RNA (2000), *6*:962–975. Cambridge University Press. Printed in the USA.

Mature mRNAs accumulated in the nucleus are neither the molecules in transit to the cytoplasm nor constitute a stockpile for gene expression

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ABSTRACT

In higher eukaryotes, the regulation of pre-mRNA processing is still poorly known. The accumulation of various mature mRNAs, which can be observed in the nuclei of mammalian cells, is suggestive of a regulatory role of transport. However, the significance of these nuclear mRNA is presently unknown. We have used a tetracycline-regulated promoter to investigate the dynamics of these pools of mRNAs upon arrest of transcription. We observed, for β-globin and LT-α genes, a slow disappearance of these mRNA from the nucleus, with an apparent half-life that is similar to their cytoplasmic half-life. In view of these dynamics, these mRNA cannot simply be mature mRNAs in transit to the cytoplasm. They could be mRNAs retained in the nucleus, provided that the regulation of mRNA stability is comparable in the nucleus and the cytoplasm. But, because of their limited stability, these nuclear mRNAs cannot constitute a significant stock for gene expression. Alternatively, they could reflect a bidirectional transport of mRNA, that is, to and from the cytoplasm, which would provide a direct explanation for the similarity in both compartments of their half-life and poly(A) tail shortening over time.

Keywords: half-life; nuclear mRNA; splicing; tetracycline; transport

INTRODUCTION

In higher eukaryotes, RNA polymerase II transcripts require extensive modifications before they can be exported as functional mRNAs to the cytoplasm. These include capping, polyadenylation, and, in most cases, the removal of numerous intervening sequences. As splicing can directly alter the coding capacity of transcripts, much attention has been focused on the contribution of this step of pre-mRNA processing to the regulation of gene expression. By contrast, the fate of mature mRNAs within the nucleus (storage, degradation or exportation to the cytoplasm) will only influence the level of expression. Consequently, although the presence of mature mRNAs within nuclear fractions has been noted for a long time, little attention has been paid to their possible significance. For instance, the presence of nuclear mRNAs was reported in landmark studies on β-globin expression in chicken erythroblast (Ross et al., 1982), and ovalbumin and ovomucoid expression in chicken oviduct (Ciejek et al., 1982). In the first study, it was noticed that mature β-globin mRNA accumulated to higher levels than the corresponding precursors although the purity of the isolated nuclei, as investigated by electron microscopy, excluded a possible cytoplasmic origin of this population. Similarly, in the second study, the accumulation of ovalbumin and ovomucoid mRNAs was found to exceed that of precursors. Moreover, about half of these mRNAs were as tightly associated with the nuclear matrix as the precursors. Thus, although it is usually assumed that splicing is the limiting step in the genesis of functional mRNA, experimental data that establish the presence of nuclear pools of mRNA have been available since the first detailed studies on eukaryotic gene expression.

Our laboratory has extended these observations by studying a set of genes in murine fibroblastic NIH3T3 cells (Gondran et al., 1999). In all cases (β-actin, c-*myc*, c-*jun*, CHO-A, and cyclophilin), mature mRNAs were much more abundant within the nucleus than partially spliced transcripts, indicating that the presence of mRNAs in the nuclear fraction is not restricted to genes expressed at very high levels. Thus, these results suggest that, in general, transport is slower than splicing.

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Indeed, if one assumes that the mRNAs that accumulate in nuclei are in transit to the cytoplasm, it is possible to estimate a kinetics of transport from the knowledge of cytoplasmic mRNA turnover. For these genes the nuclear dwell-time of the mRNA was predicted to range between 2 and 20 min. Although these estimates are not completely out of range with the time scale of pre-mRNA transcription and maturation, they are nonetheless, significantly longer than usually assumed. Moreover, these predictions would indicate that the transport rates can vary at least tenfold between genes.

Our limited knowledge of mRNA transport precludes an evaluation of these predictions and, consequently, of the biological significance of the nuclear pools of mRNA. Indeed, although considerable progress has been made in the identification of the protein transport machinery, the characterization of the mRNA export machinery is just beginning to emerge (Stutz & Rosbash, 1998). Moreover, because of the lack of appropriate experimental approaches, there are no reliable data available on the kinetics of mRNA export. Several groups have studied mRNA transport in reconstituted cell-free systems. Using isolated rat liver nuclei (Schröder et al., 1986; Schumm et al., 1989), at best 15% of the mRNA was transported out of the nucleus. Although potentially useful for the characterization of some aspects of transport, it is doubtful that this approach can be used for kinetic studies. Export out of empty nuclear vesicles can be achieved with a higher efficiency (on the order of 80%) (Riedel et al., 1987; Schröder et al., 1992), but can only recapitulate the last step of mRNA export, namely the translocation through the nuclear pore complex itself. Alternatively, a major approach has been to inject mRNA in the nuclei of *Xenopus* oocytes and to follow its transport to the cytoplasm. This strategy has proved very fruitful for the characterization of different export pathways for mRNA, tRNA and snRNA. However kinetics data have been more controversial, as the reported time required for half of the injected mRNA to leave the nucleus varies between 4 min (Dargemont & Kuhn, 1992) and 70 min (Jarmolowski et al., 1994). In addition, the oocyte is a specialized cell with a 100,000-fold more nuclear pores than a somatic cell, making the extrapolation of results to other cellular systems questionable.

Additional data on the dynamics of nuclear mRNA are therefore required for a better understanding of the contribution of mRNA export to gene expression. A first experimental approach has been to use inhibitors of RNA polymerase II, such as actinomycin D, to follow the disappearance of nuclear mRNA (Penman et al., 1968). However, because of the global shut-off of transcription, and the subsequent reorganization of the nucleus (Haaf & Ward, 1996), it is uncertain whether the strategy can provide meaningful information on mRNA dynamics. Indeed, the observation that a large pool of poly(A)+ RNA persisted in the nucleus after transcription shut-off has been interpreted as the indication of an artifactual inhibition of transport by actinomycin D, and this approach was deemed unexploitable for studying mRNA transport. To circumvent this difficulty, we have used in the present study an inducible promoter (Tet-off system; Gossen & Bujard, 1992) to specifically arrest the transcription of the gene of interest without altering other aspects of cellular metabolism. For both the β-globin and the LT-α gene, we observed the accumulation of fully spliced, polyadenylated mRNAs within the nucleus. Following transcription shut-off, these nuclear pools of mRNA decreased with the same kinetics as in the cytoplasm. This dynamic does not fit with that of transported or stored molecules, indicating the existence of a hitherto ignored aspect of mRNA metabolism.

RESULTS

Tetracycline-dependent expression of β-globin and LT-α genes

Rabbit β-globin and murine LT-α were chosen to investigate the behavior of the nuclear pool of mature mRNAs after arrest of transcription. Both genes are not naturally expressed in fibroblasts, and their expression in transfected NIH3T3 cells has been previously characterized (Fig. 1; Neel et al., 1995). β-globin contains two introns, both of them being spliced out efficiently; the cytoplasmic half-life of β-globin mRNA has been reported to be on the order of 11 h in fibroblastic cells (Schiavi et al., 1994). LT-α contains three introns, which are sequentially removed according to a 5′-to-3′ polarity (Weil et al., 1990). In addition, splicing of intron 3 is slower than that of introns 1 and 2. Fully spliced mRNA (designated as N0 in Fig. 1) as well as intron 3-containing transcripts (designated as N1 in Fig. 1) are exported to the cytoplasm. Both cytoplasmic transcripts (C0 and C1 in Fig. 1) have a half-life of 90 min in the T lymphocytic cell line CTLL-2 (Weil et al., 1990).

For both genes the complete transcribed region, including the introns, was inserted in an expression vector under the control of a Tet promoter (Dirks et al., 1994). These vectors were stably transfected into the NIHtTA-16-3 cell line (a kind gift of A. Kröger) that constitutively expresses the chimeric VP16-tTA transactivator allowing for negative regulation by tetracycline (Tet-off system) (Gossen & Bujard, 1992). Independent clones were isolated and screened for their level of expression of β-globin or LT-α mRNA. Clones with a high expression level in the absence of tetracycline (hereafter designated tTA-β-glo and tTA-LT-α) were further characterized.

