

Inefficient processing impairs release of RNA from the site of transcription

Noelia Custodio, Maria Carmo-Fonseca, Finola Geraghty¹, H.Sofia Pereira, Frank Grosveld² and Michael Antoniou^{1,3}

Institute of Histology and Embryology, Faculty of Medicine, University of Lisbon, 1699 Lisbon codex, Portugal, ¹GKT Medical School, Department of Experimental Pathology, Guy's Hospital, London SE1 9RT, UK and ²MGC Department of Cell Biology and Genetics, Erasmus University, 3015 GE Rotterdam, The Netherlands

³Corresponding author
e-mail: michael.antoniou@kcl.ac.uk

We describe here for the first time the site of retention within the nucleus of pre-mRNA processing mutants unable to be exported to the cytoplasm. Fluorescence *in situ* hybridization was used to detect transcripts from human β -globin genes that are either normal or defective in splicing or 3' end formation. Nuclear transcripts of both wild-type and mutant RNAs are detected only as intranuclear foci that colocalize with the template gene locus. The kinetics of transcript release from the site of transcription was assessed by treatment of cells with the transcriptional inhibitors actinomycin D, α -amanitin and DRB. These drugs induce the rapid disappearance of nuclear foci corresponding to wild-type human β -globin RNA. In contrast, pre-mRNA mutants defective in either splicing or 3' end formation and which fail to be transported to the cytoplasm, are retained at the site of transcription. Therefore, 3' end processing and splicing appear to be rate limiting for release of mRNA from the site of transcription.

Keywords: pre-mRNA processing/RNA transport/transcription

Introduction

Transport of mRNA from the nucleus to the cytoplasm is essential for the expression of eukaryotic genes. It is an active and highly selective process that involves *cis*-acting signals and specific *trans*-acting factors (for recent reviews see Lee and Silver, 1997; Nakielnny *et al.*, 1997; Nigg, 1997). Although much recent information has begun to define the pathways that mediate and control the nucleocytoplasmic RNA traffic in a cell, very little is known about the release of mRNAs from the site of transcription for subsequent transport to the nuclear periphery and translocation across the nuclear pore.

Several lines of evidence indicate that efficient mRNA transport involves cotranscriptional interaction with RNA-binding proteins and a correct processing of the pre-mRNA into mature mRNA (Izaurralde and Mattaj, 1992; Elliot *et al.*, 1994; Visa *et al.*, 1996a,b). In particular, a relationship between splicing and export has been clearly

established (Chang and Sharp, 1989; Legrain and Rosbash, 1989; Hamm and Mattaj, 1990). The removal of introns from pre-mRNA is catalysed by the spliceosome, a dynamic complex composed of small nuclear ribonucleoproteins (snRNPs) and numerous protein components (Moore *et al.*, 1993; Kramer, 1996). Spliceosome assembly occurs on each substrate pre-mRNA *de novo* and requires conserved recognition sequences located at the exon–intron boundaries. Pre-mRNA molecules bearing mutations that allow spliceosome assembly but impair splicing are largely retained in the nucleus, whereas mutations that disturb splicing complex formation can partially overcome the block in transport of intron-containing pre-mRNAs to the cytoplasm (Chang and Sharp, 1989; Legrain and Rosbash, 1989; Hamm and Mattaj, 1990). This led to the hypothesis that spliceosome assembly may cause retention of pre-mRNA in the nucleus, either because certain splicing factors interact with nuclear structures holding the unspliced RNA, or because the spliceosome may prevent interaction of the RNA with the export machinery (Nakielnny *et al.*, 1997). In addition to splicing, 5' capping and 3' end formation are also known to influence mRNA export. In general, both the 5' cap and the 3' poly(A) tail enhance the export rate of a transcript but they do not appear to be essential (Eckner *et al.*, 1991; Jarmolowski *et al.*, 1994). However, mutated β -globin RNAs devoid of the second intron are unable to undergo correct 3' end formation and are retained in the nucleus (Collis *et al.*, 1990; Antoniou *et al.*, 1998).

Spliceosome assembly and splicing are known to occur during ongoing transcription elongation *in vivo* (Beyer and Osheim, 1988; LeMaire and Thummel, 1990; Bauren and Wieslander, 1994; Tennyson *et al.*, 1995). Accordingly, spliceosome components and spliced RNAs have been visualized in close proximity to sites of transcription (Wu *et al.*, 1991; Xing *et al.*, 1993; Zhang *et al.*, 1994; Bauren *et al.*, 1996; Huang and Spector, 1996; Neugabauer and Roth, 1997). In addition, it has recently been discovered that the splicing and polyadenylation machinery can associate with the transcription elongation complex via the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (Steinmetz, 1997). However, it is not clear from these studies whether pre-mRNA processing must precede release for subsequent transport to the nuclear pores. Also, there is currently no information available on the kinetics of processing and release of mRNA from the site of transcription.

In order to address these questions we have used *in situ* hybridization and confocal microscopy to visualize the release of normal and mutated human β -globin RNAs from the vicinity of their cognate gene templates within the nucleus. The system we have exploited in these studies consists of human β -globin genes under the control of the locus control region (LCR) that are stably transfected

into murine erythroleukaemia (MEL) cells. The human β -globin genes within this context reproducibly express at physiological levels which are directly proportional to transgene copy number and independent of the site of integration in the host cell genome during the induced terminal differentiation of these cells (Blom van Assendelft *et al.*, 1989; Talbot *et al.*, 1989; Collis *et al.*, 1990). This pattern of gene expression demonstrates that although the transgenes are integrated at ectopic sites within the host cell genome as a tandem array, their function accurately reflects that observed from the same genes at their native chromosomal locus. Even globin loci integrated in heterochromatin regions are transcribed and processed at normal levels (Milot *et al.*, 1996).

Our data show that inhibition of transcription by actinomycin D, α -amanitin or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) causes the rapid release of wild-type human β -globin RNA from the vicinity of the site of transcription. In contrast, mutant globin pre-mRNAs that are defective in either splicing or 3' end formation are held in close proximity to the gene template in the presence of these drugs. These observations imply that efficient pre-mRNA processing is crucial and therefore rate limiting for the release of transcripts from the site of transcription.

