Kinetics of HCMV immediate early mRNA expression in stably transfected fibroblasts

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Summary

Compelling evidence supports an intimate link in time and space between eukaryotic pre-mRNA synthesis and processing and nucleocytoplasmic transport of mature mRNA. In this study, we analyzed the kinetic behavior of these processes in a quantitative manner. We used FISH and confocal scanning laser microscopy to detect transcripts produced by an inducible human cytomegalovirus immediate early (HCMV-IE) expression system. Upon induction, a large amount of pre-mRNA accumulated in nuclear foci at or near their transcription sites and, at later time, throughout the nucleoplasm. Inhibition of RNA polymerase II activity resulted in a rapid decrease in the number of transcripts in the nuclear RNA foci (half time ~two minutes), indicating that accumulated transcripts were rapidly spliced and then released. The dispersed nucleoplasmic transcripts exited the nucleus with a half time of ~10 minutes. Both processes were temperature dependent, suggesting that mRNA export is an active process. RNA polymerase II activation revealed that production of mature HCMV IE mRNAs required less than five minutes. Transcripts radiated from the gene at an average speed of ~0.13 μm^2/sec from this time on. Thus, it appears that these processes are tightly linked in time and space, with the splicing reaction as a rate-limiting factor.

Key words: Fluorescent in situ hybridization, Confocal scanning laser microscopy, RNA polymerase II, Human cytomegalovirus

Introduction

Strong evidence supports the existence of direct and indirect connections between pre-mRNA synthesis and a number of pre-mRNA processing reactions, such as 5′cap formation, splicing and polyadenylation. The C-terminal domain (CTD) of RNA polymerase II (PolII), the enzyme complex that carries out pre-mRNA transcription, plays an important role in this (reviewed in Bentley, 1999; Hirose and Manley, 2000; Zeng and Berget, 2000). The CTD consists of an evolutionarily conserved heptad repeat with a large number of potential phosphoacceptor sites (reviewed in Dahmus, 1996). The exact sites that become phosphorylated during transcription determine the order in which RNA processing factors bind to the CTD (Komarnitsky et al., 2000; Schroeder et al., 2000). Also, different regions of the CTD have been identified that independently stimulate the three major pre-mRNA processing reactions (Fong and Bentley, 2001).

At least two mechanisms indirectly link mRNA synthesis to the transport of mature mRNAs to the cytoplasm. Both of these function through the splicing reaction and determine whether a transcript can access the nuclear export pathways of the cell. One mechanism involves splicing commitment factors (organized in spliceosomes), which actively retain intron-containing pre-mRNAs in the nucleus until the splicing process removes the remaining introns and thereby the splicing commitment factors (Chang and Sharp, 1989; Legrain and Rosbash, 1989). This step may be rate limiting in some instances. In addition, export of mRNA that is defective in splicing or 3′ end processing and synthesized from mutated expression constructs is blocked at this step (Custodio et al., 1999; Johnson et al., 2000). The second mechanism concerns the selective recruitment of specific export factors, such as the TAP protein (Grüter et al., 1998), to fully processed mRNAs (Luo and Reed, 1999). This protein contains an essential domain that functions both as a nuclear export signal and as a binding domain for components of the nuclear pores (Görlich et al., 1999; Kang and Cullen, 1999; Katahira et al., 1999).

These intimate relationships between RNA synthesis, RNA processing and RNA transport are also reflected in the spatial-temporal organization of the genes and factors involved in these processes. Nascent transcripts are confined to several thousand nucleoplasmic sites or ‘transcription factories’ (Fay et al., 1997) (reviewed in Jackson et al., 2000). Each factory contains ~30 active polymerases with their associated transcripts (Jackson et al., 1998). Efficient processing of these transcripts requires the recruitment of splicing factors such as SF2/ASF to sites of active genes in vivo (Misteli et al., 1997; Misteli and Spector, 1999; Eils et al., 2000). It has been proposed that a large number of these components pre-assemble into a transcription/processing complex, followed by the recruitment or transport of this complex to the active genes (Gall et al., 1999). The large subunit of the elongating form of PolII and several splicing factors are present in numerous nuclear domains or ‘speckles’ at the light microscope resolution level in addition to a diffuse nucleoplasmic staining (Spector, 1993; Bregman et al., 1995; Mortillaro et al., 1996; Zeng et al., 1997). Moreover, the activity and localization of splicing factors is determined by their phosphorylation state (Misteli and Spector, 1998).

When a particular gene is transcribed at a high level, new transcripts may accumulate at or near the transcription site in foci that have an elongated dot or track-like appearance. This has been shown for endogenous genes such as human collagen

1α1 and β-actin (Xing et al., 1995; Femino et al., 1998) and for integrated viral genes (Lawrence et al., 1989; Dirks et al., 1997). In these cases, the active gene is often localized at one extreme of an elongated dot. This suggests that the majority of mRNAs in the elongated dot are detached from the gene. Although the majority of RNA processing is supposed to occur cotranscriptionally, elongated dots may represent sites in which pre-mRNA processing is completed or may represent an early stage of RNA export (Dirks et al., 1997; Johnson et al., 2000; Melcák et al., 2000). In rat 9G cells, which we use in this study, it has been demonstrated that nuclear foci contain HCMV IE RNA exon and intron sequences as well as splicing factors. The splicing reaction is therefore thought to continue within the elongated dot-like structures (Raap et al., 1991; Dirks et al., 1997; Snaar et al., 1999).

