FEBS Letters 421 (1998) 50–54 FEBS 19672

Nuclear accumulation of c-*myc* mRNA in phytohaemagglutinin-activated T lymphocytes treated with anti-HLA class I monoclonal antibody

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Received 20 November 1997

Abstract Anti-HLA class I monoclonal antibody 01.65 inhibits the proliferative response of PHA-activated human T lymphocytes from peripheral blood mononuclear cells. The recruitment rate in the cell cycle is slack and the G1 and S phases are prolonged. Among the early events after PHA activation, only the calcium-dependent PKC activity appears to be modified: particulate PKC is completely depleted while cytosolic residual PKC is reduced by 80% after MAb 01.65 treatment. We have carried out in greater detail the study of c-*myc* gene regulation by MAb 01.65 and the results are as follows: (i) c-*myc* RNA transcription is normally initiated and finished, suggesting a post-transcriptional regulation of c-*myc* gene expression; (ii) no alteration in c-*myc* mRNA stability has been documented; (iii) steady-state levels of c-*myc* mRNA expression by Northern blot analysis and PCR amplification are decreased in the cytoplasmic compartment, while in the nuclear compartment they appear to be increased. Nuclear accumulation of mature mRNA after MAb 01.65 and PKC inhibitor (H7 and StSp) treatment appears to be the most probable mechanism involved. The possible implications of this are discussed.

Key words: c-*myc*; Nuclear c-*myc* RNA; Monoclonal antibody; HLA class I; Protein kinase C; Protein kinase C inhibitor

1. Introduction

Anti-HLA class I monoclonal antibodies (MAbs) inhibit the proliferation of T-lymphocytes induced by Ca^{2+}-mobilising agonists, lectins or agents through either the CD3 or the CD2 pathway [1–6].

Anti-HLA class I MAb 01.65 has been selected from a panel of monomorphic anti-HLA class I MAbs for the stringency of inhibition of the proliferative response of phytohaemagglutinin (PHA)-activated T-lymphocytes [7]. The rise in cytosolic free calcium concentration, the generation of inositol phosphate and the formation of diacylglycerol (DAG) are unaffected by MAb 01.65 treatment. In contrast, membrane-translocated PKC activity is inhibited and residual cytosolic PKC is reduced [7–10]. Only the Ca^{2+}-dependent conventional protein kinase C (cPKC) is affected.

StSp and H7, two potent PKC inhibitors, are competitive with MAb 01.65 to inhibit the proliferative response of PHA-stimulated lymphocytes [11], thus indicating the existence of a common inhibitory pathway.

Treatment of PHA-activated lymphocytes with MAb 01.65 induces a slowing down of the recruitment rate into the cell cycle and a prolongation of the cell cycle time affecting either the G1 or the S phase [12,13]. A reduced mRNA steady-state level of c-*myc* has been observed in MAb 01.65-treated lymphocytes.

The c-*myc* gene expression can be regulated at either the transcriptional or the post-transcriptional level by transcription elongation block or by mRNA degeneration. PKC is also involved in c-*myc* expression. A differential effect of PKC activation on c-*myc* expression during differentiation or during proliferation has been reported, while PKC inhibition drastically reduces c-*myc* expression in HL-60 and U937 cell lines [14].

After an earlier demonstration that c-*myc* mRNA reduction was due to a post-transcriptional event we studied in greater detail the mechanism of c-*myc* mRNA depletion in the cytoplasm. c-*myc* mRNA transcriptional elongation and stability appear to be normal. Two independent sets of observations suggest that a defective nuclear export of c-*myc* mRNA is the most probable mechanism involved.

2. Materials and methods

2.1. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll/Hystopaque density gradient centrifugation. PBMC were suspended at 1×10^6 cells/ml in Iscove Dulbecco modified medium supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 U/ml gentamicin and were incubated at 37°C in a 10% CO_2 humidified atmosphere. PHA (Murex Biotech Ltd.) was administered at 0.5 μg/ml; actinomycin D (Act-D, Sigma) at 5 μg/ml; H7 ([1-(5-isoquinolinylsulphonyl)-2]methylpiperazine) (Sigma) at 25 μM [14,15].

2.2. Monoclonal anti-HLA class I antibodies

We tested the dose-dependent inhibition of [^{3}H]thymidine incorporation in PHA-activated T-lymphocytes induced by the following MAbs: 01.65 and W6/32, which are directed to framework determinants expressed on the heavy chain of HLA class I antigens [1,16]; XI-20.4, an anti-HLA-A and B [17]; R1.30, an anti-β_2-microglobulin [18]; and as control P3, an anti-IgGlk obtained from pristane-primed mice injected with X63.Ag8 murine parental myeloma cells. MAb 01.65 was selected because of the high inhibitory activity and administered at a dose of 12.5 μg/ml.

