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Vertebrate mRNAs with a 5′-Terminal Pyrimidine Tract Are Candidates for Translational Repression in Quiescent Cells: Characterization of the Translational *cis*-Regulatory Element

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The translation of mammalian ribosomal protein (rp) mRNAs is selectively repressed in nongrowing cells. This response is mediated through a regulatory element residing in the 5′ untranslated region of these mRNAs and includes a 5′ terminal oligopyrimidine tract (5′ TOP). To further characterize the translational *cis*-regulatory element, we monitored the translational behavior of various endogenous and heterologous mRNAs or hybrid transcripts derived from transfected chimeric genes. The translational efficiency of these mRNAs was assessed in cells that either were growing normally or were growth arrested under various physiological conditions. Our experiments have yielded the following results: (i) the translation of mammalian rp mRNAs is properly regulated in amphibian cells, and likewise, amphibian rp mRNA is regulated in mammalian cells, indicating that all of the elements required for translation control of rp mRNAs are conserved among vertebrate classes; (ii) selective translational control is not confined to rp mRNAs, as mRNAs encoding the naturally occurring ubiquitin-rp fusion protein and elongation factor 1α, which contain a 5′ TOP, also conform this mode of regulation; (iii) rat rpP2 mRNA contains only five pyrimidines in its 5′ TOP, yet this mRNA is translationally controlled in the same fashion as other rp mRNAs with a 5′ TOP of eight or more pyrimidines; (iv) full manifestation of this mode of regulation seems to require both the 5′ TOP and sequences immediately downstream; and (v) an intact translational regulatory element from rpL32 mRNA fails to exert its regulatory properties even when preceded by a single A residue.

The translation of vertebrate ribosomal protein (rp) mRNAs is largely regulated in a growth-dependent manner, as illustrated by their selective release from polysomes in growth-arrested cells. This has been demonstrated during transition of various cells types between growing and nongrowing states in response to a wide variety of physiological stimuli, including dexamethasone-treated mouse lymphosarcoma cells (45, 48), serum starvation of mouse fibroblasts (24, 38) or *Xenopus* kidney cells (41), and differentiation of mouse myoblasts (1) and concanavalin A-treated bovine T cells (37). Similar specific translational fluctuations have been observed for rp mRNAs during the transition from the rapidly growing state in the fetal liver to the quiescent state in the adult and upon resumption of the hepatocyte proliferation in the regenerating liver (2). The mammalian, avian, and amphibian rp mRNAs that have been rigorously analyzed thus far contain a 5′-terminal oligopyrimidine tract (5′ TOP), which consists of a C residue at the cap site, followed by an uninterrupted sequence of up to 13 pyrimidines (3, 18, 54, 69, 71). In attempt to delimit the translational regulatory element (TLRE), it has been shown that the 35-nucleotide-long 5′ untranslated region (UTR) of *Xenopus* rpS19 is sufficient to confer translational repression on a chimeric mRNA in a developmental stage-dependent manner (43). The involvement of a similar-size TLRE in the growth-dependent translational control of mammalian rp mRNAs has been established for mouse rpS16 mRNA (29, 39). Furthermore, we have demonstrated that the 5′ TOP plays a critical role in this translational control mechanism (39). The experiments described below were designed to further characterize the TLRE of the rp mRNAs. Our results suggest that this regulatory element consists of both the 5′ TOP and adjacent downstream sequences, that it is functional even in rp mRNAs with a relatively short 5′ TOP, and that its activity is dependent on its position relative to the cap site. In addition, we have demonstrated that the mechanism regulating the translation of rp mRNAs in a growth-dependent manner is evolutionarily conserved between amphibians and mammals.

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MATERIALS AND METHODS

Cell culture and DNA transfection. P1798.C7 mouse lymphosarcoma cells were grown as suspension cultures in RPMI 1640 containing 10% fetal calf serum, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. Cells (2×10^7) at a density of 5×10^5 to 7×10^5/ml were used for DEAE-dextran-mediated DNA transfection (27). Briefly, cells were incubated at room temperature with 2.5 μg of DNA per 10^6 cells in 6 ml containing 0.5 mg of DEAE-dextran (Pharmacia) per ml of Tris-buffered saline (TS in reference 27). The DNA/DEAE-dextran mixture was removed after 1 h, and the cells were treated at 37°C with growth medium containing 0.1 mM chloroquine diphosphate (Sigma). After 1 h of chloroquine treatment, the cells were transferred into 200 ml of growth medium containing 100 μg of gentamicin sulfate (Roussel Laboratories, Uxbridge, England) per ml, and at 24 h, half of the culture was treated with 0.1 μM dexamethasone (Sigma). Both treated and untreated cells were harvested 24 h later.

WHT 1249, a human lymphoblastoid cell line transformed with Epstein-Barr virus, was grown as suspension in RPMI 1640 containing 15% fetal calf serum and supplemented with 2 mM glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, and 0.1 mg of gentamicin sulfate per ml. Human skin fibroblasts (HSF) and mouse NIH 3T3 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. HSF were growth arrested by contact inhibition in confluent culture. Growth of NIH 3T3 cells was arrested by either treatment for 24 h with aphidicolin (5 μg/ml; Sigma), a specific DNA polymerase inhibitor (65), or incubation of confluent cells in medium containing 0.5% serum for 3 days (serum starvation). *Xenopus laevis* B3.2 kidney cells were grown and serum starved as described previously (41). *Xenopus* B3.2 and mouse NIH 3T3 cells were stably transfected by the DNA-calcium phosphate coprecipitation method (76) and selected for hygromycin B resistance (11) and Geneticin (Sigma) resistance, respectively.

Primer extension. Thirty-four nanograms of the oligonucleotide primer hGH-1 (5′-GAGTGGTTCGGGGAGTTGGG-3′) spanning positions 31 to 12 of the human growth hormone (hGH) gene was 5′ end labeled as described by Sambrook et al. (57). One-half microgram of poly(A)$^+$ mRNA from stably transfected mouse NIH 3T3 cells was coprecipitated with 5×10^5 to 8×10^5 cpm of the 5′-end-labeled primer, and annealing was carried out in 10 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(ethanesulfonic acid); pH 6.4]–0.4 M NaCl–1 mM EDTA–50% formamide for 8 h at 37°C. After ethanol precipitation, the annealed primer was extended at 42°C for 40 min with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) as previously described (57). The extended primer was analyzed on a 6% acrylamide–urea gel with a sequencing reaction as a marker.

DNA sequencing. Double-stranded plasmid DNA was sequenced by the dideoxy method (58), using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio).

Polysomal fractionation and RNA analysis. Protein synthesis was instantaneously arrested by pouring cells growing in suspension over frozen crushed phosphate-buffered saline (0.125 M NaCl, 10 mM NaH_2PO_4, 30 mM K_2HPO_4) as previously described (48) or treating the monolayer culture with 90 μg of cycloheximide (Sigma) per ml for 10 min prior to detachment from the plate with 0.04% trypsin in the presence of the drug. Cells were washed once with phosphate-buffered saline, and the cell pellet was kept frozen at −70°C. Lysis of cells, size fractionation of polysomes by sedimentation through sucrose gradients, extraction of the RNA from the polysomal and subpolysomal fractions, isolation of the poly(A)$^+$ mRNA fractions, and quantitative RNA (Northern) blot analysis were performed as described by Meyuhas et al. (48). Cytoplasmic extracts from rat liver were prepared as described by Aloni et al. (2). Preparation of polysomes and analysis of RNA from *Xenopus* cells were performed as previously described (41).

To assess the effectiveness of the growth arrest treatment and the selectivity of the effect on rp mRNAs, we compared in each case (even if not shown) the polysomal association of a chimeric mRNA with that of endogenous rp mRNA and non-rp mRNA from the same polysomal gradient. Only experiments in which both of these controls exhibited their typical translational behaviors (repressed and unrepressed, respectively) were included.