To measure the extent of regulation of promoter activity by tetracycline, tTA-β-glo cells were cultured in the presence of different doses of tetracycline for 72 h

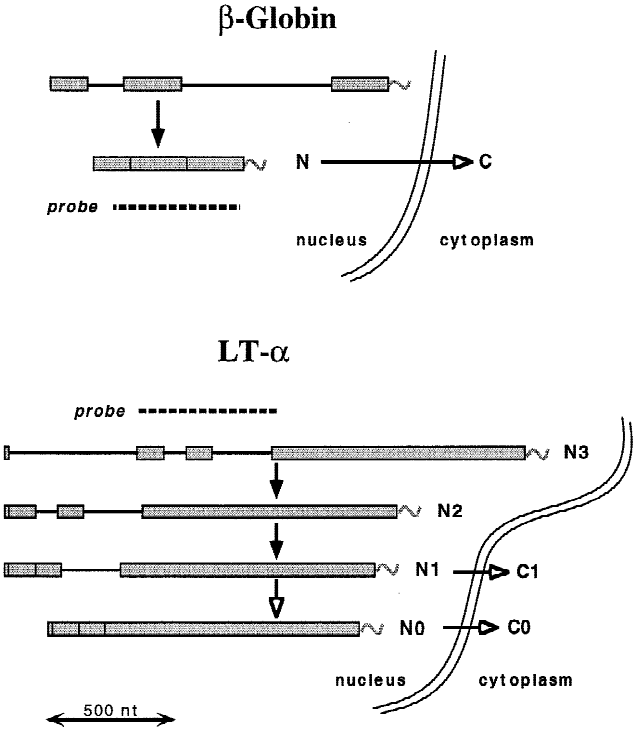


FIGURE 1. Schematic representation of β-globin and LT-α mRNA processing. Pre-mRNA, experimentally observed partially spliced mRNA, and fully spliced mRNA are represented, with black and open arrows indicating rapid and slow processing steps, respectively. Dashed lines represent the β-globin cDNA probe used for Northern blot and the LT-α genomic probe used for RNase protection assay. Scale is indicated at the bottom.

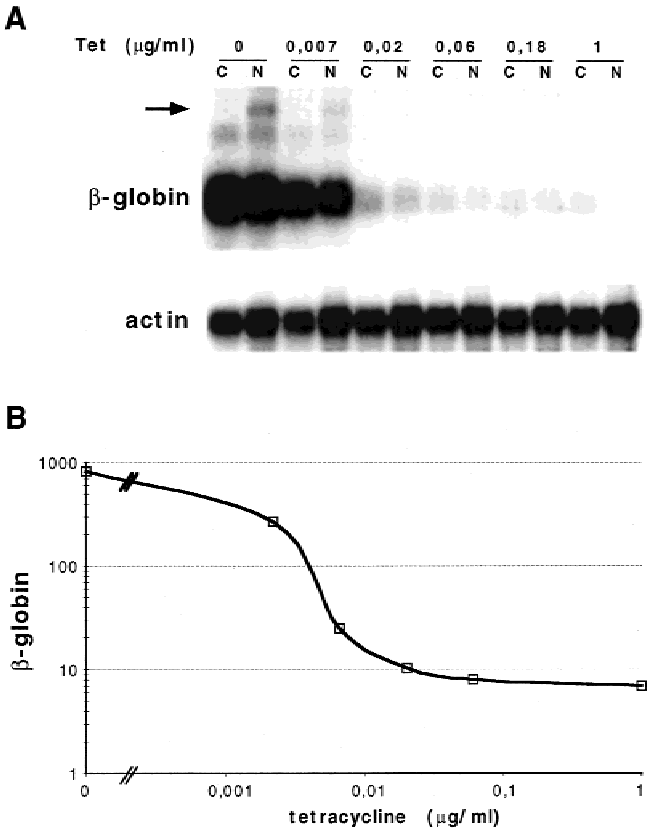


FIGURE 2. Regulation of β-globin expression by tetracycline. **A**: Northern blot analysis. tTA-β-glo cells were treated with the indicated tetracycline concentrations for 72 h. Equal amounts of nuclear (N) and cytoplasmic (C) RNA were hybridized with a β-globin probe (upper panel) represented in Figure 1, and rehybridized with a β-actin probe (lower panel). Migration of β-globin pre-mRNA is indicated with an arrow. **B**: Quantification of cytoplasmic β-globin mRNA. β-globin signal was quantified using a Molecular Dynamics Storm analyzer, corrected for equal loading using β-actin signal, and plotted as a function of tetracycline concentration. Note that both axes are on a logarithmic scale.

so that a new steady state had been reached. This incubation time corresponds to ten cytoplasmic half-lives for β-globin mRNA (see below). Cytoplasmic and nuclear RNA was extracted and analyzed by Northern blot (Fig. 2A). Quantification of cytoplasmic β-globin mRNA indicated that repression of β-globin expression was maximal with 1 μg/mL tetracycline, leading to a 120-fold decrease in mRNA accumulation (Fig. 2B). Intermediate levels of expression could be achieved using 2–60 ng/mL tetracycline. The same experiment was performed on tTA-LT-α cells using an incubation of 20 h in the presence of tetracycline, a time corresponding to ten cytoplasmic half-lives for LT-α mRNA. As the short size of LT-α intron 3 (225 nt long) does not allow for proper resolution of the two cytoplasmic transcripts (C0 and C1) on Northern blot, LT-α transcripts were analyzed by an RNase protection assay using a probe extending from exon 2 to exon 4 (Weil et al., 1990) (Fig. 3A). Maximal repression was achieved with 1 μg/mL tetracycline and resulted in an 18-fold decrease in fully spliced mRNA (C0 in Fig. 3B) and a 7-fold decrease in intron 3-containing transcripts (C1 in Fig. 3B). Unexpected fragments, indicated by asterisks, were also detected. They correspond to abnormal transcripts and, because their expression followed that of normal transcripts, their identity was not investigated further.

The 120-fold repression of β-globin and the 18-fold repression of LT-α expression provided the opportunity to investigate the dynamics of the nuclear pool of mature mRNAs after inhibition of transcription by tetracycline.

Characterization of nuclear fractions

As mRNAs are expected to accumulate in the cytoplasm, the purity of the nuclear fraction is a major concern when investigating their presence in nuclei. A common technique to prepare nuclei is by cellular fractionation in the presence of mild detergents, such as NP40. In the case of the NIHtTA-16-3 cells, this procedure led to the persistence of cytoplasmic remnants around the nuclei, as judged by light microscopy (data not shown). Use of other detergents such as a combination of Tween 20 and deoxycholate (also called Magic Wash) or Triton X-100 led to either the same results or nuclear lysis. We therefore added 10 mM EDTA to the

