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Growth-dependent and growth-independent translation of messengers for heterogeneous nuclear ribonucleoproteins

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ABSTRACT

The hnRNP A1 transcript has a relatively short 5′-untranslated region (UTR) starting with a pyrimidine tract similar to that of mRNAs encoded by the TOP [terminal oligo(pyrimidine)] genes in vertebrates. Such genes code for ribosomal proteins and for other proteins directly or indirectly involved in the production and function of the translation apparatus. As expected from the role of the pyrimidine tract in the translational regulation of TOP mRNAs, the A1 mRNA is more efficiently loaded onto polysomes in growing than in resting cells. On the other hand, a less stringent regulation with respect to that of other TOP mRNAs is observed, partially due to the presence of multiple transcription start sites within the pyrimidine tract, where transcripts with shorter TOP sequences are less sensitive to regulation. Thus, from the point of view of structural features and translation behaviour the A1 mRNA can be included in the class of TOP genes, suggesting a possible role of A1 in translation. Interestingly, a TOP-like behaviour was observed for hnRNP I mRNA but not for hnRNP C1/C2 and A2/B1 mRNAs, indicating the existence of two classes of hnRNPs with different translational regulation.

INTRODUCTION

In the nucleus of eukaryotic cells pre-mRNAs undergo a series of modifications before mature mRNAs are produced and eventually exported to the cytoplasm for translation. These post-translational modifications include 5′-end capping, splicing of introns and polyadenylation. All these reactions appear to occur shortly after or concomitant with transcription by RNA polymerase II. Throughout these maturation events pre-mRNAs and mRNAs are associated with a set of ~20 abundant proteins, collectively termed heterogeneous nuclear ribonucleoproteins or hnRNPs (1,2). Although the precise role of these proteins is still undefined, experimental evidence suggests, in addition to a structural role, a direct involvement in both splicing reactions and in assisting or promoting the export to the cytoplasm of mature mRNAs. In particular, basic hnRNP proteins belonging to the A/B group were reported to antagonize, both *in vitro* and *in vivo*, the activity of splicing factors such as ASF/SF2 in the choice between alternative 5′-splice sites and in exon skipping/inclusion (3,4). With regard to the nucleocytoplasmic distribution, hnRNPs can be divided into two groups. The first group comprises proteins such as hnRNP C1/C2 and U, which are restricted to the cell nucleus (5). The second group contains hnRNP A1, A2, B1 and K, which have been shown to shuttle rapidly between the nucleus and the cytoplasm (6). In the case of hnRNP A1 (and A2) this property is mediated by a 29 amino acid sequence in the Gly-rich auxiliary domain which acts both as nuclear localization determinant and as nuclear export sequence (6–8). This fact, along with the observation that hnRNP A1 is bound to poly(A)$^+$ RNAs in both the nucleus and the cytoplasm, led to the proposal of a direct role for this protein in the export of mature mRNAs to the cytoplasm (9). This conclusion is also supported by the observation that the hrp36 protein of *Chironomus tentans*, highly similar to mammalian A1, is translocated through the nuclear pore bound to giant Balbiani ring RNAs and appears to remain in RNP complexes after polysome assembly, suggesting a role of this protein in the translation process (10).

We previously reported isolation of the human gene for the hnRNP A1 protein (11). Sequence analysis shows a strong similarity between the A1 gene and a number of genes coding for ribosomal proteins (r-proteins) in the transcription start site region (12,13). In fact, all of these genes lack a canonical TATA box and are characterized by a transcription start site that falls within a stretch of 10–25 pyrimidines (Fig. 1). As a consequence, the corresponding mRNAs always start with a C followed by a pyrimidine tract, called 5′-TOP [5′-terminal oligo(pyrimidine)]. This sequence was shown to be involved in the coordinated growth-dependent translational regulation of all 5′-TOP-containing genes (TOP genes). The specific translational regulation of r-protein mRNAs and other TOP mRNAs can be observed in cultured cells during

