

Molecular cross-talk between the transcription, translation, and nonsense-mediated decay machineries

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Summary

It is widely believed that translation occurs only in the cytoplasm of eukaryotes, but recent results suggest some takes place in nuclei, coupled to transcription. Support for this heterodoxy comes from studies of the nonsense-mediated decay (NMD) pathway; this pathway probably uses ribosomes to proofread messenger RNAs. We find components of the machineries involved in transcription, translation and NMD colocalise, interact and copurify, and that interactions between them are probably mediated by

the C-terminal domain of the catalytic subunit of RNA polymerase II. These results are simply explained if the NMD machinery uses nuclear ribosomes to translate – and so proofread – newly made transcripts; then, faulty transcripts and any truncated peptides produced by nuclear translation would be degraded.

Key words: Exosome, Proteasome, Transcription, Translation

Introduction

It is widely believed that translation occurs only in the cytoplasm of eukaryotes, but recent results suggest some might take place in nuclei, coupled to transcription (Iborra et al., 2001; Muhlemann et al., 2001; Brogna et al., 2002; Herbert et al., 2002). For example, nascent peptides can be found at nuclear transcription sites together with the necessary components of the translation machinery, and inhibiting nuclear transcription also inhibits the production of these nascent peptides (Iborra et al., 2001; Brogna et al., 2002). Moreover, an RNA editing enzyme can induce translation within nuclei, probably at the surface of the nucleolus (Herbert et al., 2002).

Additional support for this heterodoxy comes from study of the nonsense-mediated decay (NMD) pathway; this pathway probably uses ribosomes to scan messenger RNAs for inappropriately placed (i.e. premature) termination codons, and – if detected – goes on to destroy those faulty messages (Hilleren and Parker, 1999; Wilusz et al., 2001; Maquat, 2002; Moore, 2002). In *Saccharomyces cerevisiae*, translation activates NMD, and mutations in the *UPF1*, *UPF2* and *UPF3* genes result in the specific stabilisation of mRNAs containing inappropriately positioned termination codons (Hilleren and Parker, 1999; Wilusz et al., 2001). These genes are conserved in higher eukaryotes, including man, where their products also play critical roles in NMD (Perlick et al., 1996; Lykke-Andersen et al., 2000; Serin et al., 2001). Some NMD occurs within the nuclear fraction, and it is difficult to imagine how a faulty nuclear transcript could be detected by a ribosome active only in the cytoplasm (Wilkinson and Shyu, 2002). Strikingly, faulty RNAs accumulate near nuclear transcription sites, which suggests the detection machinery – which is usually assumed

to be an active ribosome – might lie nearby (Muhlemann et al., 2001). Inhibiting mRNA export also has little effect on the elimination of faulty nuclear transcripts, which again points to the detection machinery being nuclear (Buhler et al., 2002).

We have investigated whether components of the machineries involved in transcription, translation and NMD interact. We found that they do, and that interactions between them are probably mediated by the C-terminal domain (CTD) of the catalytic subunit of RNA polymerase II. The CTD becomes hyperphosphorylated during transcriptional elongation and organises many of the functions involved in the maturation of the primary transcript, including capping, splicing and polyadenylation (Maniatis and Reed, 2002). We also show that a ‘non-nuclear’ cell-surface antigen, CD2, can be detected in nuclei, and that its degradation is closely coupled to transcription. These results are simply explained if the NMD machinery uses nuclear ribosomes to translate – and so proofread – newly made transcripts.

Materials and Methods

Immunolabelling and light microscopy

HeLa cells on coverslips were fixed in 4% paraformaldehyde, and antigens indirectly immunolabelled (Iborra et al., 2001) using (1) primary rabbit antibodies (1/100 dilution) directed against: UPF1, UPF2 and UPF3 (Lykke-Andersen et al., 2000), eIF2 α , eIF4E, eIF4G (phosphorylated on Ser¹¹⁰⁸), S6, phospho-S6 (Cell Signalling Technology, supplied through New England BioLabs, Hitchin, Herts, UK), eIF4E-BP1 (Heesom et al., 2001), QM (Santa Cruz Biotechnology, Santa Cruz, CA), eRF3 (Le Goff et al., 1997), PM-Sc175 (Mukherjee et al., 2002) and the 20S proteasome (β subunit; ICN Pharmaceuticals Ltd, Basingstoke, Hampshire, UK); and (2) monoclonal antibodies directed against: PCNA (Sigma-Aldrich,

Poole, Dorset, UK) and NUMA (Calbiochem, supplied through CN Biosciences, Beeston, Notts, UK). Nucleic acids were counterstained with 20 μ M SYTO 16 (Molecular Probes, Eugene, OR), images collected using a Radiance 2000 MP confocal microscope (BioRad Laboratories, Hemel Hempstead, Herts, UK), intensities over nucleoplasm and equivalent areas of the slide measured (EasiVision software; Soft Imaging Systems GmbH, Münster, Germany) and data exported to Excel (Microsoft) for background subtraction and analysis. To determine whether UPFs are nuclear (Fig. 1A), average intensities in equatorial confocal sections of nucleoplasm and cytoplasm abutting nuclei were determined and multiplied by the volume fraction of the two compartments (i.e. 400 and 673 μ m³ (Iborra et al., 1998)) to obtain relative contents. Cells were also grown with or without actinomycin D (5 μ g/ml; 2 hours) or leptomycin B (10 ng/ml; 12 hours). For Fig. 1C, HeLa cells were grown in bromouridine (Br-U) (2.5 mM; 15 minutes) to label nascent transcripts. They were then fixed, indirectly immunolabelled, and single equatorial optical sections through nuclei collected using a confocal microscope. Primary antibodies were mouse anti-IdU/BrdU (5 μ g/ml; Caltag Laboratories, Burlingame, CA) with or without different rabbit antibodies; secondary antibodies were donkey anti-mouse IgG tagged with Cy3 (1/200 dilution; Jackson ImmunoResearch, Bar Harbor, ME) and donkey anti-rabbit IgG tagged with Alexa 488 (1/200; prepared using a Molecular Probes kit).

