The case for nuclear translation

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Summary

Although it is frequently assumed that translation does not occur in eukaryotic nuclei, recent evidence suggests that some translation can take place and that it is closely coupled to transcription. The first evidence concerns the destruction of nuclear mRNAs containing premature termination codons by nonsense-mediated decay (NMD). Only ribosomes can detect termination codons, and as some NMD occurs within the nuclear fraction, active nuclear ribosomes could perform the required detection. The second evidence is the demonstration that tagged amino acids are incorporated into nascent polypeptides in a nuclear process coupled to transcription. The third evidence is that components involved in translation, NMD and transcription colocalize, coimmunoprecipitate and co-purify. All these results are simply explained if nuclear ribosomes scan nascent transcripts for premature termination codons at the site of transcription. Alternatively, the scanning needed for NMD might take place at the nuclear membrane, and contaminating cytoplasmic ribosomes might give the appearance of some nuclear translation. We argue, however, that the balance of evidence favours bona fide nuclear translation.

Key words: mRNA surveillance, Nonsense-mediated decay, Transcription, Translation

Introduction

In prokaryotes, ribosomes decode messages while the messages are still being made; thus, transcription is coupled to translation (Miller et al., 1970). Thirty years ago, there was a debate as to whether some translation also occurs close to the template within eukaryotic nuclei (Allen, 1978; Goidl, 1978). However, the main evidence that this is the case was the finding that isolated nuclei incorporated radiolabelled amino acids into nascent peptides and this was countered by arguments that those isolated nuclei were contaminated with cytoplasmic ribosomes. With the discovery of introns, there seemed to be a good case why eukaryotes should be different from prokaryotes: if nuclear ribosomes were to translate introns with their many termination codons, too many truncated peptides would be produced, and some of these might be toxic to the cell. As it was also clear that most translation occurs in the cytoplasm on intron-free mRNAs, this debate eventually fizzled out, leaving most researchers believing that nuclear translation is nonexistent or negligible.

The debate has recently been re-opened, largely for two reasons. One concerns the process known as mRNA surveillance or nonsense-mediated decay (NMD), which eukaryotes use to scan messenger RNAs for inappropriately placed (i.e. premature) termination codons (PTCs) and destroy faulty messages (Schell et al., 2002; Wagner and Lykke-Andersen, 2002; Vasudevan and Peltz, 2003; Baker and Parker, 2004; Maquat, 2004). Because translating ribosomes are the only known means of detecting termination codons, and because some NMD occurs within the nuclear fraction, it is attractive to suppose that the NMD scanning mechanism utilizes active nuclear ribosomes (Wilkinson and Shyu, 2002). Other observations re-igniting the debate involved high-resolution labelling of translation sites; some sites were found in nuclei and some of the labelling depended on concurrent transcription. This indicated that transcription and translation in eukaryotic cells might be coupled as in bacteria, and we have speculated that this nuclear translation might be used to proofread transcripts to see whether they contain PTCs (Iborra et al., 2001). However, other explanations of these results have been suggested (e.g. see Cosson and Philippe, 2003; Dahlberg et al., 2003; Nathanson et al., 2003; Dahlberg and Lund, 2004). Here, we examine the evidence for and against nuclear translation.

NMD: a 'nuclear' event requiring translation

A typical mammalian mRNA has 7-8 exon-exon junctions generated by splicing, and the termination codon is almost invariably found in the last exon (and so is not followed by an exon-exon junction). Introducing a stop codon into an earlier exon (so that it is now followed by an exon-exon junction) often triggers destruction of the faulty transcript through NMD (Fig. 1A) (Schell et al., 2002; Vasudevan and Peltz, 2003; Baker and Parker, 2004; Maquat, 2004). In *Saccharomyces cerevisiae*, proteins encoded by *UPF1*, *UPF2* and *UPF3* function in NMD, and mutations in these genes result in the stabilization of mRNAs containing PTCs. They are conserved in higher eukaryotes (Perlick et al., 1996), in which their products also play crucial roles in NMD; for example, tethering a UPF downstream of the normal termination codon elicits NMD (Lykke-Anderson et al., 2000). NMD involves decapping coupled to 5′-to-3′ degradation of the RNA, as well

as deadenylation and 3′-to-5′ degradation (Lejeune et al., 2003). It is widely used to regulate mRNA levels and consequently gene expression. Microarray experiments suggest levels of ~10% of yeast transcripts are affected by alterations in NMD (He et al., 2003), and perhaps a third of alternative splicing events in mammals produce substrates for NMD (Lewis et al., 2003). Failure of the system results in synthesis of truncated polypeptides, and these can have gain-of-function or dominant-negative effects (Culbertson, 1999).