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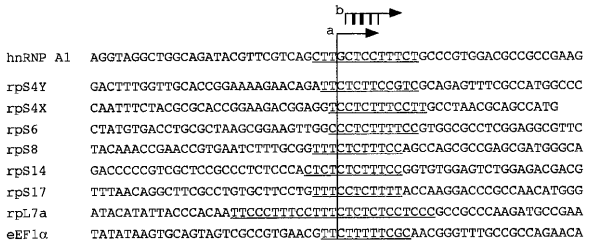

Figure 1. Comparison of the sequences surrounding the transcription start site of the human hnRNP A1 gene with those of human TOP genes (seven ribosomal proteins and the translation factor eEF1α; for references see 12). The pyrimidine tracts are underlined. The previously determined transcription start site of hnRNP A1 mRNA (11) and those of the other mRNAs are aligned under arrow a. The alternative transcription start sites of hnRNP A1 mRNA identified in this paper are indicated by arrow b. The two thicker lines correspond to the two stronger bands observed in the primer extension ladder (see Fig. 3).

the transition from the growing to the non-growing state that occurs in response to a number of physiological stimuli. For instance, in mouse fibroblasts (14,15) and *Xenopus* kidney cultured cells (16) it was shown that transfer of cells to serum-free medium (downshift) produces a rapid and coordinated displacement of r-protein mRNAs and other TOP mRNAs from polysomes to light mRNPs. Restoration of serum content (upshift) induced a similarly rapid return to the original conditions. In this paper we show that the hnRNP A1 gene is translationally regulated in a similar way and can therefore be assigned to the class of TOP genes.

MATERIALS AND METHODS

Cell culture

HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium; Sigma), 10% fetal calf serum, 50 µg/ml gentamicin, 2 mM L-glutamine. Cells were rinsed twice with PBS and detached with a limited amount of trypsin to induce a 'downshift'. After resuspension in PBS to dilute trypsin, cells were centrifuged for 5 min at 2000 *g* at 4°C, resuspended in serum-free medium and incubated at 37°C for a further 4 h.

Polysome isolation

The procedure for cell lysis, sucrose gradient sedimentation of polysomes and analysis of the polysome/mRNP distribution of mRNAs have been discussed (12). Cells were lysed directly on the plate with 300 µl lysis buffer [10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, 1% Triton-X100, 1% sodium deoxycholate, 36 U/ml RNase inhibitor (Pharmacia), 1 mM dithiothreitol] and transferred to an Eppendorf tube. After 5 min incubation on ice with occasional vortexing, the lysate was centrifuged for 8 min at 10 000 r.p.m. at 4°C. The supernatant was frozen in liquid nitrogen and stored at –70°C, to be analysed later, or immediately sedimented in a 5–70% (w/v) sucrose gradient with a low absorbance background (17). Fractions, collected while monitoring the optical density at 254 nm, were ethanol precipitated overnight at –20°C.

Extraction and analysis of RNA

Total RNA was extracted from gradient fraction pellets by the proteinase K method (18). For Northern blot analysis RNA was fractionated on formaldehyde–agarose gels and transferred to Gene Screen Plus membrane (NEN); Northern blots were done essentially according to the manual. Radioactive probes were prepared by the random primer technique (18) using as templates the inserts of plasmids containing cDNAs for human rp-L4 (19), chicken β-actin (20), hnRNP A1 (21), hnRNP C1/C2 (22), hnRNP A2/B1 (23) and hnRNP I (24). Autoradiographs were analysed with a LKB Ultroscan KL laser densitometer. Quantitation standards were included in some blots to establish the linear range response of the experiments.