In this study, we use a quantitative method based on fluorescent in situ hybridization (FISH) in combination with confocal scanning laser microscopy (CSLM) to investigate the spatial and temporal relationships of HCMV IE mRNA synthesis and nuclear export in response to inactivation and activation of PolII. On the basis of these measurements we show that nuclear HCMV IE transcripts represent two subpopulations with distinct kinetic behavior, namely those in nuclear foci (at or near the transcription site) and those dispersed throughout the nucleoplasm.

Materials and Methods

Cell culture, induction and inhibition of rat 9G cells

Rat 9G cells are transfected rat fibroblasts that contain an integrated tandem repeat of 50 copies of the HCMV immediate early gene (Boom et al., 1986; Van de Corput et al., 1998). Cells were grown to subconfluency on sterile uncoated microscope glass slides in Dulbecco's Modified Eagle (DME) medium, without phenol red, containing 4.5 mg/ml glucose and 110 g/ml sodium pyruvate, supplemented with 10% fetal calf serum (FCS), 0.03% glutamine and 1000 U/ml penicillin/streptomycin. All reagents were obtained from Life Technologies, Rockville, MD. Cells were cultured at 37°C in a 5% CO_2 atmosphere. Cells were incubated in medium containing 50 μg/ml cycloheximide for four to six hours to induce expression of the *HCMV-IE* gene. Induction occurred in approximately 30% of the cells and was S-phase dependent (Boom et al., 1986, Boom et al., 1988; Dirks and Raap, 1995). RNA polymerase II activity was inhibited by addition of either 500 ng/ml Actinomycin D (AMD) or 25 μg/ml 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) to the culture medium. Both inhibitors were purchased from Sigma (Sigma Aldrich, St. Louis, MI, USA). In some experiments, DRB was removed after various incubation periods to recover PolII activity.

Cell fixation and in situ hybridization

Cells were fixed and permeabilized as described (Dirks et al., 1993). Briefly, rat 9G cells were washed in PBS for 10 seconds and fixed with 3.7% formaldehyde (Merck, Darmstadt, Germany) containing 5% acetic acid in PBS for 15 minutes at room temperature. Cells were subsequently washed twice in PBS and stored in 70% ethanol at 4°C. Prior to hybridization, cells were washed in deionized water and pretreated with 0.1% pepsin (Sigma) in 0.01 M HCL for 30 seconds at 37°C. Cells were then washed in deionized water, dehydrated in a graded ethanol series and air-dried to prevent uncontrolled dilution of probes. Detection of HCMV-IE mRNA by DNA probes was carried out with plasmid pSS containing the 5.0 kb SphI-SalI genomic fragment of the immediate-early region (Boom et al., 1986). Probes for intron 1 and exon 4 sequences were generated by PCR from plasmid pSS with specific primers. Probes were labeled with digoxigenin-, biotin- or FITC-dUTP by nick translation (Roche Diagnostics GmbH, Mannheim, Germany) and purified over PCR purification columns according to instructions provided by the manufacturer (Qiagen, Venlo, the Netherlands). Probes were dissolved in 60% deionised formamide, 2×SSC (0.3 M NaCl, 0.03 M Na-citrate), 10 mM EDTA, 25 mM NaH_2PO_4 pH 7.4, 10% dextran sulfate and 250 ng/μl sheared herring sperm DNA at a final concentration of 5 ng/μl. Glass slides containing rat 9G cells were fixed, pretreated with pepsin and hybridized with a probe as described previously (Dirks et al., 1993). Probe and target sequences were denatured simultaneously by placing the slides on an 80°C metal plate for two minutes. Hybridizations were performed at 37°C overnight in a moist chamber.

Previously, we have shown that the hybridization patterns obtained by this procedure were very similar to those obtained when cells are submitted to a much stronger fixation procedure and examined by electron microscopy (Macville et al., 1995).

Post-hybridization washes and immunofluorescence

Cells were washed three times in 60% formamide, 2×SSC for 10 minutes each at 37°C and two times five minutes in Tris-buffered saline (TBS: 150 mM NaCl, 100 mM Tris-CL pH 7.4) containing 0.05% Tween 20 (Biorad, Hercules, California, USA) at room temperature. Digoxygenin-labeled probes were detected by anti-digoxin monoclonal antibody (Sigma Aldrich, St. Louis, MA, USA; dilution 1:1000) followed by goat anti-mouse Alexa594 (Molecular Probes Inc. Eugene, OR; dilution 1:500). Probes labeled with biotin were detected by Streptavidine-Alexa594 (Molecular Probes Inc. Eugene, OR, USA; dilution 1:400). All antibodies were diluted in TNB (TBS containing 0.5% (w/v) blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany)) and incubated for 45 minutes at 37°C. Cells were dehydrated through a graded ethanol series and mounted in Vectashield (Vector Laboratories, Inc., Burlingame, California, USA) containing 50 μg/ml 4′,6′-diamidino-2-phenyl indole (DAPI) as a DNA counterstain.