2.3. Proliferation assay

Cultures were set in 96-well flat bottom plates and 6 h before harvesting 1 μCi/ml [^{3}H]thymidine (Amersham) was added. Each determination was done in triplicate.

2.4. Nuclear run-on transcription assay

$2–5\times10^7$ cells were harvested by centrifugation and washed in PBS at 4°C. Isolation of nuclei, isolation of mRNA transcripts and hybrid-

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Abbreviations: PKC, protein kinase C; cPKC, conventional PKC: α, β_1, β_2, γ; PHA, phytohaemagglutinin; PBMC, peripheral blood mononuclear cells; MAb, monoclonal antibody; H7, [1-(5-isoquinolinylsulphonyl)-2]methylpiperazine; StSp, *N*,*N*-staurosporine; DAG, diacylglycerol

PII S0014-5793(97)01524-X

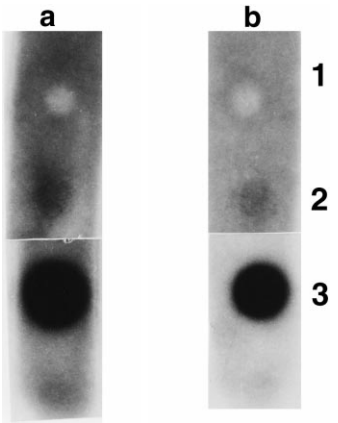

Fig. 1. Nuclear run-on analysis of the MAb 01.65 effect in c-*myc* gene transcription. a: PHA-activated PBMC. b: PHA-activated PBMC treated with MAb 01.65. 1: pBR322 DNA as negative control; 2: pMYC7.4; 3: p44-HLA as positive control.

isation of nascent transcripts were performed according to the methods described by O'Connor and Wade [19]. Dot blot was prepared utilising 2 μg linearised plasmids: pBR322, pMYC 7.4 and p44.2-HLA. Elongation of nascent mRNA transcripts was performed by digesting (a) c-*myc* cDNA from pMYC7.4 with *Cla*I to obtain a 5′ fragment corresponding to 800 bp spanning exon 2 and a 3′ fragment corresponding to 300 bp spanning exon 3; (b) H3 cDNA from pF0535 with *Pvu*II to obtain a 5′ fragment corresponding to 1600 bp and a 3′ fragment corresponding to 500 bp. 2 μg of digested cDNAs was subjected to electrophoresis on a 1% agarose gel, transferred to membrane and denatured on a pad of 3MM paper with 0.4 M NaOH for 10 min.

2.5. RNA isolation

5×10^7 cells were harvested to obtain nuclear and cytoplasmic RNA. Cells were washed in ice-cold phosphate-buffered saline and lysed in ice-cold 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$ for 5 min. The nuclear and cytoplasmic RNA was extracted as described by Chomczynski and Sacchi [20].

2.6. Northern blotting analysis

RNA (30 μg/lane) was electrophoresed in a 1.2% agarose gel containing 6% formaldehyde and electroblotted to nylon membrane (Zeta-Probe, BioRad). The blot was hybridised with 2×10^6 cpm of c-*myc* cDNA from the pMYC7.4 clone and 28S cDNA from the pCR6A1 clone labelled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using Amersham's random primer labelling kit and washed according to the manufacturer's instructions. The c-*myc* mRNA expression was normalised to the amount of 28S mRNA on the same membrane.

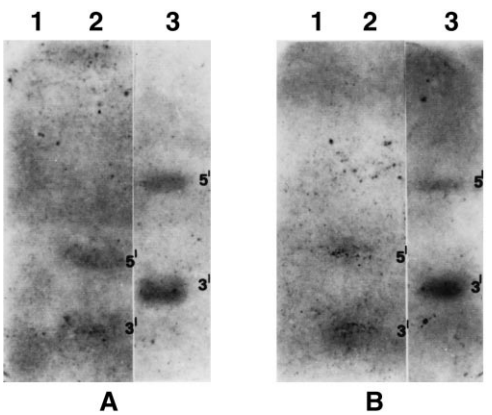

Fig. 2. Nuclear run-on analysis of the MAb 01.65 effect in c-*myc* elongation. A: PHA-activated PBMC. B: PHA-activated PBMC treated with MAb 01.65. Lane 1: pBR322 DNA as negative control. Lane 2 corresponds to c-*myc* cDNA: 5′ is the 800 bp fragment spanning exon 2; 3′ is the 300 bp fragment spanning exon 3. Lane 3 corresponds to H3 cDNA: 5′ and 3′ correspond to the 1600 bp and 500 bp fragments respectively.