Comparisons of the concentrations of transcripts with and without PTCs in isolated nuclei indicate that some NMD is nuclear (e.g. Urlaub et al., 1989; Baserga and Benz, 1992; Cheng and Maquat, 1993). Intronless transcripts are not subject to NMD (Maquat and Li, 2001), and inserting an intron into the 3′-untranslated region (UTR) of an mRNA triggers NMD (Carter et al., 1996; Thermann et al., 1998). The position of the PTC relative to the exon-exon junction is critical, and the rule concerning position can be stated in terms of the spliced mRNA: PTCs followed by what will become an exon-exon junction that is located more than 50-55 nucleotides downstream generally elicit NMD (Nagy and Maquat, 1998). [Exceptions to this rule include the T-cell receptor (TCR)-β transcript in mammals and many others in yeasts, worms and flies.] Splicing deposits an exon-junction complex (EJC) ~20-24 nucleotides upstream of (presumably) every exon-exon junction in a spliced mRNA. The EJC recruits UPF proteins that trigger NMD, which seems to be restricted to the first (‘pioneer’) round of translation (Ishigaki et al., 2001; Maquat, 2004).

A role for an active ribosome in NMD is suggested by the very nature of a stop codon; moreover, suppressor tRNAs (Losson and Lacroute, 1979; Belgrader et al., 1993), antibiotics that target active ribosomes (e.g. cycloheximide) (Lim and Maquat, 1992; Qian et al., 1993) and hairpins in the 5′-UTR (Belgrader et al., 1993) all reduce the effect of nonsense codons on mRNA degradation. However, if translation occurs solely in the cytoplasm, how is PTC detection (which then would be a cytoplasmic event) related to transcript degradation (a nuclear event)? Four models have been suggested (Frischmeyer and Dietz, 1999).

The first model involves feedback from the cytoplasm to the nucleus (Dahlberg and Lund, 2004): a PTC is recognized during cytoplasmic translation, and this generates a signal that is transmitted to the nucleus, where it induces degradation of homologous transcripts (Fig. 1B,i). However, experimental evidence is lacking, and it is difficult to envisage how the signal might be targeted to the transcript carrying the PTC.

The second model involves co-translational RNA export in which a pioneering cytoplasmic ribosome translates a newly made transcript as it exits the nucleus through the nuclear pore (Fig. 1B,ii) (Ishigaki et al., 2001; Maquat, 2004). If the transcript contains a normal termination codon, the ribosome would remodel the mRNA particle (mRNP) (with its 5′-cap-binding complex and the EJC) into the steady-state mRNP [with eukaryotic translation initiation factor 4E (eIF4E) at the cap but without the EJC]. If the mRNA contains a PTC, the ribosome detects both it and the EJC to trigger degradation at the pore. Placing a ribosome at the pore enables both scanning and degradation to be ‘nuclear’. The only experimental support for this model is indirect: the giant mRNA produced by the Balbiani ring of *Chironomus tentans* can be seen exiting the pore, where it associates with the translational initiation factor Ct-eIF4H (Bjork et al., 2003). However, we believe degradation is unlikely to occur at the pore. Introducing a PTC reduces nuclear transcript levels to a third or less (e.g. see Urlaub et al., 1989; Baserga and Benz, 1992; Cheng and Maquat, 1993); in other words, at least two out of every three transcripts are degraded – a significant fraction. If NMD were to occur at the pore, we might expect to find (at least) two out of three nuclear transcripts at pores; however, fluorescence in situ hybridization (FISH) reveals most ‘nuclear’ poly(A)$^+$ RNA to be internal; little is at the nuclear membrane (Fig. 2) (Carter et al., 1991). Quantitative analysis of RNA on its way to the cytoplasm confirms this (Iborra et al., 1998). In these experiments, cells were grown briefly in a low concentration of bromo-uridine (Br-U) so that only a few of the U residues in the newly made RNA are replaced by Br-U. This partially substituted bromo-RNA (Br-RNA) is exported like its natural counterpart, but less than 3% of the Br-RNA in the nucleus is seen passing through pores at any moment. We can stand this argument on its head: NMD could occur at pores if a significant fraction (more than two thirds) of nuclear messages were found at pores, but the above evidence is clearly against this.

The third model involves nuclear scanning for PTCs by some mechanism other than a translating ribosome (Fig. 1B,iii). However, there are no suggestions as to what this mechanism might be.

The fourth model, by contrast, invokes an active nuclear ribosome at the transcription site to detect PTCs and trigger transcript degradation; this mechanism would reduce nuclear transcript levels significantly because it would operate so early in the pathway (Fig. 1B,iv). This model is supported by the following findings: (i) nascent Ig-μ transcripts containing PTCs accumulate close to the (nuclear) gene that encodes them (Mühlemann et al., 2001); (ii) TCR-β transcripts are still degraded when their export from nuclei is inhibited (Bühler et al., 2002); and (iii) some translation appears to be coupled to transcription (see below).