Immunoprecipitation, 'pull-downs', blotting

Proteins were immunoprecipitated (Blencowe et al., 1994) from nuclear extracts (10⁷ cells/ml; Computer Cell Culture Center 4C, Seneffe, Belgium). 10⁷ Dynabeads coated with protein A (DynaL Biotech, Oslo, Norway) were washed twice in IP100 buffer (Blencowe et al., 1994), incubated (16 hours; 4°C) either with hybridoma supernatant (Srm160, SR^P) or 3 μ g antibody (all others). Antibodies used were those described above, plus ones directed against RPB8 [clone B8-1 (Jones et al., 2000)], biotin (Jackson ImmunoResearch), human ribosomal P site antigen (ImmunoVision, Springdale, AZ), Srm160 (Blencowe et al., 1994), SC35 (Sigma-Aldrich), phosphorylated SR proteins [SR^P; clone 104 (Roth et al., 1990)], histone H4 (Serotec, Kidlington, Oxford, UK) and IdU/BrdU (Caltag). After incubation, beads were rewashed, incubated for 2 hours with an equal volume of nuclear extract treated with RNase A (5 μ g/ml; 3 hours; 4°C; these conditions solubilize 93% [³H]RNA labelled during a 1-hour incubation with [³H]uridine; not shown), rewashed, and proteins resolved on acrylamide gels and detected by immunoblotting using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Bucks, UK). Biopeptides and Br-RNA were generated by allowing permeabilized cells to extend nascent peptides and transcripts by ~15 and ~500 residues, respectively (Iborra et al., 2001; Pombo et al., 1999). In some cases, 1 mg/ml cycloheximide was added to inhibit synthesis of biopeptides (results not shown). Then, 2.5 × 10⁵ cells were resuspended in 100 μ l physiological buffer (PB) (Pombo et al., 1999) plus 0.5% Triton X-100 + 0.1% Tween 20, and sonicated (Sony Soniprep 150; microprobe at level 10 for 20 seconds). Next, extracts were incubated (2 hours; 4°C) successively with 1–3 μ g antibody and 10⁷ Dynabeads, before the beads were washed four times in PB and bound proteins analysed as above. To analyse CTD interactions (Fig. 2D), glutathione-agarose (Sigma) was dissolved in PB and conjugated (16 hours; 4°C) with GST-VP16 (Lin and Green, 1991) or GST-CTD with 29 heptads (Patturajan et al., 1998), washed in PB, resuspended in PB, mixed with an equal volume of nuclear extract, incubated (2 hours; 4°C), rewashed, and bound proteins analysed as above.

Polymerase purification

HeLa cells were extracted successively with a hypotonic buffer and increasing concentrations of ammonium sulphate, treated with RNase

A (Ma et al., 1999), and released sub-nuclear RNA polymerase II further purified on a 100 μ l immuno-affinity column (Harlow and Lane, 1988) using sc899 antibodies directed against the N terminus of the largest subunit (Santa Cruz). Polymerase sensitive to α -amanitin was assayed as described previously (Roeder and Rutter, 1969). For immunoblots (Fig. 3), antibodies included sc899 (above), and those directed against XPB, cdk7 and cdk8 (Santa Cruz).

Detection of CD2

Cos-1 cells, on coverslips, in a 35 mm dish were transfected (FuGENETM 6; Boehringer Mannheim, Sussex, UK) with 1 μ g DNA encoding rat CD2 cDNA (Mizushima and Nagata, 1990; van der Merwe et al., 1993) or pEYFP-Mito (BD Clontech, Basingstoke, Hampshire, UK), grown for 24 hours, fixed and CD2 indirectly immunolabelled using the OX34 monoclonal antibody [1/2 dilution (Barclay et al., 1997)], anti-mouse IgG conjugated with horseradish peroxidase (1/50 dilution; Dako, Ely, UK) and the tyramide signal amplification Cy3 system (New England Nuclear, Zaventem, Belgium).

Stability of newly made peptides

Permeabilized cells were incubated (28°C) with BODIPY-lys-tRNA (Iborra et al., 2001; 10 μ g/ml MG-132 replaced the protease inhibitor cocktail), washed with PB-BSA (Pombo et al., 1999) (4°C) lacking protease inhibitors but supplemented with 1 mg/ml cycloheximide, re-incubated (28°C), fixed in paraformaldehyde, and fluorescence intensities measured as above.

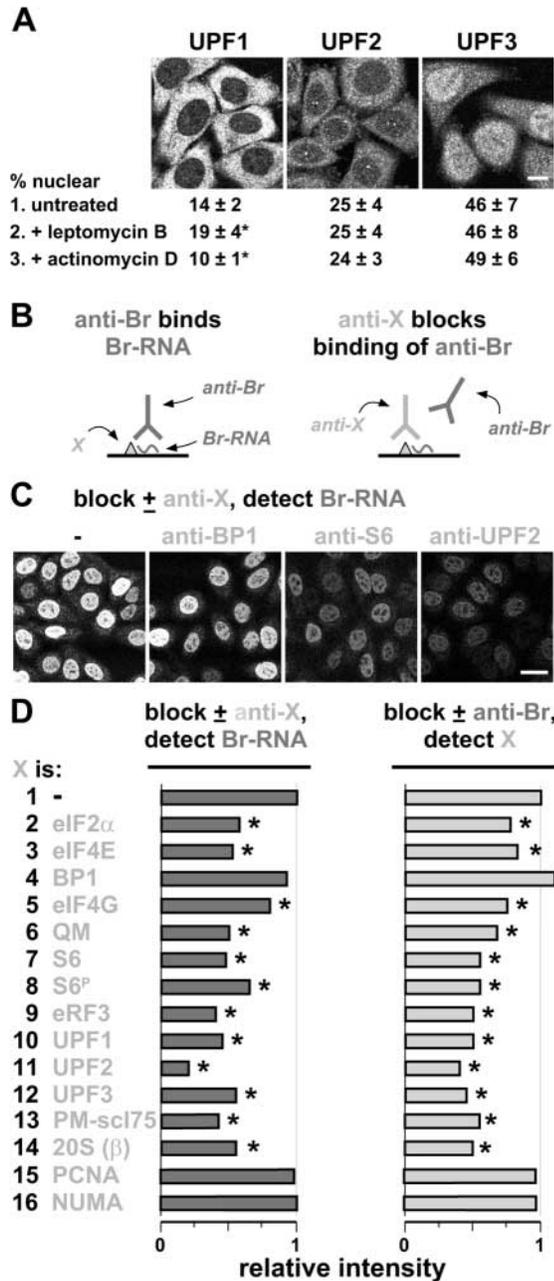
Results

Nuclear UPF1 and transcription

Immunofluorescence reveals that some of the three components of the NMD machinery (i.e., UPF1, 2 and 3) are found in human nuclei (Fig. 1A), confirming earlier results (Lejeune et al., 2002; Lykke-Andersen et al., 2000; Mendell et al., 2002). Only the nuclear fraction of UPF1 is increased by pretreatment with leptomycin B (Fig. 1A, compare rows 1 and 2), an inhibitor of CRM1-mediated nuclear export (Kudo et al., 1999); this suggests UPF1 shuttles between nucleus and cytoplasm (Shirley et al., 1998; Lejeune et al., 2002; Mendell et al., 2002). Treatment with actinomycin D, a transcriptional inhibitor, also reduces nuclear UPF1 (Fig. 1A, row 3), consistent with ongoing transcription maintaining those levels.