Microscopy and quantification of fluorescence by confocal scanning laser microscopy

Fluorescence microscopy was carried out with a Leica DM epifluorescence microscope equipped with a 100 W mercury arc lamp and appropriate filter sets. Digital images were captured with a cooled CCD camera (Photometrics, Tucson, Arizona, USA) and processed using SCIL-image (Multihouse, Amsterdam, the Netherlands) and Adobe Photoshop. Confocal images were recorded on a Leica TCS confocal scanning laser microscope (CSLM) (Leica Microsystems, Heidelberg, Germany) equipped with a 100×PL APO NA 1.4 Leica objective and an Argon/Krypton laser at an excitation wavelength of 567 nm. Image stacks of expressing and non-expressing cells in the same visual field were collected for quantitative analysis of the integrated fluorescence intensity in accumulations of HCMV IE transcripts (nuclear foci) and in the remainder of the nucleus in such a manner that saturation of the recorder (photomultiplyer tube) did not take place. The laser intensity and scanning speed were kept constant to avoid the interference of different levels of photobleaching during the measurements. The integrated fluorescence intensity in specified regions (of known area) of the nucleus was calculated in three-dimensional representations of the nucleus on the basis of the collected image stacks, which is a feature included in the Leica TCS NT software package. Additional calculations were carried out in Microsoft Excel. The integrated fluorescence intensity in the HCMV DNA integration site in non-induced cells was used as a reference, as all cells contain the same integrated tandem repeat of *HCMV* genes. All values were averaged over five to 10 cells. These cells were randomly chosen from the pool of HCMV IE expressing cells.

Differential interference contrast images were also recorded for each cell to provide information about intracellular structures.

Results

To assess the amounts of HCMV IE transcripts in the nuclei of rat 9G cells after induction by cycloheximide, we performed FISH and quantitative CSLM and calculated the integrated fluorescence intensity in 3D representations of two distinct subnuclear domains, namely RNA foci (containing accumulated transcripts at or near the transcription site) and remainder of the nucleus (see Materials and Methods). The transcription inhibitors DRB and AMD were used at concentrations specific for PolII. The adenosine analogue DRB prevented the essential conversion of hypo-phosphorylated PolIIA to elongating PolIIO through inhibition of the TFIIH-associated kinase CAK (Yankulov et al., 1995). As this mechanism is reversible, removal of DRB results in the initiation of gene expression. AMD functions through an irreversible mechanism, intercalating the DNA strands (Perry and Kelley, 1970).

After inhibition of PolII, HCMV IE transcripts exit more rapidly from nuclear foci than from the diffuse nucleoplasmic pool

As integrated fluorescence intensity in the nuclear RNA foci decreased approximately threefold within 10 minutes of