Plasmid constructions. Standard protocols were used for all recombinant DNA technology (57). The construction of pL13a-GH involved the following steps. (i) A 5-kbp *Hin*dIII fragment which contains most of the gene encoding the mouse antigen P198 (63), later identified as rpL13a (13), served as a template in PCR (35) to generate a 213-bp fragment spanning positions −186 to +27. The oligonucleotides used as primers were L13a-1 (5′-GGCCG**TCTAGA**GCCAACTCAGGCGAC-3′), corresponding to positions −186 to −161 except for the two T residues (boldface), which substituted a C residue at position −181 and an A residue at position −179 in the wild-type gene, thus creating an *Xba*I site (underlined); and L13a-2 (5′-CTCCG**AGATCT**TCGGCAGCCGCCTGG-3′), corresponding to positions +27 to +2 except for the dinucleotide AG (boldface), which substituted a CC dinucleotide at position +21 in the wild-type gene, thus eliminating the initiation codon starting at position +19. In addition, this replacement created a *Bgl*II recognition site (underlined). (ii) The PCR-generated fragment was cleaved with *Xba*I and *Bgl*II, and the resulting 203-bp fragment was cloned between the *Xba*I and *Bam*HI sites of the promoterless plasmid p0GH, which contains the hGH gene (60).

Construction of the pS16wt(1-15)-GH chimera involved digestion of construct s-CAT (32) with *Bam*HI and *Eco*RI to remove the bacterial chloramphenicol acetyltransferase gene and simian virus 40 sequences. A 2.1-kbp *Bam*HI-*Eco*RI fragment containing the hGH gene was excised from p0GH and inserted between the corresponding sites in s-CAT.

pS16wt(1-10)-GH was constructed as follows. (i) By using oligonucleotide primers S16-4 (5′-CG**GGATCCGGAAAAGG GAAGCGCGG**-3′; boldface letters correspond to nucleotides +10 to −9 of the rpS16 gene preceded by an underlined *Bam*HI recognition site) and a commercial oligonucleotide, 17-mer(A) (5′-GTAAAACGACGGCCAGT-3′, corresponding to nucleotide 379 to 395 of pUC18, supplied by International Biotechnology, Inc.), a 236-bp fragment was generated from a construct of the rpS16 gene (32) by PCR. (ii) The PCR-generated fragment was cleaved with *Hin*dIII and *Bam*HI, and the resulting 217-bp fragment was cloned between the *Hin*dIII and *Bam*HI sites of the promoterless plasmid p0GH.

pL32wt(1-34)-GH was constructed as follows. First, a 236-bp *Acc*I-*Sac*II fragment spanning positions −159 to +77 of the mouse rpL32 gene was isolated (20). The ends of this fragment were made blunt by T4 DNA polymerase, and it was ligated into filled-in (Klenow enzyme) *Xba*I sites of pUC18 to yield pL32a (the 5′ end at the *Acc*I site was adjacent to the *Hin*dIII site in the pUC18 polylinker). Using oligonucleotide primers L32b (5′-CG**GGATCCGGCAGCCACCTCGTAGG**-3′; boldface letters correspond to nucleotides +34 to +18 of the rpL32 gene preceded by an underlined *Bam*HI recognition site) and 17-mer(A), a 269-bp fragment was generated from pL32a by PCR.

pL32wt(1-12)-GH was constructed by digestion of pL32-1 (46) with *Hin*dIII. The ends were made blunt by filling in with Klenow enzyme and then cut with *Bam*HI. A 2.1-kbp fragment containing the hGH gene was excised from p0GH by excision with *Eco*RI, filling in of the ends with Klenow enzyme, digestion with *Bam*HI, and insertion between the *Bam*HI and the blunted *Hin*dIII sites of pL32-1.

The construction of pL32(S16)-GH involved the following steps. (i) Oligonucleotides S16-1 (5′-GGTCGCGGCGCT GCGGTGTG-3′, spanning positions +9 to +28 of the rpS16 gene) and S16-2 (5′-**GATCCACACCGCAGCGCCGCGAC CGCGC**-3′; boldface letters correspond to nucleotides +29 to +9 of the rpS16 gene preceded by an underlined *Bam*HI protruding 5′ terminus and followed by an underlined *Hae*II protruding 3′ terminus) were annealed as detailed in the protocol for the Sequenase kit (U.S. Biochemical). The result-

ing double-stranded oligonucleotide included *Bam*HI- and *Hae*II-compatible ends. (ii) A 195-bp *Hin*dIII-*Hae*II fragment, containing nucleotides −159 to +15 of the rpL32 gene, was isolated from pL32a. (iii) The fragments described in steps i and ii were simultaneously ligated (three-piece ligation) between *Hin*dIII and *Bam*HI sites of the promoterless plasmid p0GH. The resulting chimeric plasmid contains the hGH gene driven by the rpL32 promoter (nucleotides −159 to +15) immediately followed by nucleotides +9 to +29 of the rpS16 gene.

The pAct(28)-GH chimera was constructed by excising a 0.4-kbp *Hin*dIII-*Rsa*I fragment from pact-GH (46). This fragment, spanning positions −370 to +28 of the rat β-actin gene, was inserted between a *Hin*dIII site and 5 bp upstream to the *Pst*I site in p0GH (the sequence context of the latter resulted from a cloning artifact).

The construction of pAct(L32)-GH1 involved two steps. (i) A 50-bp fragment was generated by PCR using pL32a as a template and the oligonucleotide primers L32b (see above) and L32a (5′-GCTCTAGA**CTTCTTCCTCGGCGCTG**-3′; boldface letters represent nucleotides +1 to +17 of the rpL32 gene preceded by an underlined *Xba*I recognition site). (ii) The PCR-generated fragment was cut by *Bam*HI and inserted between a *Bam*HI site and a blunted (Klenow enzyme) *Xba*I site, separating the rat β-actin gene sequence from that of the hGH gene in pAct(28)-GH. This construct includes a rat β-actin gene sequence spanning positions −370 to +28 followed by a 30-bp polylinker sequence and 34 bp corresponding to nucleotides +1 to +34 of the mouse rpL32 gene.

pAct(L32)-GH2 is a derivative of pAct(L32)-GH1 with a shorter (13-bp) polylinker sequence separating the rat β-actin gene sequence from that of mouse rpL32. Its construction involved digestion of pAct(L32)-GH1 with *Xba*I, filling the ends with Klenow enzyme, and then cleavage with *Hin*dIII. The β-actin gene sequence and part of the polylinker (17 bp) were substituted by a 0.4-kbp *Hin*dIII-*Rsa*I fragment spanning positions −370 to +28 of the rat β-actin gene from pAct-3 (46). The resulting plasmid contains the first 34 bp of the rpL32 gene located 42 bp downstream of the transcription start site of the rat β-actin gene.

pCM3(L32)-GH was constructed through the following steps. (i) A 2.15-kbp *Xba*I-*Eco*RI fragment, containing the first 34 transcribed nucleotides of the mouse rpL32 gene followed by a 13-bp linker sequence and the hGH gene, was excised from pAct(L32)-GH1, and the ends of the fragment were made blunt by the Klenow enzyme. (ii) The rpS16 sequences spanning positions +30 to +2050 were excised from the rpS16 mutant CM-3 (33) by digestion with *Eco*RI and *Sac*I. The vector ends were made blunt by T4 DNA polymerase, and the fragment was ligated with the 2.15-kbp *Xba*I-*Eco*RI hGH gene fragment. The resulting plasmid contains the first 34 transcribed nucleotides of the rpL32 gene located 41 nucleotides downstream of the transcription start site of the mutated S16 promoter.

pL32($-1_{C\rightarrow A}$)-GH is similar to pL32wt(1-34)-GH except for replacement of the C residue at position −1 by an A residue. Site-directed mutagenesis was carried out by the megaprimer method (59). Briefly, an approximately 250-bp fragment was generated by PCR using plasmid pL32wt(1-34)-GH as a template and the two oligonucleotides L32m(−1) (5′-CGAG GAAGAAGTAAGAGGCGGCG-3′, corresponding to positions +11 to −12 of the rpL32 gene, with the underlined T residue substituting a G residue at position −1) and PUC-1 (5′-GTTTTCCCAGTCACGACGTT-3′, corresponding to nucleotides 361 to 380 of pUC18) as primers. The resulting double-stranded DNA fragment (megaprimer) together with the oligonucleotide hGH-3 primer (5′-CGTCCCATCTA CAGGTCGCT-3′, corresponding to a sequence within the first intron of the hGH gene) and the template pL32wt(1-34)-GH were used in a second PCR. The reaction conditions in both rounds were similar except for inclusion of 4% dimethyl sulfoxide in the second one. The resulting 415-bp fragment was cleaved by *Hin*dIII and *Bam*HI, and the resulting 221-bp fragment was cloned between the *Hin*dIII and *Bam*HI sites of the promoterless plasmid p0GH.