Colocalization of the translation and NMD machineries with nascent RNA

We next investigated whether components of the various machineries colocalize. Colocalization is usually demonstrated by immunolabelling two different antigens with red and green fluors, which are then seen as yellow in a 'merged' image, indicating that the two antigens lie within ~200 nm – the limit of resolution of the light microscope. Therefore, we used a higher-resolution approach widely used by immunologists to map epitopes within one protein (Mason and Williams, 1986); it exploits the ability of one antibody to block access of another to its target, but only if that target lies within a few nanometers (Fig. 1B). We have used this approach previously to show that access of an antibody targeted against Br-RNA is blocked by another against polymerase II [but not by one against polymerase III (Pombo et al., 1999)]. HeLa cells were grown



briefly in Br-U to label nascent transcripts, fixed, and the resulting nascent Br-RNA indirectly immunolabelled. In the absence of a blocking antibody, about 80% of the nucleoplasmic signal is then due to polymerase II, the remainder to polymerase III (Fig. 1C, left) (Iborra et al., 1998; Pombo et al., 1999). However, this intensity is reduced by co-incubation with anti-UPF2 (Fig. 1C, right), consistent with anti-UPF2 preventing anti-Br-RNA from binding to Br-RNA lying within ~10 nm, the diameter of an anti-UPF molecule. Anti-UPF2 reduces the nucleoplasmic signal but not the nucleolar signal generated by polymerase I (not shown). This indicates that UPF2 lies near Br-RNA made by one of the nucleoplasmic polymerases (i.e. II and III) but not the nucleolar polymerase (i.e. I), and that the antibody does not bind non-specifically to the fluorochrome to quench its

Fig. 1. Immunofluorescence reveals components involved in translation and NMD are found near nascent transcripts in HeLa nuclei. (A) Some UPF1, 2 and 3 is nuclear. Cells were fixed, UPF1, 2, or 3 indirectly immunolabelled with Cy3, and equatorial sections through nuclei collected using a confocal microscope. (Rows 1-3) Fraction cellular intensity found in nuclei for cells grown with or without leptomycin B or actinomycin D. *Difference relative to value in row 1, significant at 99.999% level (Student's *t*-test). Although the nuclear signal given by UPF1 and 2 is faint, quantitative analysis reveals it constitutes a significant fraction of the total. Scale bar: 10 μ m. (B) Anti-Br binds to Br-RNA (left), but not when blocked by anti-X bound to its target (right). (C) Some antibodies block access of anti-Br to nascent Br-RNA. HeLa cells were grown briefly in Br-U to label nascent transcripts, and fixed; then the resulting nascent Br-RNA was indirectly immunolabelled with Cy3, and single equatorial optical sections through nuclei collected using a confocal microscope. In the absence of a blocking antibody, nuclear fluorescence marks nascent Br-RNA (left), which is reduced by co-incubation with anti-UPF2 (right); other antibodies have intermediate effects (middle). Scale bar: 20 μ m. (D) Colocalization revealed by antibody blocking: various antibodies prevent access of anti-Br to Br-RNA (left), and vice versa (right). Fluorescence intensities over >300 nuclei in images like those in the right-hand panel in (C) were expressed relative to those in the left-hand panel. *Difference relative to value in row 1, significant at 99.999% level (Student's *t* test).

fluorescence. An antibody to a ribosomal subunit (anti-S6) blocks access to nucleoplasmic Br-RNA (but again not to nucleolar Br-RNA) to a lesser extent than anti-UPF2. In contrast, an antibody against 4E-BP1 has no effect (Fig. 1C). Binding protein 1 (BP1) is an abundant protein that binds to eIF4E and inactivates it, therefore it would not be expected to be near an active ribosome. Antibodies against other proteins (e.g. PCNA, NuMA) not involved in the pathways analysed also have no effect (Fig. 1D). Antibodies directed against other components involved in translation – including initiation factors (eIF2 α , eIF4E, eIF4G), ribosomal antigens (QM, S6, S6^P), a release factor (eRF3), as well as in NMD (i.e. UPF1, 2, 3), RNA degradation (i.e. the exosomal protein, PM-Scl75) and protein degradation (i.e. the β subunit of the 20S proteasome) also block access of anti-Br to nucleoplasmic Br-RNA (Fig. 1D, left), but not to nucleolar Br-RNA (not shown). Conversely, anti-Br blocks access of the same set of antibodies (with the exception of anti-BP1, PCNA, and NUMA) to their targets (Fig. 1D, right). This shows that these components lie a few nanometers apart in nuclei. [The close association of eIF4E and the proteasomal subunit with Br-RNA confirms results seen by electron microscopy (Iborra et al., 2001).] Note that blocking is incomplete, so some yellow is seen in 'merged images' obtained after labelling antigens with red and green fluors (not shown).

Only low concentrations of translation factors have been detected in nuclei by immunolabelling after fixation with glutaraldehyde (Bohnsack et al., 2002); however, such fixation reduces antibody access. Thus, 40% of the hyperphosphorylated CTD of RNA polymerase II (CTD^P; below) was nuclear in glutaraldehyde-fixed cells compared to 93% after fixation using our method (values corrected for compartment volumes); corresponding percentages for eIF2 α , eIF4E, eIF4G, eRF3, QM, and S6 were 55 and 82, 23 and 57, 30 and 59, 20 and 59, 1 and 10, and 5 and 20 (not shown).