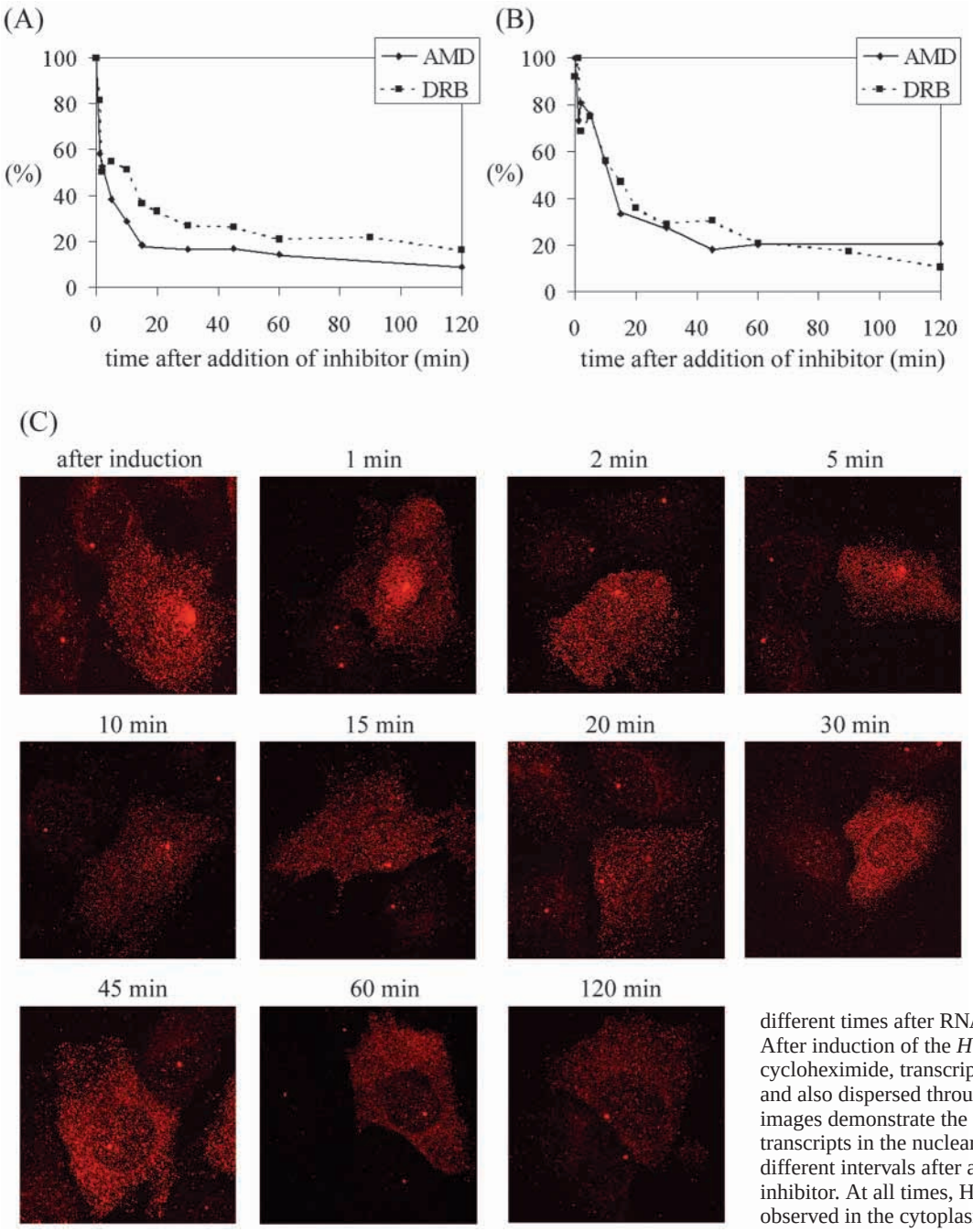

Fig. 1. Inhibition of RNA polymerase II activity by DRB or AMD treatment. (A) Integrated fluorescent intensity of HCMV IE transcripts accumulated in the nuclear foci relative to the integration site. Addition of either RNA polymerase II inhibitor resulted in a rapid decrease (half time ~2 minutes) in the amount of HCMV IE transcripts accumulated in nuclear foci. (B) Integrated fluorescent intensity of HCMV IE transcripts dispersed through the nucleoplasm relative to negative nuclei. The amount of HCMV IE mRNAs in the remainder of the nucleoplasm decreased at a much slower rate (half time ~10 minutes). (C) CSLM images of cells at different times after RNA polymerase II inactivation. After induction of the *HCMV IE* gene expression by cycloheximide, transcripts were present in nuclear foci and also dispersed throughout the nucleoplasm. These images demonstrate the decrease in the amount of transcripts in the nuclear foci and in the nucleoplasm at different intervals after addition of a RNA polymerase II inhibitor. At all times, HCMV IE transcripts were observed in the cytoplasm.

inhibition of PolII activity, the half time of this process is approximately two minutes (Fig. 1A; Table 1). The ratio of transcribed intron 1 sequences and transcribed total *HCMV-IE* sequences remained constant throughout the foci at all time-points (data not shown). This suggests that the release of transcripts from the RNA foci correlated with the completion of the splicing reaction (Dirks and Raap, 1995; Snaar et al., 1999). After ~30 minutes, the level of fluorescence in the track was comparable to that in the integration site, indicating that no or only a few transcripts are present at the transcription site. The integrated fluorescence intensity of the dispersed nuclear transcripts decreased with much slower rates (Fig. 1B; Table 1) after PolII inactivation (half time of ~10 min). After ~120 minutes, the nuclei of these cells reached an integrated fluorescence intensity closely resembling that of HCMV-IE negative cells. The inhibitory mechanism of PolII did not seem to influence the kinetics of these processes, as the integrated fluorescent intensity decreased with similar kinetics whether PolII was inhibited by AMD or by DRB (Table 1).

Activation of PolII results in rapid accumulation of HCMV IE transcripts at the transcription site. Transcripts then exit from the transcription site in a radial pattern

Coordinated activation of *HCMV IE* gene transcription was achieved by the removal of DRB from the incubation medium of rat 9G cells. As described above, a large majority of HCMV-IE transcripts had exited from the nucleus after 120 minutes of