Primer extension

Primer extension was performed on total, polysomal and sub-polysomal RNAs fractionated on sucrose gradients as described above. The reaction was performed using the primer previously described (11). Briefly, 10 pmol gel purified 20mer 5′-AAGAG-AGACTTTAACGATGC-3′, complementary to nt –33 to –13 with respect to the ATG, were end-labelled with 50 µCi [γ-^{32}P]ATP (5000 Ci/mM; Amersham) using T4 polynucleotide kinase (Promega). Aliquots of 2.5×10^5 c.p.m. labelled primer were resuspended together with the RNA fractions in 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, 400 mM NaCl, 80% formamide, denatured for 10 min at 85°C and annealed at 54°C for 16 h. The annealed RNA–primer complexes were ethanol precipitated and dried at room temperature. Primer extension was performed in a final volume of 20 µl containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM each dNTP, 1 µl RNasin (20 U/ml; Promega) and water up to 20 µl. To this was added 50 U M-MuLV reverse transcriptase (Perkin Elmer) and the reaction incubated for 30 min at 37°C. The reaction was stopped by adding 1 µl 0.5 M EDTA, pH 8.0 and 1 µl DNase-free RNase A (5 mg/ml; Boehringer) and incubated for an additional 30 min at 37°C. The reaction products were added with 150 µl 0.1 M NaCl, phenol extracted twice and ethanol precipitated. The pellet was dried and resuspended in 10 µl 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, heat denatured and loaded onto an 8% sequencing denaturing gel.

RESULTS

Translational regulation of hnRNP A1 mRNA in HeLa cells during nutritional changes

In order to see whether the hnRNP A1 gene is regulated at the translational level similarly to the 5′-TOP genes, we analysed by Northern blot experiments the polysome/mRNP distribution of hnRNP A1-specific mRNAs in growing and resting HeLa cells. HeLa cells, grown in the presence or absence of serum, were lysed and the cytoplasmic extracts fractionated through 5–70% sucrose gradients (see Materials and Methods). Eight fractions were collected from each gradient while recording the absorbance profile. As shown in Figure 2, most of the ribosomes purified from HeLa cells after serum starvation are in the form of monomers, whilst most ribosomes are associated in polysomes when cells are grown in the presence of 10% serum. The RNA was then extracted from each fraction and analysed by agarose gel electrophoresis and Northern blot hybridization. As a control, RNAs were first probed with the cDNA of the non-TOP β-actin

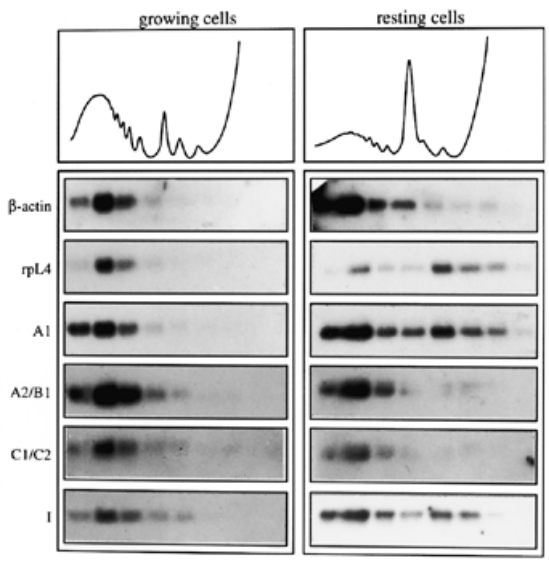

Figure 2. Polysome/mRNP distribution in growing and resting cells of the mRNAs for hnRNP A1, A2/B1, C1/C2 and I. The distribution of β-actin and rp-L4 mRNAs is shown for comparison. (Upper) Optical density profiles of sucrose gradients of cytoplasmic extracts prepared from exponentially growing and down-shifted cells (5–70% sucrose right to left). (Lower) Northern blot analysis of the RNAs extracted from sucrose gradient fractions.