The structures of all constructs described above were confirmed by DNA sequencing.

Molecular probes. The isolated fragment probes used in the Northern blot analysis were a 1.15-kb *Pst*I fragment containing mouse α-actin cDNA (49); a 0.97-kb fragment bearing the rpL32 processed gene 4A, joined to the 5′ and 3′ flanks of 3A (17); a 0.7-kb *Eco*RI fragment containing *X. laevis* rpL14 cDNA (10); a 0.8-kb *Pst*I fragment containing *X. laevis* calmodulin cDNA (15); a 1.05-kb *Eco*RI-*Bam*HI fragment containing the ubiquitin coding region and the 3′ UTR from the human polyubiquitin gene UbB (7); a 1.8-kb *Bgl*I fragment containing murine elongation factor-1α (EF-1α) cDNA (kindly provided by L. I. Slobin); a 0.95-kb *Pst*I fragment containing rat rpS4 cDNA (23); a 0.8-kb *Hin*dIII fragment containing hGH cDNA (provided by T. Fogel, Bio-Technology General); a 0.29-kb *Eco*RI-*Hin*dIII fragment containing the cDNA insert of mouse S16 derived from a subclone in pUC18 (47); a 0.65-kb *Bam*HI fragment containing human rpP2 cDNA (56); a 2-kb *Hin*cII fragment containing exons 3 to 8 of the gene encoding mouse antigen P198 (63); a 0.62-kb *Pst*I fragment containing human superoxide dismutase I (SOD) cDNA (62); and a 0.51-kb *Sac*II-*Xba*I fragment containing a mouse rpL30 processed gene derived from p1cXba (75).

RESULTS

Functional conservation of the TLRE between amphibians and mammals. The translation of *X. laevis* rp mRNAs is selectively repressed during early embryogenesis, even though an intensive mitotic activity occurs during these developmental stages (reference 3 and references therein). Interestingly, this translational behavior is quite opposite that reported for cultured cells from *Xenopus* kidney (41) or mammals, in which the translation efficiency of rp mRNAs parallels the growth status. Nevertheless, in both classes, the TLRE resides within the first 20 to 40 nucleotides of the rp mRNAs and includes a 5′ TOP (references 3 and 54 and references therein). To examine whether the *Xenopus* rp mRNAs can respond to mammalian growth signals, we transiently transfected mouse P1798 lymphosarcoma cells with the *Xenopus* rpL14 gene (9). The results presented in Fig. 1a indicate that the unloading of ribosomes from the *Xenopus* rpL14 mRNA, upon growth arrest by dexamethasone treatment, is similar to that observed for the endogenous rpL32 mRNA. Likewise, *X. laevis* B3.2 kidney cells stably transfected with the mouse rpL32 gene exhibit a repressed translation of both the respective mammalian mRNA and the endogenous rpL14 mRNA upon growth arrest in serum-deprived medium (Fig. 1b). This response is of a selective nature, as the mRNAs encoding mouse actin and *Xenopus* calmodulin were efficiently translated regardless of the growth status of the cells. It appears, therefore, that similar *trans*-acting factors are capable of recognizing TLREs from amphibian and mammalian rp mRNAs. Noteworthy, the seemingly smaller size of the various transcripts in the subpolysomal fractions of B3.2 cells seem to result from a technical problem, which was not reproduced in other experiments using this cell line (40).

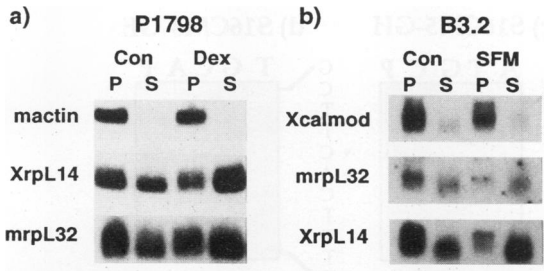

FIG. 1. Evolutionary conservation of the translational control mechanism among vertebrates. (a) Polysome-subpolysome distribution of *Xenopus* rpL14 mRNA in murine cells. P1798 cells were transiently transfected with pL14 containing the *Xenopus* rpL14 gene (9). Cytoplasmic extracts from untreated (Con) or 24-h dexamethasone-treated (Dex) cells were centrifuged through sucrose gradients and separated into polysomal (P) and subpolysomal (S) fractions. Poly(A)$^+$ mRNA from equivalent aliquots of these fractions was analyzed by Northern blot hybridization with the cDNA probes indicated at the left. (b) Polysome-subpolysome distribution of mouse rpL32 mRNA in *Xenopus* cells. B3.2 cells were stably transfected with p3A/Acc3.7 containing the mouse rpL32 gene (20). Cytoplasmic extracts from exponentially growing cells (Con) or cells maintained in serum-free medium (SFM) were prepared and manipulated as described previously (41). RNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization with *Xenopus* cDNAs for calmodulin (Xcalmod) and rpL14 and mouse rpL32. In both panels, X and m refer to *X. laevis* and mouse rp cDNAs, respectively. The stringency at which the blots were washed (15 mM NaCl–1.5 mM sodium citrate–0.25% sodium dodecyl sulfate) completely eliminated cross-hybridization between the *Xenopus* probe and the corresponding mouse mRNA and vice versa.

Several nontypical rp mRNAs which contain a 5′ TOP are also subject to growth-dependent translational control. Transition from growing to nongrowing states in cultured cells is associated with a decrease, by at least one-third, of the fraction of ribosomes engaged in translation (38, 48). Since rp mRNAs collectively comprise about 8% of the cellular mRNA (47), it is conceivable that the utilization of other mRNAs is also repressed. Indeed, two murine non-rp mRNAs, encoding laminin receptor-like protein and a 21-kDa protein, were shown to be translationally repressed in nongrowing cells (78) and later to contain a 5′ TOP (reference 4 and references therein; 16). To directly address the question of whether other mRNAs equipped with a 5′ TOP are similarly regulated, we have monitored the translational behavior of human mRNAs encoding ubiquitin and EF-1α.

The mammalian UbA gene comprises a unique example of a naturally occurring rp chimeric gene. It encodes a monomer of ubiquitin fused to a ribosomal protein of either 52 or 80 residues (UbA_{52} and UbA_{80}) (8). The 228-nucleotide-long ubiquitin coding unit in the corresponding mRNA separates the 5′ TOP from the rp coding sequence which resides at the 3′ end. The other two members of the ubiquitin family, UbB and UbC, encode polyubiquitin of three or nine direct repeats of mature ubiquitin, respectively (reference 8 and references therein). Unlike the former, the mRNAs transcribed from the polyubiquitin genes lack a 5′ TOP (see Fig. 2a for a schematic representation of the various ubiquitin genes). To address the question of whether the unique organization of the UbA mRNA confers on it a distinct translational behavior, we analyzed the polysomal distribution of the various ubiquitin mRNAs in exponentially growing human WHT 1249 lymphoblastoid cells. These cells exhibit a selectively repressed translation of rp mRNAs, as demonstrated by the efficient transla-