2.7. Reverse transcription and PCR

The reverse transcription was performed in a 20 μl volume using the Invitrogen cDNA cycle kit. The following primers were used for PCR amplification: forward primer A: 5′-GAGACATGGTGAACCA-GAGT-3′; reverse primer B: 5′-GTCGAGGAGAGCAGAGAATC-3′; reverse primer E: 5′-AGTTGTGCTGATGTGTGGAG-3′.

2.8. Southern blotting

Southern blotting was done by standard procedure on 2% agarose gel and hybridised with end-labeled probe F: 5′-CCAAGCTCGTCT-CAGAGAAG-3′ [γ-^{32}P]ATP (Amersham) according to Maniatis [21]. The autoradiograms were scanned using a LKB laser densitometer.

3. Results

3.1. Nuclear run-on transcription assay

To investigate the effect of MAb 01.65 on c-*myc* mRNA

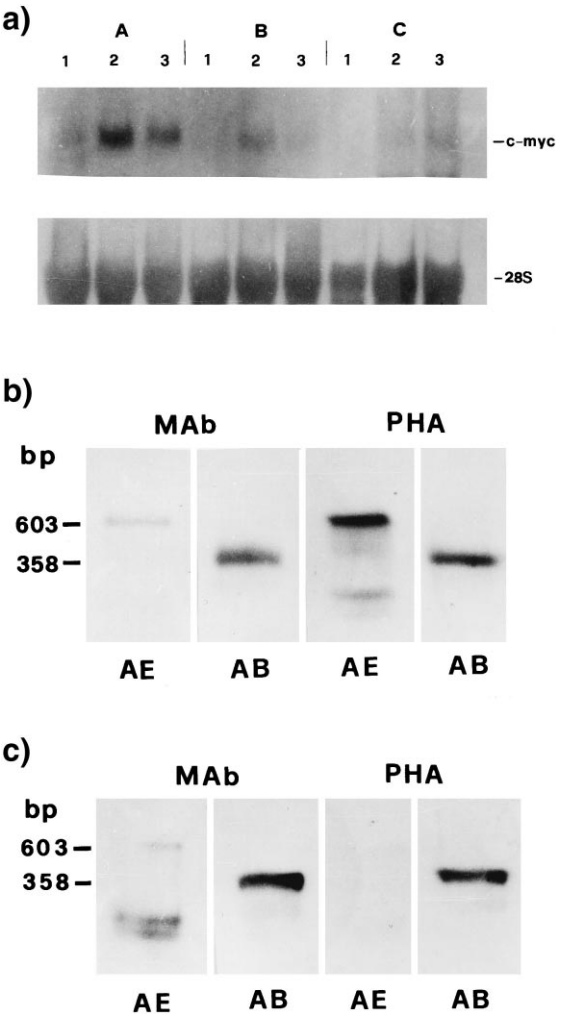

Fig. 3. a: Northern blot analysis of the total (A), cytoplasmic (B) and nuclear RNA (C) of PBMC (1), PHA-activated PBMC (2) and PBMC treated with MAb 01.65 (3). The blot was hybridised with the ^{32}P-labeled c-*myc* probe and with ^{32}P-labeled 28S probe as quantitative control. Cytoplasmic (b) and nuclear (c) c-*myc* RNA levels were determined by RT-PCR in PHA-activated PBMC (PHA) and PBMC treated with MAb 01.65 (MAb). The AB (358 bp) and AE (603 bp) products were obtained after 30 cycles, subjected to Southern blotting and hybridised with ^{32}P-labeled F-probe.