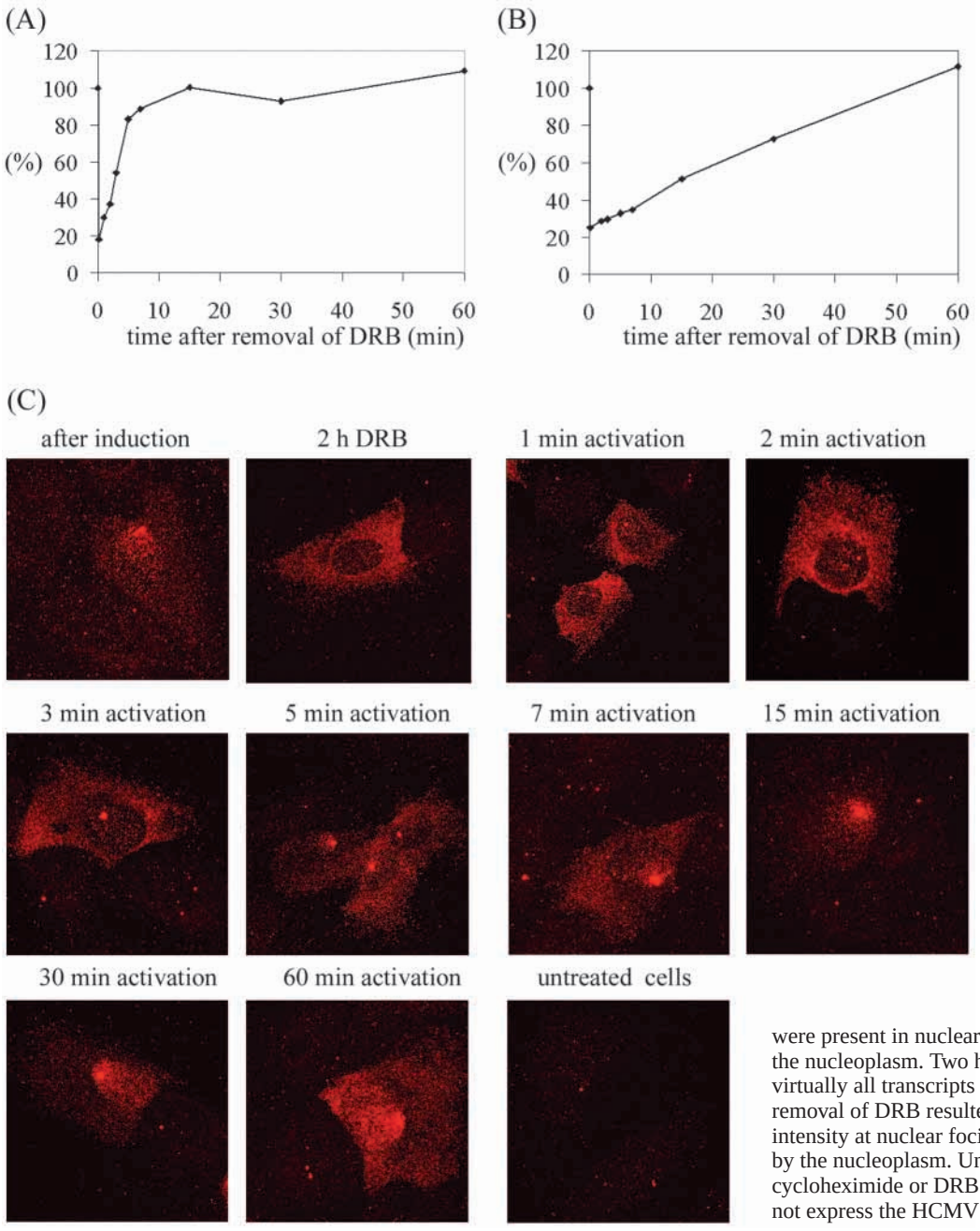

Fig. 2. Activation of RNA polymerase II by removal of DRB. (A) Integrated fluorescent intensity of HCMV IE transcripts accumulated in the nuclear foci relative to the integration site. Removal of DRB resulted in a rapid accumulation of HCMV IE mRNAs at nuclear foci. (B) Integrated fluorescent intensity of HCMV IE transcripts dispersed through the nucleoplasm relative to negative nuclei. The amount of HCMV IE mRNAs in the remainder of the nucleoplasm increased at a slower rate after DRB removal. Transcripts radiating from the nuclear foci were observed from approximately 5 minutes onwards. (C) CSLM images of cells at different times after RNA polymerase II inactivation. After induction of the *HCMV IE* gene expression by cycloheximide, transcripts were present in nuclear foci and also dispersed throughout the nucleoplasm. Two hours of DRB inhibition removed virtually all transcripts from the nucleus. Subsequent removal of DRB resulted in a fast increase of fluorescent intensity at nuclear foci (~3 minutes and later), followed by the nucleoplasm. Untreated cells were not subjected to cycloheximide or DRB treatment; therefore these cells did not express the HCMV IE genes.

DRB inhibition. In this manner, it became feasible to analyze the kinetics of HCMV-IE transcription before the steady-state situation (accumulated transcripts in foci and transcripts dispersed through the nucleoplasm) was established. These experiments demonstrated that the HCMV-IE integration site gained RNA fluorescence at one minute after PolII activation (Fig. 2A; Table 1). Activation of transcription was therefore extremely rapid. Accumulation of transcripts in a nuclear focus or dot larger than the integration site was found after approximately three minutes. The continuing increase in fluorescent intensity with time resulted after approximately five minutes in the radial dispersion of HCMV IE transcripts from the nuclear RNA foci into the surrounding nucleoplasm. The amount of radiating transcripts increased at longer times (seven to 15 minutes), as did the average distance of the transported transcripts from the transcription site. The amount of transcripts in the remainder of the nucleoplasm also increased with time but at a slower rate (Fig. 2B; Table 1). Approximately 30 minutes after PolII activation, HCMV-IE transcripts were present throughout the nucleus. Transcripts were also detectable in the cytoplasm of newly HCMV-IE-expressing cells at this time. Accumulation of large amounts of transcripts in the entire nucleoplasm and in the cytoplasm occurred at longer intervals (60 minutes) of PolII activity.

