Accumulation of mature mRNA in the nuclear fraction of mammalian cells

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Received 9 August 1999

Abstract Little is known about the nuclear mRNA content of mammalian cells. In this study, we analyzed by Northern blotting with a panel of probes the nuclear and cytoplasmic fractions derived from several rodent cell lines. For most of the genes under study, mature mRNAs could easily be detected in the nuclear fraction and accumulated to higher levels than the corresponding precursors. In addition, significant differences in the nucleo-cytoplasmic partition of mature mRNAs were observed between genes as well as between cell types (NIH 3T3, CTLL-2, D3-ES, PC-12), indicating that this nuclear accumulation of mRNA is regulated. Thus, while it is usually considered that splicing is the limiting step of pre-mRNA processing, these results point towards transport or nuclear retention of mRNA as a key determinant of nuclear mRNA metabolism.

Key words: Nuclear mRNA metabolism; Nuclear retention; mRNA transport; Nuclear matrix

1. Introduction

In eukaryotes, the existence of the nuclear envelope introduces a physical separation between the two end-steps of gene expression: transcription and translation. Moreover, in higher eukaryotes, most primary transcripts contain numerous introns and have to be extensively processed within the nucleus before they can be productively exported. It has been proposed for a long time that this complexity of mRNA genesis in higher eukaryotes could provide the basis for novel regulations of gene expression [1]. However, our knowledge of regulations acting on pre-mRNA splicing or nucleo-cytoplasmic transport of specific transcripts is still limited [2–4].

The difference in the size distribution of in vivo pulse-labelled RNA polymerase II transcripts between nuclear and cytoplasmic fractions has suggested that mostly primary and partially spliced transcripts were present in the nucleus. Accordingly, it is commonly assumed that once their processing has been completed, mature mRNAs are rapidly and efficiently exported from the nucleus [5,6]. In this model, the emphasis is put on the splicing machinery as the major source of nuclear retention and it is assumed that there are fewer mature mRNAs than precursors in the nucleus. However, because the size of introns usually greatly exceeds that of exons, the labelling profile is strongly biased in favor of the longer molecules and the actual proportion of mature mRNAs in the nucleus remains to be analyzed.

In this study, we have searched for the presence of mature mRNAs within the nuclear fraction of several rodent cell lines. For most of the genes that we have analyzed, and independently of the structure of the primary transcript, mature mRNAs could easily be detected in the nucleus. We also observed that the ratio of cytoplasmic to nuclear mRNA accumulation (the 'nucleo-cytoplasmic partition') varied significantly between genes and cell types. These results indicate that for some mRNAs, transport is much slower and/or inefficient than anticipated and is regulated by cellular physiology.

2. Materials and methods

2.1. Genes and probes

The β-actin probe used in Northern analysis was the 600 bp *Pst*I-*Taq*I fragment corresponding to the 5′ end of the murine cDNA cloned in the Bluescript plasmid (Stratagene). The murine c-*myc* gene contains two introns of 1.6 and 1.2 kb. The probe used is a 1 kb *Rsa*I-*Hin*dIII genomic fragment encompassing exon 3 cloned in Bluescript. The murine cyclophilin I gene contains four introns of 2.4, 0.15, 0.2 and 1.4 kb. The probe used is a near full length cDNA cloned in pSP65. CHO-A belongs to a set of randomly chosen cDNAs from the CHO cell line which have been studied by J. Darnell's laboratory. The CHO-A probe used is a 1.4 kb near full length cDNA [7]. The histone H4 probe is a 392 bp *Alu*I genomic fragment cloned in pSP65 (Promega). Probes for β-actin, c-*myc*, cyclophilin and histone H4 were obtained by in vitro transcription, probes for c-*jun* and CHO-A were obtained by random priming of DNA synthesis on purified inserts.