Results

Localization of human β -globin RNA in MEL cells

The wild-type human β -globin gene (β WT) within the microlocus LCR expression cassette (Figure 1A; Collis *et al.*, 1990) was transfected into MEL cells and stable clones or populations selected with G418, as described (Antoniou, 1991). One of these clones, MEL β WT, which harbours ~14 copies of the transgene as a tandem array (data not shown), was used to perform *in situ* hybridization experiments. Expression of the human β -globin transgenes was induced by adding dimethylsulfoxide (DMSO) to the culture medium for 1–4 days during which the MEL cells undergo terminal erythroid differentiation (Antoniou, 1991; Antoniou *et al.*, 1993). Uninduced and induced cells were hybridized under non-denaturing conditions with a probe complementary to the transcribed sequence of the human β -globin gene (RNA probe, Figure 1A). Uninduced cells were devoid of any hybridization signal (data not shown), whereas cells induced for 4 days contain a fluorescent focus in the nucleus and additional cytoplasmic staining (Figure 1B, a–c). The intensity of staining of the nuclear foci reached maximum levels after 2 days of induction whereas the cytoplasmic labelling peaked after 4 days of differentiation (data not shown). This is consistent with biochemical data showing that the rate of globin gene transcription in MEL cells attains maximum levels 36–48 h after induced differentiation and cytoplasmic accumulation peaks after 4 days (see Antoniou, 1991). Labelling of total cellular DNA with TO-PRO-3 confirmed the intranuclear localization of the human β -globin RNA foci (Figure 1B, b). In addition, superimposition of confocal optical sections revealed that intranuclear β -globin RNA is detectable only as a single focus within the nucleus with no other sites of accumulation (Figure 1B, c).

In order to determine whether the RNA foci observed

in the nuclei of induced cells represent sites of transcription of the transfected human β -globin genes, double-hybridization experiments were performed. Cells were sequentially hybridized for human β -globin RNA under non-denaturation conditions with a probe complementary to the transcribed sequence of the gene (RNA probe; Figure 1A), followed by detection of the corresponding gene locus by hybridizing under denaturing conditions with a probe complementary to the plasmid cassette used for transfection (DNA probe; Figure 1A). As expected for a clonal population of stably transfected cells, hybridization with the DNA probe produces a fluorescent focus in each nucleus (Figure 1B, e). In contrast, RNA foci are only detected in ~20–40% of the cells (Figure 1B, d). A similar result is observed when immunofluorescence is performed using an antibody specific for human β -globin (data not shown; see Fraser *et al.*, 1993), indicating that expression of human β -globin is restricted to a subset of the transfected population. This is probably due to a combination of the asynchronous nature of the cell cultures and position-effect variegation (see Milot *et al.*, 1996). Nevertheless, the overlay of red (DNA hybridization) and green (RNA hybridization) images shows that the focal signals overlap in the nucleus (Figure 1B, f). Given the limits of resolution of light microscopy, these results indicate that the foci labelled by the RNA probe are likely to correspond to the sites of human β -globin gene transcription.

Since the RNA probe is complementary to the full-length β -globin transcript, it does not allow nascent and terminating or terminated transcripts to be distinguished. Therefore, to identify specifically those RNA molecules that have been elongated towards the end of the transcription unit and that are either terminating or terminated, we used a 3' RNA probe (Figure 1A; see Materials and methods). The 3' RNA probe spans the poly(A)-addition site and will, as a result, only hybridize to those β -globin RNAs that have been extended to this terminal region of the gene. In addition to cytoplasmic labelling, this probe produces a focal intranuclear signal which colocalizes with the focus produced by the full-length RNA probe (Figure 1B, g–i). These data show that our *in situ* hybridization procedure has the sensitivity to detect terminating or terminated β -globin RNA chains at the site of transcription.

β -globin pre-mRNA is spliced at the site of transcription

We next asked whether splicing of β -globin pre-mRNA occurs while the transcripts are still in the vicinity of the site of transcription. In order to address this question, splice junction oligonucleotide probes (SJ I/II and SJ II/III), which are complementary to the ends of the three exons and span the two introns of the human β -globin gene (Figure 2A), were employed. The oligonucleotides used were the same as described previously (Zhang *et al.*, 1996) and had been shown to be incapable of forming stable hybrids with unspliced RNA. MEL β WT cells that harbour the wild-type human β -globin gene were induced to differentiate and hybridized with a mixture of the SJ I/II and SJ II/III probes. The results (Figure 2B, a and d) show a cytoplasmic signal with additional labelling of intranuclear foci. Double-hybridization experiments using these splice junction oligonucleotide probes and the full-length RNA probe (Figure 1A) demonstrate that the signals

