore, there are po-
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Fig. 10. JunD mRNA translation is cap-dependent and does not contain an IRES. A, dicistronic plasmid constructs. A region of the EMCV virus IRES element or the 5’ region of JunD was inserted into the intercistronic space of a dicistronic construct with or without a hairpin sequence. B, constructs shown in A that lack a hairpin were transfected into HeLa cells, Cos-1 cells, or CHO cells. The cells were harvested 24 h later, and the firefly (FFLuc) and Renilla (RenLuc) luciferase activities were determined. C, constructs shown in A that contain an inhibitory synthetic hairpin structure were transfected into CHO cells. Luciferase activities were determined as in B.

potentially four isoforms of JunD that can be expressed. The other non-AUG codon, an ACG codon upstream of the JunD-FL start site, is in-frame with the μORF. Mutation of individual upstream start codons led to increased expression from the adjacent downstream codon but never significantly changed the expression level of ΔJunD. However, mutation of upstream start sites in combination led to a progressive increase in ΔJunD expression.

According to the scanning model of translation, the eukaryotic initiation factor 4F complex (eIF4F), consisting of eIF4E, eIF4G, and eIF4A, binds to the 7-methylguanosine cap of mRNA and recruits the 43 S ribosomal complex (comprised of the 40 S ribosomal subunit, methionyl-tRNA\(^{Met}\), and several eukaryotic initiation factors). The 43 S ribosomal complex scans linearly through the 5’-UTR in a 5’ to 3’ direction until it reaches the first AUG surrounded by a good Kozak sequence, where translation begins (22, 30). The most important nucleotides for efficient translational initiation are an A in the –3 position or a G in the +4 position, where the A of the start codon is position +1 (26). A small subset of cellular mRNAs has been reported to express protein products from more than one start codon, but this is generally due to the poor context of start codons allowing ribosomes to bypass and reach initiation codons further downstream. For example, the mRNAs for C/EBPα and C/EBPβ each give rise to multiple isoforms by use of translation start sites surrounded by poor Kozak sequences (31). Alternatively, some mRNAs such as basic fibroblast growth factor-2 and c-Myc utilize non-AUG codons for translational initiation, a situation that also leads to translation of multiple protein isoforms (32, 33). We have determined that the JunD-FL and ΔJunD isoforms are generated by alternative translational initiation at AUG codons residing 144 nucleotides apart within the JunD mRNA (Fig. 3). The JunD S1 AUG is surrounded by a good Kozak sequence with a purine at position –3 and a guanine at position +4 (Fig. 2). Nevertheless, ribosomes efficiently bypass the S1 AUG codon of JunD and reach the ΔJunD (S3) start site in the presence or absence of the JunD 5’-UTR.

The JunD 5’-UTR suppresses JunD-FL expression and alters the ratio of JunD-FL to ΔJunD (Fig. 7B). The JunD 5’-UTR, like the other Jun family 5’-UTRs, is long (more than 100 nucleotides) and G/C-rich. The JunD 5’-UTR has the highest G/C content of any Jun mRNA UTR (88% for the human and 83% for rodent) and is a Class I 5’-UTR (a classification scheme based on structural complexity) with a predicted free energy of folding of ~74 and ~84 kcal/mol for rat and human, respectively (Fig. 6) (34). In vitro and in vivo experiments have shown that Class I 5’-UTRs are inhibitory to translation. For example, synthetic UTRs with free energies of folding less than ~50 kcal/mol inhibit translation by trapping the 43 S ribosome complex at the 5’ end of the mRNA and blocking its access to downstream AUG codons (35, 36). In addition, the highly structured 5’-UTRs of ornithine decarboxylase, protein kinase-Cε, and the N-methyl-D-aspartate Receptor (NR2A) have all been shown to inhibit translational initiation by scanning ribosomes (37–39). Interestingly, the JunD 5’-UTR inhibits JunD-FL expression but does not affect ΔJunD protein levels, suggesting that ribosomal bypass becomes more efficient in the presence of the 5’-UTR (Fig. 7). This result cannot be explained simply by alteration of the S1 Kozak sequence, because a construct containing the first 24 nucleotides of 5’-UTR upstream of the JunD-FL start site and the wild type S1 (JunD-FL) Kozak sequence did not alter the ratio of JunD-FL/ΔJunD (data not shown).