2.2. Cell lines and cell culture

NIH 3T3 cells were maintained in DMEM containing 7.5% fetal calf serum (FCS). For experiments with asynchronously growing cells, cultures were used when reaching a density of 10^6 cells per 85 mm dish. CTLL-2 is an interleukin-2 (IL-2)-dependent murine T-lymphocytic cell line which was maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, pH 7.3, 5% FCS and 10% rat spleen cell conditioned medium as a source of IL-2 [8]. D3-ES is a culture of totipotent embryonic stem cells derived from the inner cell mass of blastocysts obtained form the 129sv mouse strain [9]. They were grown as described [10] on gelatinized dishes in DMEM containing non-essential amino acids, 15% FCS and 1000 U/ml of leukemia inhibitory factor. PC-12 is a rat pheochromocytoma cell line which was maintained in DMEM containing 10% FCS.

2.3. Isolation of nuclear and cytoplasmic fractions

Adherent cells were washed three times in ice-cold phosphate-buffered saline (PBS) and harvested by scraping with a rubber policeman. CTLL-2 cells were collected by centrifugation and washed three times in ice-cold PBS. Except when otherwise indicated, nuclear fractions were prepared according to the following procedure (method 1). Lysis of the plasma membrane was achieved by incubating 10^7 cells for 5 min on ice in 500 μl of lysis solution (10 mM Tris-hydrochloride pH 7.4, 10 mM NaCl, 10 mM $MgCl_2$) containing the indicated concentration of Nonidet P-40 (NP-40). After a 5 min centrifugation at $500\times g$, the nuclear pellet was resuspended in 500 μl lysis buffer, vigorously pipetted and further incubated for 5 min on ice. This procedure was repeated 2–3 times until nuclei appeared to be free

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PII: S0014-5793(99)01175-8

of cytoplasmic remnants by phase contrast microscopic examination. Preliminary experiments were carried out to determine the maximal NP-40 concentration which yielded intact nuclei (NIH 3T3: 0.5%, CTLL-2: 0.5%, ES: 0.05%, PC-12: 2%). The nuclear pellet was lysed in 5.5 M guanidinium thiocyanate, while the supernatants were pooled to generate the cytoplasmic fraction and adjusted to 4 M guanidinium thiocyanate. RNAs were prepared by centrifugation over a cesium chloride cushion as previously described [8]. Method 2 is derived from Belgrader et al. [11]. Briefly, cells were lysed with 0.3% NP-40 in a hypotonic buffer (10 mM Tris pH 7.4, 2 mM $MgCl_2$, 1 mM $CaCl_2$) and nuclei were pelleted twice through a 0.25 M sucrose pad. Method 3 includes a 'magic wash' step [12], cells were lysed in 0.5% NP-40 in 10 mM Tris pH 8.4, 140 mM NaCl, 1.5 mM $MgCl_2$. Nuclei were washed once in the magic wash solution (42 mM Tris pH 8.3, 8.5 mM NaCl, 2.6 mM $MgCl_2$, 0.6% Tween 40, 0.3% sodium deoxycholate). RNAs were prepared from the nuclear, cytoplasmic and magic wash fractions as above.

2.4. Northern analysis

Northern blots were performed according to standard procedures [8]. Briefly, 4 μg of total RNA per lane was electrophoresed through 1.2% agarose gels containing formaldehyde and transferred in 150 mM NH_4Ac onto an uncharged nylon membrane (Amersham Hybond N). Hybridization was performed overnight in 50% formamide at 60°C for RNA probes and at 42°C for DNA probes. Signals were quantified with a Fuji Bioimager BAS 1000 and detected by autoradiography.

3. Results

3.1. Analysis of the nuclear and cytoplasmic fractions of proliferating NIH 3T3 cells

To search for the presence of mature mRNAs in the nucleus of mammalian cells, we analyzed by Northern blotting the nuclear and cytoplasmic fractions of NIH 3T3 cells prepared by detergent lysis of the plasma membrane (method 1, see Section 2). The accumulation of specific transcripts was analyzed with equal amounts (4 μg) of total nuclear and total cytoplasmic RNA. An ethidium bromide staining of the nylon filter after transfer of the electrophoresed RNA is presented in Fig. 1A. The abundance of the rRNA precursors (45S and 32S) and the ratio of 18S to 28S rRNA provide an indication of the selectivity of the fractionation [13].