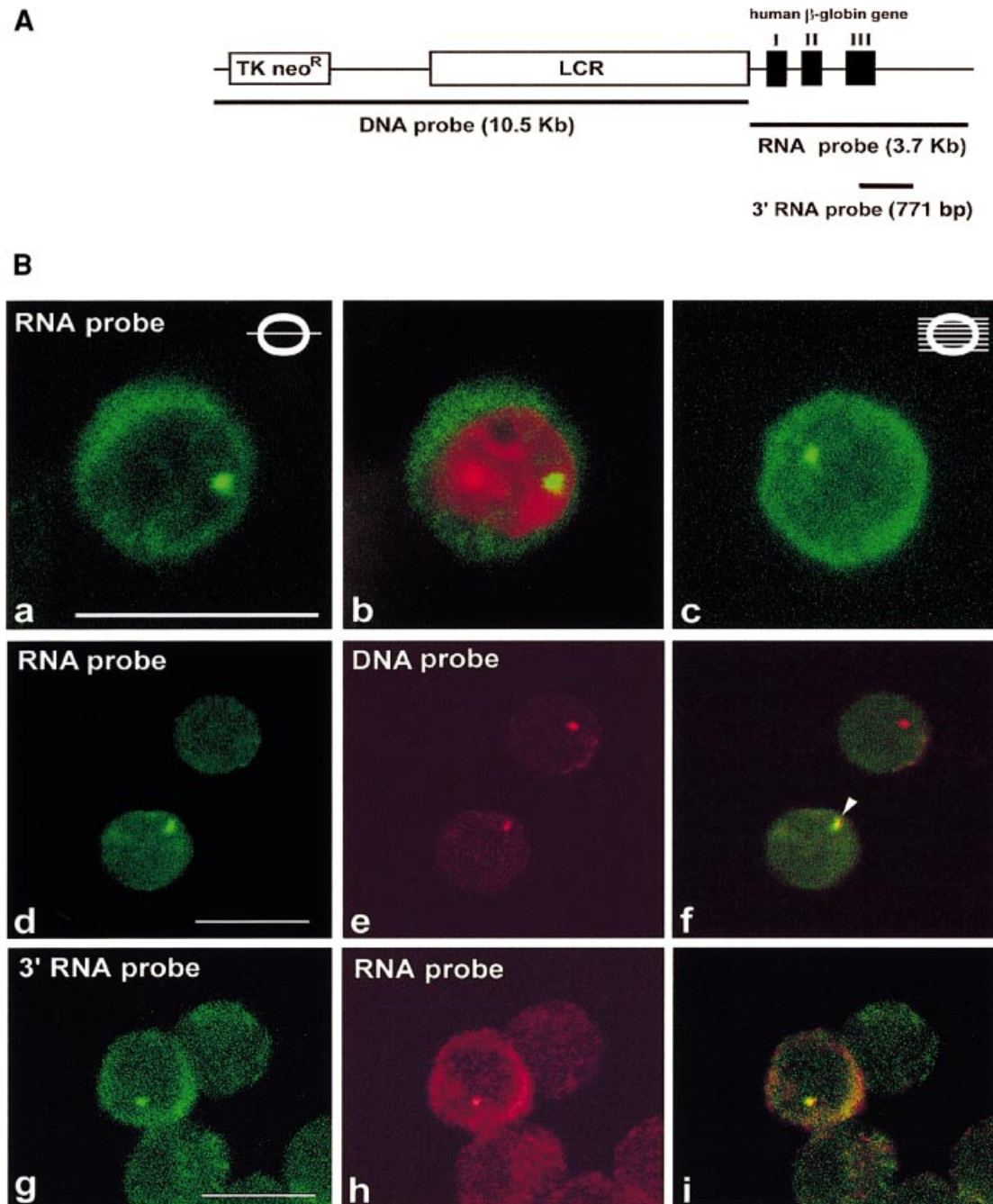


Fig. 1. (A) Schematic illustration of the human wild-type β -globin construct. The wild-type human β -globin gene (β WT) is within the micro-locus control region (LCR) expression cassette (Collis *et al.*, 1990; see Materials and methods). Human β -globin gene exons are shown as black rectangles. The TK neo^R gene confers resistance to G418 in stably transfected MEL cells. The extent of the probes used for *in situ* hybridization to detect human β -globin RNA (RNA probe; 3' RNA probe) and site of transgene integration (DNA probe) are also shown. (B) *In situ* detection of human β -globin RNA in transfected MEL cells. MEL cells transfected with the β WT construct (MEL β WT) were fixed either in formaldehyde and permeabilized with Triton X-100/saponin (a–c) or in formaldehyde/acetic acid and digested with pepsin (d–i). (a) and (b) depict a single confocal section through a cell hybridized with the RNA probe (2 ng/ μ l) that is complementary to the entire length of the human β -globin transcription unit (green staining); total DNA was labelled with the dye TO-PRO-3 (Molecular Probes) (red staining). (c) depicts a superimposition of 10 optical sections through another cell hybridized with the RNA probe (consecutive sections are separated by 0.7 μ m). The cells were induced to undergo erythroid differentiation for 4 days. (d)–(f) depict the simultaneous detection of human β -globin RNA and the transfected gene locus. MEL β WT cells were induced for 2 days, and hybridized with the RNA probe labelled with digoxigenin (4 ng/ μ l) (e, green staining). The cells were then fixed in formaldehyde, denatured and hybridized with the DNA probe labelled with dinitrophenyl (2 ng/ μ l) (d, red staining). Fluorescein- and Texas Red-coupled antibodies revealed the sites of hybridization of the RNA and DNA probes, respectively. Superimposition of red and green images shows that the DNA and RNA foci in the nucleus colocalize (f). (g)–(i) show sequential hybridization with the 3' RNA probe (3 ng/ μ l) labelled with digoxigenin (revealed with fluorescein, g), and the full-length RNA probe (4 ng/ μ l) labelled with DNP (detected with Texas Red, h). (i) depicts a superimposition of the two images. Cells were induced for 4 days. Bar, 10 μ m.