Overexpression of eIF4E, the limiting component for a functional eIF4F complex, has been shown to relieve translational suppression of mRNAs that contain extensive secondary structure within their 5’-UTR (40). For example, overexpression of eIF4E up-regulates Pim-1 protein expression, but this effect is
overcome by mutating eIF4E phosphorylation sites (41). We found that overexpression of eIF4E in Swiss-3T3 cells did not relieve the translational repression exerted by the JunD 5′-UTR (data not shown). Furthermore, treatment of 3T3 cells with the drug rapamycin, which blocks the eIF4E binding to the 5′ cap of mRNA, also had no effect on JunD-FL and ΔJunD expression (data not shown). These negative results indicate that the effects of the extensive secondary structure within the JunD 5′-UTR on translation are not regulated by eIF4E levels.

Currently, there are three models describing how ribosomes can bypass an upstream start codon, 1) internal ribosome entry, 2) linear but leaky scanning, and 3) non-linear scanning (ribosomal shunting) (42, 43). Internal ribosome entry, a mechanism first discovered in viruses, occurs when ribosomes bind to an internal ribosome entry sequence and begin translation independently of the mRNA 5′ cap (44). Several cellular mRNAs have now been characterized as containing bona fide IRES elements. For example, the c-myc proto-oncogene was found to contain an IRES element that is utilized during apoptosis (45, 46). The p58 and p110 PITSLRE protein kinases that are generated by alternative translational initiation from a single mRNA and ornithine decarboxylase were found to contain IRES elements that are cell cycle-regulated (47, 48). In addition, a recent report indicated that the chicken c-Jun 5′-UTR possesses an IRES (21). When tested in dicistronic constructs, the JunD 5′ region only increased cap-independent translation 1.5–3-fold compared with a no insert control plasmid. This was a small increase when compared with the EMCV positive control (Fig. 10) (29). Expression from the EMCV IRES control was consistent with previous studies, being up-regulated 10-fold when compared with the no insert construct (24, 49). Insertion of a synthetic hairpin sequence into a dicistronic vector was shown previously to inhibit expression of the 5′-ORF while simultaneously increasing expression of the 3′-ORF in the presence of an IRES (21). Insertion of a hairpin into the JunD dicistronic construct did not alter the ratio of firefly/Renilla luciferase activity above the negative control. However, the activity of the EMCV sequence, a characterized IRES, was strongly amplified by insertion of the hairpin structure.

Although the results from the dicistronic IRES assays appeared negative, we undertook one additional analysis to further test the JunD mRNA for IRES activity. The ornithine decarboxylase mRNA contains two UUUC sequences upstream of the initiation codon that were demonstrated to contribute to ornithine decarboxylase IRES activity (48). The JunD mRNA has a similar UUUC sequence located 24 nucleotides upstream of the ΔJunD start site. Mutation of this JunD UUUC sequence to AAAA had no detectable impact on ΔJunD protein levels (data not shown). Taken together, these data present strong evidence that the JunD mRNA does not contain an IRES and that the mRNA is being translated in a cap-dependent manner.

Either the scanning model or the ribosomal shunting model could be used to explain increased ribosomal bypass of the S1 AUG in the presence of the JunD 5′-UTR. In the leaky scanning model the 5′-UTR would permit fewer ribosomes to scan the JunD mRNA but would allow an increased fraction of ribosomes to leak past the S1 AUG, resulting in decreased JunD-FL expression while maintaining ΔJunD levels. In the ribosome shunting model, ribosomes would repositioned from the 5′-UTR downstream of the S1 AUG, leading to initiation at the S3 AUG. The two models as applied to the JunD mRNA are not mutually exclusive and differ primarily in that shunting would lead to “leaking” past multiple translation initiation sites. Our results are consistent with both mechanisms functioning in the regulation of JunD mRNA translation.