Co-immunoprecipitation of the machineries

This tight colocalization suggests the various components might interact, so we tested if the translational initiation factor, eIF4G, did so with the hyperphosphorylated CTD of RNA polymerase II (CTD^P), which is recognised by the H5 antibody (Patturajan et al., 1998). Beads coated with anti-eIF4G were incubated with an extract of HeLa nuclei, and protein:eIF4G complexes binding to the beads pelleted; then, bound proteins were resolved by electrophoresis, blotted, and the blot probed with the H5 antibody. CTD^P was detected, indicating the initiation factor co-immunoprecipitates with the active polymerase. Analogous experiments show that another initiation factor (eIF4E), NMD proteins (UPF1, 2, 3) and nascent peptides (bio-peptide) all co-immunoprecipitate with CTD^P (Fig. 2A; results for UPF3 not shown). Antibodies directed against another polymerase subunit (RPB8), which can immunoprecipitate the whole polymerising complex (Jones et al., 2000), provide positive controls. Normal mouse serum, or peptides lacking biotin, provide negative controls (Fig. 2A); inhibiting synthesis of bio-peptides with cycloheximide also provides another negative control (not shown). The CTD^P and two NMD proteins also co-precipitate with ribosomal subunits (S6, ribosomal P site antigen) and several nuclear proteins associated with newly made transcripts [Srm160, SC35, phosphorylated SR proteins-SR^P (Lejeune et al., 2002; Reichert et al., 2002)], however, they do not co-precipitate with the abundant histone, H4 (Fig. 2B). Similarly, components involved in NMD (UPF1, 2, 3) and translation (eIF4E, eIF4G, S6, QM) co-precipitate with nascent Br-RNA and RPB8, but not with unbrominated ('control') transcripts (Fig. 2C). As some proteins aggregate in the non-physiological buffers used to prepare nuclear extracts (Kimura et al., 1999), immunoprecipitations like those in Fig. 2A were repeated using cell extracts prepared in a 'physiological' buffer (as in Fig. 2C), with similar results (not shown). Taken together, these results indicate that the three machineries interact in cell and nuclear extracts. Note that Lejeune et al. (Lejeune et al., 2002) concluded that eIF4E and UPF2 do not interact with the polymerase. However, their conclusion was based on a different experimental system. For example, we immunoprecipitated eIF4E (or UPF2) and probed for the CTD^P; they immunoprecipitated the polymerase [using an anti-CTD^P that can displace proteins from the CTD (Kim et al., 1994)] and probed for eIF4E (or UPF2). Alternatively, where we do immunoprecipitate the polymerase, we use an anti-RPB8 (rather than an anti-CTD) again because the latter might displace proteins.

A 'pull-down' experiment suggests the CTD mediates these interactions. Beads coated with glutathione S-transferase (GST) coupled to a truncated CTD with 29 heptads (Patturajan et al., 1998) were incubated in a nuclear extract supplemented with ATP; then, GST-CTD becomes phosphorylated (detected by the appearance of H5 reactivity in immunoblots, not shown). It also interacts with representatives of the translation (S6) and NMD machineries (UPF1), while a control - GST-VP16 (Lin and Green, 1991) - does not (Fig. 2D).

Co-purification with RNA polymerase II

We next determined if these components co-purify with RNA polymerase II. This polymerase has been isolated in different

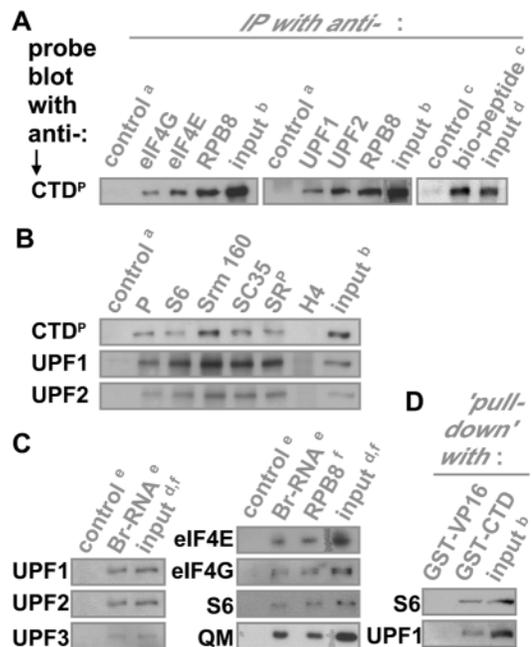


Fig. 2. Interactions between the different machineries. Various antibodies (A-C) or proteins (D) bound to beads were incubated with HeLa nuclear extract, and proteins immunoprecipitating (IP) with bound antibodies or proteins analysed by immunoblotting. ^aNormal mouse serum; ^b1/5 extract used for IP applied directly to lane; ^cbeads coated with anti-biotin, and extract from permeabilized cells containing nascent peptides tagged without ('control') and with biotin ('bio-peptide'); ^d1/10 extract used for IP applied directly to lane; ^ebeads coated with anti-Br, and extract from permeabilized cells containing nascent transcripts tagged without ('control') and with bromine ('Br-RNA'); ^fextract from permeabilized cells containing nascent Br-RNA. (A) CTD^P co-immunoprecipitates with another subunit of the polymerase (RPB8), translation initiation factors (left), NMD proteins (middle) and nascent biotin-peptides (right). (B) CTD^P and NMD proteins co-immunoprecipitate with proteins in ribosomes and newly made transcripts, but not histone H4. (C) Components involved in NMD (left) and translation (right) co-immunoprecipitate with nascent Br-RNA and RPB8. (D) The CTD interacts with S6 and UPF1, but not VP16.

complexes (Hampsey and Reinberg, 1999), often by sonicating a nuclear pellet to release the active enzyme (Maldonado et al., 1996). We extracted cells successively with hypo- and hypertonic buffers, before release with RNase A (Ma et al., 1999). Immunoblotting reveals the forms of the polymerase present at different stages in this established purification procedure. The cytosol contains the hypo-phosphorylated form (II_A) of the largest subunit (Fig. 3A, lane 1), while 0.014-0.65 M ammonium sulphate extracts more hyper-phosphorylated II_O (Fig. 3A, lanes 3,4). After the final RNase treatment, the polymerase (lane 5) proved to be the most active that we have ever isolated using other procedures (activities obtained by this procedure are given in the legend to Fig. 3A); it was purified ~570-fold, rich in an even more hyper-phosphorylated form (II_O*; Fig. 3A) (Kim et al., 2002), and migrated in a sizing column as a complex of >1.5 MDa (not shown). This enzyme was then immuno-affinity purified using an antibody directed against the N terminus of the largest subunit, as others directed

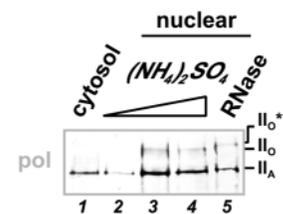
against the CTD can displace associated proteins (Kim et al., 1994). Representative examples of the different machineries (i.e. eIF4E, S6, UPF1 and 2), remain with the polymerase on purification another 14-fold, as TFIIF (XPB, cdk7), and cdk8 that is found in some forms of the holoenzyme (Hampsey and Reinberg, 1999), are not retained (Fig. 3B, compare lanes 1 and 3). The absence of the abundant cytoplasmic protein, cdk7, also suggests there was little cytoplasmic contamination (Dubois et al., 1997). These results show that representatives of the translation and NMD machineries remain with the polymerase as it is purified ~8,000-fold.