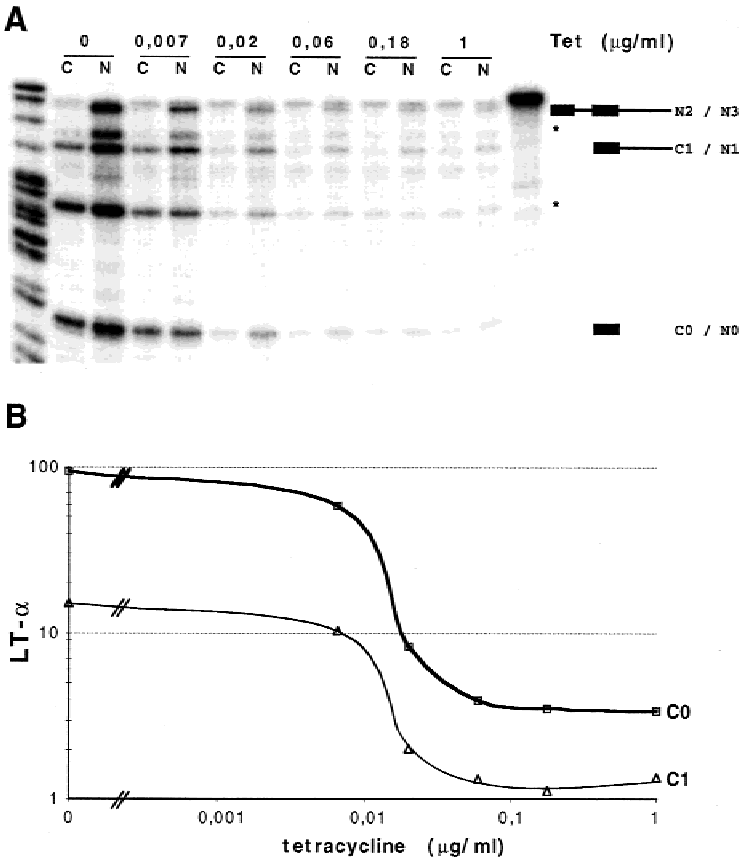


FIGURE 3. Regulation of LT-α expression by tetracycline. **A**: RNase protection assay analysis. tTA-LT-α cells were treated with indicated tetracycline concentrations for 20 h. Equal amounts of nuclear (N) and cytoplasmic (C) RNA were analyzed using the LT-α probe represented in Figure 1. Migration of the riboprobe fragments protected by partially and fully spliced transcripts is indicated on the right. See Figure 1 for nomenclature of the transcripts. Unexpected protected fragments are indicated with asterisks. **B**: Quantification of cytoplasmic LT-α mRNA. LT-α signals were quantified using a Molecular Dynamics Storm analyzer, corrected for the number of labeled uridine in each fragment, and plotted as a function of tetracycline concentration. Concentration of mRNA samples was first checked by Northern blot analysis with β-actin probe. Note that both axes are on a logarithmic scale.

NP40 lysis buffer to release mRNA from the polysomes that could be present in the cytoplasmic remnants. No morphological difference was observed by light microscopy, but the percentage of RNA present in the nuclear fraction dropped from around 25% to 11% of total cell RNA, a value that is comparable with what has been observed in other fibroblastic cell lines.

Nuclei prepared in the presence or absence of EDTA were analyzed by electronic microscopy (Fig. 4). The extent of cytoplasmic remnants was similar in EDTA-treated and EDTA-untreated nuclei. However, ribosomes were no longer detectable after EDTA treatment. Otherwise, the structural content of the nuclei showed no difference between the two samples as anticipated from previous biochemical analysis of EDTA-treated nuclei (Long et al., 1979).

EDTA-treated nuclei were also analyzed for the presence of cytoplasmic mRNA using in situ hybridization with oligo(dT) probe and confocal microscopy. Nuclei were deposited on glass coverslips, fixed with paraformaldehyde, and hybridized with a fluorescent FITC-oligo(dT) probe. FITC-oligo(dA) was used as a negative control. Figure 5 presents a gallery of three nuclei, representative of different levels of cytoplasmic contamination. In each case an optical section through the mid-plane of the cell is presented. No particular accumulation of fluorescence could be observed in the perinuclear region, as previously described on intact cells (Taneja et al., 1992; Bassell et al., 1994; Huang et al., 1994). To estimate the amount of cytoplasmic polyadenylated mRNA present in our nuclear fraction, 14–20 sections were collected for each nucleus and fluorescence was quantified in both the nuclear and cytoplasmic area, as detailed in Materials and methods. Results were summed to obtain the cytoplasmic contamination per nucleus. Nuclei 1, 2, and 3 showed a contamination of 10, 4, and 1%, respectively (Table 1). Taking into account the proportion of nuclei similar to nuclei 1, 2, and 3 (24, 42, and 34%, respectively), the average level of contamination of the nuclear fraction by cytoplasmic poly(A)+ RNA was determined to be on the order of 4%.

In the following study all nuclear fractions were prepared in the presence of EDTA. Total RNA was purified from nuclear and cytoplasmic fractions of tTA-β-glo cells and analyzed by Northern blot (Fig. 2A, Tet 0 μg/mL). When equal amounts of RNA were analyzed, comparable levels of globin mature mRNA were observed in both fractions (1.2-fold more in the cytoplasm). Thus, the cytoplasmic contamination of the nuclear fraction (4%) should contribute for only 5% of the nuclear signal. Taking into account the relative abundance of nuclear and cytoplasmic RNA per cell, 8% of the mature β-globin mRNA is present in the nucleus. The same

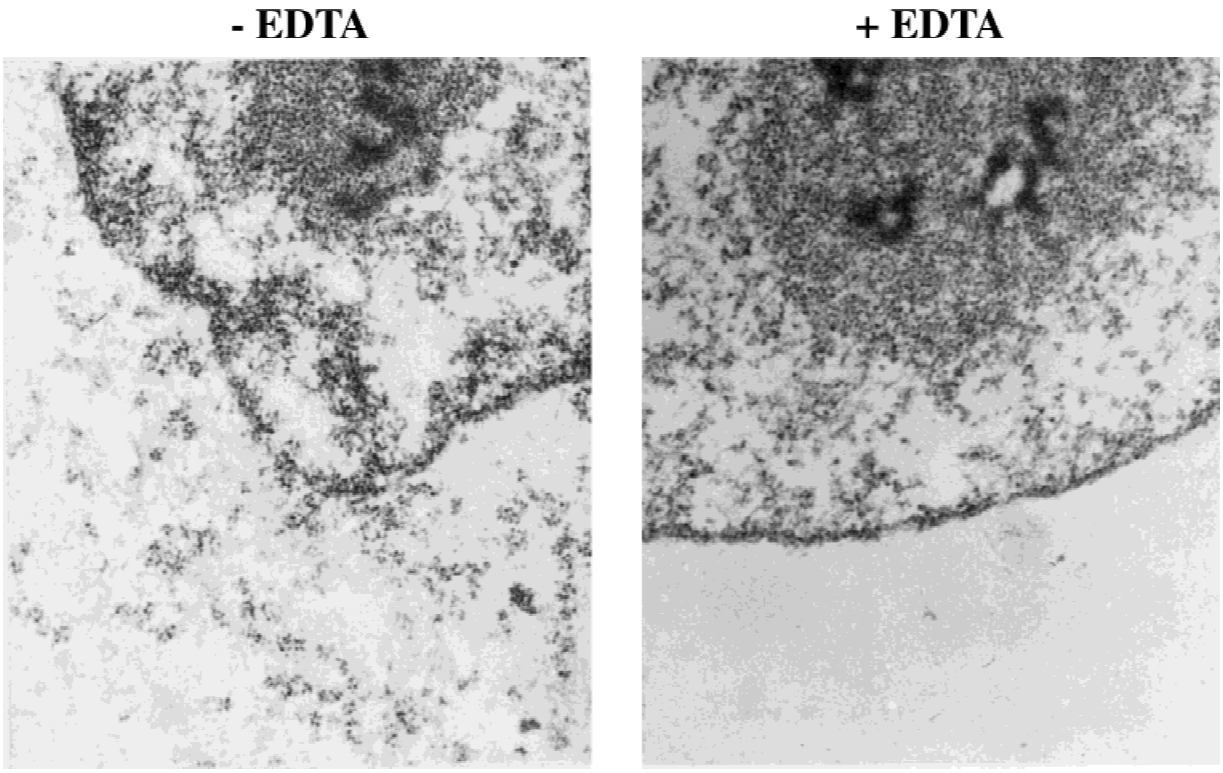


FIGURE 4. Electron microscopic analysis of nuclear preparations. Nuclei of tTA-β-glo cells were prepared in the presence or absence of EDTA. Samples were stained with uranyl acetate and lead citrate and analyzed with an electron microscope at 17,000× magnification. For both samples, the boundary between a nucleus and surrounding cytoplasmic remnants is shown. A nucleolus is visible in the upper part of the image.

experiment was performed on tTA-LT-α cells (Fig. 3A, Tet 0 μg/mL). Fully spliced LT-α mRNA was more abundant in the nuclear fraction than in the cytoplasmic one (1.8-fold), so that 17% of the fully mature mRNA of the cell is nuclear. In contrast to β-globin, and as previously described (Weil et al., 1990; Neel et al., 1995), LT-α partially spliced transcripts (N2, N3, and N1) could be readily observed in the nuclear fraction, indicating limiting rates of splicing. For comparison, the nucleocytoplasmic partition of an endogenous gene was also analyzed and 17% of actin mRNA was found in the nucleus.