PTCs might also alter the pattern of splicing (Aoufouchi et al., 1996; Mühlemann et al., 2001; Wang et al., 20002a; Wang et al., 20002b). As splicing is a nuclear event – much of it occurring co-transcriptionally (see below) – this also implies that detection must take place close to the transcription site (Wilkinson and Shyu, 2002; Vasudevan and Peltz, 2003). However, recent work suggests that the effects may not be as large as originally suspected (Lytle and Steitz, 2004).

Direct evidence for nuclear translation coupled to transcription

Recent work has indicated that some nascent peptides are found in nuclei, providing direct evidence for nuclear translation (Iborra et al., 2001). These experiments used HeLa cells permeabilized in a ‘physiological’ buffer, which were allowed to extend nascent polypeptides in biotin-lysine-tRNA (or BODIPY-lysine-tRNA). Under these conditions, most of the label in newly made protein is found in the cytoplasm, but 9-15% is nuclear. Nuclei isolated in a conventional (hypotonic) buffer give similar results (Iborra et al., 2001).

The nuclear labelling is unlikely to result from import of cytoplasmic peptides for several reasons. First, in these

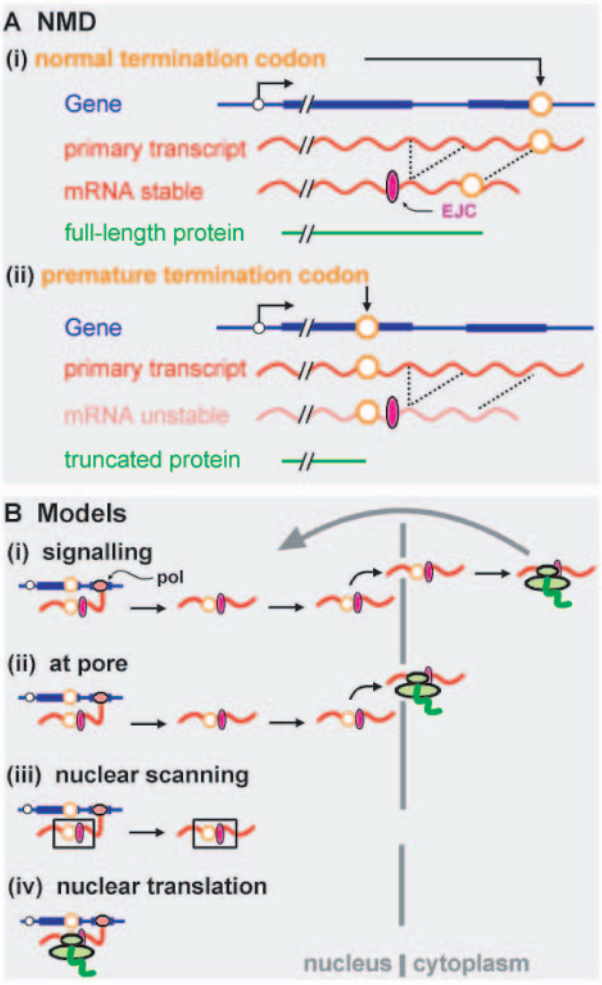

Fig. 1. Nonsense-mediated decay. (A) The phenomenon. The gene (with promoter, parts of the first and penultimate exons, and last exon), primary transcript, spliced mRNA (with an exon junction complex – EJC – deposited 5′ of the exon-exon junction) and protein are shown. UPF proteins associate with the EJC (not shown). (i) Transcription of a gene with a termination codon in the correct place leads to a spliced and stable mRNA, and a full-length protein. (ii) Moving the termination codon 5′ to what will become an exon-exon junction leads to an unstable mRNA and a truncated protein. (B) Models for NMD. In each case, an mRNA encoding a PTC is made at the transcription site by a polymerase (pol). The mRNA with its EJC then passes through the nucleoplasm to dock at the membrane, before exiting through the pore to the cytoplasm. The mRNA is destroyed once the NMD machinery detects the PTC. (i) A cytoplasmic ribosome detects the PTC, and transmits a signal to the nucleus that leads to degradation of homologous transcripts containing the PTC. (ii) NMD occurs as the transcript bearing the PTC emerges into the cytoplasm. (iii) Some unknown nuclear mechanism (black box) recognizes the PTC and destroys the mRNA. (iv) A ribosome detects the PTC in the nascent transcript at the transcription site.

studies, nascent peptides are extended by ⩽15 residues; thus, few would be extended sufficiently to be completed and released from the ribosome during labelling (because proteins typically contain ~350 residues). Second, if cytoplasmic proteins do enter the nucleus, some should be seen at the