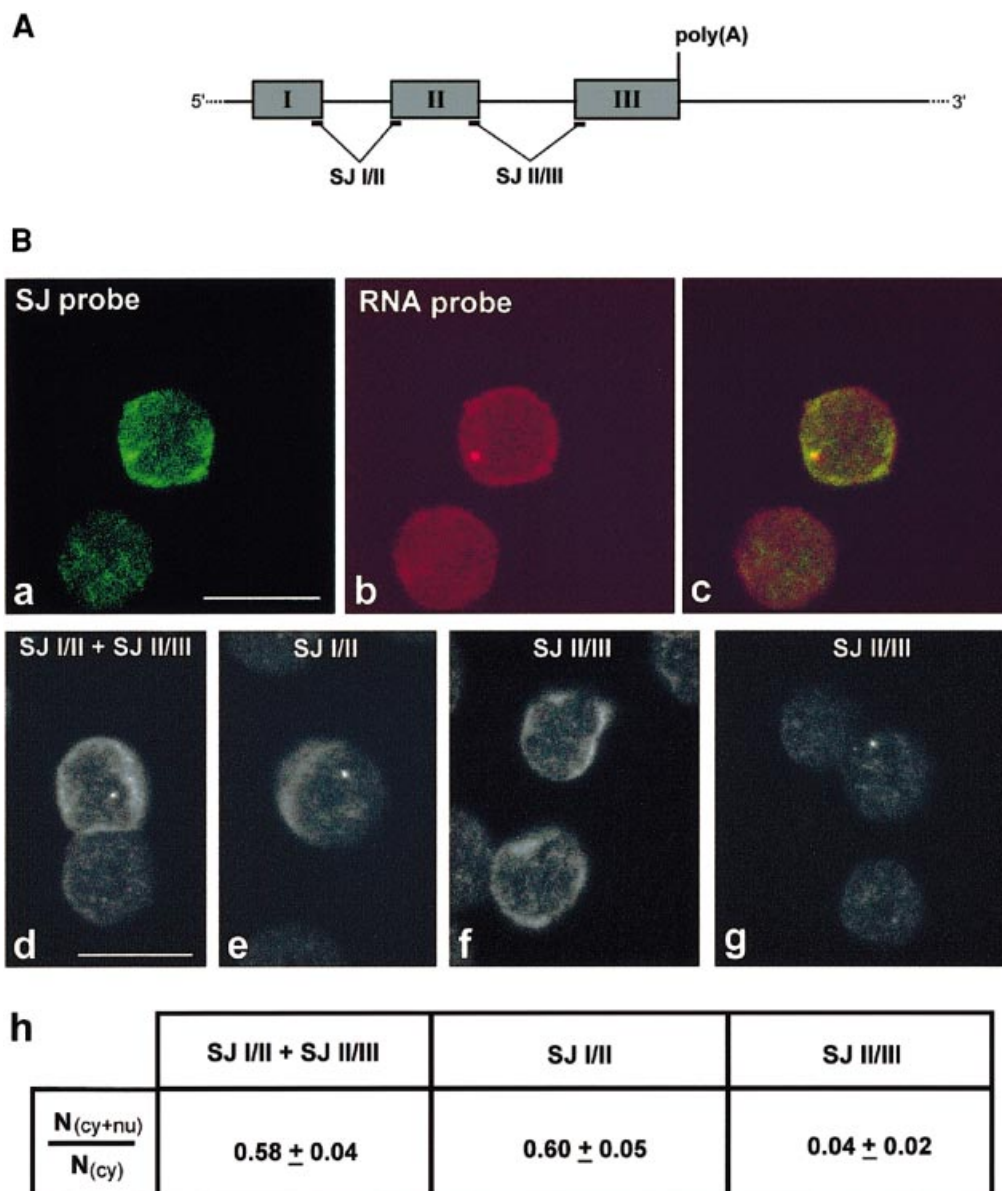


Fig. 2. Spliced human β -globin RNAs are detected at the site of transcription. (A) Schematic illustration of the splice junction (SJ) probes. The SJ I/II probe is complementary to the last 12 nucleotides of exon I and the first 12 nucleotides of exon II, whereas the SJ II/III probe hybridizes to the last and first 12 nucleotides of exons II and III, respectively. (B) MEL β WT cells were induced for 2 days, fixed in formaldehyde/acetic acid, digested with pepsin and hybridized with a mixture of splice junction oligonucleotide probes (SJ I/II and SJ II/III, 1 ng/ μ l each) labelled with DNP and revealed with fluorescein (a). The cells were then fixed in formaldehyde and hybridized with the full-length RNA probe (4 ng/ μ l) labelled with digoxigenin and revealed with rhodamine (b). (c) depicts a superimposition of the two images. In (d)–(g) MEL β WT cells were induced for 4 days, fixed in formaldehyde/acetic acid, digested with pepsin and hybridized with either a mixture of the splice junction oligonucleotide probes SJ I/II and SJ II/III (d), SJ I/II (e) or SJ II/III (f). As a positive control, MEL β IVSI cells were induced for 4 days and hybridized with the SJ II/III probe (g). The proportion of labelled cells containing nuclear foci was estimated according to the formula: $[N_{(cy + nu)}/N_{(cy)}]$. (h) depicts means \pm SE (three separate experiments were performed for each probe and a total of 100–200 cells were analysed). Bar, 10 μ m.

colocalize in the nucleus (Figure 2B, b and c), indicating that splicing of globin pre-mRNA is taking place in close proximity to the site of transcription. However, different results were obtained when the splice junction oligonucleotide probes were used separately. The SJ I/II probe which spans exons I and II, produces an intranuclear hybridization signal (Figure 2B, e) similar to that obtained with the mixture of the two probes (Figure 2B, d). This indicates that splicing of intron I is taking place in the vicinity of the site of transcription. In contrast, the exon II–III spanning SJ II/III probe fails to label nuclear foci in the

vast majority of cells (Figure 2B, f). In the cytoplasm, both probes produce a strong hybridization signal (Figure 2B, d–f). Potential technical artefacts with the SJ II/III probe were controlled for by hybridization to MEL β IVSI cells. These cells harbour a mutant human β -globin gene which lacks the second intron and therefore possesses a fusion of exons II and III (Figure 4B, upper panel; see below). MEL β IVSI cells hybridized with SJ II/III show clearly labelled intranuclear foci (Figure 2B, g) demonstrating that this probe is functioning normally. (Note the absence of cytoplasmic labelling in MEL β IVSI cells,

consistent with the finding that this mutant RNA fails to be exported from the nucleus; Antoniou *et al.*, 1998 and see below.)

A quantitative analysis of wild-type MEL β WT cells hybridized with the splice junction SJ probes either separately or in combination was conducted (Figure 2B, h). The proportion of cells that are labelled in the cytoplasm and also contain a nuclear focus is similar for the mixture of the two probes (58%) or the exon I–II spanning SJ I/II probe alone (60%). In contrast, the majority (96%) of cells labelled in the cytoplasm by the exon II–III spanning SJ II/III probe is devoid of a detectable intranuclear focus. These data suggest that the first intron of human β -globin RNA is spliced while the transcript is still at the gene locus, whereas removal of the second intron takes place either immediately prior to rapid release from the site of transcription or at some other location within the nucleus after transport from the region of the gene template.