Dynamics of the nuclear pool of mRNA following arrest of transcription

To investigate the fate of mature β-globin transcripts after arrest of transcription, tTA-β-glo cells were grown in the presence of 1 μg/mL tetracycline for up to 45 h. Nuclear and cytoplasmic RNA were extracted, analyzed by Northern blot and the β-globin signal quantified (Fig. 6A). Cytoplasmic β-globin mRNA decreased with a half-life of about 7 h. In agreement with a rapid splicing, nuclear β-globin pre-mRNA could no longer be detected 1 h after addition of tetracycline (data not shown). By contrast, the nuclear population of mature mRNAs decreased only very slowly. Earlier time points were then analyzed to investigate whether a subpopulation of nuclear mRNAs could have been rapidly exported (Fig. 6B). At all times, the decrease in the nuclear mRNA population was monophasic and paralleled that of the cytoplasmic mRNA. Thus, the large majority of the nuclear mature mRNAs were apparently not rapidly exported from the nuclei. Overall, the decrease in nuclear mature mRNA was similar to that of cytoplasmic mature mRNA, with a 7 h half-life.

To check whether this result was specific for this tTA-β-glo clone or reflected its high level of expression, two other independent NIHtTA β-globin transfectants were similarly analyzed (Fig. 6C). In all cases, and although one clone expressed 10 times less β-globin mRNA than the others, the same dynamics were observed in the nuclear and cytoplasmic compartments. Therefore the slow disappearance of nuclear β-globin mRNA is not related to the level of expression of the gene.

The same experiment was performed with tTA-LT-α cells that were incubated for up to 2 h in the presence of 1 μg/mL tetracycline. Nuclear and cytoplasmic RNA were extracted, analyzed by an RNase protection assay and the amount of the different LT-α transcripts quantified (Fig. 6D). The cytoplasmic fully spliced mRNA (C0) decreased with a half-life of 2 h,

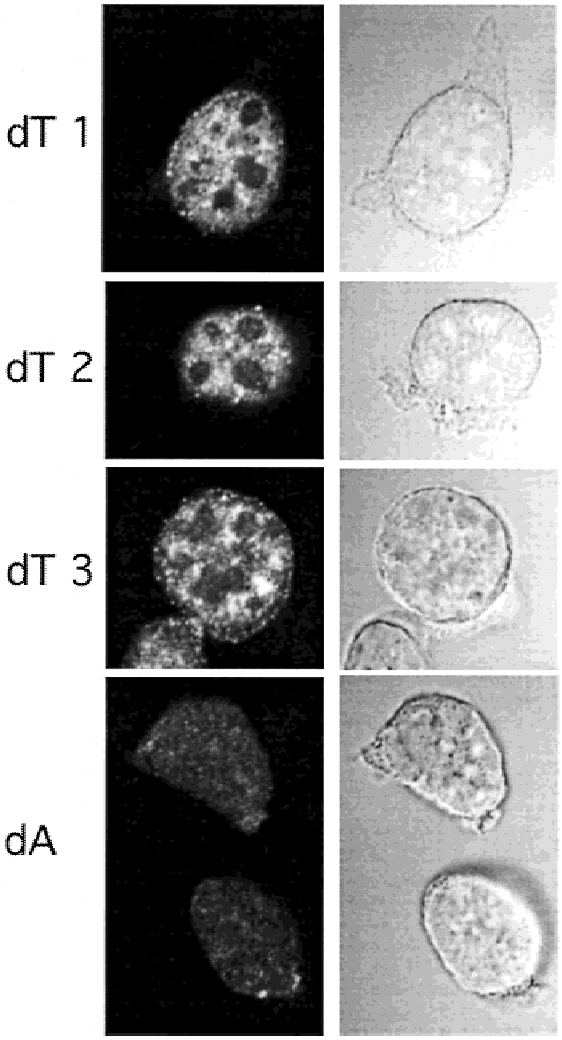


FIGURE 5. In situ hybridization of nuclei prepared with EDTA. Nuclei of tTA-β-glo cells were prepared in the presence of EDTA. Samples were hybridized with a fluorescent oligo(dT) or oligo(dA) probe and fluorescence was analyzed with a confocal microscope. Three nuclei hybridized with oligo(dT), representative of various extents of cytoplasmic contamination (see text), and two hybridized with oligo(dA) are shown. In each case an optical section through the mid-plane of the cell is presented on the left and the differential interference contrast image on the right.

whereas intron 3-containing transcripts (C1) were slightly more stable. This was confirmed in 4 h time-course experiments (data not shown). In the nucleus, pre-mRNAs (N2 and N3) disappeared rapidly, with a combined half-life of about 13 min. This rapid disappearance of the precursors confirmed a rapid inhibition of transcription, within a few minutes of tetracycline addition. As for β-globin, no rapid decrease in the nuclear population of fully spliced mRNA (N0) was observed following arrest of transcription. Indeed, after a delay that can be accounted for by the splicing of the pool of precursors, the decrease in nuclear mature mRNA paralleled the decrease in cytoplasmic mature mRNA, with a 2 h half-life.

Characterization of the poly(A) tail of β-globin nuclear mRNA

In the preceding analysis, nuclear β-globin mRNA had a migration on gel identical with that of the cytoplasmic one, suggesting that it was fully processed mRNA (both spliced and polyadenylated). The metabolism of the poly(A) tail was further investigated in a reinduction experiment designed to roughly synchronize the transcripts. Indeed, it has been previously reported that if limiting doses of tetracycline are used, a reinduction from the tet-off promoter/regulator system could be observed within 2 h after washing off the tetracycline from the culture medium (Xu et al., 1998). tTA-β-glo cells were grown for 3 days in the presence of 80 ng/mL of tetracycline, a concentration just sufficient to achieve maximal inhibition of transcription. Cells were then extensively washed and further incubated in the absence of tetracycline. A Northern blot analysis of the nuclear and cytoplasmic RNA prepared at different time postwashing is presented Fig. 7A. Resumption of β-globin mRNA accumulation could be detected from 2 h on. Importantly, in the nucleus, the newly synthesized transcripts appeared to migrate more slowly than most of the transcripts present in unrepressed cells (Fig. 7A, lane C), suggesting that they have a longer poly(A) tail. To confirm this, the 3 h, 6 h, and unrepressed samples were submitted to poly(A) tail digestion using oligodT/RNase H treatment (Fig. 7B). After poly(A) tail removal, the nuclear and cytoplasmic β-globin mRNAs had the expected migration for the 713-nt fully spliced transcripts according to the molecular weight markers (Fig. 7B, lane MW) (note that introns 1 and 2 are 126 and 573 nt long, respectively). Furthermore, the shift in mobility due to the poly(A) tail digestion indicated that at 3 h postwashing, the β-globin mRNAs had a poly(A) tail of about 200 nt, which ranged from 150 to 200 nt at 6 h and from 30 to 200 nt in unrepressed cells (see Fig. 7B, bottom panel, for an intensity adjusted picture). Thus, as expected, newly synthesized transcripts had a long poly(A) tail that slowly decreased over time, in agreement with the deadenylation rate of 20 nt/h that has been observed for other mRNAs (Mercer & Wake, 1985). More importantly, this deadenylation was observed not only in the cytoplasmic compartment, but also in the nuclear compartment.

Is there a relation between transcription and mRNA export rates?

Our results concerning the existence of a long-lived nuclear population of polyadenylated mRNAs were reminiscent of those obtained with general inhibitors of transcription (Penman et al., 1968; Huang et al., 1994). However interpretation of these latter experiments has been controversial (see Introduction and Discussion), it was therefore of interest to relate these two approaches.

TABLE 1. Quantification of the fluorescence following in situ hybridization of isolated nuclei with an oligo(dT) probe.