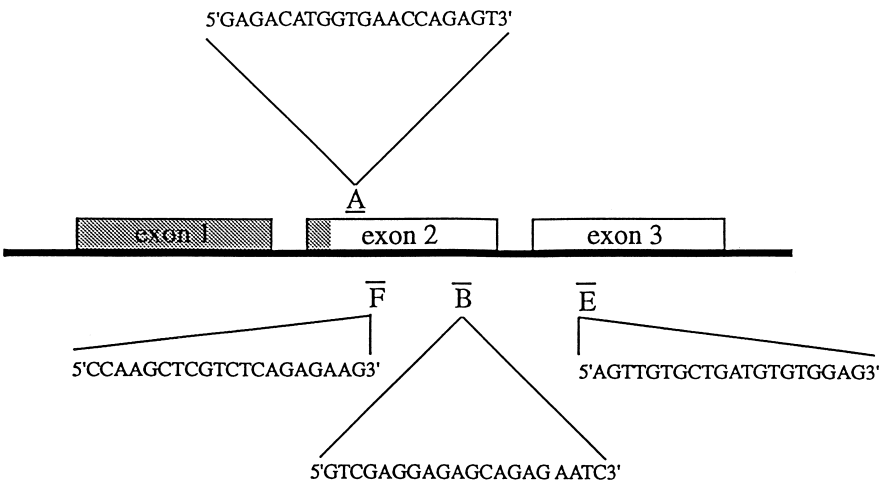

Fig. 4. Genomic map of the c-*myc* gene depicting the positions of the three exons and the relative positions of the oligodeoxynucleotides A, B, E used to amplify DNA by PCR. The oligodeoxynucleotide F was used to label the amplification products. By PCR we could obtain the following amplification products: 1, forward A plus reverse B = 358 bp; 2, forward A plus reverse E = 603 bp on mRNA post-splicing; 3, forward A plus reverse E = 1978 bp on RNA pre-splicing.

transcription, run-on transcription assays were performed. Nuclei were isolated from PBMC following activation with PHA alone or PHA plus MAb 01.65 after 12 h incubation. As shown in Fig. 1, the intensities of autoradiographic spots were comparable in PHA-activated and in MAb 01.65-treated PBMC suggesting that the transcriptional initiation was unmodified. We also studied the transcriptional elongation of c-*myc* gene. Nascent radiolabeled RNA transcripts were isolated and hybridised to filters containing (1) pBR322 DNA as negative control; (2) c-*myc* cDNAs corresponding to exon 2 (5′ fragment) and to exon 3 (3′ fragment); (3) H3 cDNA probe as positive control. Comparison of the band intensities (Fig. 2) between exon 2 and exon 3 of c-*myc* cDNA suggested that the transcriptional elongation was carried out normally in both PHA-activated PBMC and MAb 01.65-treated PHA-activated PBMC.

3.2. *c-myc mRNA stability is not altered in MAb 01.65-treated PBMC*

Experimental evidence confirms that the c-*myc* mRNA from control culture cells is labile and decays with a $t_{1/2}$ of 10–25 min [22]. We examined the effect of MAb 01.65 treatment on c-*myc* mRNA stability in PHA-activated PBMC. MAb 01.65 treatment induced 67% inhibition of c-*myc* mRNA steady-state level as compared to the PHA control confirming previous results [23]. 5 min Act-D treatment caused 60% reduction of the mRNA level in either MAb 01.65-treated or untreated control cultures. mRNA was scarcely detectable after 20 min culture with Act-D. On the basis of densitometric evaluation of specific mRNA bands, we obtained an exponential regression curve and the mRNA decay slope was almost identical in both mRNAs from PHA control and MAb-treated cultures: the estimated mRNA $t_{1/2}$s were 2.2 min and 2.4 min for control and MAb 01.65-treated PBMC respectively.

3.3. *Nuclear and cytoplasmic c-myc transcripts*

Several investigations have described nuclear post-transcriptional regulation involving changes in the accumulation of primary (unspliced) transcripts. Nevertheless, a mechanism involving inhibition of pre-RNA splicing could be excluded since unspliced c-*myc* RNA in the nuclei of MAb 01.65-treated cells was not detectable.

We examined by Northern blotting c-*myc* mRNA expression both in the nuclear and in the cytoplasmic compartment to check whether the proved down-regulation by MAb 01.65 was due to nuclear accumulation. We confirmed a decrease in cytoplasmic c-*myc* mRNA accumulation and a significant increase of c-*myc* mRNA in the nuclear preparation (Fig. 3a). These results, supported by densitometric scanning, suggest an accumulation of mature c-*myc* mRNA in the nucleus.

Reverse transcription and PCR was used to confirm the above results. Fig. 4 shows the relative position of the oligodeoxynucleotide sequences that we used. As shown in Fig. 3b,c, after 30 amplification cycles AE product was already detected in MAb 01.65-treated nuclear RNA preparation while it was still undetectable in PHA control nuclear samples. After 35 amplification cycles AE was also detected in PHA control samples, but in a significantly reduced amount as compared to MAb 01.65-treated RNA preparation (Fig. 5a,b). Cytoplasmic c-*myc* mRNA estimate indicated that the AE product is decreased in MAb-treated preparation, confirming the previously reported decrease of cytoplasmic c-*myc* mRNA by Northern blot. Arbitrary optical densitometry units of AB were used to normalise AE values.