Incubation at 4°C interrupts processing and transport of HCMV IE mRNA in a nearly complete, but reversible manner

Rat 9G cells were incubated at 4°C to determine if pre-mRNA processing and nucleocytoplasmic mRNA transport were energy-dependent processes. These experiments demonstrated that the integrated fluorescence intensity of pre-mRNA transcripts at the transcription site remained relatively constant over a period of two hours when PolII inhibition was carried out at 4°C (Fig. 3A; Table 1). A similar situation was observed for the amount of diffusely distributed nucleoplasmic transcripts (Fig. 3B; Table 1). Subsequent return of the rat 9G cells to an incubation temperature of 37°C resulted in a rapid decrease in the amount of transcripts accumulated at the transcription site and in the amount of dispersed nucleoplasmic transcripts. Fick's law predicts a decrease in diffusion rate of only ~10% as a result of a decrease in temperature from 37°C to 4°C. This can therefore not explain the magnitude of the difference that was observed in these experiments. We therefore concluded that the interruption of pre-mRNA processing at the transcription site and of mRNA export to the nucleus was fully reversible. This was also supported by the kinetics of these decreases in integrated fluorescence intensity, as they were very similar to the kinetics for PolII inhibition at 37°C (Fig. 3A,B; Table 1).

Discussion

In the current study, we have analyzed the kinetics of HCMV IE mRNA synthesis, processing and transport in rat 9G cells. Initiation of PolII activity in rat 9G cells by DRB removal was shown to result in a rapid onset of mRNA synthesis (within two minutes). This can occur through two mechanisms. Hypophosphorylated PolIIA may be rapidly recruited to a pre-initiation complex at the moment the gene becomes activated.

Table 1. Overview of kinetic values calculated form the integrated fluorescence intensities of HCMV-IE transcripts in response to PolII inhibition or activation

Treatment	Incubation temperature (°C)	Domain*	Half time (min)
DRB	37	RNA foci	2
		Nucleus	10
AMD	37	RNA foci	2
		Nucleus	10
DRB	4	RNA foci	>200
		Nucleus	>200
DRB removal	37	RNA foci	2
		Nucleus	30

* Indicates the subnuclear domain in which the HCMV-IE transcripts are found, either accumulated at or near the site of transcription (indicated as RNA foci) or dispersed throughout the remainder of the nucleus (indicated as 'Nucleus').

Alternatively, a pre-initiation complex containing PolIIA may already be present at the site of the inactive gene. In either case, the removal of DRB allows transcription initiation and the subsequent phosphorylation of PolIIA to elongating PolIIO. PolIIA did not colocalize with the transcriptionally inactive *HCMV IE* gene in rat 9G cells (Snaar et al., 1999), which renders the second hypothesis less likely.

The kinetics of HCMV IE activation in rat 9G cells are in agreement with the kinetics of *β-actin* gene activation in rat kidney cells (Femino et al., 1998). In both cell types, the number of transcripts at the transcription site increases in a linear fashion during the first five minutes of gene activation. At this time, transcripts become visible in nuclear RNA foci or elongated dots. Mature mRNAs are detected in our system at nuclear sites distinct from, but surrounding, the nuclear RNA foci from five minutes onwards. This implies that both transcription and pre-mRNA processing of the newly transcribed ~6 kb HCMV IE messengers can be completed within five minutes. Comparison with the reported rates of PolII transcription, which are 1.7-2.5 kb/min for human PolII (Tennyson et al., 1995) and ~1.1-1.4 kb/min for rat PolII (Femino et al., 1998), suggests that most or all of this time will be required for mRNA transcription. Pre-mRNA processing must therefore be mostly cotranscriptional in these early phases of transcription.

Inactivation of RNA PolII by DRB or AMD results in the disappearance of accumulated HCMV IE transcripts with a calculated half time of approximately two minutes. Melcák et al. (Melcák et al., 2000) observed a similar reduction in the amount of Epstein-barr virus mRNA, as judged qualitatively by the length of the elongated dots that accumulated at transcription sites in Namalwa cells after DRB or AMD inhibition. As efficient release of transcripts from the spliceosome requires the completion of pre-mRNA processing (Custodio et al., 1999), the decrease in the rate of integrated fluorescence intensity appears to mirror the rate at which the remaining introns are removed from the accumulated transcripts. Several protein factors may be involved in this transient immobilization of transcripts at and near the transcription site. It may involve splicing commitment factors, but it may also be a function of RNA PolII, which remains