and of the TOP ribosomal rp-L4 proteins. The same filter was then hybridized with cDNAs of four different hnRNPs, namely hnRNP A1, hnRNP A2/B1, hnRNP C1/C2 and hnRNP I. The results of the different hybridizations are shown in Figure 2. As expected, the non-TOP β-actin mRNA was mostly (>90%) associated with polysomes in both growing and resting cells. In contrast, while 95% of the TOP rp-L4 mRNA was loaded onto polysomes in growing cells, only ~25% was loaded in resting cells, the remaining 75% being shifted to light mRNPs. The A1 mRNA showed an intermediate behaviour between the non-TOP β-actin mRNA and the typical TOP rp-L4 mRNA. In fact, A1 mRNA loading onto polysomes was almost complete (>90%) in growing cells but only partial (65%) in resting cells. This result indicates that the A1 gene is actually regulated at the translation level, although less stringently than other typical TOP mRNAs. It should be noted that in this particular experiment differential loading of the rp-L4 mRNA on polysomes was particularly effective, 90% and 25% in growing and resting cells respectively. These figures can vary somewhat, 70–90% and 20–40% respectively being typical figures in different experiments. This variability is not unexpected, as growth stimulation depends on poorly controlled factors such as the particular serum batch used, the manufacturing company and also the cell concentration in the culture dish. To minimize these problems we always compared parallel cultures under stimulated and unstimulated conditions and the analysis of different mRNAs was done by reprobing the same filter.

In contrast to A1 mRNA, the polysome/mRNPs distribution of A2/B1 mRNA did not vary with growth conditions, >90% being on polysomes in both resting and growing cells, as expected from the fact that its transcription start site does not map within a pyrimidine tract (25). We also controlled the behaviour of two additional hnRNP mRNAs: that of the acidic hnRNP C1/C2 proteins and that of hnRNP I/PTB (polypyrimidine tract binding protein). While >95% of the hnRNP C1/C2 mRNA was loaded on polysomes even under serum-free conditions, the hnRNP I-specific mRNA was associated with polysomes in a growth-dependent manner (~85% in exponentially growing cells versus ~55% in resting cells).

HnRNP A1 mRNAs starting at adjacent sites are subject to different translational regulation

We have previously found, by primer extension and RNase protection experiments, that the major transcription start site of the hnRNP A1 mRNA in HeLa cells covers a limited number of adjacent nucleotides (11). Two minor start sites were also detected at about –20 and –40 nt upstream of the major start sites, but they will not considered here since they are used in <5% of the cases. Translational regulation of TOP mRNAs strictly depends on sequences at the 5′-end, thus it is conceivable that A1 mRNAs differing at their 5′-end, even by a few nucleotides, might be differentially regulated. To explore this possibility, cytoplasmic extracts from growing and resting HeLa cells were fractionated on sucrose gradients as described above. Fractions from each gradient, corresponding respectively to polysomes (P) and to the sub-polysomal mRNPs (S), were pooled and the extracted RNA analysed by primer extension with a 20mer primer complementary to nt –33 to –13 with respect to the AUG. Phosphorimager quantitation of radioactive signals obtained in four independent experiments revealed that, on average, 71.25 ± 5.65% of A1 mRNA was loaded onto polysomes in growing cells, compared with 27 ± 1.74% in resting cells. The pattern shown in Figure 3A is consistent with the previously described microheterogeneity of transcription start sites clustered within a limited number of adjacent nucleotides (11). Notice that the first of the five most represented bands is located 1 nt downstream with respect to the previously reported transcription start site (see Figs 1 and 3). This discrepancy is due to the fact that in the previous paper an unrelated molecular weight marker ladder was used, while here the size markers are sequencing reactions carried out on the hnRNP A1 genomic clone (11) with the same oligonucleotide primer used for RNA analysis. As can be seen, the 5′-end of the longest hnRNP A1 mRNA perfectly matches a true TOP sequence (5′-CUCCUUUCU-3′). Phosphorimager quantitation of the experiment in Figure 3A and of three more experiments showed that A1 mRNAs with shorter TOP sequences are loaded onto polysomes more efficiently, under both growing and resting conditions (Fig. 3). The fact that identification of the 5′-end by this technique might have a ±1 nt uncertainty does not hinder this conclusion.