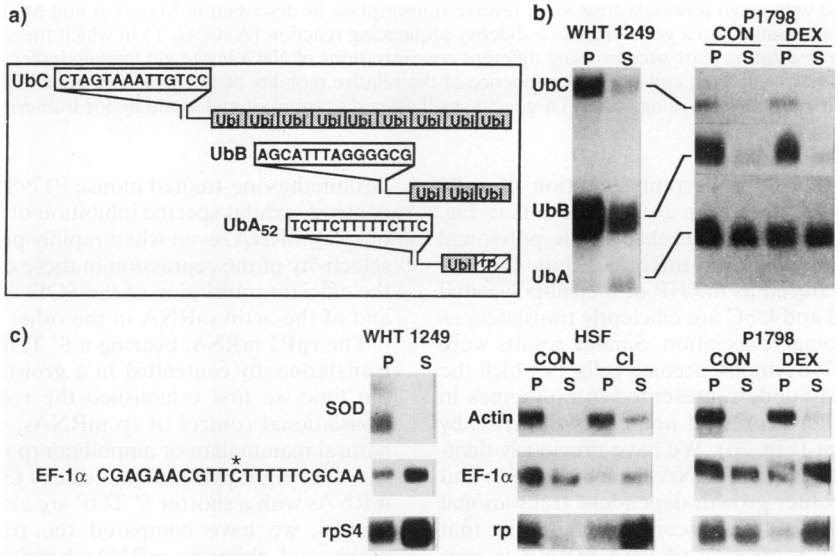

FIG. 2. Translational control of mRNAs encoding ubiquitin and EF-1α. (a) Schematic representation of the human ubiquitin mRNAs. The dotted and stippled boxes represent ubiquitin coding units (Ubi) and a tail of rp sequences, respectively. The boxed sequences depict sequences around the transcription start site of the corresponding human genes (6–8, 74). The putative transcription start site in each of these genes was deduced from analysis of the sequence homology between the 5′-end region of the active gene with that of the respective processed pseudogene. (b and c) Polysome-subpolysome distribution of various mRNAs under different growth conditions. Cytoplasmic extracts from human WHT 1249 cells, from untreated (Con) or 24-h dexamethasone-treated (Dex) P1798 cells, and from exponentially growing (Con) or contact-inhibited (CI) HSF were centrifuged through sucrose gradients and separated into polysomal (P) and subpolysomal (S) fractions. Poly(A)$^+$ mRNA from equivalent aliquots of these fractions was analyzed by Northern blot hybridization with a UbB probe in panel b and with the probes indicated at the left in panel c. rp refers to the probes used to detect rpS16 mRNA in HSF and rpL32 mRNA in P1798 cells. The sequence at the left in panel c represents the nucleotides around the transcription start site (marked by an asterisk) of the human EF-1α gene.

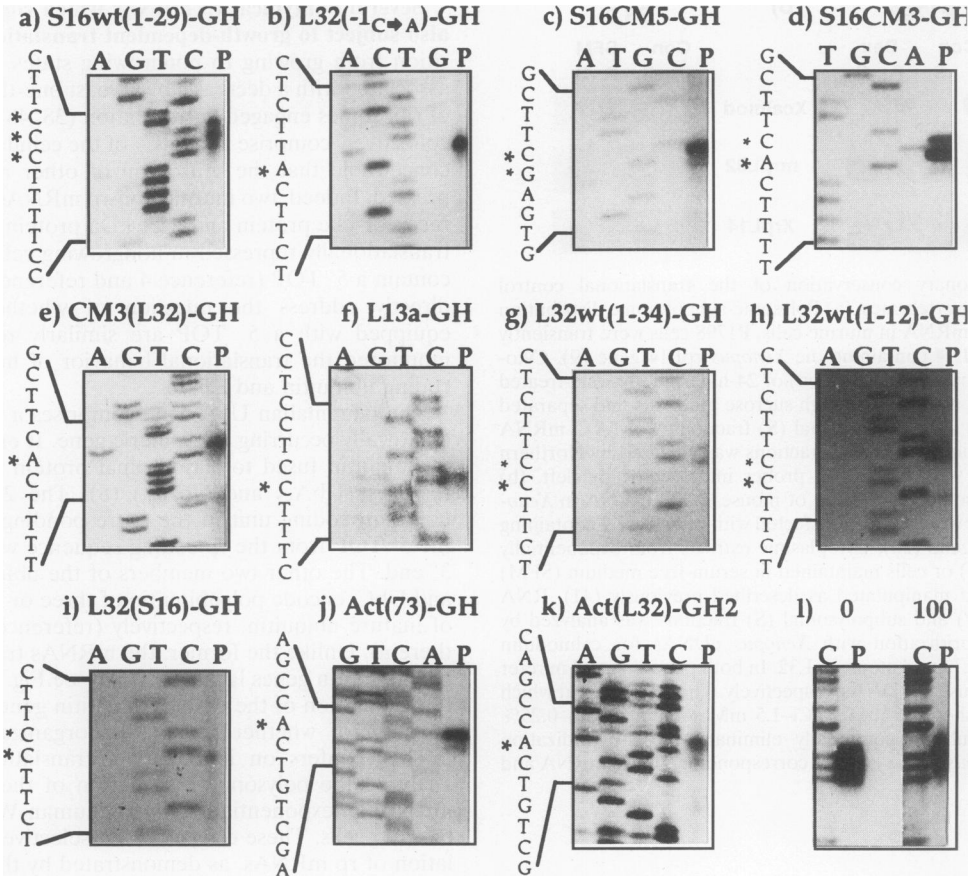

FIG. 3. Determination of the transcription start sites of various hGH chimeric mRNAs by primer extension. A 5′-end-labeled synthetic oligonucleotide complementary to nucleotides +31 to +12 of the hGH gene was annealed to 0.5 mg of poly(A)$^+$ mRNA from stably transfected NIH 3T3 cells and extended with avian myeloblastosis virus reverse transcriptase as described in Materials and Methods. The extended product (P) was analyzed on a 6% acrylamide–urea gel alongside a dideoxy sequencing reaction (A, C, G, T) in which the same primer (unlabeled) was used. (a to k) Analysis of transcription start sites by using different concentrations of NaCl in the gel loading buffer: 100 mM (a, b, and f), 25 to 50 mM (e, j, and k), and 0 mM (c, d, g, h, and i). (l) Dependence of the relative mobility of the primer-extended DNA on the concentration of NaCl (0, 0 mM; 100, 100 mM) in the gel loading buffer. Large and small asterisks represent major and minor transcription start sites, respectively.

tion of SOD mRNA and the underrepresentation of rpS4 mRNA in the polysomal fraction (Fig. 2c). The results in Fig. 2b indeed demonstrate a striking difference in the polysomal engagement of the various ubiquitin mRNAs. Thus, the majority of UbA mRNA is stored as mRNP in the subpolysomal fraction, while both UbB and UbC are efficiently translated, as indicated by their polysomal association. Similar results were observed with mouse P1798 lymphosarcoma cells, in which the UbA mRNA is selectively underrepresented in polysomes in untreated cells and further repressed upon growth arrest by dexamethasone treatment (Fig. 2b). We have previously demonstrated that the 5′ UTRs of the mRNAs encoding rpS16 and rpL30 are sufficient to confer growth-dependent translational control on hGH mRNA (39). It is conceivable, therefore, that the selective translational repression of UbA mRNA is conferred by the 5′ TOP, rather than the rp coding region, which is located at the 3′ end of this hybrid mRNA.

The human polypeptide chain EF-1α is encoded by an mRNA which bears a typical 5′ TOP: $_{+1}$CTTTTTC$_{+7}$ (72). To examine whether this mRNA is also regulated in a growth-dependent manner, we have monitored its polysomal association in various cell lines. The results presented in Fig. 2c indicate that the translation of EF-1α mRNA is repressed when HSF stop proliferating upon contact inhibition or in dexamethasone-treated mouse P1798 cells. WHT 1249 cells, in contrast, exhibit specific inhibition of EF-1α mRNA, as well as of rpS4 mRNA, even when rapidly proliferating (Fig. 2c). The selectivity of the repression in these cell lines is exemplified by the efficient translation of the SOD mRNA in WHT 1249 cells and of the actin mRNA in the other two cell lines.