Nuclear expression of a 'non-nuclear' protein

The above results are consistent with the various machineries interacting *in vitro*; we next investigated whether they might also interact *in vivo*. We chose to analyze a protein that would not be expected to be found in nuclei, unless some were made there. CD2 is a cell-surface antigen found on leucocytes; it contains a signal sequence plus transmembrane domain, and so is inserted into the endoplasmic reticulum (ER) when it is made in the cytoplasm (Barclay et al., 1997). If translation occurs solely in the cytoplasm, little, if any, should be found in nuclei. But if all messages are proofread by nuclear ribosomes, some should be found in nuclei. To increase the chance of detecting even small amounts of nuclear CD2, plasmids encoding a control protein (EYFP-Mito) and CD2 were transfected into monkey cells that do not normally express CD2, and allowed to replicate for 24 hours to give cells with many CD2 genes. A minority of untransfected cells are essentially unlabelled, as they do not express CD2 (Fig. 4A). Transfected cells (with mitochondria that fluoresce yellow) express CD2, especially in the ER and at the cell surface. Some faint nuclear labelling is also seen, and quantitative analysis shows that this is, on average, ninefold higher than that in control cells expressing only EYFP-Mito (Fig. 4B). This nuclear signal could be due to CD2 that has been inappropriately imported, to CD2 made in nuclei, to out-of-focus flare from the cytoplasm lying above and below the nucleus (even though this is a confocal section), or to a combination of all these factors.

CD2 made in nuclei cannot be inserted into the ER during synthesis so it probably misfolds to be degraded quickly by the proteasome, as components of this complex both interact with the transcription machinery (Thomas and Tyers, 2000) and lie close to nascent Br-RNA (Fig. 1D) (Iborra et al., 2001). Then, lactacystin, a proteasomal inhibitor (Fenteany and Schreiber, 1998), should prevent degradation and so increase nuclear CD2; this proves to be so. Each point in the scatter plots in Fig. 4C indicates the relative amounts of CD2 in nuclear and cytoplasmic areas of images like that in Fig. 4B. Brief exposure to lactacystin slightly increases cytoplasmic labelling, but more than doubles nuclear labelling (Fig. 4C, upper row; arrowheads give averages). This makes it unlikely that much of the nuclear signal is due to import of CD2 made in the cytoplasm, or from out-of-focus flare from cytoplasmic signal, as the two compartments have roughly equal volumes. Significantly, inhibiting transcription with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) or actinomycin D prevents this increase (Fig. 4C), which is expected if nuclear translation generating this CD2 is coupled to transcription. Neither drug has any effect in the absence of lactacystin (Fig. 4C, left). As exposure

A Initial purification



B Immuno-affinity purification (input is RNase-treated fraction)

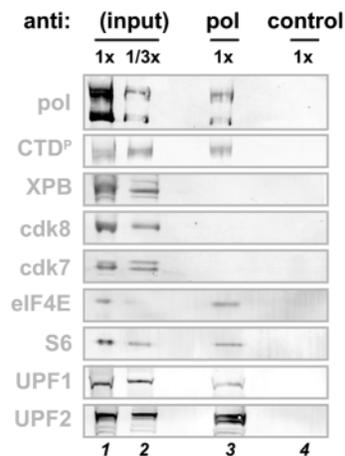


Fig. 3. Association detected by co-purification. (A) Immunoblot (using antibody directed against N terminus of the largest subunit) showing form II_A and II_O content in fractions released from HeLa cells treated successively with a hypotonic buffer ('cytosol', lane 1), and 0.035, 0.14, and 0.65 M (NH₄)₂SO₄ ('nuclear', lanes 2-4) and RNase A ('RNase', lane 5). After RNase treatment the fraction contains an especially hyper-phosphorylated form (II_O*). Polymerizing activities (pmol UTP incorporated/min/μg protein) sensitive to α -amanitin were: 2.7 [polymerase purified by a conventional procedure (Maldonado et al., 1996)], 0.3 (cytosol), 0.4 (0.035 M), 0.2 (0.14 M), 0.3 (0.65 M), 3.5 (sub-nuclear) and 0.1 (negative control lacking DNA). (B) Immunoblots of the RNase-treated fraction before ('input' with 1 \times and 1/3 \times loadings in lanes 1 and 2) and after immunopurification using either antibodies directed against the N terminus of the largest catalytic subunit ('anti-pol', lane 3) or normal mouse serum (lane 4). eIF4E, S6, UPF1 and UPF2 co-immunopurify with the forms II_A and II_O (pol) and the CTD^p; in contrast, XPB, cdk7, and cdk8 do not.

to the transcriptional inhibitors is too short for message synthesis, export, translation and protein import (Jackson et al., 2000), this places protein degradation close in time to transcription.

If translation occurs solely in the cytoplasm, lactacystin could raise cytoplasmic CD2 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. However, these *in vivo* results are simply explained if all inhibitors have their expected effects. Translation coupled to transcription generates misfolded nuclear CD2 that is immediately degraded by nearby proteasomes; in lactacystin, less CD2 is destroyed and this increases CD2 levels. And when