DISCUSSION

In this paper we extend our previous analysis of expression of the hnRNP A1 gene (26) and demonstrate the existence of a translational control mechanism which determines a lower fraction of A1 mRNA loaded onto polysomes in resting cells compared with proliferating cells. HnRNP A1 mRNAs starting at adjacent nucleotides are subject to different translational regulation, supporting the concept that the observed phenomenon depends on the length of the pyrimidine tract at the 5′-end of the mRNA. On the basis of these data the A1 gene can be assigned to the class of TOP genes that include all genes coding for r-proteins and some of those coding for translation factors and nucleolar proteins, all

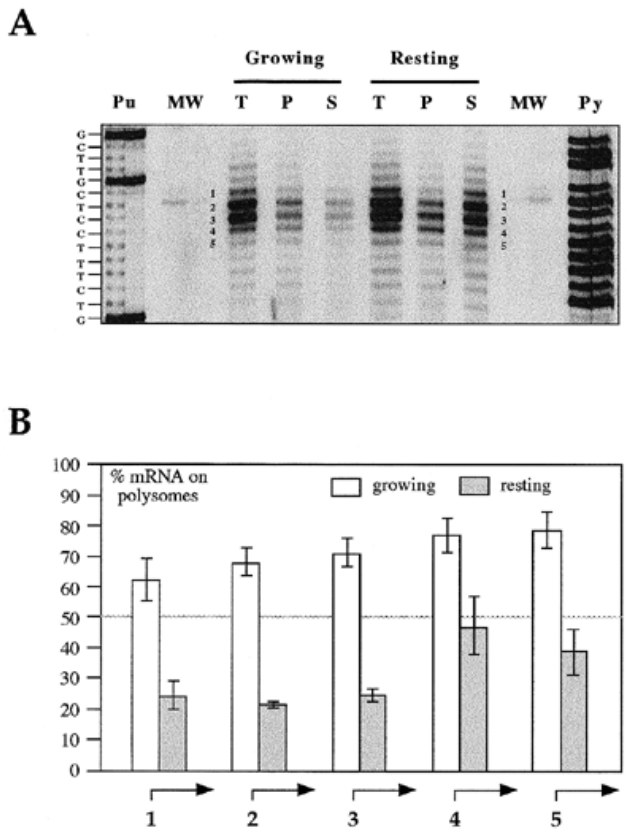

Figure 3. Primer extension analysis carried out on total HeLa cell RNA (T) or on polysomal (P) and sub-polysomal (S) mRNAs purified either from exponentially growing or resting cells. Primer extension was performed as described in Materials and Methods using a primer complementary to nt –33 to –13 with respect to the AUG. (**A**) Primer extension products were run on an 8% sequencing gel, along with sequencing reactions for purines (Pu) or pyrimidines (Py) obtained by extending the same primer on the hnRNP A1 genomic clone (11). MW, the 51 nt band of the molecular weight marker pBR322 DNA digested with *Hae*III. The five major transcription start sites are indicated by numbers on the left and on the right of the primer extensions. The sequence of the entire region is shown on the left of the panel. (**B**) Phosphorimager quantification of the radioactive signals corresponding to the five major transcription start sites obtained in four independent experiments. The percentages of mRNA loaded on polysomes in exponentially growing and resting cells for each transcription start site are indicated: rectangles represent the mean values, while standard deviations are indicated by error bars.

of them involved, directly or indirectly, in the synthesis and function of the translational apparatus (12,13).