The rpP2 mRNA, bearing a 5′ TOP of only five residues, is translationally controlled in a growth-dependent manner. At the time we first established the role of the 5′ TOP in the translational control of rp mRNAs, we were unaware of any natural mammalian or amphibian rp mRNAs with a 5′ TOP of less than eight pyrimidine residues (39). To establish whether mRNAs with a shorter 5′ TOP are also subject to translational control, we have compared the translational behaviors of native and chimeric mRNAs bearing a 5′ TOP of eight or fewer residues. It has been previously shown that rpS16 mRNA from mouse myeloma MPC 11 cells possesses a 5′ TOP of eight pyrimidines (20). Primer extension analysis of S16wt(1-29)-GH mRNA from stably transfected NIH 3T3 cells (Fig. 3a) and from transiently transfected P1798 cells (5) has revealed a triple transcription initiation site at three consecutive C residues, with the middle one at position +1. It should be noted, that the relative mobility of the primer extended DNA reflects the NaCl concentration in the gel loading buffer. A high

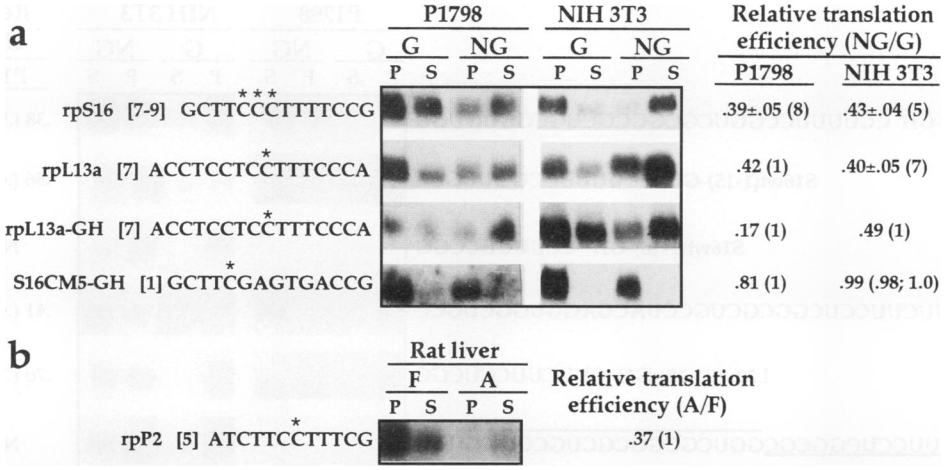

FIG. 4. Relationship between the length of the 5′ TOP and the translational repression of the respective mRNAs upon growth arrest of cultured cells (a) or upon development of rat liver (b). P1798 and NIH 3T3 cells were transiently and stably transfected, respectively, with the indicated hGH chimeric genes. Cytoplasmic extracts were prepared from growing (G) cells (untreated P1798 and NIH 3T3 cells) or fetal (F) rat liver and from nongrowing (NG) cells (24-h dexamethasone-treated P1798 cells and growth-arrested NIH 3T3 cells) or adult (A) rat liver and manipulated as described in the legend to Fig. 1. Poly(A)$^+$ mRNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization. Analysis of the endogenous rpS16 mRNA or rpL13a mRNA and the chimeric hGH mRNA from P1798 and NIH 3T3 cells, as well as the rpP2 mRNA from rat liver, was performed with the respective probes. Sequences at the left represent the nucleotides around the transcription start site (marked by asterisks). The number of transcribed residues in the 5′ TOP is indicated in brackets. The extent of ribosomal loading on the various mRNAs was determined by densitometric scanning of the autoradiograms. The relative translation efficiency of these mRNAs in nongrowing cells (NG or A) is presented as the ratio of the percentage of mRNA engaged in polysomes to that of the mRNA in growing cells (G or F) and is expressed as an average ± standard error of the mean of the number of determinations in parentheses or the average with the individual values in parentheses if only two determinations are available. Results concerning translation efficiency in NIH cells were based on an isolated clone for S16CM5-GH and an isolated clone or a polyclonal culture for L13a-GH. The autoradiogram of rpS16 mRNA in NIH 3T3 cells represents the most extreme case (NG/G = 0.28) among the five experiments used for the statistical calculation.

concentration (≥100 mM) leads to a retarded mobility relative to that of the corresponding fragment in the sequencing reaction which is loaded on the gel in a buffer containing 24 mM NaCl (Fig. 3l). A lower salt concentration (0 mM) in the gel loading buffer might lead to a relative accelerated mobility of the extended DNA (see, for examples, Fig. 3c, d, g, h, and i).

The mouse transplantation antigen P198 has been recently identified as rpL13a (13). We have performed primer extension analysis of an L13a-GH chimeric mRNA from stably transfected NIH 3T3 cells. This latter mRNA starts with an L13a sequence spanning positions +1 to +27. The transcription start site in this mRNA was identified at a C residue followed by six pyrimidines (Fig. 3f). Similar results were obtained by primer extension analysis of endogenous mouse fetal rpL13a mRNA, using a 20-mer oligonucleotide complementary to codons 19 to 13 (5). Thus, it appears that the 5′ TOP of rpL13a includes seven pyrimidines and not three as previously reported (63). The transcription of the rat rpP2 gene initiates in the middle of a stretch of 10 pyrimidines leading to an mRNA with a 5-pyrimidine-long 5′ TOP (14). This transcription start site was reconfirmed by primer extension analysis of rat liver mRNA, using a 25-mer oligonucleotide complementary to positions +285 to +261 of the rat rpP2 gene (5). The polysomal association of endogenous mRNAs encoding rpS16 and rpL13a and the chimeric mRNA encoding rpL13a-GH was analyzed in P1798 cells and NIH 3T3 cells, while that of endogenous rpP2 was analyzed in rat liver. The unloading of ribosomes from rpP2 mRNA upon development of rat liver does not reflect a general translational repression, as previous hybridization of the same blot with a probe for SOD showed an efficient translation of the respective mRNA in both developmental stages (2). Our results demonstrate that the translations of all these mRNAs, with 5′ TOPs ranging from nine to five pyrimidines, are equally repressed (by a factor of two- to threefold) upon growth arrest of P1798 or NIH 3T3 cells or during transition of rat liver into the quiescent state in the adult life (Fig. 4 and reference 2). S16CM5-GH is a mutant in which five of the eight pyrimidines in the rpS16 5′ TOP were substituted by purines (33). Our primer extension analysis indicates that the resulting mRNA initiates at a C residue corresponding to position −1 in the wild-type gene (Fig. 3c). Nevertheless, although containing a C residue at the cap site, this mRNA is not subject to growth-dependent translational control in both P1798 cells and NIH 3T3 cells (Fig. 4 and reference 39). It appears, therefore, that a single C residue at the cap site is not sufficient to render an mRNA translationally controlled. Nonetheless, the translation of a native rp mRNA with as few as five pyrimidines in its 5′ TOP is repressed in growth-arrested cells. It should be noted that throughout this report, we have referred to an mRNA as subject to translational repression whenever its polysomal association dropped by a factor of 2 in resting cells.

Are sequences downstream of the 5′ TOP involved in TLRE function? It has been previously demonstrated that the 5′ TOP plays a critical role in the translational control of rp mRNAs (37, 39). To elucidate whether the TLRE extends beyond the 5′ TOP, we used several chimeric constructs in which the hGH sequence was preceded by 5′ UTRs of various lengths from mRNAs encoding rpS16 and rpL32.

The chimeric mRNA, transcribed from S16wt(1-29)-GH (referred to as S16-GH in reference 39), bears the first 29 nucleotides of rpS16 mRNA in its 5′ terminus. The translation of this mRNA is repressed to the same extent (2- to 2.5-fold) as that of the endogenous rpS16 mRNA in nongrowing P1798 and NIH 3T3 cells (Fig. 4 and 5). Clearly, this is a specific response, as the mRNA of SOD is efficiently translated in both