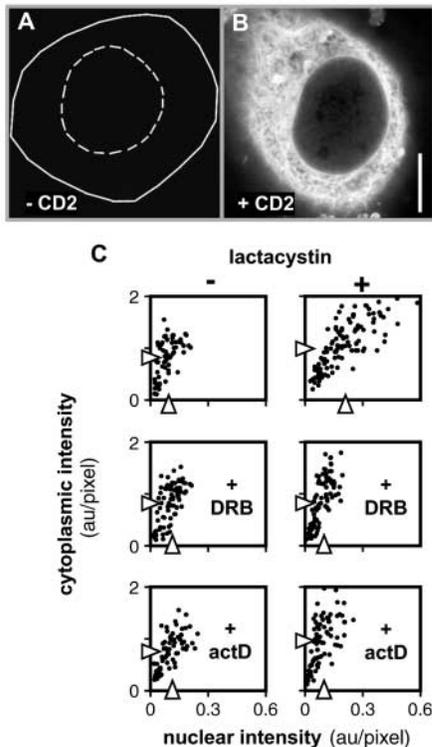


Fig. 4. The effects of inhibitors on CD2 levels in the nucleus and cytoplasm. Cos-1 cells were transfected with plasmids encoding a fluorescent control protein (EYFP-Mito, an enhanced yellow fluorescent protein fused with a mitochondrial targeting sequence from subunit VIII of cytochrome c oxidase) with or without rat CD2, grown for 24 hours, and treated with or without 20 μ M lactacystin for 15 minutes. After fixation, CD2 was indirectly immunolabelled with Cy3 (red), and an equatorial (confocal) section through nuclei collected. Only Cy3 fluorescence is shown. In some cases, 100 μ M DRB or 5 μ g/ml actinomycin D (act D) was added 15 minutes before lactacystin. (A) Cell transfected with plasmids encoding only the control protein (lines mark cell and nuclear peripheries). It contains intensely fluorescing mitochondria (not visible), and no CD2 (average nuclear and cytoplasmic intensities of 0.009 and 0.04 arbitrary units/pixel respectively). (B) Cell transfected with plasmids encoding CD2 (appears white) and the control protein (yellow, not visible); most CD2 is in the ER, however, some faint fluorescence is found in nuclei, and quantitative analysis shows this to have 9 \times the intensity of that seen in an equivalent area in A. 24% (untransfected) cells in this population expressed no EYFP-Mito or CD2 (i.e., with CD2 labelling like that in A); this population was not analysed further. Scale bar: 10 μ m. (C) Intensities in individual cells. Each panel contains results from >75 cells transfected with CD2, each point indicates intensity (arbitrary units/pixel) in nuclear and cytoplasmic areas of one cell, and each arrowhead the average intensity.

transcription, and so nuclear translation (Iborra et al., 2001), is inhibited, the supply of nuclear CD2 is reduced and lactacystin then has little effect.

Instability of newly made nuclear peptides

If nuclear ribosomes proofread new transcripts, we might expect many of the resulting peptides to be faulty and to be degraded quickly; some might misfold (membrane proteins

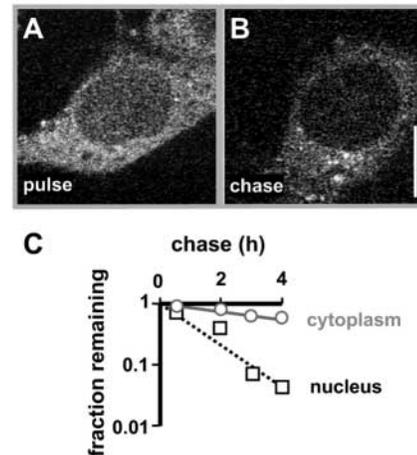


Fig. 5. Instability of newly made nuclear peptides. HeLa cells were permeabilized, allowed to extend nascent peptides in BODIPY-lys-tRNA for 10 minutes in 10 μ g/ml MG-132, washed, reincubated in 1 mg/ml cycloheximide for various times, fixed, and confocal sections through the centre of nuclei collected. (A) Cell after initial pulse in BODIPY-lys-tRNA. (B) As A, but after 2 hours chase. Scale bar: 10 μ m. (C) Fall in intensity of nuclear and cytoplasmic regions of >50 cells after chases of different times. Lines fit exponential decays; $t_{1/2}$ of 120 (nucleus) and 690 (cytoplasm) minutes.

like CD2), others might be truncated (those arising through mis-splicing to give faulty mRNAs with altered reading frames and premature termination codons). We might also expect more peptides made in the cytoplasm to fold correctly, and so to be more stable as they are generated from proofread transcripts in the appropriate cellular location; a pulse-chase experiment showed this to be so. Permeabilized HeLa cells were allowed to extend nascent peptides by \sim 20 residues in both a reversible proteasomal inhibitor, MG-132, and the tagged translational precursor, BODIPY-lys-tRNA; \sim 16% of the resulting fluorescent BODIPY-peptides are nuclear (Fig. 5A) (Iborra et al., 2001). After removing inhibitor and precursor, nuclear BODIPY-peptides disappear sixfold more rapidly during a subsequent chase than cytoplasmic ones (Fig. 5B,C); therefore, at steady state <3% cellular peptides may be made in nuclei, and the values may be even lower in vivo.

Various authors have argued that any nuclear translation machinery cannot support the synthesis of \sim 16% cellular protein (Bohnsack et al., 2002; Dahlberg et al., 2003; Nathanson et al., 2003). However, these results provide a reconciliation, as less than 3% survives to contribute to the steady-state pool.

Discussion

Coordinating the function of eight different machineries

Previous work suggests that transcription, translation and NMD might be closely coupled (Iborra et al., 2001; Muhlemann et al., 2001; Brogna et al., 2002; Buhler et al., 2002). This heterodox idea was difficult to accept if the first process occurred only in nuclei, and the last two only in the cytoplasm. We now show that components of these three machineries colocalize (Fig. 1), interact (Fig. 2), and co-purify

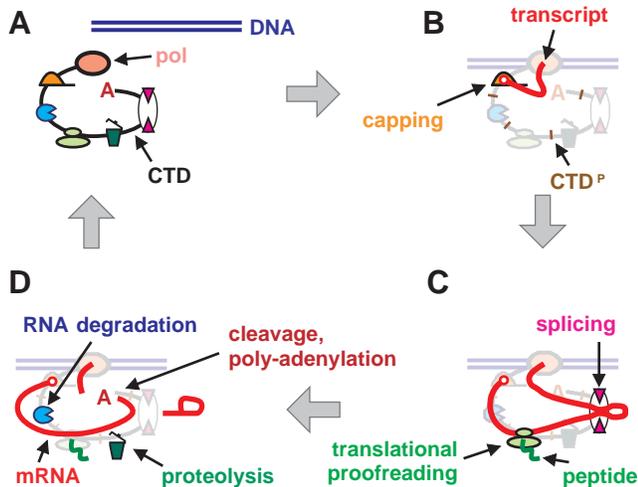


Fig. 6. A model for transcript production. (A) The CTD has the potential to associate with sites involved in capping (Fong and Bentley, 2001), transcript degradation (Andrulis et al., 2002; Libri et al., 2002; Lykke-Andersen et al., 2002), translational proofreading (Iborra et al., 2001; Wilkinson and Shyu, 2002), proteolysis (Iborra et al., 2001), splicing and polyadenylation (labelled A) (Maniatis and Reed, 2002). (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, 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 that may contain many termination codons). (D) Once introns are removed (lariat), the transcript is cleaved, poly-adenylated, and exported to the cytoplasm; but if errors are detected, the faulty transcript and peptide produced during proofreading are degraded by nucleases and proteasomes.