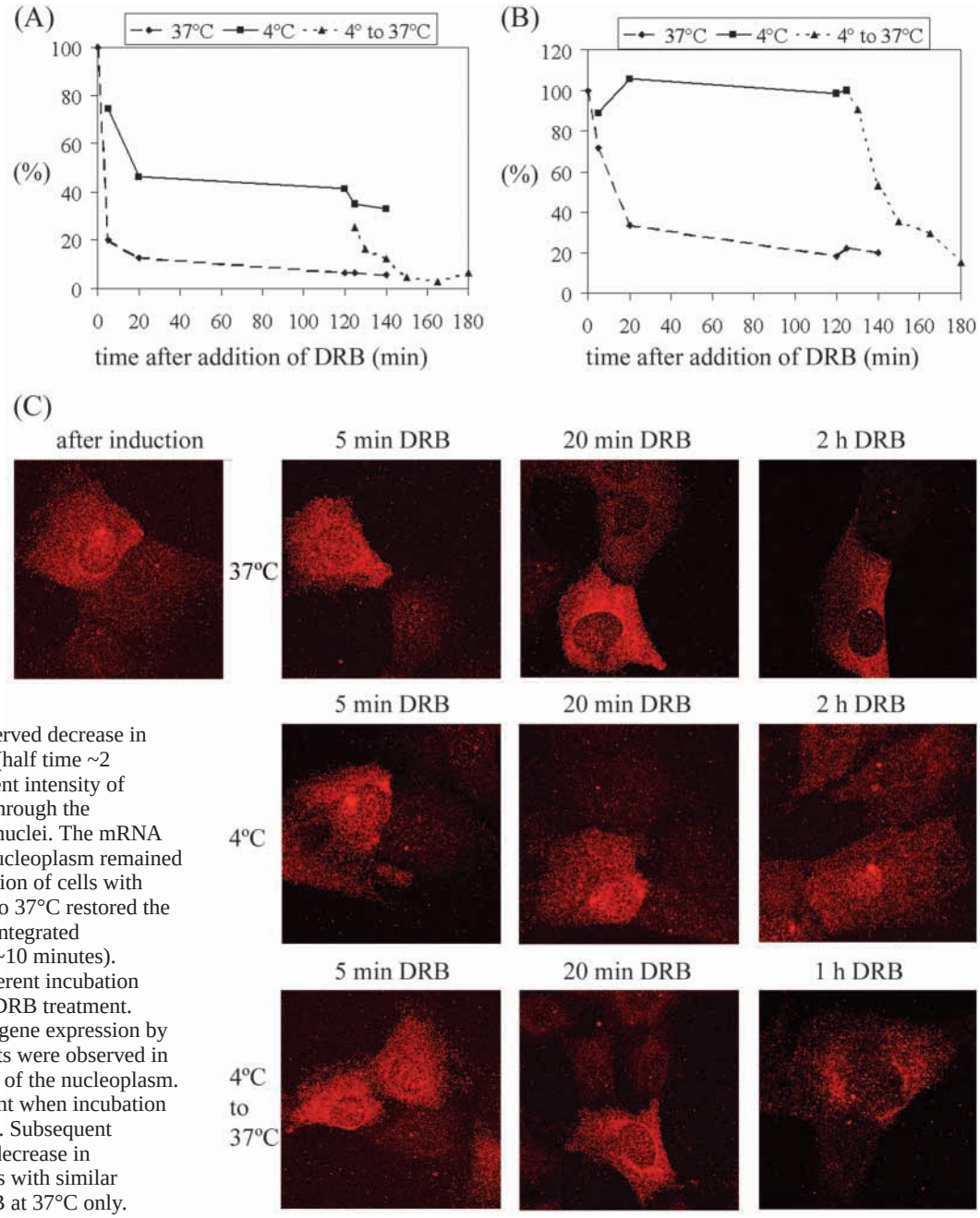

Fig. 3. Effect of incubation temperature on the amount of HCMV IE transcripts after RNA polymerase II inhibition by DRB. (A) Integrated fluorescent intensity of HCMV IE transcripts accumulated in the nuclear foci relative to the integration site. The mRNA amount in the nuclear foci remained relatively constant during incubation of cells with DRB at 4°C. Shifting cells back to 37°C restored the previously observed decrease in integrated fluorescence intensity (half time ~2 minutes). (B) Integrated fluorescent intensity of HCMV IE transcripts dispersed through the nucleoplasm relative to negative nuclei. The mRNA amount in the remainder of the nucleoplasm remained relatively constant during incubation of cells with DRB at 4°C. Shifting cells back to 37°C restored the previously observed decrease in integrated fluorescence intensity (half time ~10 minutes). (C) CSLM images of cells at different incubation temperatures during intervals of DRB treatment. After induction of the *HCMV IE* gene expression by cycloheximide at 37°C, transcripts were observed in nuclear foci and in the remainder of the nucleoplasm. These transcripts remained present when incubation with DRB was carried out at 4°C. Subsequent incubation at 37°C resulted in a decrease in integrated fluorescence intensities with similar kinetics to cells treated with DRB at 37°C only.

present in a complex with splicing factors after termination of transcription (Snaar et al., 1999). The subsequent release of spliced transcripts from spliceosomes may involve putative RNA helicases (Company et al., 1991; Ohno and Shimura, 1996; Schwer and Meszaros, 2000) and the small GTPase Ran (Grimm et al., 1997). Concomitant with RNA synthesis and processing, the cap-binding proteins, the poly(A)-binding protein PABP2 and various other mRNA-binding proteins associate with mRNAs to mediate their transport (Visa et al., 1996; Calado et al., 2000; Huang and Steitz, 2001; Le Hir et al., 2001; Lei et al., 2001). The capacity of these processes may also be exceeded by the transcription rate of the *HCMV IE* gene, possibly resulting in the saturation of the entire transport system. Interestingly, yeast strains carrying mutations in mRNA nuclear export factors were shown to accumulate mature mRNAs in foci that coincide with sites of transcription (Jensen et al., 2001).