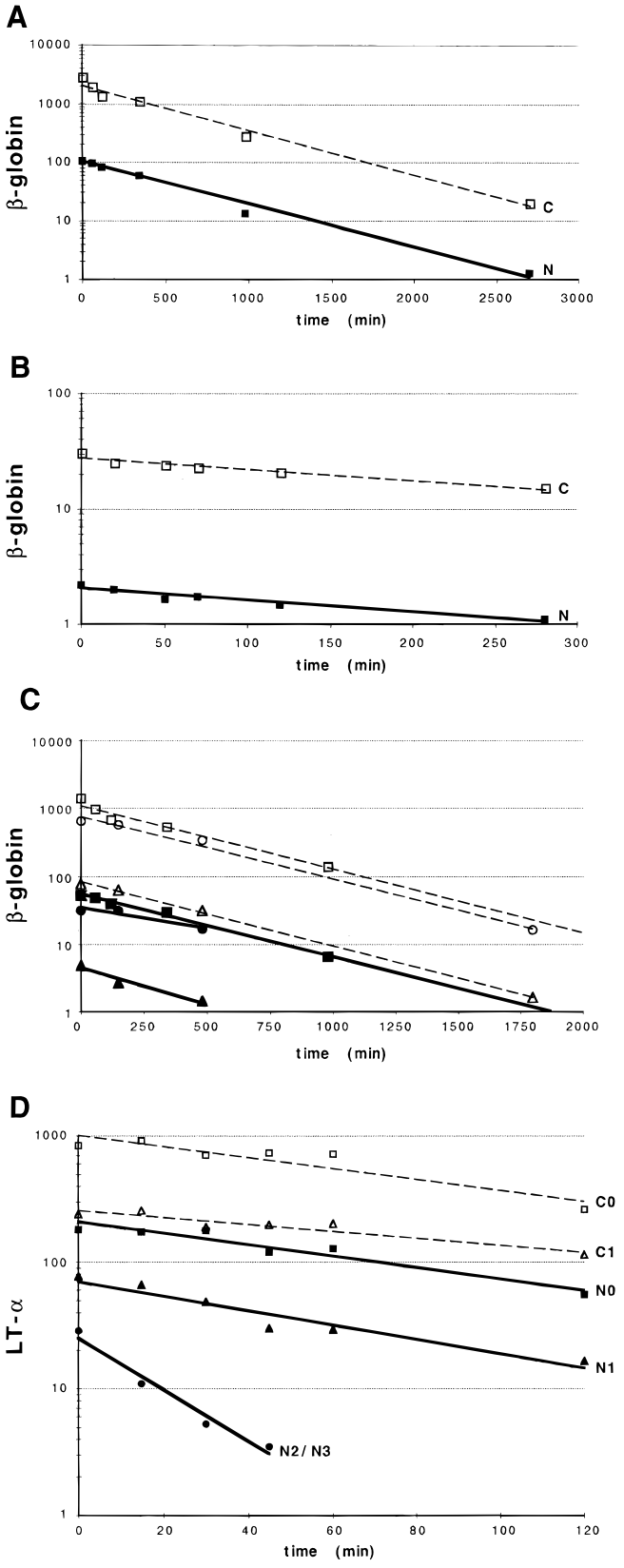
	Exp. dT1 signal		Exp. dT2 signal		Exp. dT3 signal	
Optical section	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
1	110032	77163	262288	56970	215180	7150
2	154868	84660	349338	37107	324254	4686
3	200811	94162	368431	28365	410368	7284
4	237636	87465	380184	22020	445130	6180
5	277310	69568	374490	17360	443100	
6	278376	59328	344034	14000	424780	
7	310000	47880	323676	6760	392512	
8	324408	11355	297308		365977	
9	301840	8304	258552		346408	
10	280638		231136		318157	
11	265090		198198		282983	
12	236124		163392		221061	
13	207720		133035		158775	
14	174096		100521		114736	
15	152124		72672			
16	128363					
17	102657					
18	78225					
19	60216					
20	48384					
Cytoplasmic contamination (%)	9.6		3.4		0.6	

Nuclear and cytoplasmic RNA from tTA-β-glo cells incubated in the presence of 10 μg/mL actinomycin D were analyzed by Northern blot and the signal quantified as above (Fig. 8A). In spite of a rapid disappearance of β-globin pre-mRNA, which was no longer detectable after 20 min (data not shown), mature β-globin mRNA persisted in the nucleus. In both the nuclear and the cytoplasmic compartments, the observed mRNA half-life was similar to that determined with tetracycline. The same analysis was performed in actinomycin D-treated tTA-LT-α cells and extended to a quantification of pre-mRNA. As in the presence of tetracycline, the half-life of LT-α precursors (N3 and N2) was on the order of 13 min and fully spliced mRNAs had the same half-life in the nucleus (N0) and in the cytoplasm (C0). The full concordance between the results of gene-specific and general inhibition studies argues against a rapid block of mRNA transport by actinomycin D, and therefore suggests that the persistence of fully spliced and polyadenylated mRNAs in the nucleus is true for most genes.

These results, however, raised the possibility of a coupling between transcription and mRNA export. To investigate this issue, we used the opportunity to modulate the expression level from the tet-off promoter with low doses of tetracycline to search for a relationship between the rate of transcription and the rate of mRNA export. We first considered whether the nucleocytoplasmic partition was influenced by the expression level. As detailed previously (Gondran et al., 1999), using the simplest model, this partition reflects the balance between mRNA export and mRNA degradation in the cytoplasm. Nuclear and cytoplasmic RNA obtained from tTA-β-glo cells incubated for 72 h in the presence of various amount of tetracycline (Fig. 2) were used for analysis. The percentage of β-glo mRNA present in the nucleus was plotted as a function of β-globin expression (Fig. 9A). Although the activity of tTA promoter varied over two orders of magnitude, no significant difference was observed in this percentage. The degradation rate of β-globin mRNA expressed at different levels was assessed by incubating further the cells in the presence of 1 μg/mL tetracycline to obtain maximal inhibition. No difference in β-globin mRNA stability was observed (data not shown), leading to the conclusion that there is no difference in the transport rate.

To address this issue dynamically, a second approach was used. If the transport rate is related to the transcription rate, nuclear β-globin mRNA may leave the nucleus faster after a 10-fold reduction in expression than after a 120-fold reduction. tTA-β-glo cells were incubated for up to 42 h in the presence of 40 ng/mL tetracycline to obtain an intermediate level of β-globin expression. Nuclear and cytoplasmic RNAs were then analyzed (Fig. 9B). At 42 h, the cytoplasmic β-globin mRNA level had nearly reached an equilibrium, with a 13-fold decrease in expression. In this experiment a lag in the decrease of cytoplasmic β-globin mRNA was observed, probably due to a stimulation of transcription by serum during the complete exchange of

the culture medium that is required to achieve an accurate concentration of tetracycline (see expression of actin mRNA in Fig. 9B). Importantly, the nuclear β-globin mRNA decreased with the same 7 h half-life as after a complete shut-off of transcription. In conclusion, we found no indication for a relationship between transcription and transport rates.



DISCUSSION

In this study, we have derived cell lines that express the β-globin or LT-α gene under the control of a tetracycline-regulated promoter (Gossen & Bujard, 1992). A 120-fold and an 18-fold regulation of the expression level was achieved for β-globin and LT-α, respectively, and the rapid disappearance of mRNA precursors confirmed that the expression shut-off took place within a few minutes. As we have previously reported for endogenous genes, fully processed (i.e., spliced and polyadenylated) β-globin and LT-α mRNAs accumulated in the nuclear fraction of these cell lines, the nuclear pool amounting to 8% and 17% of the cellular accumulation of these mRNAs, respectively. In situ hybridization data reported here as well as by other groups do not show an increased accumulation of poly(A)+ mRNA in the perinuclear region, indicating that these molecules are likely to be nucleoplasmic rather than nuclear-associated. Moreover, it has been previously reported that β-globin mRNA does localize diffusely through the cytoplasm and not in the perinuclear region in transfected fibroblasts (Veyrune et al., 1996).

Following inhibition of transcription with tetracycline, these nuclear pools slowly decreased with a half-life of 7 h for β-globin and 2 h for LT-α. As export to the cytoplasm and degradation within the nucleus are expected to contribute independently and additively to the turnover of these nuclear mRNAs, these half-lives are unexpectedly long.

Nuclear pools of mRNA and export to the cytoplasm

mRNAs in the process of being exported constitute an obvious source of mature mRNAs in the nucleus. Following transcription shut-off, these mRNAs should disappear from the nucleus under the combined action of transport and nuclear degradation. Although the actual transport rates are not known, it is possible to derive an

FIGURE 6. Dynamics of the transcripts following arrest of transcription with tetracycline. **A**,**B**: tTA-β-glo cells (**A**: long kinetics, **B**: short kinetics) were treated with 1 μg/mL tetracycline for indicated periods of time. Equal amounts of nuclear and cytoplasmic RNA were analyzed by Northern blot using β-globin and β-actin probes, as for Figure 2. Results were corrected for the relative size of the nuclear and cytoplasmic compartments and plotted as a function of time. **C**: The same experiment was repeated on two independent NIHtTA-16-3 clones expressing a high (circles) or low (triangles) level of β-globin mRNA in the absence of tetracycline. The results obtained with the first tTA-β-glo clone (exp. A) were included for comparison (squares). **D**: The same experiment was performed on tTA-LT-α cells and RNA was analyzed by RNase protection assay, as for Figure 3.