We used a panel of probes derived from genes which generate primary transcripts of diverse structures such as histone H4 (intronless, polyA−), c-*jun* (intronless, polyA+) and β-actin, c-*myc*, cyclophilin as representatives of more 'classical' pre-mRNAs. We also included CHO-A, although its identity is unknown, as a representative of the genes studied by J. Darnell's laboratory [7,14]. With each probe (Fig. 1B), the predominant signal in the nuclear fraction co-migrated with the mature mRNA (the two c-*jun* messages are due to alternative polyadenylation sites). While c-*myc* and cyclophilin contain introns which are large enough to significantly alter the migration of the corresponding transcripts, only in the case of c-*myc* could a low level of a potential precursor be detected (Fig. 1B). The genomic organization of CHO-A is unknown, but the existence of slowly migrating precursors has been reported [7]. For β-actin, which contains two small introns, a reverse transcriptase PCR analysis was performed and confirmed that the predominant species was indeed the fully spliced mRNA (data not shown). Leaving aside the c-*jun* and histone H4 genes which do not contain introns, these observations indicated that, for this set of genes, mature mRNAs accumulated to higher levels than partially spliced species in the nuclear fraction.

The nucleo-cytoplasmic partition of these transcripts (C/N) was measured in three independent Northern blot experiments. The quantification presented Fig. 1C is the mean value ± S.E.M. of C/N and takes into account the relative amount of cytoplasmic and nuclear RNA, so that the value represents the actual distribution of transcripts within the cell. Genes which gave a strong nuclear signal in the Northern analysis like β-actin, c-*myc* and c-*jun* have nucleo-cytoplasmic partition values around 15, while cyclophilin, which was hardly detectable in the nucleus, has a corresponding value of 65. CHO-A and histone H4 gave intermediate partitions with C/N ratios around 30. In summary, significant differences in

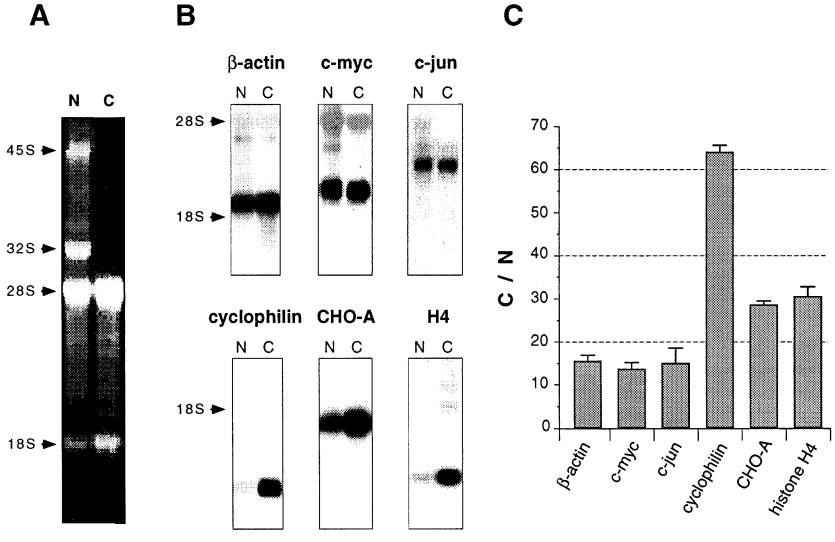

Fig. 1. Northern blot analysis of the nuclear and cytoplasmic fractions of proliferating NIH 3T3 cells. 4 μg of total RNA from either the nuclear (N) or cytoplasmic fraction of proliferating NIH 3T3 cells was analyzed by Northern blotting. A: Ethidium bromide staining of the nylon filter. B: Autoradiograms of the filter following hybridization with the indicated probes. Migration of the 18S and 28S rRNAs is indicated on the left. C: Relative accumulation in the cytoplasmic and nuclear fractions of mature mRNAs (C/N). These values (mean ± S.E.M.) were derived from three experiments and take into account the respective sizes of the nuclear and cytoplasmic fractions. These figures were derived by quantification of the Northern blots with a Fuji Bioimager (only signals corresponding to mature mRNAs were considered).