Monitoring the decrease in nuclear fluorescence after treatment of rat 9G cells with DRB revealed that HCMV-IE mRNA export takes place with a half time of ~10 minutes. Previously, mRNA export from oocyte nuclei had been reported with half times of 2.4 minutes (Dargemont and Kühn, 1992) and 30 minutes (Jarmolowski et al., 1994). The half-time value measured in this study is consistent with the value measured by Jarmolowski et al. (Jarmolowski et al., 1994), considering the fact that the diameter of an oocyte nucleus is at least five times larger than the diameter of a rat fibroblast nucleus. Differences in transport rates may also be caused by

discrepancies in the nuclear complexity and the compactness in these cell types. Transport of in vitro transcribed mRNA in oocyte nuclei was analyzed upon microinjection. Interestingly, Jarmolowski et al. (Jarmolowski et al., 1994) measured a lag time of 20-25 minutes before export of mRNA. It is likely that this period was needed to assemble a transport-competent complex. Apparently, the formation of such a complex is much more efficient when coupled to transcription, as HCMV-IE transcripts are exported from the gene within five minutes of the initiation of transcription. This is also consistent with data presented by Pallavicini et al. (Pallavicini et al., 1994), who studied c-myc expression under control of a heat shock promoter in CHO-like cells. Upon heat shock, c-myc transcripts started to accumulate in cell nuclei within 10 minutes and remained there, but after returning the cells to 37ºC it took them one hour to be transported to cytoplasm. This suggests that either splicing or the formation of transport-competent complexes does not occur during heat shock.

As previously described, HCMV-IE transcripts radiate in all directions upon release from the transcription site and are eventually present throughout the nucleoplasm in a punctate pattern (Dirks and Raap, 1995; Macville et al., 1996). Transcripts are detected at increasing distances from the transcription site at increasing transcription times and throughout the nucleoplasm after approximately 30 minutes. A diffusion rate of ~0.13 μm^2/sec is calculated for this process. Much faster rates of RNA transport have been reported by Politz et al. (Politz et al., 1999) using hybridizing oligo (dT) labeled with chemically masked fluorescein to nuclear poly(A) RNA followed by spot photolysis and recording the distribution of fluorescence. After uncaging fluorescein, fluorescence spread within 30 seconds in all directions throughout the nucleus at a calculated diffusion rate of 0.6 μm^2/sec. It was therefore concluded that mRNA export is a process of free diffusion, which does not require energy. It should be noted, however, that there exist many different types of poly(A) RNA in the cell nucleus that are poorly characterized. For example, it is still not known what type of poly(A) RNA is present in speckle domains containing splicing factors and other processing and transcription factors. Earlier, Politz et al. (Politz et al., 1998) measured a broader range of diffusion rates by fluorescence correlation microscopy, suggesting that different pools of poly(A) RNA that do not all necessarily leave the cell nucleus but may move at fast speed may indeed exist. The mRNAs in transit to the cytoplasm may move at a much slower speed because they are packed in an RNP particle (Singh et al., 1999). Miralles et al. (Miralles et al., 2000) demonstrated that Balbiani Ring RNP particles do not always diffuse freely through the nucleoplasm. Instead, they have been observed to interact with fibers of specific structure and composition (Miralles et al., 2000). Notably, poly(A) RNA was shown to move in nuclei at a rate consistent with free diffusion but they did not accumulate in the cytoplasm. We observed that fluorochrome-labeled poly (dT) hybridized to poly (A) RNA in living cells was poorly exported towards the cytoplasm, indicating that hybridized mRNAs may escape from the regular transport mechanism (C. Molenaar, H.J.T. and R.W.D., unpublished).

Furthermore, our results and those of others indicate that mRNA export is at some stage energy dependent because transport is blocked when cells are incubated at 4ºC or at slightly lower temperature (Dargemont and Kühn, 1992; Jarmalowski et al., 1994; Melcák et al., 2001). Consistent with these observations, Calado et al. (Calado et al., 2000) reported that no transport of PABP2 to the cytoplasm takes place at 4°C. Another argument opposing a free diffusion model of RNA export is the presence of nucleoplasmic structures that at some stage mediate RNA export by transient interactions [for example see Strambio-de-Castillia et al. (Strambio-de-Castillia et al., 1999)]. In conclusion, this study demonstrates that levels of transcription, pre-mRNA processing and transport of HCMV IE mRNA are rapidly regulated in response to inhibition or activation of PolII. These processes are tightly linked in time and space. Furthermore, our results indicate that nucleocytoplasmic transport of these transcripts may occur in an energy-dependent active process.

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