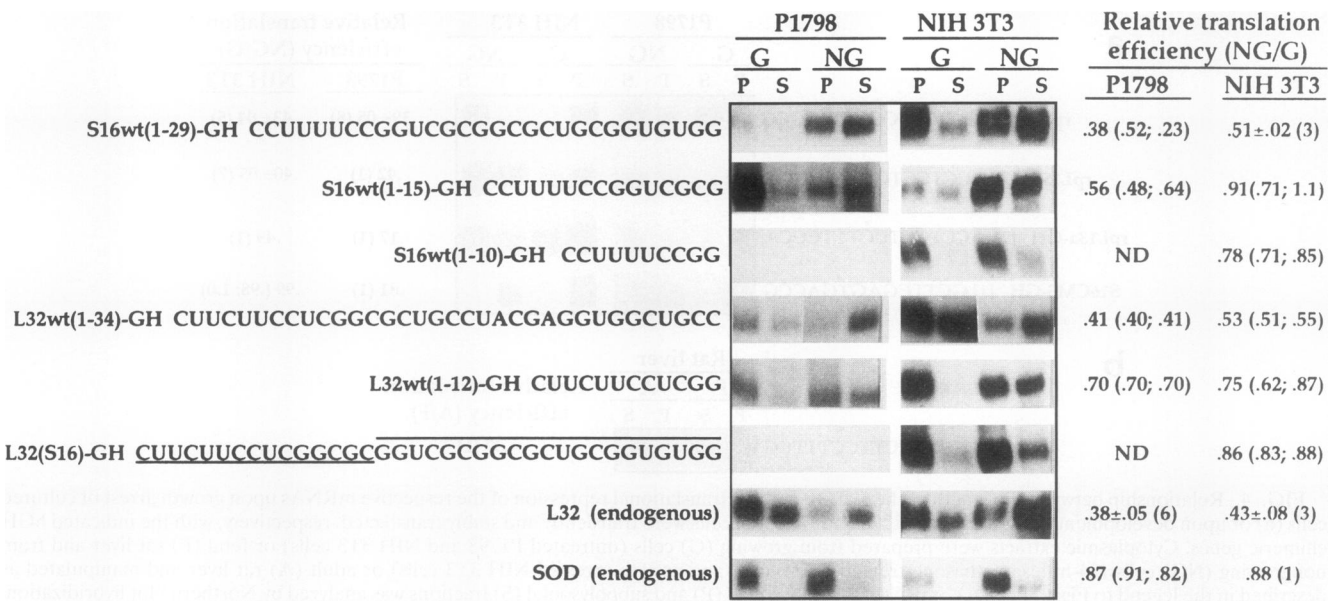

FIG. 5. Involvement of sequences downstream of the 5′ TOP in the translational control of rp mRNAs. P1798 and NIH 3T3 cells were transiently and stably transfected, respectively, with the indicated hGH chimeric genes. Cytoplasmic extracts were prepared from growing (G) cells (untreated P1798 and NIH 3T3 cells) and from nongrowing (NG) cells (24-h dexamethasone-treated P1798 cells and growth-arrested NIH 3T3 cells) and manipulated as described in the legend to Fig. 1. Poly(A)$^+$ mRNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization with the hGH probe for the respective chimeric mRNA and the SOD or rpL32 probe for the endogenous mRNA. The relative translation efficiency in nongrowing cells was determined and expressed as described in the legend to Fig. 4. Results for translation efficiency in NIH cells were based on an isolated clone, and a polyclonal culture for S16wt(1-29)-GH, a single isolated clone each for S16wt(1-15)GH, L32wt(1-34)-GH, and L32wt(1-12)-GH, a polyclonal culture for S16wt(1-10)-GH, and two independent clones for L32(S16)-GH. The mouse SOD mRNA, unlike the human counterpart, appears as a single species (62). The nucleotide sequences at the left depict the 5′-terminal rp sequences in the chimeric hGH mRNA. The underlined and overlined nucleotides in L32(S16)-GH represents sequences from L32 and S16, respectively. ND, not determined.

cell lines regardless of their growth status (Fig. 5 and reference 39). When the nucleotides spanning positions +16 to +29 of the rpS16 sequence were deleted [S16wt(1-15)-GH in Fig. 5), the repressive effect was diminished in the transiently transfected P1798 cells and completely abolished in stably transfected NIH 3T3 cells. Likewise, the 10 5′-terminal nucleotides of rpS16 mRNA (which includes the entire 5′ TOP) failed to confer translational repression on hGH mRNA in growth-arrested NIH 3T3 cells [S16wt(1-10)-GH in Fig. 5]. Primer extension analysis of the transcripts, derived from these latter two constructs, revealed that deletion of rpS16 sequences downstream to position +10 does not alter the transcription start sites relative to the endogenous rpS16 mRNA (5). Notably, the apparent expression of the S16wt(1-10)-GH gene refutes a previous notion that deletion of sequences downstream to position +10 abolished completely the activity of the rpS16 promoter (32).

The dependence of the translational repression on sequences downstream of the 5′ TOP is evident also in experiments with hGH mRNA preceded by either the first 34 [L32wt(1-34)-GH] or 12 [L32wt(1-12)-GH] nucleotides of rpL32 mRNA. While the former exhibited a repressed translation in growth-arrested cells, similar to that of the endogenous rpL32 mRNA, the responsiveness of the latter was substantially reduced (Fig. 5). The transcription start site of the respective transcripts coincides with that of the endogenous rpL32 mRNA (Fig. 3g and h and reference 75). The hGH mRNAs transcribed from the L32wt(1-12)-GH and S16wt(1-10)-GH genes preceded by a full-length 5′ TOP followed by just two G residues from rpL32 and rpS16 mRNAs, respectively. Thus, it appears that full manifestation of the translational control of these mRNAs requires sequences downstream of the 5′ TOP. To test whether sequences immediately downstream of the 5′ TOP of different rp mRNAs are interchangeable, we replaced the sequence spanning positions +16 to +34 of rpL32 5′ UTR with nucleotides +9 to +29 of rpS16 to yield L32(S16)-GH. The resulting mRNA, which initiates at the normal rpL32 transcription start site (compare Fig. 3i and g) was refractory to growth arrest, as was the mRNA with the truncated TLRE [compare L32(S16)-GH with L32wt(1-12)-GH in Fig. 5]. The significance of these results is discussed below.

Dependence of the activity of the TLRE on its distance from the cap site. The evolutionary conservation of the 5′ TOP and the cytidine residue at the cap site in every mRNA rigorously analyzed so far, and in such widely separated species as *X. laevis* and *Homo sapiens*, together with the regulatory role assigned to this element, suggests that its exact location and general structure are vital for its function. Indeed, mouse rpS16 mRNA, with its 5′ TOP situated 69 nucleotides downstream of the cap site, failed to exhibit its regulatory properties during myoblast differentiation (29). However, it was recently reported that mouse rpL32 mRNA is translationally regulated in a growth-dependent manner, even though its 5′ TOP was situated 42 nucleotides downstream of a G residue at the cap site (37).

To establish whether the activity of the TLRE is dependent on its location relative to the cap site, we analyzed several chimeric mRNAs in which the mouse rpL32 TLRE (a sequence spanning positions +1 to +34) is preceded by sequences of different lengths and compositions. hGH mRNA, preceded by the first 28 transcribed nucleotides of rat β-actin