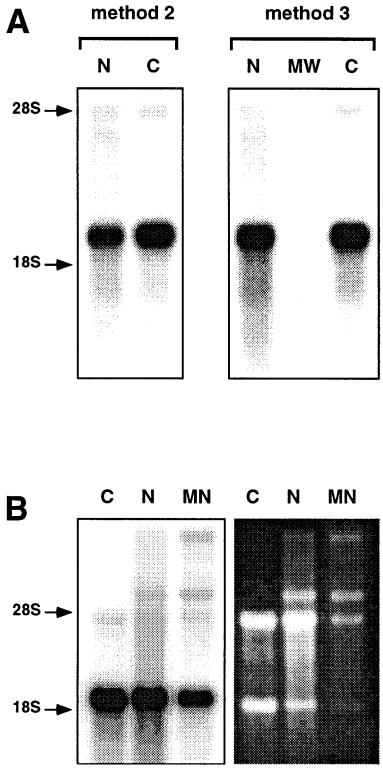

Fig. 2. Northern blot analysis of nuclear and cytoplasmic fractions prepared by different protocols as well as a nuclear matrix preparation. A: Nuclear and cytoplasmic fractions were prepared from proliferating NIH 3T3 cells by the hypotonic/sucrose gradient (method 2) and magic wash (method 3) procedures (see Section 2). 2 μg of each fraction (N, nuclear; C, cytoplasmic; MW, magic wash) was analyzed by Northern blotting with a β-actin probe. B: Nuclear matrix was prepared by a standard protocol (see Section 2). 2 μg of each fraction was analyzed as in A. An ethidium bromide staining of the filter is presented on the right. Migration of the 18S and 28S rRNA is indicated on the left.

the nucleo-cytoplasmic partition can be observed between genes.

Nuclear fractions prepared by detergent lysis might contain some cytoplasmic contamination. Because for β-actin, c-*myc* and c-*jun* the observed signals in both fractions are comparable, this potential contamination should not affect our conclusion, unless one envisions a specific artifact for these mRNAs. To address this eventuality, we used different fractionation protocols that attempt at 'cleaning' the nuclei. In method 2, we used a combination of hypotonic lysis and repeated centrifugation over a sucrose cushion [11]. In method 3, we used stronger detergents (deoxycholate and Tween 20, 'magic wash') to strip off the outer nuclear membrane [12]. Fig. 2A presents a Northern blot analysis of the fractions obtained by methods 2 and 3. In both cases, a strong signal corresponding to mature β-actin mRNA was observed in the nuclear fraction. Quantification of these signals for method 2 and 3 yielded C/N ratios 20% above and 10% below that of method 1, respectively, indicating a good agreement between the different. Finally, we used a nuclear matrix preparation to assess the strength of the interactions of β-actin mRNA within the nucleus. One third of the mRNA could not be extracted after Triton X-100, DNAse treatment and high salt extraction (Fig. 2B). Thus, a significant proportion of nuclear β-actin mRNA was tightly associated with the nuclear matrix.

3.2. Analysis of the nuclear and cytoplasmic fractions of CTLL-2, ES and PC-12 cells

We next considered whether the partition between the nucleus and the cytoplasm of a given mRNA varied between cell types. We carried out the same analysis on nuclear and cytoplasmic fractions prepared from CTLL-2, an IL-2-dependent T-lymphocytic cell line, D3-ES cells, a strain of totipotent embryonic stem cells, and PC-12, a rat pheochromocytoma cell line. In all cases, exponentially growing cultures were analyzed. For each cell type, experiments were first performed to determine the optimal NP-40 concentration to efficiently isolate nuclei (see Section 2). The percentage of total RNA recovered in the nuclear fraction was around 15% for these three cell types.