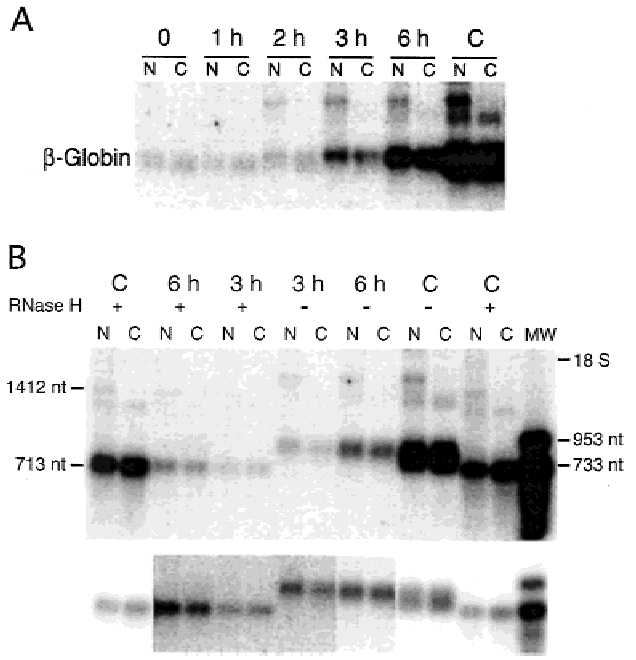


FIGURE 7. Polyadenylation of nuclear transcripts. **A**: Resumption of β-globin transcription following tetracycline removal. After a 72 h culture in the presence of 80 ng/mL of tetracycline, tTA-β-glo cells were reincubated in the absence of tetracycline for indicated periods of time. Control cells (lane C) were not preincubated with tetracycline. Equal amounts of nuclear (N) and cytoplasmic (C) RNA were analyzed by Northern blot using a β-globin probe. **B**: Poly(A) tail analysis. RNA from indicated samples before (− RNase H) or after (+ RNase H) poly(A) tail digestion was analyzed by Northern blot using a β-globin probe, along with a radioactive RNA molecular weight marker (MW). Both a straight representation of the hybridization (upper panel) and an intensity adjusted image (lower panel) are presented.

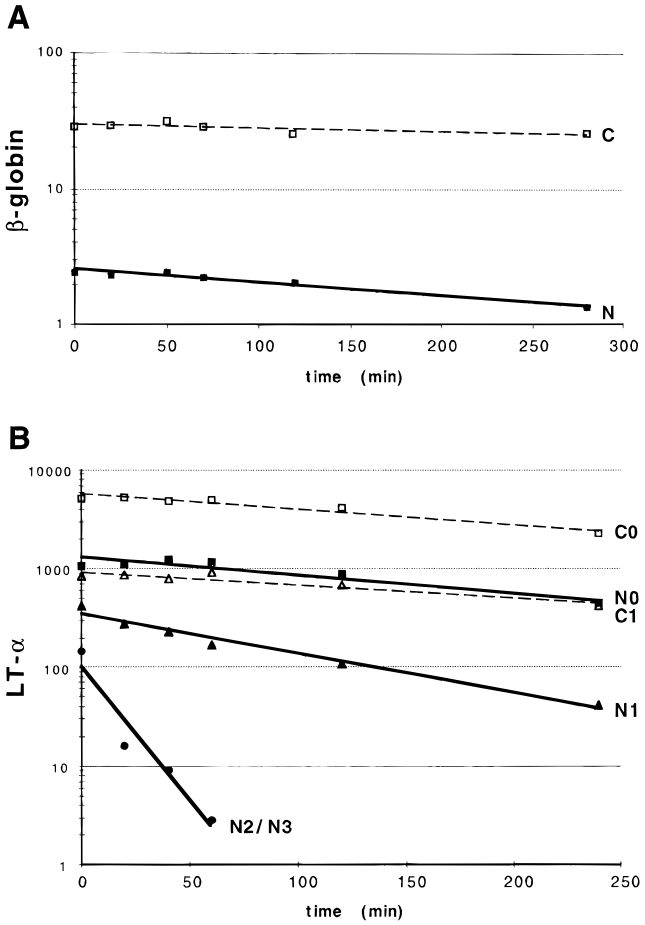


FIGURE 8. Dynamics of the transcripts following arrest of transcription with actinomycin D. tTA-β-glo cells (**A**) and tTA-LT-α cells (**B**) were treated with 10 μg/mL actinomycin D for indicated periods of time. Nuclear and cytoplasmic β-globin (**A**) and LT-α (**B**) transcripts were analyzed, as for Figure 6, except that β-actin normalization was omitted because it is unwarranted.

upper limit of the nuclear dwell-time of mRNAs in transit, from the knowledge of the flux of molecules in the cytoplasm and the size of the nuclear population (Gondran et al., 1999). Using this approach, we have predicted nuclear dwell-time ranging from 2 to 20 min for endogenous genes, such as β-actin, c-*myc*, c-*jun*, cyclophilin, or CHO-A (Gondran et al., 1999). Similarly, the results of the present study can be used to predict nuclear dwell-times of 35 and 25 min for β-globin or LT-α mRNAs, respectively. Thus, the observed monotonous decrease in nuclear mRNAs with its 7 h and 2 h half-lives cannot reflect the actual export rate of these messages. Moreover, it is also inconsistent with the usual assertion that Pol II transcripts are extremely unstable in the nucleus.

The above conclusion on transport is only dependent on the assumption that it can proceed unabated after transcription shut-off. A mechanistic coupling between transcription and polyadenylation as well as transcription and splicing has been proposed (Mortillaro et al., 1996; Yuryev et al., 1996; Cramer et al., 1997; McCracken et al., 1997). In these cases, the RNA polymerase II presumably loads essential factors onto the nascent transcript, which subsequently contribute to polyadenylation and splicing. In this scheme, however, one can assume that once a given transcript has been synthesized, the transcription machinery is no longer required for the subsequent steps. Accordingly, we observed that splicing of cleaved and polyadenylated precursor mRNAs proceeded after transcription arrest. Nevertheless, we used the opportunity to achieve intermediate expression levels with low doses of tetracycline to search for a correlation between transcription rate and mRNA transport. Over a range of two orders of magnitude in expression levels, we found no change in the nucleocytoplasmic partition of mRNA, the cytoplasmic half-life also being unchanged. Similarly, the same half-life was observed for β-globin nuclear mRNA when the expression level was reduced 13-fold instead of a maximal reduction of 120-fold. Therefore, we found no evidence to support a coupling between the rates of transcription and transport.

In summary, our results exclude that mRNA in the process of being exported constitutes the bulk of the nuclear pool of β-globin or LT-α mRNA. Indeed, be-