Actinomycin D causes a rapid release of β -globin transcripts from the site of transcription

The observed intranuclear foci of β -globin RNA appear to represent newly synthesized transcripts in the vicinity of the gene locus. If this is indeed the case, treating cells with transcription inhibitors should lead to the disappearance of these nuclear foci as a result of transport away from the site of transcription of previously synthesized RNA molecules. This in turn may provide insight into the kinetics of transcript release from the site of transcription. In order to test this idea, we initially used actinomycin D, a drug that acts very rapidly *in vivo* (Darnell *et al.*, 1971) and exerts its effects by binding to the DNA template, thereby interfering with the elongation of the growing RNA chain (Kersten *et al.*, 1960; Goldberg *et al.*, 1962).

The results show that when MEL β WT cells are treated with actinomycin D for 5 min and hybridized with the full-length RNA probe (Figure 1A), the intranuclear foci are no longer detected (Figure 3a and b). However, cytoplasmic labelling remains visible, confirming that these cells were transcribing the transfected, wild-type human β -globin genes before exposure to the drug (Figure 3b). Quantitative analysis reveals that actinomycin D causes a highly significant decrease in the proportion of cells containing a visible focus in the nucleus (Figure 5A, MEL β WT). Similar results were obtained with the 3' RNA probe (Figure 1A) during a time course experiment which shows that within 1 min of exposure to the drug, the proportion of cells with a visible focal signal in the nucleus decreases to approximately one-third (Figure 3c). Thus, in the presence of actinomycin D, β -globin RNAs have a half-life of <1 min at the site of transcription.

β -globin RNA processing mutants are retained at the site of transcription

The data presented thus far establish that actinomycin D induces a rapid release of newly synthesized β -globin RNA from the site of transcription (Figure 3). We next analysed the effect of this drug on RNA processing mutants defective in cytoplasmic transport.

We have previously generated stably transfected MEL cell pools harbouring a human β -globin gene possessing a 5' splice site mutation (GT→AC) of the second intron

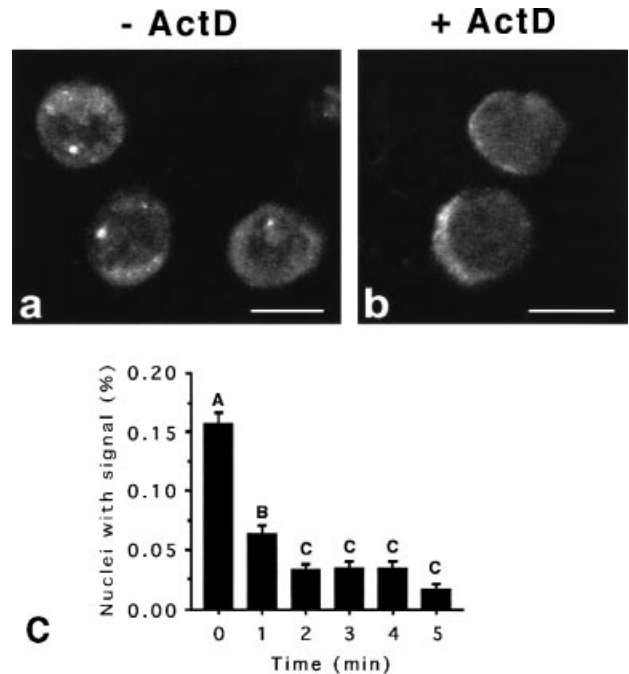


Fig. 3. Actinomycin D induces a rapid release of β -globin RNA from the site of transcription. (a) and (b) MEL β WT cells were induced to undergo erythroid differentiation for 2 days and treated with actinomycin D. The cells were fixed in formaldehyde, permeabilized with Triton X-100/saponin and hybridized with the full-length or 3' RNA probes. (a) Cells untreated with actinomycin D. (b) Cells treated with actinomycin D for 5 min before fixation and hybridization. Bar, 10 μ m. (c) Kinetics of release of β -globin RNA from the site of transcription in the presence of actinomycin D. MEL β WT cells were induced for 2 days, fixed in formaldehyde/acetic acid, digested with pepsin and hybridized using the 3' RNA probe. The cells were either untreated (time 0) or treated with actinomycin D for 1, 2, 3, 4 or 5 min. The proportion of cells with a nuclear RNA signal was estimated in three independent experiments for each time point. A total of 12 microscopic fields corresponding to a total of 300–400 cells were analysed per experiment. There were no significant differences between experiments, allowing them to be pooled. The mean proportion of cells with nuclear foci is plotted (mean \pm SE) and the values compared using a one-way analysis of variance, ANOVA (SAS Institute, 1990). A significant decrease in the mean proportion of positive cells over the 5 min period is observed [$F_{(5,239)} = 88.72$, $p < 0.0001$]. In order to determine which time slots differed significantly, a Student–Newman–Keuls (SNK; SAS Institute, 1990) was used as an *a posteriori* test. This showed three groupings of values (SNK, $p < 0.05$). At time 0 the mean proportion of positive cells is significantly greater than at all other time slots; the mean value at time 1 is also significantly greater than the remaining time slots and there are no significant differences between mean values at times 2, 3, 4 and 5. Different letters above the histogram bars are used to represent statistically significant differences between means.