These data all together suggest that the observed decrease of c-*myc* mRNA accumulation in the cytoplasm after MAb 01.65 treatment of PHA-activated PBMC was due to a defective c-*myc* late mRNA nuclear export.

3.4. *H7 affects the proliferative response of PHA-activated PBMC and c-myc expression*

H7 inhibited in a dose-dependent manner [^{3}H]thymidine incorporation of PHA-activated PBMC. The IC_{50} dose (12.25 μm) was used in cultures where cytoplasmic and nuclear c-*myc* mRNA were measured by Northern blot (Fig. 5c). The results showed that cytoplasmic accumulation of c-*myc* mRNA after 12 h treatment with H7 was reduced. In the nuclear compartment, in contrast, an increase of mature c-*myc* mRNA was detected, though of lower intensity than in MAb 01.65-treated cells.

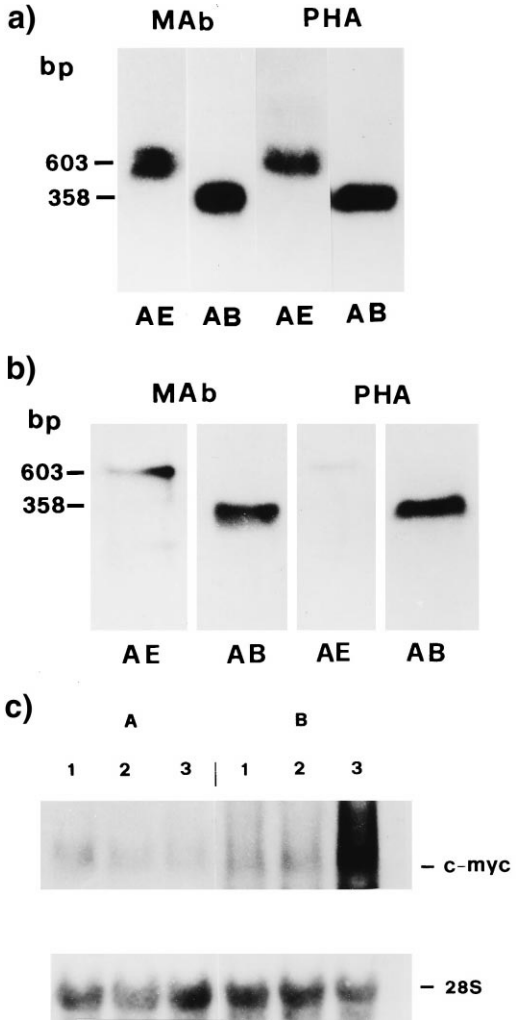

Fig. 5. The AB (358 bp) and AE (603 bp) products were obtained after 35 cycles of amplification by RT-PCR in the cytoplasmic (a) and nuclear (b) compartments of PHA-activated PBMC (PHA) and PBMC treated with MAb 01.65 (MAb). Blots were subjected to Southern blotting and hybridised with ^{32}P-labeled F-probe. c: Northern blot analysis of RNA in the cytoplasmic (A) and nuclear (B) compartments of PBMC activated with PHA (1), PHA plus H7 (2), PHA plus MAb 01.65 (3). The blot was hybridised with the ^{32}P-labeled c-*myc* probe. Normalisation was performed with ^{32}P-labeled 28S probe.

4. Discussion

During the study of the reduced proliferative response of PHA-activated T-lymphocytes treated with anti-HLA class I MAbs we became interested to the mechanisms leading to deregulation of c-*myc* mRNA expression. We have demonstrated, by Northern blot, a decrease of c-*myc* mRNA in the cytoplasmic compartment of anti-HLA class I MAb-treated PHA-activated T-lymphocytes [23]. Run-on experiments show clearly that c-*myc* RNA transcription is initiated and no difference is observed between MAb 01.65-treated and control samples. This finding suggests that post-transcriptional mechanisms of regulation of gene expression might be involved.

c-*myc* mRNA levels can be controlled to transcription elongation by a block during differentiation. We have observed that the c-*myc* exons 2 and 3 are transcribed in MAb 01.65-treated cultures as in PHA control. It is, therefore, unlikely that a transcription elongation block is responsible for the MAb 01.65-induced decrease of c-*myc* mRNA accumulation in the cytoplasm.