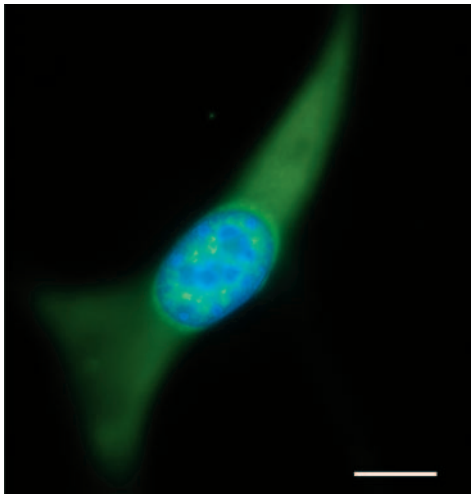

Fig. 2. Poly(A)$^+$ RNA in the nucleus and cytoplasm. Little poly(A)$^+$ RNA is found at the nuclear periphery in a mouse fibroblast (NIH 3T3). Poly(A)$^+$ RNA was detected by in situ hybridization using biotinylated poly(dT)$_{54}$ and streptavidin conjugated with Alexa594 (pseudo-coloured green); DNA was counterstained with DAPI (blue). Image kindly provided by Meg Byron, John McNeil and Jeanne Lawrence. Bar, 10 μm.

nuclear periphery, but no labelling is observed there (biotin-labelled peptides are concentrated internally at transcription sites). Finally, similar nuclear labelling is obtained when isolated nuclei lacking >95% of cytoplasmic ribosomes are used. Again, no peripheral labelling is seen, and so the nuclear labelling is unlikely to result from ribosomes that remain attached either to the nuclear membrane or to transcripts being exported through pores (a requirement of model ii in Fig. 1B). The importance of this result does not rest on the purity of nuclei, since extranuclear ribosomes are unlikely to be responsible because reducing their numbers significantly has no effect on the signal.

In bacteria, translation can be coupled to transcription, and inhibiting transcription immediately reduces translation. This is also true in the studies of mammalian cells discussed above. Thus, the nuclear signal increases >2-fold when the four nucleotide triphosphates needed for transcription are added, and this stimulation can be blocked by a transcriptional inhibitor (α-amanitin) or chain terminator (3′dATP). It is also still seen when Br-UTP replaces UTP. In this case (i.e. a permeabilized cell where endogenous pools have been washed away), Br-RNA is made in which every U is replaced by Br-U; this Br-RNA cannot leave transcription sites, unlike its partially substituted counterpart (Iborra et al., 1998). As a result, cytoplasmic translation of nuclear RNA made in vitro cannot generate the increased signal. Finally, nascent peptides colocalize with nascent transcripts.

All these results suggest some translation occurs co-transcriptionally. Further evidence is the demonstration that [^{35}S]methionine/cysteine is incorporated in a cycloheximide-sensitive manner into transcriptionally active puffs on polytene chromosomes of *Drosophila* (Brogna et al., 2002). If proteins were only made in the cytoplasm, it is difficult to imagine why the newly made ones should be targeted to sites of transcription.

Indirect evidence for nuclear translation

Transcription sites contain the required machinery

As ribosomes are made in nucleoli, it is unsurprising that they are found in nuclei; however, it is surprising that they are found close to transcription sites, which contain an RNA polymerase II catalytic subunit that has a hyper-phosphorylated C-terminal domain (CTD^P). Ribosomal RNA, >20 ribosomal proteins and two translation factors (IF2/eIF5B) colocalize with the CTD^P in the puffs on the polytene chromosomes of *Drosophila*, and some of these components are recruited to the relevant loci when transcription is activated by heat shock or ecdysone (Brogna et al., 2002). Moreover, nascent RNA in HeLa nuclei lies only a few nanometers away from components involved in translational initiation/release (eIF2α, eIF4E, eIF4G and eRF3), ribosomes (QM, S6 and $S6^P$), NMD (UPF1, UPF2 and UPF3α) and RNA/protein degradation (PM-Scl75 of the exosome, and the β subunit of the 20S proteasome) (Iborra et al., 2001; Iborra et al., 2004).

Interactions between the transcription, translation and NMD machineries

Representative components of the machineries for transcription, translation and NMD also co-precipitate and co-purify (Iborra et al., 2004). Thus, initiation factors (eIF4E and eIF4G), ribosomal subunits (S6 and ribosomal P site antigen), NMD proteins (UPF1, UPF2 and UPF3α) and nascent peptides all co-immunoprecipitate from human extracts with the CTD^P, and some also do so with nascent RNA and another subunit of the polymerase (RPB8). Selected components (eIF4E, S6, UPF1 and UPF2) also co-purify ~8,000-fold with the polymerase. Interactions are probably mediated by the CTD^P, because a ribosomal subunit (S6) and an NMD factor (UPF1) can be 'pulled down' from nuclear extracts with it. This extends the list of partners of the CTD^P, which is already known to interact with the machineries involved in capping, splicing and poly-adenylation (see below).