Nuclear and cytoplasmic fractions were analyzed by a Northern blot (Fig. 3A). As in NIH 3T3 cells, the predominant signal in the nuclear fraction co-migrated with mature mRNAs. The results of the quantification of three independent experiments are presented in Fig. 3B. The low level of c-*myc* expression in PC-12 and of c-*jun* in CTLL-2 and ES cells prevented a reliable determination of their nucleo-cytoplasmic

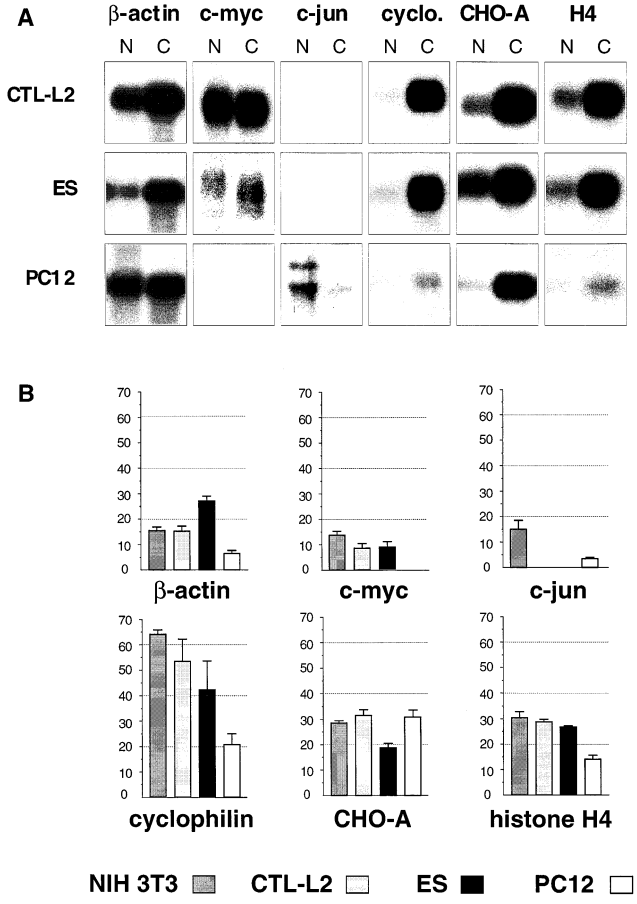

Fig. 3. Northern blot analysis of the nuclear and cytoplasmic fractions of CTLL-2, ES and PC-12 cells. A: Autoradiograms of filters containing the nuclear and cytoplasmic RNAs from the different cell lines following hybridization with the indicated probes. Only the relevant part of the autoradiograms corresponding to the migration of the mature mRNAs is presented, the overall hybridization pattern being similar to that of Fig. 1B. B: Relative accumulation in the cytoplasmic and nuclear fractions of mature mRNAs (C/N). The values were derived from three experiments as in Fig. 1C.

Table 1
Cytoplasmic half-lives and prediction of the nuclear turn-over

mRNA	C/N	Cytoplasmic half-life (min)[a]	Apparent transport rate (s^{-1})[b]	Predicted nuclear dwell-time (min)[c]
β-Actin	13	270	0.58×10^{-3}	20
c-*myc*	13	20	7.7×10^{-3}	1.5
c-*jun*	15	30	5.8×10^{-3}	2
Cyclophilin	65	120	5.8×10^{-3}	2
CHO-A	30	600	0.58×10^{-3}	20
Histone H4	26	25	11×10^{-3}	1

[a]As measured in the presence of actinomycin D.
[b]As derived from the cytoplasmic and nuclear accumulations by assuming a pseudo-first order transport reaction: $k_T = C/N \times k_d$, where k_T and k_d are the apparent transport and cytoplasmic degradation constants, respectively.
[c]Using the same assumptions as in b, $t_{1/2N} = N/C \times t_{1/2C}$.