c-*myc* mRNA belongs to the group of messengers encoding proteins such as cytokines and oncogenes, associated with G0/G1 transition, that tend to be short-lived [24]. mRNA stability is an important method to regulate gene expression. Actinomycin D tests have shown that c-*myc* mRNA was decreased in the MAb 01.65-treated PBMC cultures, as compared to untreated control cultures. However, the decay appears to follow the same velocity and the $L_{50} > 15'$ is approximately the same as in control samples. No apparent increased instability of c-*myc* mRNA is therefore detectable in MAb 01.65-treated cultures. Both RT-PCR and Northern blot data confirm that the c-*myc* mRNA level is decreased in the cytoplasm and conversely increased in the nucleus of MAb 01.65-treated PBMCs. Identical results were obtained when PHA-activated PBMC were treated with the PKC inhibitor H7. The accumulation of mature c-*myc* mRNA in the nucleus possibly depends on a modification of the mRNA nuclear export system, induced by either MAb 01.65 or H7.

Three lines of evidence possibly trace a link between the MAb 01.65 effect on PHA-activated T-lymphocytes and the deregulated c-*myc* mRNA expression.

(1) The inhibition of calcium-dependent PKC by anti-HLA class I MAbs that we have described and characterised previously [9,10].

(2) A dual effect of PKC on c-*myc* expression has been described. PKC activation by phorbol esters induces HL-60 and U-937 cells to differentiate along the monocytic lineage [5,25] and the expression of c-*myc* is reduced [26,27]. In contrast, activation of PKC in resting cells induced expression of c-*myc*. In addition to StSp, H7 reduces c-*myc* expression to undetectable levels within 60 min in HL-60 and U-937 cells [14,26,28]. Inhibitors related to cyclic nucleotide-dependent protein kinases have no effect on c-*myc* mRNA levels [26].

(3) Recent compelling evidence shows the involvement of specific hnRP proteins in cellular mRNA transport. These proteins are also present outside the nucleus and may have some cytoplasmic function and regulation [29,30]. It has been suggested that mRNA is exported from the nucleus as a result of its association with the 'shuttling' hnRP proteins. Specific factors could mediate the export of different classes of RNA [31]. Recently Schäffer et al. [32] identified a 110 kDa nuclear protein thought to be involved in mRNA translocation through the nuclear pore and it is localised in the nuclear envelope. P110 is phosphorylated by PKC and fragmented before or during mRNA export.

Malviya and Block [33] underline the importance of PKC present in the nucleus in contrast to signals transduced by cytoplasmic PKC leading to a nuclear effect. Nuclear PKC is involved in the regulation of activity of proteins that are PKC substrates and are involved in DNA replication or processing RNA. Substrates involved in nuclear RNA processing and export are poly(A) polymerase [34] and a 106 kDa nuclear pore complex protein [35]. Phosphorylation of these proteins by PKC leads to a decrease in nuclear envelope NTPase activity required for mRNA export from the nucleus. The study of viral RNA transport has provided insight into the mechanisms of mRNA nuclear export [36]. Proteins that regulate the nuclear export of mRNA have been identified in at

least three viral systems: REV of HIV-1; the complex of adenovirus E1B 55Kda and E434Kda and the NS1 protein of influenza virus. The latter protein inhibits the nuclear export of spliced RNA binding to poly(A) sequences in vitro and in vivo. However, it has been shown that post-translational regulation takes place at the level of mRNA transport. NS1 is a phosphoprotein that is loosed after phosphorylation function and spliced mRNAs are released in the cytoplasm [14,37]. Protein kinase inhibitors and methyltransferase inhibitors preventing phosphorylation determine loss of function of NS1 protein and nuclear accumulation of late viral mRNAs.

All together these data suggest that post-transcriptional modification of phosphorylated nuclear proteins is a fundamental step to a correct mRNA nuclear exportation.

Kinase inhibitors, such as H7 and MAb 01.65, might prevent the phosphorylation of an hitherto unknown protein(s) involved in c-*myc* mRNA export, determining the nuclear accumulation.

Acknowledgements: This work was supported by CNR Target Project 'Ingegneria Genetica', P.F. 'Applicazioni cliniche della Ricerca Oncologica' (ACRO), CNR Target Project 'Biotechnology and Bioinstrumentation' and Associazione E. and E. Rulfo for Medical Genetics, Parma, Italy.

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