Nuclear expression of a non-nuclear protein

The following findings are also consistent with co-transcriptional proofreading (Iborra et al., 2004). CD2 is a leukocyte cell-surface antigen that has a signal sequence and a transmembrane domain; it is inserted co-translationally into the endoplasmic reticulum (ER). $CD2^-$ cells were transfected with multi-copy plasmids encoding CD2. If translation occurs solely in the cytoplasm, little CD2 should be found in nuclei; if mRNAs are proofread by nuclear ribosomes, some should be nuclear and this should misfold in the absence of the ER to be degraded by proteasomes. Large amounts of CD2 are seen in the ER and at the cell surface, but some is nuclear. Inhibiting the proteasome with lactacystin increases nuclear CD2 levels, and inhibiting transcription with 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) or actinomycin D prevents this increase. This experiment has the drawback that it relies on over-expression and inhibitors, and – if translation were only cytoplasmic – lactacystin could raise cytoplasmic CD2 levels and so increase import. However, cytoplasmic levels remain unchanged, and it is difficult to explain the effects of DRB and actinomycin D – which inhibit transcription in unrelated ways – unless they have similar and undocumented effects on import and/or protein degradation. But these in vivo results are simply explained if all inhibitors have their expected effects. Nuclear translation coupled to transcription would generate misfolded (nuclear) CD2 that is immediately degraded by nearby proteasomes; in lactacystin, degradation is inhibited, and CD2 levels increase. And when transcription – and so nuclear translation – is inhibited, less CD2 is produced.

Arguments against nuclear translation

Following publication of recent work providing evidence for nuclear translation, several arguments against it have been put forward, frequently focusing on potential limitations of the supporting work. In the sections below, we examine some of the main criticisms.

The strength of the nuclear signal

Nathanson et al. (Nathanson et al., 2003) claim to have repeated the experiments of Iborra et al. (Iborra et al., 2001) and found a tenth of the nuclear signal; however, they repeated a minority of the experiments, generally using conditions that differed in at least three crucial respects. (In the single experiment in which the same conditions were used, the same result was obtained.)

First, they used a different buffer, and most nuclear processes are known to be sensitive to the buffers used. For example, their buffer supports significantly less transcription, which implies that some transcription complexes are disrupted (or do not work as efficiently); thus, it might also disrupt (or make less efficient) any associated translation. [Transcription rates in an earlier version of the buffer used by Iborra et al. are 14.6 pmoles nucleotides/10^6 cells/minute (Jackson et al., 1988), compared with 1.26 pmoles/10^6 cells/minute in that used by Nathanson et al.; moreover, the improved version used by Iborra et al. further doubles the transcription rate (Pombo et al., 1999).]

Second, Nathanson et al. did not add protease inhibitors. It is obviously good practice to limit product degradation when measuring synthetic rates; it is especially important to do so when proofreading is an issue. Thus, some peptides made during nuclear proofreading will misfold (membrane proteins that cannot be inserted into a lipid bilayer), others may be truncated (because the ribosome encounters a PTC introduced by mis-splicing), and still others will be junk peptides (because they are encoded by transcripts miscopied from non-genic DNA). We also expect such newly made peptides are degraded rapidly by proteasomes at nuclear transcription/translation sites (Iborra et al., 2001; Iborra et al., 2004; Gillette et al., 2004). In the cytoplasm, a smaller fraction will be degraded; proofreading has culled many faulty transcripts, and proteins destined for membranes can be inserted directly to fold correctly. Therefore, it is no surprise that adding protease inhibitors gives a higher nuclear signal. It turns out that newly made nuclear peptides are indeed degraded more rapidly than their cytoplasmic counterparts (Iborra et al., 2004). Because of this, less than 3% of total cellular protein seen under steady-state conditions should be made in nuclei, despite ~16% of all translation occurring there.

Third, Nathanson et al. also used a 50-fold higher concentration of amino acids. Under these conditions,

ribosomes should translate further, increasing the chance of encountering PTCs and generating truncated peptides that are rapidly degraded (to reduce the nuclear signal). We would argue that Nathanson et al. did not repeat the experiments reported by Iborra et al.

Cytoplasmic contamination

As with the early work, it has been argued that the nuclear signal reported is a consequence of cytoplasmic contamination. However, the issue here is not whether there is contamination (there is always some), but whether that contamination affects the nuclear signal. Since a >20-fold variation in the number of cytoplasmic ribosomes has no effect (Iborra et al., 2001), we would argue that contamination cannot be responsible.