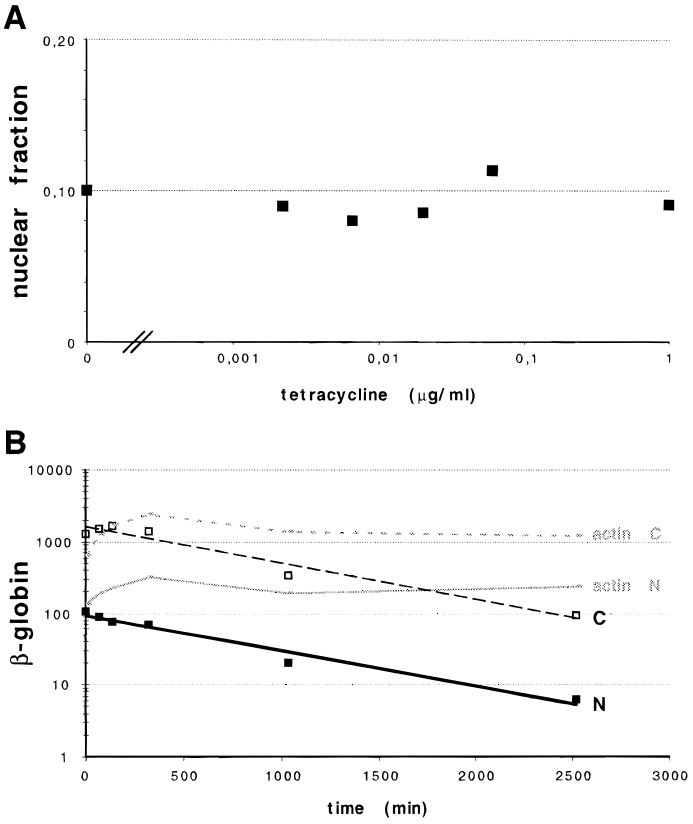


FIGURE 9. Accumulation and dynamics of nuclear β-globin mRNA as a function of the expression level. **A**: Nucleocytoplasmic partition. The Northern blot experiment presented in Figure 2A was reanalyzed. Both nuclear and cytoplasmic β-globin mRNA were quantified as for Figure 2B and the percentage of β-globin mRNA present in the nucleus was plotted as a function of tetracycline concentration. **B**: Dynamics of the transcripts following a diminution of transcription. tTA-β-glo cells were treated with 40 ng/mL tetracycline for the indicated periods of time. Equal amounts of nuclear and cytoplasmic RNA were analyzed by Northern blot using β-globin and β-actin probes, as for Figure 2A. β-globin and β-actin signals were quantified, and results were corrected for the relative size of the nuclear and cytoplasmic compartments and plotted as a function of time.

cause we have been unable to observe a rapid efflux of at least some mRNA following transcription shut-off, the population of mRNA in transit should amount to less than 10% of the nuclear pools of β-globin and LT-α mRNAs.

Nuclear pools of mRNA and storage

Nuclear mRNAs could therefore be molecules stored within the nucleus as proposed by Huang et al. (1994), their dynamics reflecting their degradation in this compartment rather than their export to the cytoplasm. These molecules could either have escaped from the transport pathway or be actively retained within the nucleus.

One particular system where nucleocytoplasmic transport of an mRNA has been studied is the salivary glands of *Chironomus tentans*, where the Balbiani ring (BR) mRNA can be identified by electronic microscopy. Dynamic studies following bromo-uridine labeling of mRNA demonstrated the full turnover of half of the nucleoplasmic BR mRNA within 120 min (Singh et al., 1999). That bromo-uridine could not be detected at this time in the other half was attributed to a technical limitation. It is also possible that some of these unlabeled BR mRNA may be stable and incompetent for transport. Indeed recent studies have shown that a portion of nucleoplasmic BR mRNA is attached to thin connecting fibers that merge into a fibrogranular cluster (Miralles et al., 2000). The mobility of this complexed mRNA should be much reduced compared to free BR mRNA and may correspond to resident mRNA.

In our study, the similarity of the half-lives in the nuclear and cytoplasmic compartments of β-globin and LT-α mRNA is unexpected. Moreover, the limited stability of the nuclear pools does not fit with the usual concept of storage, which would imply some protection from degradation. Recent studies have, however, revealed the presence of components of the mRNA degradation machinery in both the nucleus and the cytoplasm. For instance, similar complexes of nucleases (exosomes) have been detected in the nuclear and cytoplasmic compartments (Allmang et al., 1999), whereas some of the proteins implicated in the control of mRNA degradation can shuttle between these compartments (Zhang et al., 1993; Loflin et al., 1999). Thus, it is conceivable that the same control of mRNA degradation operates in the nucleus and the cytoplasm, including the progressive deadenylation which, in the cytoplasm, precedes degradation. This similar regulation of mRNA stability in the nucleus and the cytoplasm would prevent a distinct role of nuclear mRNA as a stockpile for gene expression. Accordingly, the accumulation of mRNA within the nucleus would be more appropriately described as resulting from an inefficient transport.

A bidirectional transport?

Another possibility that would directly account for the similarity of the nuclear and cytoplasmic mRNA half-life, as well as the poly(A) tail length, would be that the nuclear and cytoplasmic compartments are in equilibrium. This could simply be achieved if mRNA transport were bidirectional, that is, if in addition to mRNA export, a retrograde transport from the cytoplasm to the nucleus also takes place. As has been observed for proteins, the actual partition of mRNA would then depend on the detail of their interactions in both compartments. For example, it has been proposed that RNA could be retained within the nucleus through an association with nucleus-restricted hnRNP, such as hnRNP C (reviewed in Izaurralde & Adam, 1998). Similarly, mRNA could be anchored to the cytoplasm through association with ribosomes during active translation. Accordingly, in a previous study (Gondran et al., 1999) we have observed that, even though the existence of a nuclear pool of mRNA is a common phenomenon, its size relative to the cytoplasmic pool varies significantly between transcripts.

In the case of BR mRNA, the translocation of BR through the nuclear pore has been observed by electron microscopy and led to the following conclusions: (1) the 5′ end of the mRNA is exported first (Visa et al., 1996), (2) the protein cover of the mRNA is significantly reduced in the cytoplasm, and (3) translation can take place on a partly translocated mRNA (Daneholt, 1997). The complexity of the translocation process for BR and the single orientation of BR mRNA observed during its passage through the nuclear pore complex suggest that, for BR, transport is predominantly or exclusively unidirectional. The extreme size of BR mRNA (30 kb), as well as the particular ultrastructure of polytene cell nuclei make it difficult to assess the generality of this observation.

Yet, the fact that the nuclear import of large nucleic acid molecules can take place is confirmed by DNA transfection experiments or by the life cycle of influenza virus during which the viral nucleoprotein NP mediates the import of genomic RNA to the nucleus via a classical NLS pathway (O'Neill et al., 1995). A current view of mRNA export is that it is controlled by the protein cover of the transcript and that a mature mRNA could carry a multiplicity of signals that could promote export or retention within the nucleus. The fact that the localization signals of several hnRNP proteins, like hnRNP A1 and hnRNP K, are functional for both export and import suggests a possible pathway for a retrograde transport of mRNA (Michael, 2000).

Such a dynamic equilibrium between nuclear and cytoplasmic compartments could create the conditions for a retrocontrol of gene expression by cellular mRNA. If specific mRNAs could reach back to their site of transcription, they could play a regulatory role, as has been postulated for snRNAs (Frey et al., 1999). More generally, the presence within the nucleus of an image of the cytoplasmic mRNA compartment could constitute a sensing mechanism of cellular metabolism and be involved in a broad transcriptional regulation.

A bidirectional transport would also shed a new light on the mechanism of nonsense-mediated mRNA decay (NMD), which seems to involve a coordination between nuclear and cytoplasmic mRNA metabolism. On one side, the presence of a premature termination codon (PTC) in an open reading frame causes destabilization of the mRNA, provided that the PTC is located upstream of a functional intron. Yet, in most cases, destabilization occurs after splicing, as the abundance of pre-mRNA is not modified and the PTC can be recognized as such when overlapping an exon–exon junction (Carter et al., 1996). On the other side, destabilization is intimately linked to translation, as it is abolished in the presence of suppressor tRNA or translation inhibiting 5′ stem-loop structure (Belgrader et al., 1993). Recently, a model has been proposed to account for the intron and translation dependence of NMD, based on the possibility that following splicing, proteins may remain bound to the exon–exon junction. During translation the presence of such a signature downstream of a stop codon could induce the degradation of the mRNA (Thermann et al., 1998). The remaining issue is that, in several instances, mRNA abundance is decreased not only in the cytoplasm but also in the nucleus. A bidirectional transport would explain this coordinated degradation which otherwise would necessitate the presence in the nucleus of a translation apparatus able to detect nonsense codons.