(Figure 4A, upper panel; Antoniou *et al.*, 1998). Biochemical analysis indicates that *in vivo* this mutant RNA (here referred to as 'single splice site mutant', β SM), is correctly 3' cleaved and polyadenylated at normal rates but is not spliced and not transported to the cytoplasm (Antoniou *et al.*, 1998). In addition, *in vitro* this same mutant RNA is able to support at least partial spliceosome assembly (Lamond *et al.*, 1987). In the present study, a clone transfected with β SM (MEL β SM) was used for *in situ* hybridization. After 4 days of induced erythroid differentiation, hybridization of MEL β SM cells with the full-length RNA probe (Figure 1A) reveals one focal signal per nucleus (Figure 4A, a). However, no cytoplasmic staining is detected, consistent with the biochemical data

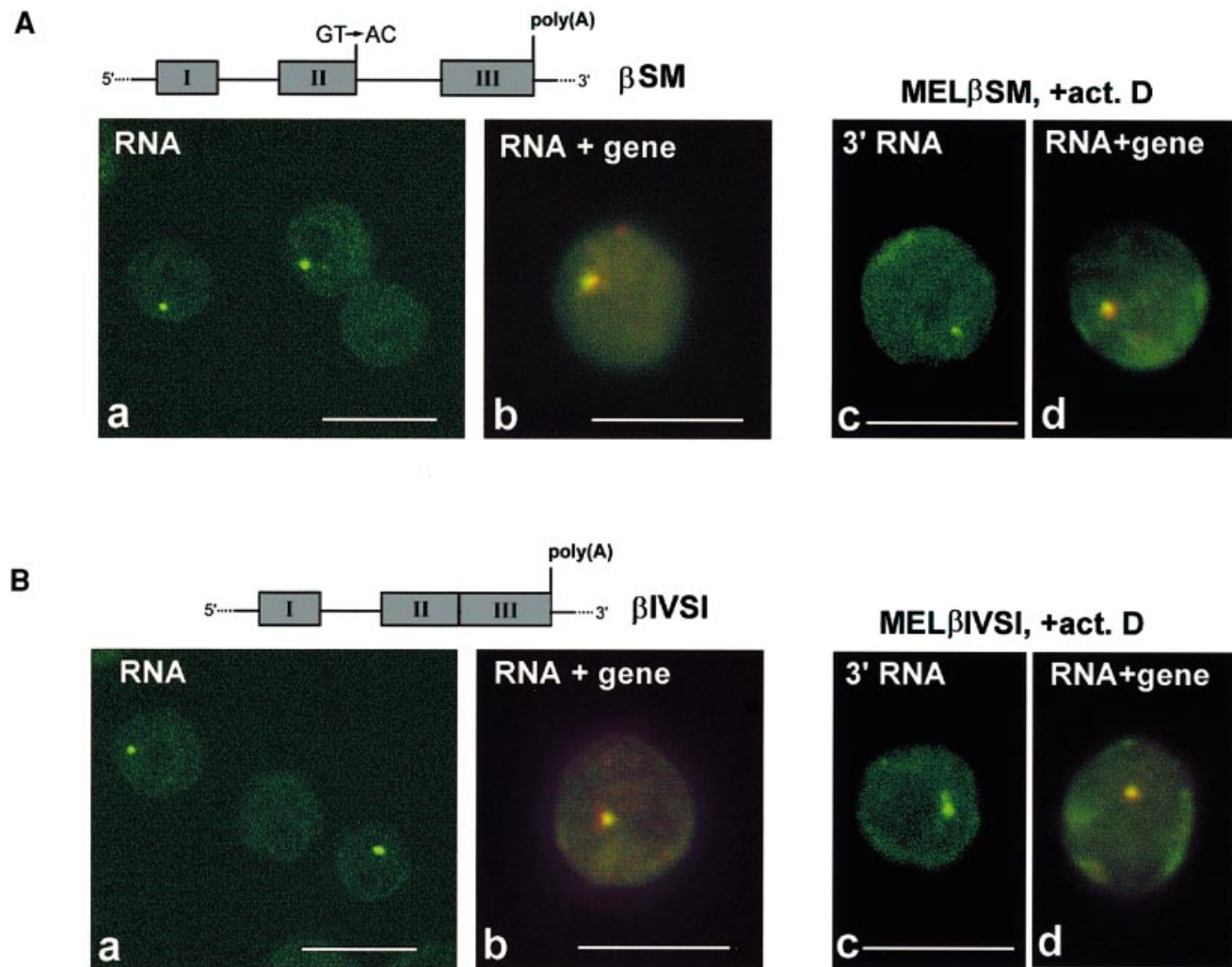


Fig. 4. β -globin RNA mutants for splicing and 3' end processing are retained near the site of transcription. (A) MEL β SM cells transfected with the construct β SM that contains a 5' splice donor site mutation (GT→AC) of the second intron (upper illustration) were induced to undergo erythroid differentiation with DMSO for 4 days. (a) Hybridization with the RNA probe. Note that a focal signal is readily visible in the nucleus, but no cytoplasmic staining is detected. (b) Double-labelling of a cell with the RNA and DNA probes. Note that the RNA focus is localized in close vicinity to the site of transcription. (c) and (d) MEL β SM cells treated with actinomycin D for 5 min. Hybridization with the 3' RNA probe reveals an intranuclear focus (c), and double-hybridization using the RNA and DNA probes shows that the RNA focus colocalizes with the site of transcription (d). (B) A similar set of experiments was performed using MEL β IVSI cells, which contain a β -globin construct that lacks completely the second intron (IVS-II), possessing only IVS-I (β IVSI; upper panel illustration). Cells were induced with DMSO for 4 days. (a) Hybridization with the RNA probe shows intranuclear foci but no cytoplasmic staining. (b) Double-hybridization using the RNA and DNA probes confirms that the RNA focus is localized near the site of transcription. (c) and (d) After treatment with actinomycin D for 5 min, hybridization with the 3' RNA probe reveals the presence of intranuclear foci (c) and double-hybridization demonstrates that the RNA is retained near the site of transcription (d). Bar, 10 μ m. Note: no cytoplasmic staining is seen in any of the panels depicted as the transcripts from these mutant human β -globin genes are defective in transport. The staining observed at the rim of the nuclei shown in (A, c/d and B, d) is due to non-specific background hybridization and trapping of the probe. This is shown by the fact that a similar pattern can be seen when the same probes are used with untransfected, uninduced, (negative control) MEL cells (data not shown).

demonstrating that these RNA molecules fail to be exported from the nucleus (Antoniou *et al.*, 1998). Double-hybridization experiments show that the foci corresponding to this mutant β SM RNA, colocalize with the gene template (Figure 4A, b).