Over-permeabilization

Another argument advanced is that over-permeabilization might lead to entry of cytoplasmic ribosomes into the nucleus, which then generate the nuclear signal. More than 95% of the isolated nuclei studied by Iborra et al. (Iborra et al., 2001) were sufficiently intact to exclude a 500 kDa dextran conjugated with FITC (which is smaller than a ribosome). Moreover, the signal does not depend on the integrity of the nuclear membrane, because it remains the same when nuclei are isolated without washing with Triton. The permeabilized cells used for most of these experiments even exclude a 40 kDa dextran conjugated with fluorescein isothiocyanate (FITC) (Fig. 3). However, could nuclei contain all the translation machinery except for one small component that enters during permeabilization? If this were so, the translation machinery would still have to initiate; however, inhibiting initiation (but not elongation) with aurintricarboxylic acid has no effect on nucleoplasmic labelling. Moreover, such entry would have to be targeted, because in these studies labelled peptides concentrate at transcription sites, and this seems unlikely. And in any case, why – on permeabilization – should relatively more of the ribosomes (or missing component) travel inwards specifically to a transcription site, and not outwards down the steeper concentration gradient into the huge excess of buffer? Finally, this argument becomes equivalent to that concerning cytoplasmic contamination (above): the 5% or less of the overpermeabilized cells would still have to contribute nuclear signal to the 95% or more of the appropriately permeabilized cells.

Low nuclear concentration of components of the translational machinery

Bohnsack et al. used green fluorescent protein (GFP) tagging and immunolocalization to show that many components involved in protein synthesis are present in nuclei at 1% or less of cytoplasmic levels, and argued that this is too little to generate the nuclear signal seen (Bohnsack et al., 2002). Because the factors shuttled between nucleus and cytoplasm, and accumulated in nuclei when export was inhibited with leptomycin B, a nuclear presence is not the issue. Although care was taken to limit overexpression in the GFP-tagging experiments, there presumably must have been some. In addition, the authors presented no evidence that the tagged components are functional and that their distributions reflect

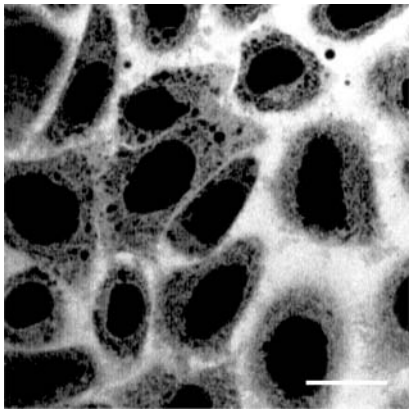

Fig. 3. The nuclei (but not cytoplasm) of permeabilized HeLa cells exclude a 40 kDa dextran conjugated with fluorescein isothiocyanate (FITC). Hela cells were permeabilized in saponin in a 'physiological' buffer, washed in the same buffer, incubated (15 minutes, 27.5°C) in the precursors required for translation (as for Fig. 2 of Iborra et al., 2001), a 40 kDa dextran-FITC added, and cells imaged on a confocal microscope. FITC fluorescence is illustrated. Bar, 10 μm.

those of the endogenous proteins (tagged proteins might be incorporated inefficiently into large translating complexes to accumulate in the cytoplasm). Moreover, immunolocalization was carried out after glutaraldehyde fixation, which preferentially reduces antibody access to targets in dense chromatin. If so, lighter fixation should give higher nuclear signals, and it does (Iborra et al., 2004). For example, 40% of CTD^P appears nuclear by immunolabelling after glutaraldehyde fixation compared with 93% after lighter fixation (similar effects are seen with eIF2α, eIF4E, eIF4G, eRF3, QM and S6). In any case, this kind of argument cannot be decisive in the absence of quantitative information on how much of the protein is active, whether the concentration limits synthesis, and so on.

Antibodies used were raised against epitopes in human ribosomes and were not specific for *Drosophila* ribosomes

Brogna et al. localized ribosomal proteins and translation factors by using more than 20 different antibodies (Brogna et al., 2002). It would be surprising if so many antibodies crossreacted with proteins that all happened to be in the same place. Moreover, Iborra et al. obtained similar results in human nuclei by using antibodies raised against human antigens (Iborra et al., 2004).

Functional ribosomes in the nucleus

After staining with heavy metals, cytoplasmic ribosomes are seen as electron-dense granules. The nucleus is full of similar granules, but it is impossible to decide whether these are ribosomes, other RNPs or chromatin. Therefore, such electron micrographs do not provide evidence for or against the existence of ribosomes in nuclei. However, there is a widespread belief that ribosomes only become functional once they reach the cytoplasm. This belief seems to stem from various observations. First, TIF6 interacts with the large ribosomal subunit to inhibit its association with the smaller one