This finding offers a plausible explanation for the previously reported dependence of A1 protein levels on cell proliferation state (27,26). A1 belongs to a subset of hnRNPs, termed 'core' proteins, that can be isolated after mild RNase digestion in the form of 30–40 S particles associated with RNAs of ~700 nt (28,29). In proliferating cells particle 'core' proteins exist in a fixed stoichiometry: 3A1:3A2:3C1:1B1:1B2:1C2 (27). Protein A1 is unique among the 'core' proteins in that its level is the only one which decreases drastically when the cells reduce their growth rate [e.g starving HeLa cells (30), resting rat liver cells (31) or resting lymphocytes (28)]. As a consequence, the stoichiometry of the 'core' proteins in resting cells is 1A1:3A2:3C1:1B1:1B2:1C2. We have previously studied expression of the hnRNP A1, A2/B1 and C1/C2 genes by measuring the level of respective mRNAs in different cell lines under different growth conditions (26). From that analysis we concluded that expression of both the A1 and A2/B1 genes, but not of C1/C2, is controlled at the transcriptional level in a cell proliferation-dependent manner. Coordinated transcription of the A1 and A2/B1 genes does not explain the different A1:A2 protein ratios observed in proliferating versus resting cells, thus suggesting the existence of further levels of gene expression control. The data reported in this paper can account for the discrepancy observed between mRNA and protein levels. In fact, A2/B1 mRNA appears to be translated to the same extent in both resting and proliferating HeLa cells, while A1 mRNA is more efficiently utilized in growing cells compared with resting cells. This different behaviour could be due to the fact that the transcription start site of the A2 mRNA does not map within a pyrimidine tract. Interestingly, A1 is not the only hnRNP protein subject to this control, since a similar differential loading on polysomes was observed in the case of hnRNP I mRNA. Thus hnRNP genes can be grouped into two classes according to their translational regulation, a novel parameter that could help in understanding the role of individual hnRNP proteins in RNA metabolism. On the basis of these results and considerations we also anticipate that hnRNP I (but not hnRNP C) mRNA could have a 5′-TOP sequence.

At present it is unknown why A1 and A2, in spite of their apparently close structural and functional relatedness, are translationally controlled in such a different way. As a matter of fact, A2 shares with A1 the ability to affect alternative splicing *in vitro* (3) and to shuttle continuously between the nucleus and the cytoplasm (9). On the other hand, a specialized function of the two proteins is suggested by the fact that A2, in contrast to A1, is heavily phosphorylated in a cell cycle-dependent manner (32). Moreover, at steady-state ~60% of A2 can be recovered in the cytoplasm of HeLa cells, while most of A1 appears to be localized in the cell nucleus (33). Altogether, these considerations point to a different role of the two proteins in RNA metabolism.

At present the functional significance of this translational regulation is only a matter of speculation. As a consequence of this control, the intracellular levels of A1 would increase very rapidly after a proliferation stimulus, thus prior to or in parallel with the new wave of RNA transcription. This temporal order could be relevant for the nucleocytoplasmic trafficking of mRNAs that has been suggested to be assisted by A1 (6): the more RNA needs to be exported, the higher the amount of A1 required. It is also possible that a switch in the alternative splicing of a subset of genes is required when cells re-enter the cycle. Another open possibility is that in its cytoplasmic state A1 has a direct role in RNA translation, as suggested by the observation that in *C.tentans* hrp36, a homologue of A1, remains associated with Balbiani ring RNA during translation (10). This hypothesis is further strengthened by the recently reported observation (34) that both A1 and hnRNP I can prevent initiation of translation from internal start codons and promote cap-dependent translation in a rabbit reticulocyte lysate. Interestingly, both proteins are subject to the same translational control.

The presence of staggered transcription start sites for hnRNP A1 mRNA has given us the opportunity to compare the effect of slightly different 5′-UTRs on translational regulation of otherwise identical mRNAs within the same cell. We have found that translational regulation of the various A1 mRNA forms becomes progressively less stringent as the length of the pyrimidine tract

shortens. This indicates that a full canonical 5′-TOP sequence is necessary for normal translational regulation of TOP mRNAs and provides an explanation for the observation that translational regulation of A1 mRNA is less stringent than that of other TOP mRNAs. To our knowledge this is the first time that such an effect has been observed on a single endogenous RNA. It was previously reported that differences do exist between similarly regulated TOP mRNAs in their association with polysomes. For instance, we previously observed that in *Xenopus* embryos between stages 16 and 32 the rp-L18 mRNA loaded on polysomes increases from 29 to 62%, the rp-S24 mRNA from 44 to 76% (35) and nucleolin mRNA from 62 to 82% (36). These differences were attributed to sequence differences in their 5′-TOP region and possibly in other regions of the 5′-UTR relevant to translation regulation. Now this interpretation has to be reconsidered, since in all those cases the exact initiation nucleotide was not determined.

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