(Fig. 3); moreover, newly made CD2 (a 'non-nuclear' cell-surface antigen) can be found in nuclei, and its degradation is closely coupled to transcription (Fig. 4). As inhibiting the proteasome increases nuclear CD2 levels (Fig. 4C), and as proteasomal and exosomal subunits lie close to nascent transcripts (Fig. 1D) (Iborra et al., 2001), it seems likely that the machinery for degrading faulty transcripts and proteins also lies near the other machineries. Then, all these results are simply explained if the nuclear complex that makes the transcript and goes on to cap, splice and polyadenylate it (Maniatis and Reed, 2002) also contains the appropriate translation, NMD and degradative machineries (Andrulis et al., 2002; Libri et al., 2002; Lykke-Andersen, 2002) (Fig. 6). If so, a ribosome in this complex could 'proofread' a transcript immediately it is made to check it for an appropriately placed cap, tail and termination codon. If all are correctly placed, the message would be exported to the cytoplasm; if not, the NMD machinery would trigger degradation of the transcript, while the proteasome would destroy any misfolded or truncated peptide generated by the ribosome.

The model illustrated in Fig. 6 provides an answer to one powerful argument that translation cannot occur in nuclei (e.g. Bohnsack et al., 2002). In prokaryotes, messages can be translated as they are made, but in eukaryotes ribosomes must be prevented from translating introns; this would generate nonsensical proteins, and those ribosomes would probably also terminate at one of the many intronic stop codons. The

argument runs that eukaryotes elegantly prevent such unproductive translation by confining transcription and splicing to one side of the nuclear envelope, and translation to the other. However, in Fig. 6, the nuclear ribosome is also spatially separated from the intron, and can only translate a spliced message. This model also reinforces the idea that message production should not be viewed as a sequence of spatially and temporarily separated processes (Maniatis and Reed, 2002); rather, one message-producing complex would contain eight linked machines involved in polymerization, capping, splicing, polyadenylation, translation, NMD and degradation (of faulty proteins and RNA).

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References

- Andrulis, E. D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P. and Lis, J. T. (2002). The RNA processing exosome is linked to elongating RNA polymerase II in *Drosophila*. *Nature* **420**, 837-841.
- Barclay, A. N., Brown, M. H., Law, S. K. A., McKnight, A. J., Tomlinson, M. G. and van der Merwe, P. A. (1997). *The Leucocyte Antigen Factsbook*. Second edition, San Diego, USA: Academic Press Inc.
- Blencowe, B. J., Nickerson, J. A., Issner, R., Penman, S. and Sharp, P. A. (1994). Association of nuclear matrix antigens with exon-containing splicing complexes. *J. Cell Biol.* **127**, 593-607.
- Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E. and Gorlich, D. (2002). Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *EMBO J.* **21**, 6205-6215.
- Brogna, S., Sato, T. A. and Rosbash, M. (2002). Ribosome components are associated with sites of transcription. *Mol. Cell* **10**, 93-104.
- Buhler, M., Wilkinson, M. F. and Muhlemann, O. (2002). Intranuclear degradation of nonsense codon-containing mRNA. *EMBO Rep.* **3**, 646-651.
- Dahlberg, J. E., Lund, E. and Goodwin, E. B. (2003). Nuclear translation: what is the evidence? *RNA* **9**, 1-8.
- Dubois, M. F., Vincent, M., Vigneron, M., Adamczewski, J., Egly, J. M. and Bensaude, O. (1997). Heat-shock inactivation of the TFIIF-associated kinase and change in the phosphorylation sites on the C-terminal domain of RNA polymerase II. *Nucl. Acids Res.* **25**, 694-700.
- Fenteany, G. and Schreiber, S. L. (1998). Lactacystin, proteasome function, and cell fate. *J. Biol. Chem.* **273**, 8545-8548.
- Fong, N. and Bentley, D. L. (2001). Capping, splicing, and 3' processing are independently stimulated by RNA polymerase II: different functions for different segments of the CTD. *Genes Dev.* **15**, 1783-1795.
- Hampsey, M. and Reinberg, D. (1999). RNA polymerase II as a control panel for multiple coactivator complexes. *Curr. Opin. Genet. Dev.* **9**, 132-139.
- Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. New York, USA: Cold Spring Harbor Laboratory Press.
- Heesom, K. J., Gampel, A., Mellor, H. and Denton, R. M. (2001). Cell cycle-dependent phosphorylation of the translational repressor eIF-4E binding protein-1 (4E-BP1). *Curr. Biol.* **11**, 1374-1379.
- Herbert, A., Wagner, S. and Nickerson, J. A. (2002). Induction of protein translation by ADAR1 within living cell nuclei is not dependent on RNA editing. *Mol. Cell* **10**, 1235-1246.
- Hilleren, P. and Parker, R. (1999). Mechanisms of mRNA surveillance in eukaryotes. *Annu. Rev. Genet.* **33**, 229-260.
- Iborra, F. J., Jackson, D. A. and Cook, P. R. (1998). The path of transcripts from extra-nucleolar synthetic sites to nuclear pores: transcripts in transit are concentrated in discrete structures containing SR proteins. *J. Cell Sci.* **111**, 2269-2282.
- Iborra, F. J., Jackson, D. A. and Cook, P. R. (2001). Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**, 1139-1142.
- Jackson, D. A., Pombo, A., Iborra, F. J. (2000). The balance sheet for transcription: an analysis of nuclear RNA metabolism in mammalian cells. *FASEB J.* **14**, 242-254.
- Jones, E., Kimura, H., Vigneron, M., Wang, Z., Roeder, R. G. and Cook,