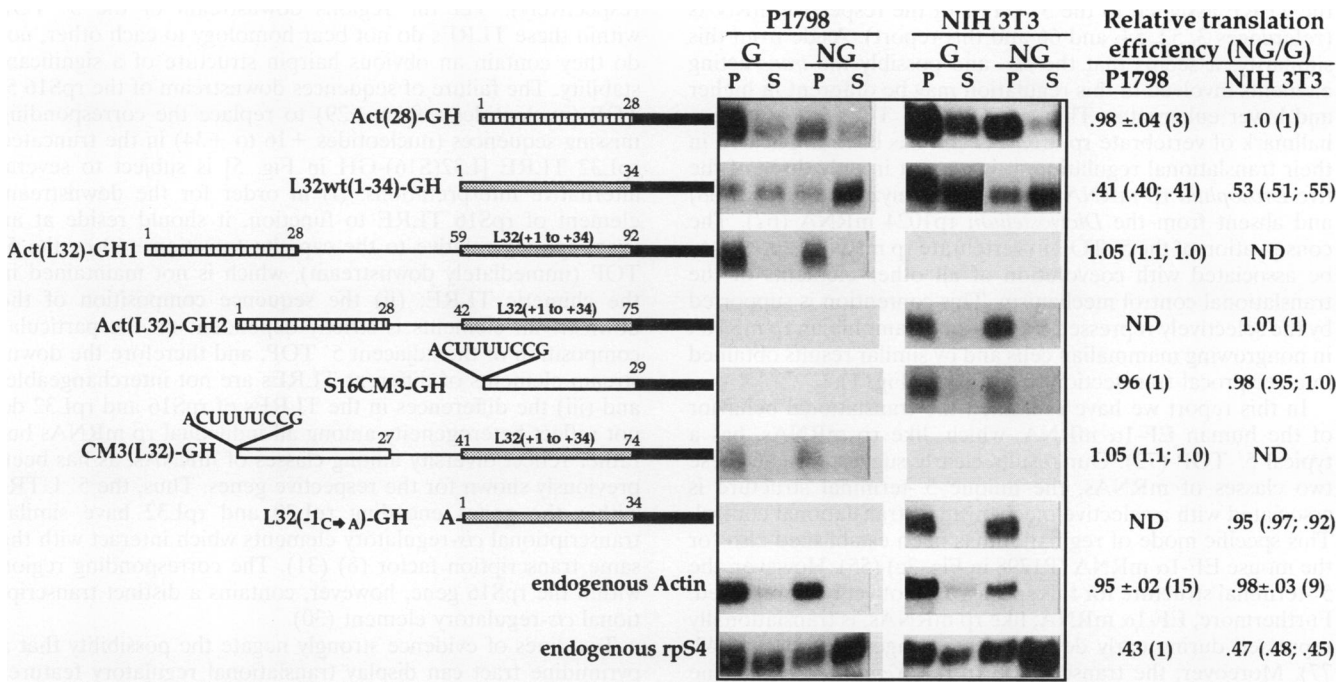

FIG. 6. Effect of the TLRE position on the translational control of chimeric mRNAs. P1798 and NIH 3T3 cells were transiently and stably transfected, respectively, with the indicated hGH chimeric genes. Poly(A)$^+$ mRNA from polysomal (P) and subpolysomal (S) fractions was analyzed by Northern blot hybridization with the hGH probe for the respective chimeric mRNA and the actin or rpS4 probe for the endogenous mRNA. The relative translation efficiency in nongrowing (NG) cells was determined and expressed as described in the legend to Fig. 4. The value of translation efficiency of S16CM3-GH mRNA in P1798 cells is from reference 39. Results for translation efficiency in NIH cells were based on an isolated clone for L32wt(1-34)-GH and Act(L32)-GH2, two independent clones for S16CM3-GH, and a polyclonal culture for Act(28)-GH and L32($-1_{C\rightarrow A}$)-GH. The filled, open, stippled, and hatched bars represent the hGH gene and the 5′ UTRs of mouse rpS16, mouse rpL32, and rat β-actin mRNAs, respectively. Polylinker sequences are denoted by a solid line. The oligopyrimidine sequences near the 5′ terminus of S16CM3-GH is presented in the corresponding schemes. G, growing cells; ND, not determined.

mRNA, is efficiently translated, regardless of the growth status of P1798 or NIH 3T3 cells [Act(28)-GH in Fig. 6]. The respective mRNA starts at the A residue at position +1 (5). This residue coincides with cap site of a similar transcript containing the first 73 nucleotides of rat β-actin [Act(73)-GH in Fig. 3j] and the human β-actin mRNA (50), rather than the C residue previously assigned as the cap site in rat β-actin mRNA (51). The 34-nucleotide-long TLRE of rpL32 mRNA can confer translational control on hGH mRNA, provided that its TOP (CUUCUUCCUC) is located at the 5′ terminus [L32wt(1-34)-GH in Fig. 6]. Insertion of this TLRE into Act(28)-GH so that the respective 5′ TOP is located at a distance of 59 or 42 nucleotides from the cap site [in Act(L32)-GH1 or Act(L32)-GH2, respectively] failed to confer translational control on the resulting mRNAs (Fig. 6). The major cap site in Act(L32)-GH2 mRNA is at the same A residue as in Act(73)-GH (compare Fig. 3k and j).

S16CM3-GH is a mutant in which the C residue at position +1 was replaced by an A residue (33) and transcription largely initiates at this A residue (Fig. 3d and e). The S16CM3-GH mRNA is efficiently translated in a growth-independent manner in either cell line (39) (and S16CM3-GH in Fig. 6). This lack of translational repression in nongrowing cells is evident, even though a tract of seven pyrimidines (CUUUUCC) follows immediately the A residue at the cap site. Insertion of the rpL32 TLRE at a distance of only 42 nucleotides downstream of the cap site failed to resume the translational control to the slightly modified rpS16 TLRE [compare CM3(L32)-GH and S16CM3-GH in Fig. 6].

To test whether the translational regulatory mechanism will tolerate a shift of the TLRE by a single nucleotide from its 5′-terminal position, we replaced the C residue at position −1 of L32wt(1-34)-GH by an A residue. This substitution led to a shift of the transcription start site to the new A residue, leaving the TLRE intact (unlike the case of S16CM3-GH mRNA, in which the 5′ TOP was shortened by one residue) but separated from the cap site by one residue (Fig. 3b). The resulting transcript was efficiently translated regardless of the growth status of the transfected NIH 3T3 cells [L32($-1_{C\rightarrow A}$)-GH in Fig. 6]. Our results suggest, therefore, that an intact rpL32 TLRE is not functional when removed from the cap site by even a single nucleotide.

It should be noted that in previous reports, the transcription start sites in mRNAs encoding S16CM5 and S16CM3 were assigned to residues which do not coincide with those demonstrated here (Fig. 3c and d and reference 33). This discrepancy might reflect (i) the different methods used (S1 nuclease mapping versus primer extension, respectively), (ii) the different constructs used (the mutation is within the context of the entire rpS16 gene versus a hGH chimeric gene, respectively), or (iii) the different genomic contexts (episomal plasmid versus stably integrated gene, respectively).

DISCUSSION

Control of rp gene expression at the translational level appears to be a prevalent regulatory mechanism in *Dictyostelium discoideum*, *Drosophila melanogaster*, and vertebrates with

the TLRE assigned to the 5′ UTRs of the respective mRNAs (references 3, 52, 54, and 66 and this report). Aside from this similarity, it seems that the *cis*- and possibly the *trans*-acting elements involved in this regulation may be different in higher and lower eukaryotes. Thus, while the 5′ TOP has become a hallmark of vertebrate rp mRNAs and has been implicated in their translational regulation, it is present in only three of the five *Drosophila* rp mRNAs rigorously analyzed so far (52, 68) and absent from the *Dictyostelium* rp1024 mRNA (67). The conservation of the 5′ TOP in vertebrate rp mRNAs appears to be associated with coevolution of all other elements of the translational control mechanism. This contention is supported by the selectively repressed translation of amphibian rp mRNA in nongrowing mammalian cells and by similar results obtained in a reciprocal transfection experiment (Fig. 1).

In this report we have examined the translational behavior of the human EF-1α mRNA, which, like rp mRNAs, has a typical 5′ TOP (72). Our results clearly suggest that in these two classes of mRNAs, the unique 5′-terminal structure is associated with a selective mechanism of translational control. This specific mode of regulation has been established also for the mouse EF-1α mRNA (P1798 in Fig. 2c) (55). However, the 5′-terminal structure for this species has not yet been reported. Furthermore, EF-1α mRNA, like rp mRNAs, is translationally repressed during early developmental stages of *X. laevis* (42, 77). Moreover, the transcription of the *Xenopus* EF-1α gene starts at a C residue followed by eight pyrimidines (42).

The evolutionary conservation of eight or more pyrimidine residues in the 5′ TOPs of 14 of 17 vertebrate (amphibian, avian, and mammalian) rp mRNAs rigorously analyzed thus far (3, 14, 18, 54, 69, 71) might reflect a minimal requirement for an optimal regulatory function. Nevertheless, data presented here indicate that the translation of endogenous mRNAs bearing a 5′ TOP of seven (mouse rpL13a) or just five (rat rpP2 and) residues is down regulated in growth-arrested cells to the same extent as that of rp mRNAs with a longer 5′ TOP. One plausible explanation for the exceptionally short 5′ TOP in rpP2 mRNA is that a unique nucleotide composition downstream of the 5′ TOP provides an appropriate context, which compensates for its shorter than normal 5′ TOP. This notion is further supported by our observation that the 5′ TOP, although necessary, is not sufficient for full manifestation of the growth-dependent translational control, at least in the case of the mRNAs encoding rpL32 and S16. Thus, the ability of the 34- and 29-nucleotide-long 5′ TLREs, respectively, of these mRNAs to confer translational control on hGH was impaired upon deletion of the 3′ portion of this region, even if the 5′ TOP remained intact. It can be argued that this disturbed regulation results from shortening the distance between the 5′ TOP and the initiation codon within the hGH mRNA [from 94 to 72 and from 89 to 68 in L32wt(1-12)-GH and S16wt(1-10)-GH, respectively]. However, this possibility seems unlikely, as even smaller spacing between these two elements (ranging from 22 to 70 nucleotides) is common for the native rp mRNAs already shown to be translationally regulated in a growth-dependent manner, for example, mRNAs encoding rpL32, rpS16, rpL30, rpL13a, and rpP2 (14, 20, 63, 73, 75). Moreover, reexpansion of the spacing between the 5′ TOP and the translation initiation codon by insertion of 21 nucleotides from the rpS16 TLRE (nucleotides +9 to +29) did not resume the translational control (Fig. 5). Our interpretation is, therefore, that sequences downstream of the 5′ TOP are involved in the translational control of mRNAs encoding rpS16 and rpL32.