In contrast with the results obtained with the wild-type β WT construct (Figure 3), focal signals of 5' splice site mutant β SM RNA remain visible in the nuclei of cells treated with actinomycin D for 5 min (Figure 4A, c and d; Figure 5A, MEL β SM). Furthermore, the β SM RNA foci colocalize with the signal produced by DNA hybridization for the transgene template, suggesting that these mutant transcripts are not released from the vicinity of the site of transcription (Figure 4A, d).

As 3' end processing also plays an important role in transport of β -globin RNA to the cytoplasm (Collis *et al.*, 1990; Antoniou *et al.*, 1998), we extended our analysis to cells transfected with a construct that lacks completely the second intron (IVS-II), possessing only IVS-I (β IVSI; Figure 4B, upper panel). Despite possessing normal 3' end processing signal sequences, this mutant is unable to undergo correct 3' end formation, producing an RNA species that is not cleaved and fails to reach the cytoplasm (Collis *et al.*, 1990; Antoniou *et al.*, 1998). The *in situ* hybridization results show that β IVSI RNA is detected in close proximity to the site of transcription but not in the cytoplasm (Figure 4B, a and b). In addition, intranuclear foci of β IVSI mutant transcripts that colocalize with

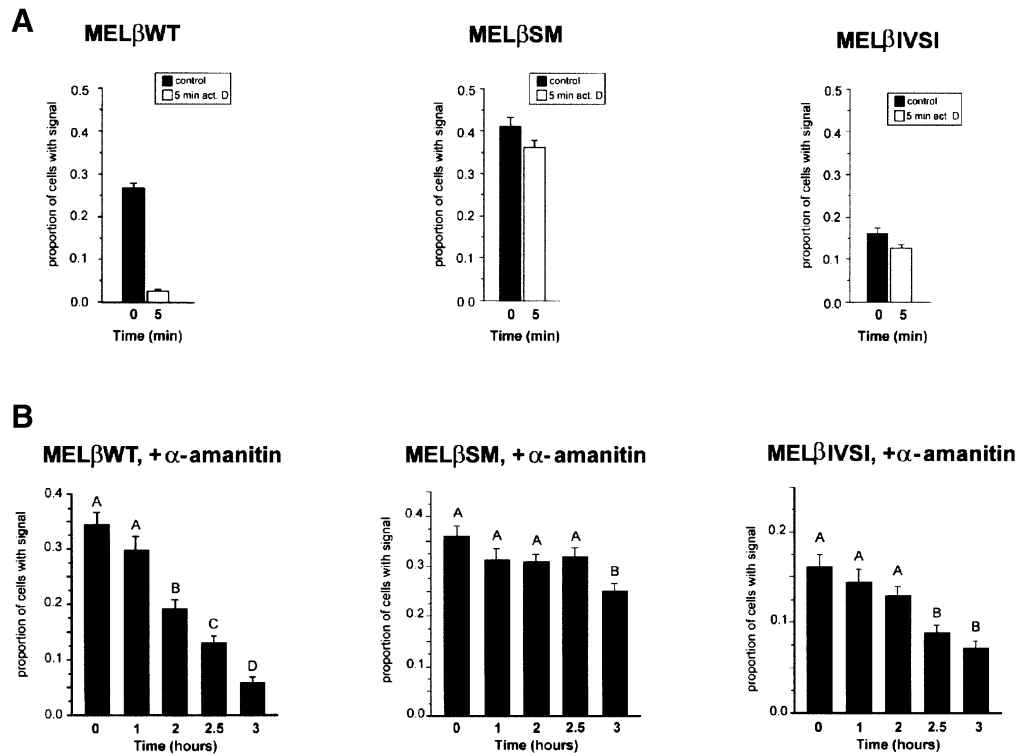


Fig. 5. Quantitative analysis of the effect of transcription inhibitors on β -globin RNA release. (A) MEL β WT, MEL β SM and MEL β IVSI cells were induced to undergo erythroid differentiation for 2 days, untreated or treated with actinomycin D for 5 min and hybridized with the full-length RNA probe. The proportion of cells with nuclear foci was assessed within ~10 randomly selected microscopic fields corresponding to a total of 300–400 cells per experiment. Three separate experiments were conducted which showed no significant difference between sets of data allowing values to be pooled. Means \pm SE are plotted. The values were compared using a Student's *t*-test (SAS Institute, 1990). Treatment with actinomycin D induces a highly significant decrease in the mean proportion of MEL β WT cells with a hybridization signal in the nucleus [Student's *t*-test, $t_{(48,6)} = 18.94$, $p = 0.0001$] but not with MEL β SM cells [$t_{(68)} = 2.02$, $p = 0.05$]. MEL β IVSI cells show a slight decrease [$t_{(51,2)} = 2.43$, $p = 0.02$] in the mean proportion of nuclei with a signal in the presence of this drug. (B) The effect of α -amanitin on release of human β -globin transcripts from the site of transcription was studied on MEL β WT, MEL β SM and MEL β IVSI cells that had been induced for 2 days. The statistical analysis was performed as described in the legend to Figure 3c. Two independent experiments were conducted for each cell type. In each experiment, 10 microscopic fields were counted for every time point. There were no significant differences between experiments, allowing them to be pooled. Statistically significant different means within each cell type (SNK, $p < 0.05$) are represented by different letters.

the gene locus remain visible following treatment with actinomycin D for 5 min (Figures 4B, c and d, and 5A, MEL β IVSI).

Therefore, in marked dissimilarity to wild-type β -globin transcripts that rapidly disappear from the site of transcription below a detection threshold upon actinomycin D treatment (Figure 3), the 5' splice site β SM and 3' end formation β IVSI processing mutant RNA molecules are retained near the gene locus. Importantly, after 30 min of actinomycin D treatment the number of MEL β IVSI or MEL β SM cells with RNA foci in the nucleus was reduced by 50% and after 1 h to <1% (data not shown). This may reflect either degradation of the arrested RNAs or a protracted release from the site of transcription.