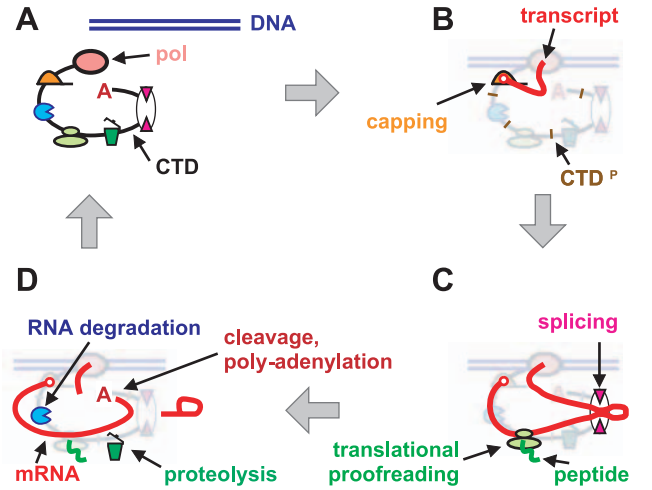

Fig. 4. A model for transcript production (Iborra et al., 2004). (A) The CTD has the potential to associate with sites involved in capping, transcript degradation, translational proofreading (involving the translational and NMD machineries), proteolysis, splicing and polyadenylation. It remains unclear whether all bind to the CTD simultaneously, or whether they attach and detach as needed. [Wetterberg et al. (Wetterberg et al., 2001) have analysed the association of the splicing machinery during the synthesis of an exceptionally long mRNA.] (B) Transcription began as the template bound to the polymerizing complex and was reeled in as the transcript was extruded; the CTD is now hyper-phosphorylated (CTD^P), and a cap has been added. (C) The transcript continues to be extruded through a splicing site as the ribosome/NMD machinery begins proofreading the now-spliced message (and so does not read introns, which might contain many termination codons). (D) Once introns are removed (lariat), the transcript is cleaved and poly-adenylated, and is ready to leave for the cytoplasm. If errors are detected, the faulty transcript and peptide produced during proofreading are degraded by nucleases and proteasomes.

and is only removed in the cytoplasm (Senger et al., 2001). However, this tells us nothing about possible nuclear association and activity. Second, yeast 20S rRNA matures to 18S only in the cytoplasm. The fragment cleaved off 20S rRNA accumulates in strains lacking Xrnp1 (the cytoplasmic exonuclease) but not Rat1p (its nuclear counterpart; Venema and Tollervey, 1999). Although this is consistent with cytoplasmic maturation, it is also consistent with many other explanations (e.g. a minority of Xrnp1 might be nuclear). Third, 40S and 60s ribosomal subunits are exported from nuclei independently (Trotta et al., 2003). Again, this tells us nothing about whether they are active in nuclei. Fourth, the giant mRNA produced in a Balbiani ring on a polytene chromosome of *Chironomus tentans* associates with Ct-eIF4H at the pore (Bjork et al., 2003). It might therefore only be translated in the cytoplasm. However, there might be sufficient Ct-eIF4H at transcription sites to allow translation, or another factor might be used (i.e. the nuclear and cytoplasmic machineries might differ) (Ferraiuolo et al., 2004).

It has also been argued that functional ribosomes are not found in the nuclei of *Xenopus* oocytes (Cosson and Philippe, 2003). Stage VI oocytes are usually used for micro-injection studies, but these are transcriptionally inactive. As nuclear translation might be coupled to transcription, Cosson and Philippe micro-injected plasmids encoding luciferase or CD2 into the transcriptionally active nuclei of smaller stage V oocytes, and monitored whether one or other protein appeared in the nucleus before the cytoplasm. Although the proportion of luciferase activity associated with the nuclear fraction decreases with time (which is consistent with nuclear translation), the signal seen was attributed to cytoplasmic contamination. CD2 appears in the nucleus and cytoplasm at the same time, but then increases more rapidly in the cytoplasm (which is also consistent with nuclear translation). However, most nuclear CD2 is at the periphery, and so was again attributed to cytoplasmic contamination. We would argue that this kind of experiment is necessarily indecisive; incubations of $\geqslant$3 hours were required, which gives plenty of time for newly made proteins to diffuse away from synthesis sites.

tRNA import

One suggestion is that tRNAs cannot enter intact nuclei and thus, in the experiments showing nuclear translation, the nuclei must have been damaged. There seems to be no direct evidence that tRNAs cannot cross the nuclear membrane, and Iborra et al. (Iborra et al., 2001) provide functional evidence that they can. Note that the machinery for charging tRNAs is nuclear (Lund and Dahlberg, 1998).

Evolution of the nuclear membrane

It seems sensible to prevent ribosomes from translating introns with their many termination codons, and it is suggested that the nuclear membrane evolved to prevent this by separating transcription/splicing from translation (Bohnsack et al., 2002). However, a nuclear ribosome is prevented from translating an intron if placed in the transcription complex where it cannot contact the unspliced transcript. Indeed, we have previously put forward a model that places the ribosome in just such a position (see below).