- P. R.** (2000). Isolation and characterization of monoclonal antibodies directed against subunits of human RNA polymerases I, II, and III. *Exp. Cell Res.* **254**, 163-172.
- Kim, Y. J., Bjorklund, S., Li, Y., Sayre, M. H. and Kornberg, R. D.** (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599-608.
- Kim, Y. K., Bourgeois, C. F., Isel, C., Churcher, M. J. and Karn, J.** (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. *Mol. Cell. Biol.* **22**, 4622-4637.
- Kimura, H., Tao, Y., Roeder, R. G. and Cook, P. R.** (1999). Quantitation of RNA polymerase II and its transcription factors in an HeLa cell: little soluble holoenzyme but significant amounts of polymerases attached to the nuclear substructure. *Mol. Cell. Biol.* **19**, 5383-5392.
- Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M. and Horinouchi, S.** (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl. Acad. Sci. USA* **96**, 9112-9117.
- Le Goff, X., Philippe, M. and Jean-Jean, O.** (1997). Overexpression of human release factor 1 alone has an antisuppressor effect in human cells. *Mol. Cell. Biol.* **17**, 3164-3172.
- Lejeune, F., Ishigaki, Y., Li, X. and Maquat, L. E.** (2002). The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. *EMBO J.* **21**, 3536-3545.
- Libri, D., Dower, K., Boulay, J., Thomsen, R., Rosbash, M., Jensen, T. H.** (2002). Interactions between mRNA export commitment, 3'-end quality control, and nuclear degradation. *Mol. Cell. Biol.* **22**, 8254-8266.
- Lin, Y. S. and Green, M. R.** (1991). Mechanism of action of an acidic transcriptional activator in vitro. *Cell* **64**, 971-981.
- Lykke-Andersen, J., Shu, M. D. and Steitz, J. A.** (2000). Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell* **103**, 1121-1131.
- Lykke-Andersen, J.** (2002). Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. *Mol. Cell. Biol.* **22**, 8114-8121.
- Ma, H., Siegel, A. J. and Berezney, R.** (1999). Association of chromosome territories with the nuclear matrix. Disruption of human chromosome territories correlates with the release of a subset of nuclear matrix proteins. *J. Cell Biol.* **146**, 531-542.
- Maldonado, E., Drapkin, R. and Reinberg, D.** (1996). Purification of human RNA polymerase II and general transcription factors. *Meth. Enzymol.* **274**, 72-100.
- Maniatis, T. and Reed, R.** (2002). An extensive network of coupling among gene expression machines. *Nature* **416**, 499-506.
- Maquat, L. E.** (2002). Nonsense-mediated mRNA decay. *Curr. Biol.* **12**, R196-R197.
- Mason, D. W. and Williams, A. F.** (1986). Kinetics of antibody reactions and the analysis of cell surface antigens. In *Handbook of Experimental Immunobiology* (ed. D. M. Weir), pp. 38.1-38.17. Oxford, UK: Blackwell Scientific Publications.
- Mendell, J. T., ap Rhys, C. M. and Dietz, H. C.** (2002). Separable roles for rent1/hUpf1 in altered splicing and decay of nonsense transcripts. *Science* **298**, 419-422.
- Mizushima, S. and Nagata, S.** (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**, 5322.
- Moore, M. J.** (2002). Nuclear RNA turnover. *Cell* **108**, 431-434.
- Muhlemann, O., Mock-Casagrande, C. S., Wang, J., Li, S., Custodio, N., Carmo-Fonseca, M., Wilkinson, M. F. and Moore, M. J.** (2001). Precursor RNAs harboring nonsense codons accumulate near the site of transcription. *Mol. Cell* **8**, 33-44.
- Mukherjee, D., Gao, M., O'Connor, J. P., Raijmakers, R., Pruijn, G., Lutz, C. S. and Wilusz, J.** (2002). The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J.* **21**, 165-174.
- Nathanson, L., Xia, T. and Deutscher, M. P.** (2003). Nuclear protein synthesis: a re-evaluation. *RNA* **9**, 9-13.
- Patturajan, M., Schulte, R. J., Sefton, B. M., Berezney, R., Vincent, M., Bensaude, O., Warren, S. L. and Corden, J. L.** (1998). Growth-related changes in phosphorylation of yeast RNA polymerase II. *J. Biol. Chem.* **273**, 4689-4694.
- Perlick, H. A., Medghalchi, S. M., Spencer, F. A., Kendzior, R. J. and Dietz, H. C.** (1996). Mammalian orthologues of a yeast regulator of nonsense-transcript stability. *Proc. Natl. Acad. Sci. USA* **93**, 10928-10932.
- Pombo, A., Jackson, D. A., Hollinshead, M., Wang, Z., Roeder, R. G. and Cook, P. R.** (1999). Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. *EMBO J.* **18**, 2241-2253.
- Reichert, V. L., le Hir, H., Jurica, M. S. and Moore, M. J.** (2002). 5' exon interactions within the human spliceosome establish a framework for exon junction complex structure and assembly. *Genes Dev.* **16**, 2778-2791.
- Roeder, R. G. and Rutter, W. J.** (1969). Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature* **224**, 234-237.
- Roth, M. B., Zahler, A. M. and Gall, J. G.** (1990). A monoclonal antibody that recognizes a phosphorylated epitope stains lampbrush chromosomes and small granules in the amphibian germinal vesicle. *J. Cell Biol.* **111**, 2217-2223.
- Serin, G., Gersappe, A., Black, J. D., Aronoff, R. and Maquat, L. E.** (2001). Identification and characterization of human orthologues to *Saccharomyces cerevisiae* upf2 protein and upf3 protein (*Caenorhabditis elegans* SMG-4). *Mol. Cell. Biol.* **21**, 209-223.
- Shirley, R. L., Lelivelt, M. J., Schenkman, L. R., Dahlseid, J. N. and Culbertson, M. R.** (1998). A factor required for nonsense-mediated mRNA decay in yeast is exported from the nucleus to the cytoplasm by a nuclear export signal sequence. *J. Cell Sci.* **111**, 3129-3143.
- Thomas, D. and Tyers, M.** (2000). Transcriptional regulation: kamikaze activators. *Curr. Biol.* **10**, R341-R343.
- van der Merwe, P. A., McPherson, D. C., Brown, M. H., Barclay, A. N., Cyster, J. G., Williams, A. F. and Davis, S. J.** (1993). The NH2-terminal domain of rat CD2 binds rat CD48 with a low affinity and binding does not require glycosylation of CD2. *Eur. J. Immunol.* **23**, 1373-1377.
- Wilkinson, M. F. and Shyu, A. B.** (2002). RNA surveillance by nuclear scanning? *Nat. Cell Biol.* **4**, E144-E147.
- Wilusz, C. J., Wang, W. and Peltz, S. W.** (2001). Curbing the nonsense: the activation and regulation of mRNA surveillance. *Genes Dev.* **15**, 2781-2785.