Clearly, a relatively short sequence is sufficient to confer translational control on hGH mRNA (the first 34, 29, and 27 nucleotides of mRNAs encoding rpL32, rpS16, and rpL13a, respectively). Yet the regions downstream of the 5′ TOP within these TLREs do not bear homology to each other, nor do they contain an obvious hairpin structure of a significant stability. The failure of sequences downstream of the rpS16 5′ TOP (nucleotides +9 to +29) to replace the corresponding missing sequences (nucleotides +16 to +34) in the truncated rpL32 TLRE [L32(S16)-GH in Fig. 5] is subject to several alternative interpretations: (i) in order for the downstream element of rpS16 TLRE to function, it should reside at an exact spacing relative to the cap site (position +9) or the 5′ TOP (immediately downstream), which is not maintained in the chimeric TLRE; (ii) the sequence composition of the downstream elements is strictly dependent on the particular composition of the adjacent 5′ TOP, and therefore the downstream elements of different TLREs are not interchangeable; and (iii) the differences in the TLREs of rpS16 and rpL32 do not reflect heterogeneity among all individual rp mRNAs but rather reflect diversity among classes of mRNAs, as has been previously shown for the respective genes. Thus, the 5′ UTRs within the genes encoding rpL30 and rpL32 have similar transcriptional *cis*-regulatory elements which interact with the same transcription factor (δ) (31). The corresponding region within the rpS16 gene, however, contains a distinct transcriptional *cis*-regulatory element (30).

Two lines of evidence strongly negate the possibility that a pyrimidine tract can display translational regulatory features regardless of its position: (i) the strictly conserved location of the oligopyrimidine tracts in the 5′ termini of all vertebrate rp mRNAs and (ii) the presence of seemingly perfect pyrimidine tracts in internal positions within the 5′ UTRs of many mRNAs that are not translationally regulated. One such an example is the mRNA encoding mouse hypoxanthine phosphoribosyltransferase, which contains in its 5′ UTR two such tracts, CCUCCUCC and CUUCCUCCUC (44), of which the latter has 90% homology with the 5′ TOP of mouse rpL32 mRNA (Fig. 5). Nonetheless, the translation of this mRNA is not repressed in nongrowing cells (48). Indeed, our results for the rpL32 TLRE (nucleotides +1 to +34) clearly demonstrate that although this sequence is sufficient to confer translational control on hGH mRNA, it fails to exert this effect when preceded by even a single nucleotide (Fig. 6). Evidently, this loss of function occurs regardless of the base composition of the sequence separating the 5′ TOP from the cap site (Fig. 6). Hence, our observations together with those of Hammond et al. (29) strongly support the notion that the TLRE is functional in a position-dependent manner. This conclusion is not unprecedented, as similar dependence has been previously demonstrated for ferritin mRNA. This mRNA contains a conserved 28-nucleotide region in its 5′ UTR, which has been referred to as the iron-responsive element (IRE). This element folds into a stem-and-loop structure and is recognized by a 90-kDa translational regulatory protein (IRE-binding protein) (70). Displacement of the IRE 67 nucleotides or more downstream of the cap site leads to a complete loss of the translational control function of this element (26). It appears, therefore, that the results obtained with the Rous sarcoma virus-L32 chimeric mRNA (37) might reflect an exceptional case in which the Rous sarcoma virus long terminal repeat sequences upstream of the 5′ TOP of rpL32 mRNA provide an appropriate context that compensates for its internal location or an alternative 5′ TOP.

The repressed translation of rp mRNAs frequently observed in nongrowing cells does not necessarily indicate that the translation efficiency of these mRNAs is strictly dependent on the cellular growth status. Thus, the translation of mRNAs encoding ribosomal proteins EF-1α and UbA_{52} is repressed in

exponentially growing human WHT 1249 lymphoblastoid cells. Likewise, the translation of rp mRNAs is selectively repressed during early *Xenopus* embryogenesis, despite the rapid cell proliferation characterizing this developmental stage (3). Uncoupling between fluctuations in translation efficiency and growth status has been demonstrated also in insulin-treated chicken embryo fibroblasts and mouse myoblasts. Under these circumstances, the enhanced translation efficiency of rp mRNAs was not accompanied by a parallel change in the proliferation rate (19, 28). Presumably, the activity of a putative *trans*-acting factor, which is involved in controlling the translation efficiency of 5′ TOP-containing mRNAs, is modulated in a growth-dependent manner. However, it seems to be independently regulated under certain developmental and hormonal conditions. The exceptional behavior of WHT 1249 cells might result from their viral transformation, their unique developmental stage (lymphoblastoids) in the course of B-cell differentiation, or an as yet unknown cause.

Conceivably, the 5′ TOP and perhaps additional sequences within the TLREs of the mRNAs encoding ribosomal proteins UbA_{52} and EF-1α are recognized by a protein(s) which is involved in their unique translational control. The nature (general translation initiation factor or a specific factor) or the mode of regulation (repressor or activator) of this presumptive *trans*-acting factor is presently obscure. A simple model to account for the selective translational control of rp mRNAs posits the participation of the limiting initiation factor eIF-4E, which binds specifically to the mRNA cap structure (reference 21 and references therein). If this factor had a particularly low affinity for rp mRNAs, because their unique 5′ termini, a decrease in its activity or content could lead to a selective diminution in the utilization of these mRNAs. Indeed, we have observed parallel fluctuations in the utilization of rp mRNAs and in the abundance of eIF-4E (2, 61). Moreover, supplementing reticulocyte lysate with the initiation complex eIF-4F, which contains eIF-4E, could selectively increase the translation efficiency of rp mRNAs, which was otherwise repressed (29). We have recently examined the proposed model, and our results have clearly demonstrated that (i) the presence of an intact 5′ TOP does not diminish the affinity for the cap-binding protein and (ii) overexpression of eIF-4E in its active (phosphorylated) form does not relieve the repressed translational of rp mRNA in resting cells, thus excluding its key role in the repression of these mRNAs (61).

The involvement of oligopyrimidine tracts in cellular processes is not confined to the translational control described here. These sequences have been previously implicated also in the cap-independent translation of picornavirus RNAs (references 36 and 64 and references therein) and in the splicing of pre-mRNAs (22). Interestingly, a 57-kDa protein which specifically binds to polypyrimidine tracts (PTB) and has been purified from nuclei and cytosol was suggested to be engaged in the splicing process and translation of picornavirus (25, 34, 53). The sequence of a tryptic peptide was used to isolate three classes of cDNAs encoding different forms of PTB (25). It is noteworthy that a cytoplasmic protein of about 56 kDa from mouse and *Xenopus* cells has been recently shown to specifically bind to oligopyrimidine sequences (12, 37). It remains, however, to be established whether this protein is involved in the translational control of rp mRNA.

Several lines of evidence suggest that any of the known PTBs are either irrelevant or insufficient for the selective translational control of 5′ TOP-containing mRNA: (i) the oligopyrimidine tract in rp mRNAs is strictly localized to the 5′ terminus and loses its regulatory properties when separated by a single adenosine from the cap site, while both PTB and the 56-kDa protein have been shown to bind to oligopyrimidine tracts in internal positions (22, 37); and (ii) the binding activity of the 56-kDa protein, unlike the translational control of rp mRNAs, is not regulated in a growth-dependent manner in mouse cells (37) or developmentally regulated in *X. laevis* (12). The present demonstration that also sequences downstream of the 5′ TOP are essential for the translational control of rp mRNAs suggests that additional *trans*-acting factors might be involved in this regulatory mechanism. Nevertheless, establishment of the physiological significance of a 5′ TOP-binding protein, or other TLRE-binding proteins, will require functional analysis of their roles in the translational control of the target mRNAs.

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