An integrated model for transcription, nuclear translation and NMD

RNA polymerase II interacts with the capping, splicing and polyadenylation machineries to generate a ‘standard’ message (Maniatis and Reed, 2002; Neugebauer, 2002). RNA polymerase II also interacts with machineries that proofread the message (see above), destroy unwanted transcripts (Andrulis et al., 2002; Libri et al., 2002; Lykke-Andersen, 2002) and degrade proteins that might arise during proofreading (Verma et al., 2000; Ferdous et al., 2002; Gillette et al., 2004). The recent work discussed above indicates the nuclear translation machinery might also interact with the transcriptional machinery. We have therefore proposed an integrated model for transcript production in which the CTD organizes the various machineries involved (Fig. 4) (Iborra et al., 2004). In this model, a transcript is extruded from the polymerase through sites that cap, splice, proofread and poly-adenylate it. As the ribosome/NMD machinery proofreads the spliced message, it does not read introns with their many termination codons. If errors are detected, the faulty transcript and peptide produced during proofreading are degraded by associated nucleases and proteasomes.

This organization brings several advantages. The local concentration of the relevant machines should increase reaction

rates (through mass action and by allowing efficient 'channeling' from one complex to another) and facilitate regulation (e.g. through cooperative effects). It is also easy to see how this organization might have evolved. In the proto-eukaryote, transcription and translation would be coupled (as in bacteria). As introns appeared and genomes became more complex, mis-splicing and inappropriate transcriptional initiation become significant, and this drives 'pioneer' ribosomes towards a proofreading role (leaving others in the cytoplasm to concentrate on mass production).

We speculate that proofreading plays a bigger role than hitherto imagined. There is always pressure to increase efficiency. Higher efficiency can be achieved by increasing precision during manufacture, frequent checking, and low throughput, but both multi-national corporations and Nature usually adopt a more economical compromise – a moderate throughput with acceptable error rates. Consumers often think those rates are set too high and, to our eyes, Nature also seems profligate; for example, in mammals, ~95% of a typical genic transcript is discarded during splicing, and ~30% of proteins made by the cytoplasmic ribosome misfold and are degraded as soon as they are made (Schubert et al., 2000). We believe it likely that other steps in message production are as error prone, with the cell relying on proofreading at every step to weed out faulty products.

Conclusions

The case for nuclear translation now rests on three types of evidence. The first is indirect. Some NMD occurs within the 'nuclear' fraction and, because translating ribosomes are the only known means of detecting termination codons, it is easy to imagine that scanning utilizes active nuclear ribosomes. This contrasts with the currently favoured model, which has come to the fore largely by default – if translation occurs only in the cytoplasm, and if NMD is a 'nuclear event', then cytoplasmic ribosomes must scan messages as those messages emerge from nuclear pores. However, there is little evidence for this model, and we have argued that too few transcripts pass through pores at any moment to account for the degradation levels seen. Moreover, there is general agreement that, if translation does occur in nuclei, then nuclear translation/scanning provides the natural explanation of how NMD could be a nuclear event.

The second type of evidence is more direct. When cells are permeabilized and nascent polypeptides extended by a few residues in the presence of a tagged precursor, some of the resulting tagged polypeptides are found in nuclei. Significantly, some nuclear incorporation depends on concurrent transcription. Criticism has naturally focused on whether the nuclear signal seen in such experiments is an artefact resulting from permeabilization. Here, we have argued that it is not. But we note that none of these criticisms addresses what we consider to be the strongest evidence – the dependence of the nuclear signal on ongoing transcription.

The third type of evidence is again indirect. Components involved in translation (e.g. ribosomal proteins and rRNA, initiation and elongation factors) and NMD (e.g. UPF1, UPF2 and UPF3) colocalize, co-immunoprecipitate and co-purify with the transcription machinery. There is no equivalent information on the association of the translation/NMD machinery with the cytoplasmic face of the pore.

But there is as yet no smoking gun: decisive evidence for the coupling of transcription and translation is lacking. Better evidence would include the demonstration of a local concentration of a (newly made) cell membrane protein immediately next to the (nuclear) gene that encoded it (and the nascent RNA). But this is a difficult experiment. Most active genes are probably associated at any moment with only one engaged polymerase (Jackson et al., 2000) and one proofreading ribosome (Iborra et al., 2001); therefore, only one (nascent) protein molecule would be there to be detected. Unfortunately, few methods provide sufficient sensitivity. Multiple gene copies must be analysed, and the resulting hyper-expression increases the background of inappropriately localized protein in nuclei, raising the question of whether any nuclear signal reflects cytoplasmic synthesis.

Despite the lack of decisive evidence, we believe the simplest interpretation consistent with the data is that translation can occur in nuclei. Many questions remain. For example, how much nuclear translation occurs, what is the significance of the nuclear signal seen outside transcription sites, how many and which types of transcript are proofread by the NMD machinery, to what extent do the nuclear and cytoplasmic translation machineries differ, does one specialize in proofreading and the other in mass production, and how much NMD occurs in nuclei?

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