partition. For c-*jun* in PC-12 cells, only the predominant signal corresponding to the smaller transcript was taken into account. Significant variations could be observed between cell types as illustrated by β-actin and cyclophilin. The partition coefficient for β-actin was significantly larger in ES cells and smaller in PC-12 than in other cell types. For cyclophilin, it varied between 65 in NIH 3T3 cells and 20 in PC-12. In PC-12 cells, the nucleo-cytoplasmic partition was lower for all the mRNAs except for CHO-A. Thus, significant differences in the nucleo-cytoplasmic partition of a given transcript could be observed between cell types.

3.3. Evaluation of the mRNA transport rates

By themselves, steady state accumulations provide no information on the underlying kinetics. One major kinetic parameter is the cytoplasmic mRNA half-life since it determines the flux of molecules exported from the nucleus to the cytoplasm. Thus, one would predict the presence of more nuclear mRNA for transcripts with a rapid cytoplasmic turn-over. We used actinomycin D to determine the cytoplasmic half-life of the mRNA under study in proliferating NIH 3T3 cells. Fig. 4 presents the results of the quantification of cytoplasmic mRNA by Northern blot following 1 and 2 h of actinomycin D treatment. The corresponding half-lives (Table 1) varied between 20 min for c-*myc*, c-*jun*, histone H4 and 10 h for CHO-A. β-actin and cyclophilin had half-lives of 4 h 30 min and 2 h, respectively. These values are in good agreement with those obtained in previous studies, irrespectively of the technique used: equilibrium labelling [14] or inhibition of transcription [15].

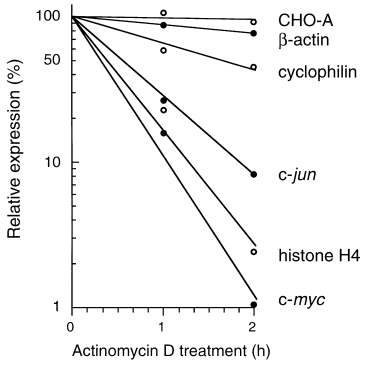

Fig. 4. Determination of cytoplasmic mRNA half-lives in proliferating NIH 3T3 cells. Cytoplasmic fractions of NIH 3T3 cells incubated for 0, 1 and 2 h in the presence of 10 μg/ml of actinomycin D were analyzed by a Northern blot as in Fig. 1. The intensity of the signal was quantitated with a Fuji Bioimager and expressed as a percentage of the value in the absence of actinomycin D.

Interestingly, there was no correlation between these cytoplasmic half-lives and the observed nucleo-cytoplasmic partitions (Table 1). This is clearly illustrated by the case of β-actin and c-*myc* mRNA which have cytoplasmic half-lives which differ by more than one order of magnitude while their nucleo-cytoplasmic partitions are identical. The transcription rate should not be a major determinant of the partition as, in first approximation, it should affect similarly both compartments. Therefore, to compensate this difference in turn-over, β-actin and c-*myc* mRNA must differ by their nuclear metabolism (i.e. by a potential nuclear retention and by their actual transport), leading to a difference in their apparent transport. On the basis of the results of Table 1, we can divide the genes under study between those with a rapid apparent transport (c-*myc*, c-*jun*, cyclophilin, histone H4) and a slow apparent transport (β-actin, CHO-A).

To further analyze these results, a simple formalism can be used to derive apparent transport rates and nuclear dwell-times. The two underlying hypotheses are that (i) mRNAs which accumulate in the nucleus are the precursors of the cytoplasmic mRNA and (ii) nucleo-cytoplasmic transport and cytoplasmic degradation can be described by pseudo-first order reactions. Then, the apparent transport rate (k_T) and a nuclear dwell-time can be derived from the nucleo-cytoplasmic partition (C/N) by $k_T = C/N \times k_d$ and $t_{1/2} = 0.693/k_T$, where k_d is the cytoplasmic degradation rate [16]. The predicted nuclear dwell-times vary between less than 2 min for the 'rapidly exported' transcripts (c-*myc*, c-*jun*, cyclophilin, histone H4) and 20 min for the slowly exported ones (β-actin, CHO-A) (Table 1).