Generality of our observations

This study extends previous works on the accumulation of mature mRNAs in the nucleus of mammalian cells. Specifically, the fact that the nucleocytoplasmic partition does not change when the expression level varies over two order of magnitude definitely establishes that these nuclear mRNAs do not reflect a saturation of the export machinery. In addition, taken together, these studies suggest that the accumulation of nuclear mRNAs is a common feature of gene expression. Our conclusion that, for β-globin and LT-α, these nuclear mRNAs are not the direct precursors of cytoplasmic mRNAs also probably applies to many genes. Indeed, the most striking observation made with actinomycin D (Penman et al., 1968), α-amanitin, or DRB (Huang et al., 1994) is the persistence in the nucleus of a large population of polyadenylated RNA. In the latter study, these molecules have been detected by in situ hybridization and are therefore undoubtedly intranucleoplasmic. These studies were difficult to interpret because of the general inhibition of RNA metabolism, the use of drugs with a wide range of poten-

tial side effects (Haaf & Ward, 1996), and the lack of characterization of nuclear RNA beyond polyadenylation. However, in our study, which circumvents these limitations, we observe similar dynamics for the β-globin and LT-α nuclear mRNAs. We therefore propose that the bulk of poly(A)+ mRNAs that are present within the nucleus are, similarly, mature mRNAs that are not in the process of being exported and have comparable half-lives in the nuclear and cytoplasmic compartments. In this case, these nuclear mRNAs would be the signature of a general and unexpected characteristic of transport, inefficiency or bidirectionality.

MATERIALS AND METHODS

Expression vectors

The tTA expression vector has been described in Dirks et al. (1994). The poliovirus IRES has been removed from the vector by a *Not*I-*Hin*dIII deletion. To obtain the tTA-β-glo expression vector, genomic rabbit β-globin sequences from nt 421 to nt 2030 (GenBank accession number K03256) have been introduced at the *Sal*I site of the tTA polylinker. This includes the complete transcribed region, as well as the downstream region required for polyadenylation. For the tTA-LT-α expression vector, genomic murine LT-α sequences from nt 1203 to nt 3106 (GenBank accession number Y00467) have been introduced at the *Eco*RI site of the tTA polylinker. This includes the complete transcribed region, except for the last 100 bp of exon 4, the polyadenylation site being provided by the vector. pSVtkNeo contains the neomycin resistance gene under the control of tk promoter.

Cell culture

NIHtTA-16-3 cell lines were routinely maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 0.8 μg/mL puromycin, and 125 μg/mL hygromycin (Boehringer Mannheim, France). For transfections, 5×10^5 cells were plated on 85-mm-diameter dishes and cotransfected with 9 μg of tTA-β-glo or tTA-LT-α plasmid and 1 μg pSVtkNeo plasmid DNA by a standard calcium phosphate procedure (Sambrook et al., 1989). After 16 h the medium was replaced with fresh medium and after 48 h replaced again with fresh medium supplemented with 0.8 μg/mL geneticin sulfate (Life Technologies, France). After 21 days geneticin-resistant clones were isolated and plated in 24-well plates before being routinely maintained in 85-mm-diameter dishes in the presence of geneticin.

For expression studies, 0–1 μg/mL tetracycline (Boehringer Mannheim, France) or 10 μg/mL actinomycin D (Boehringer Mannheim, France) was added to the culture medium for indicated periods of time. For the experiment using 40 ng/mL tetracycline, the whole medium was replaced to achieve an accurate concentration of the drug. In all experiments cells were harvested at subconfluence.

Nuclear and cytoplasmic RNA

Nuclear and cytoplasmic fractions were obtained by NP40 lysis. Cells were rinsed, scraped, and rinsed again, all with ice-cold phosphate-buffered saline. Cells were resuspended in lysis buffer (0.5% NP40, 0.14 M NaCl, 10 mM Tris, pH 8.4, 1.5 mM $MgCl_2$, with or without 10 mM EDTA, pH 8) for 5 min at 0 °C. After centrifugation at 470 g for 5 min at 4 °C, the supernatant containing the cytoplasmic fraction was harvested. The nuclear pellet was washed twice with lysis buffer and both supernatants were added to the cytoplasmic fraction.

RNA was extracted from nuclear and cytoplasmic fractions by denaturation in guanidine thiocyanate prior to ultracentrifugation over a cesium chloride cushion (Berger & Kimmel, 1987) and quantified by spectrophotometry.

Poly(A) tail digestion was performed by hybridization of 4 μg of RNA with 0.5 μg of oligo$(dT)_{20}$, followed by digestion with 1 U of RNase H (Life Technologies, France), as previously described (Weil et al., 1990).

For Northern blot analysis, 4 μg RNA was electrophoresed on 1.2% formaldehyde-agarose gels, transferred on nylon membrane, and hybridized with ^{32}P-labeled riboprobes, as described previously (Neel et al., 1995). For analysis of the poly(A) tail, electrophoresis was on 1.6% formaldehyde-agarose gel. β-globin riboprobe contains the complete rabbit β-globin cDNA sequence from exon 1 to exon 3 (GenBank accession number V00878). Actin riboprobe contains a Taq1 fragment of murine β-actin cDNA (GenBank accession number X03672) corresponding to exon 2 to exon 4. RNA molecular weight markers are two ^{32}P-labeled transcripts synthesized from β-globin plasmids of the laboratory.

For RNase protection assay, 4 μg RNA was hybridized with 0.5 ng ^{32}P-labeled riboprobe, digested with RNases A and T1, and electrophoresed on 5% urea-polyacrylamide gels, as described previously (Weil et al., 1990). LT-α probe contains nt 1733–2226 (GenBank accession number Y00467) of murine LT-α gene from exon 2 to exon 4, including introns 2 and 3. Protection of fully and partially spliced transcripts has been described in detail previously (Weil et al., 1990).

Electron microscopy

Pellets of isolated nuclei were fixed with 1.6% glutaraldehyde (Taab Laboratory Equipment Limited, United Kingdom) in 0.1 M Sörensen phosphate buffer, pH 7.3–7.4, for 1 h at 4 °C and postfixed with 2% aqueous osmium tetroxide (Pelanne Instruments, France) prior to being dehydrated in ethanol and embedded in Epon. Ultrathin sections were collected on 200-mesh copper grids coated with Formvar and carbon and stained with uranyl acetate and lead citrate prior to being observed with a Philips 400 transmission electron microscope, at 80 kV, at 4,600–17,000× magnification.

In situ hybridization

Isolated nuclei were deposited on glass coverslips on ice and fixed with 4% paraformaldehyde (Merck) in phosphate buffered saline for 15 min at room temperature prior to being dehydrated in 70% ethanol for at least 30 min at 4 °C. Nuclei were then rehydrated in phosphate buffered saline for 5 min, prehybridized in 15% formamide and 2× SPE for 10 min at room temperature and hybridized in 15% formamide, 2× SPE, 10% dextran sulfate (Sigma Aldrich, France), 0.5 mg/mL tRNA, and 2.5 μg/mL fluorescent oligo(dT) or oligo(dA) probe for 1–3 h at 37 °C. Coverslips were washed in 15% formamide

and 2× SPE for 20 min, then in 1× SPE for 10 min at room temperature and mounted in Citifluor (Citifluor, United Kingdom).

Oligo(dT)$_{45}$ and oligo(dA)$_{45}$ probes were synthesized and labeled with two FITC and one FITC at the 5′ and 3′ extremities, respectively, and purified by HPLC (Genaxis Biotechnology, France). The distribution of fluorescence was analyzed with an LSM 510 confocal microscope attached to an axiovert microscope equipped with an argon-neon laser (Carl Zeiss, Inc.). Optical sections were recorded with a 63× lens at 0.35 μm z-intervals and at an effective pixel size of 0.1 μm^2. The fluorescence intensity was at least 10-fold higher with the oligo(dT) than with the oligo(dA) control probe.

Quantification of the fluorescence was performed on three nuclei (designated dT1, dT2, and dT3) considered to be representative of those with high, medium, and low levels of cytoplasmic contamination. For each optical section, the signal within the nucleus and outside of it were integrated with NIH Image software, as detailed in Table 1. The cytoplasmic contamination was calculated from the sum of the intranuclear and extranuclear signals.

ACKNOWLEDGMENTS

We are grateful to Francine Puvion and Laurence Majbruch for performing the electronic microscopy analysis, and to Alice Dautry and Raymond Hellio for their efficient help with confocal microscopy. The confocal microscope was purchased with a donation from Marcel and Liliane Pollack. Cell line and vector for tetracycline-regulated expression were kindly provided by Andrea Kröger and Hansjörg Hauser. This work was supported by Centre National de la Recherche Scientifique and by the European Union (Biotech project 970324).

Received February 28, 2000; returned for revision April 10, 2000; revised manuscript received April 25, 2000

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