We have identified a total of six translation initiation events that can occur within the intact wild-type JunD mRNA at AUG and non-AUG codons. Non-AUG translation initiation sites have similar optimal requirements for the flanking sequences as AUG codons. For example, a G at position +4 is important for productive interaction with ribosomal complexes (50). Analysis of the known functional non-AUG codons reveals that most are present in sequences that contain a G at +4 and an A in the −3 position (51). Each of the three alternative start codons identified in the JunD mRNA also contains a G at +4 and a purine at −3, suggesting that each of these codons is in a good context for ribosomal binding and initiation. A CUG start codon (A1) in the 5′-UTR can support translation and would generate a 37-amino acid peptide that would be in-frame with the μORF. An ACG (A2) positioned eight nucleotides downstream of the A1 codon is also functional for initiation and would generate a 23-amino acid N-terminal extension of JunD-FL. Finally, within the JunD-FL-coding sequence there is a functional CUG codon (A3) that is also in-frame with JunD-FL. Initiation from A3 would yield a JunD protein lacking the first 13 amino acids (versus 48 residues lacking in ΔJunD) (Fig. 8). All together, these data indicate that there are potentially four JunD isoforms and two peptides generated from the JunD mRNA. Although we easily detected the expression of these additional JunD isoforms from transiently transfected expression plasmids, only the two main protein isoforms of JunD were detected in all tissues and cell lines tested thus far (Fig. 1) (20). BLAST searches with the predicted A1 product and A2 peptide extension of JunD-FL yielded no significant matches. Whether cellular contexts exist where expression of the A1 or A3 protein products would be up-regulated to detectable (or functional) levels is not known. An example of such regulation has been reported for a human fibroblast growth factor 2 isoform that is translated through initiation at a CUG codon and is strongly up-regulated in transformed cells but is undetectable in normal skin fibroblasts (52).

Upstream ORFs have been shown to affect translation of downstream ORFs (53). For example, expression of C/EBPα and C/EBPβ are inhibited by an upstream ORF in a manner dependent on intercistronic length and sequence (54). Mutation of individual JunD mRNA upstream start codons, including the S1 and S2 AUG codons that were efficiently used for translation, did not significantly affect ΔJunD protein levels. Individual start codon mutations did lead to increased expression from the next downstream start site, but this increase did not correspond to the protein levels from the upstream start codon usage. For example, mutation of the S1 start codon did not cause the S2 protein product levels to increase to the sum of the translation occurring at S1 and S2 in the wild-type mRNA (data not shown). In addition, the two main JunD isoforms were consistently expressed at approximately equal ratios from the intact or singly mutated JunD mRNAs, and we have not found conditions that cause this ratio to vary. These data suggest that a large fraction of ribosomes arrive at initiation codons through a non-linear mechanism (shunting) and that upstream start codons (and ORFs) individually do not strongly affect ΔJunD expression. However, mutation of multiple upstream start codons led to a progressive increase in initiation from S3, generating ΔJunD, suggesting that these codons can repress ΔJunD expression in a cumulative manner that most likely results from sequestration of ribosomes. These data indicate that the upstream codons, both AUG and non-AUG, are important for regulating ΔJunD protein levels within the cell.

Recent studies indicate that JunD-FL and ΔJunD are differentially regulated through interactions with other nuclear proteins. For example, the Menin tumor suppressor protein binds
directly to JunD-FL through the first 48 amino acids and suppresses JunD-FL transcriptional activity (55, 56). ΔJunD does not bind Menin, and its transcriptional activity is unaffected by Menin overexpression. In addition, the Jun-N-terminal kinases (JNKs) bind and activate JunD-FL more efficiently than ΔJunD even though both JunD isoforms contain a JNK-docking domain and three JNK phosphorylation target sites. Undoubtedly, given the high level of conservation of the JunD mRNA sequence elements regulating expression of both JunD isoforms, additional functional differences between the JunD-FL and ΔJunD proteins will come to light. Finally, in addition to JunD-FL and ΔJunD, we have identified two new JunD isoforms that, even if expressed at low levels, may expand the functional repertoire of JunD and activator protein 1 activity.

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