4. Discussion

Although, in eukaryotes, the generation of cytoplasmic mRNA requires extensive pre-mRNA processing and an active transport, the in vivo dynamics of these events for specific transcripts are still poorly known [7,14]. In the present study, we have used a Northern blot approach to analyze and quantify the nuclear and cytoplasmic pattern of expression of a set of genes. We report two main observations: (i) for all the genes analyzed, mature mRNAs accumulate to a higher level in the nuclear fraction than pre-mRNA, (ii) for several genes, comparable signals are detectable in the nuclear and cytoplasmic fractions.

One potential limitation of this study comes from the use of

detergent lysis to prepare nuclear fractions. So far, and until the appropriate technology for quantitative in situ detection of specific RNA molecules is available (see [17] for a discussion of some of the technical limitations of fluorescent probes), biochemical fractionation is nonetheless the best approach for quantification. We have used several protocols that gave comparable results in terms of nucleo-cytoplasmic partition of β-actin mRNA, indicating the robustness of our analysis. Moreover, the available in situ data are compatible with a significant accumulation of mRNAs in the nuclear fraction. Thus, when either oligodT [18–21] or gene specific probes [22–25] were used for in situ hybridization, signals could be detected in both compartments.

If, as it is generally assumed, mature transcripts are rapidly exported from the nucleus, they should accumulate at much lower levels in the nucleus than in the cytoplasm. Our results do fit this prediction inasmuch as in proliferating NIH 3T3 cells, the C/N ratio is greater than 15 for all the genes under study. However, this greater cytoplasmic accumulation of mRNA reflects the respective sizes of the nuclear and cytoplasmic fractions and not a lower concentration of mature transcripts in the nucleus. Moreover, for β-actin, c-*myc* and CHO-A, no significant accumulation of primary or partially spliced transcripts was observed, establishing that mature mRNAs were the predominant nuclear species. A similar observation has been made incidentally for several other genes including thymidine kinase [26], growth hormone [27] and transfected β-globin expression vectors [28]. Thus, exportation from the nucleus rather than splicing appears to be limiting for the expression of a large number of genes. As has been previously reported for ovalbumin [29], we have observed the presence of significant amounts of mature β-actin mRNA in nuclear matrix preparations, indicating the existence of strong interactions with nuclear components. Therefore, whereas the splicing machinery is a source of nuclear retention of pre-mRNA, some other component of the nuclear matrix is involved in the retention of mature mRNA.

Our results provide an unexpected view of the nuclei of mammalian cells in which mature mRNAs, rather than high molecular weight precursors, accumulate. Moreover, while for some transcripts, the apparent transport kinetics fit with the usual perception of a rapid mRNA export (a 1–2 min nuclear dwell-time), for others, transport appears unexpectedly slow. Two mechanisms could generate a slow apparent transport. First, transport itself could be slow for some transcripts like β-actin. Second, mRNAs could be retained in the nucleus. This could be achieved through the interaction of mRNA which nucleus-restricted hnRNP such as hnRNP C [30] or through their participation to the nuclear skeleton [31,32]. These two modes of nuclear accumulation are mutually non-exclusive and could be simultaneously operating. Interestingly, in an in vivo labelling study, CHO-A was the gene which exhibited the longest lag between the nuclear and cytoplasmic accumulation of label [14], suggesting that the transport of this mRNA was slow. Further studies will be required to determine the actual transport kinetics of these transcripts and to ascertain the possible role of a nuclear retention.

Acknowledgements: We thank Miles Wilkinson for suggestions on the genes to investigate and the CHO-A and cyclophilin probes. The histone H4 probe was provided by D. Schumperli. We thank members of the laboratory and E. Puvion for helpful discussions. This work was supported by the CNRS and the ARC (Grant 6307 to F.D.).

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