Since actinomycin D may induce pleiotropic effects on cells, we also assessed the effect of α -amanitin on the kinetics of human β -globin RNA release from the site of transcription. α -Amanitin is a cyclic peptide which binds with high affinity to the large subunit of RNA polymerase II (Cochet-Meilhac and Chambon, 1974; Lutter, 1982) thereby inhibiting transcription (Kedinger *et al.*, 1970; Lindell *et al.*, 1970). Unlike actinomycin D, α -amanitin penetrates slowly into cultured cells and requires a number of hours to inhibit transcription *in vivo* (Nguyen *et al.*, 1996). We therefore performed a time course analysis

of the effect of α -amanitin on MEL cells transfected with the wild-type human β -globin gene (Figure 5B, MEL β WT). The results show that after 1 h of treatment the proportion of cells with intranuclear foci remains essentially unaltered, whereas significant decreases are observed following exposure to the drug for 2, 2.5 and 3 h.

When a similar analysis was performed on MEL cells transfected with either the 5' splice site mutant (MEL β SM) or the mutant lacking IVS-II and defective in 3' end formation (MEL β IVSI), the proportion of cells with visible nuclear foci remained unaltered during the first 2 h of α -amanitin treatment (Figure 5B, MEL β SM and MEL β IVSI). However, after 2.5 h there is a significant decrease in the proportion of labelled MEL β IVSI cells, whereas no change is detected in MEL β SM cells. After 3 h of treatment the proportion of labelled MEL β IVSI cells remains unchanged, while a significant decrease is detected for the first time in MEL β SM cells. Therefore, the disappearance of the intranuclear signal of β -globin RNA induced by α -amanitin occurs with significantly slower kinetics in cells transfected with the mutant β SM and β IVSI gene constructs than in those cells harbouring the β WT transgene. Similarly, treatment of MEL β WT, MEL β SM and MEL β IVSI cells with the purine nucleoside analogue DRB, a specific inhibitor of processive transcrip-

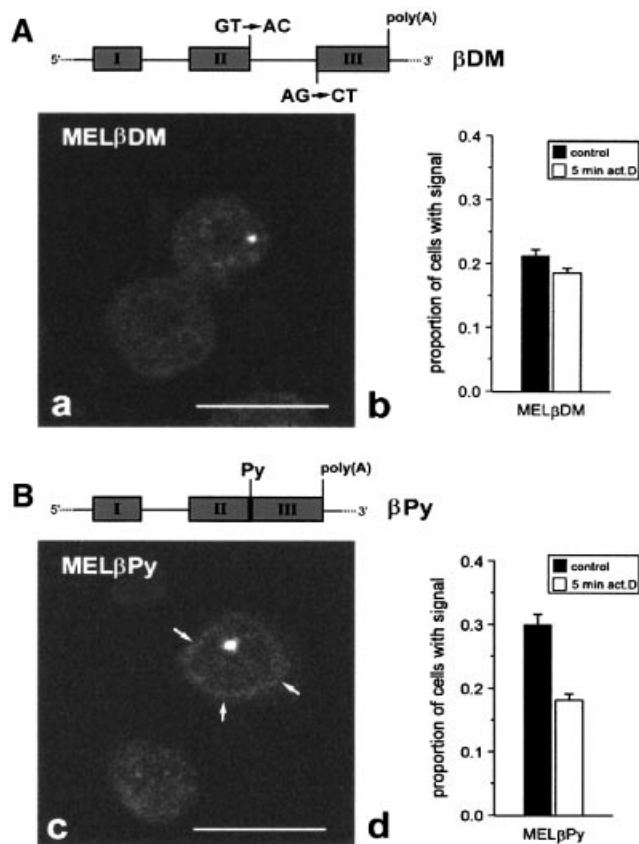


Fig. 6. Retention at the site of transcription correlates with the ability of β -globin RNA mutants to support at least partial spliceosome assembly. (A) (a) MEL cells transfected with the β DM construct which contains a 5' splice donor (GT \rightarrow AC) and a 3' splice acceptor (AG \rightarrow CT) double mutation of IVS-II. These MEL β DM cells were induced to undergo erythroid differentiation for 4 days and hybridized with the full-length RNA probe. Note: a focal signal is readily visible in the nucleus, but no cytoplasmic staining is detected. (b) Quantitative analysis of the effect of actinomycin D on RNA release. Cells were induced for 2 days and hybridized with the full-length RNA probe, as described in the legend to Figure 5c. The results show that actinomycin D does not significantly affect the mean proportion of cells with signal in the nucleus [Student's *t*-test, $t_{(58)} = 2.04$, $p = 0.05$]. (B) (c) MEL β Py cells harbour the β Py construct, which bears a 21 bp polypyrimidine tract substitution of IVS-II. After 4 days of erythroid differentiation these cells were hybridized with the full-length RNA probe. Note the presence of an intranuclear focus with additional staining of the cytoplasm (arrows). (d) Quantitative analysis shows that actinomycin D induces a highly significant decrease in the mean proportion of MEL β Py cells with visible intranuclear foci [Student's *t*-test, $t_{(48.6)} = 6.24$, $p = 0.0001$]. Cells were either fixed in formaldehyde and permeabilized with Triton X-100/saponin (a and c) or fixed in formaldehyde/acetic acid and digested with pepsin (b and d).

tion that blocks phosphorylation of the CTD of RNA polymerase II (see Bentley, 1995), resulted in a significantly faster disappearance of intranuclear β WT RNA signal compared with that observed with the two mutants (data not shown).

In order to gain further insight into the mechanism of retention of transcripts at the site of transcription caused by defects in RNA processing, experiments were conducted with MEL β DM and MEL β Py cells. The 'double mutant' β DM construct contains a 5' (GT \rightarrow AC) and 3' (AG \rightarrow CT) splice site mutation (Figure 6A, upper panel). In the β Py construct, the second intron was removed and

replaced by a 21 bp sequence corresponding to the polypyrimidine (Py) tract of IVS-II and devoid of all other splicing signals (Figure 6B, upper panel). Biochemical analysis has shown that β DM and β Py transcripts are, as expected, not spliced at the IVS-II position but are still able to undergo 3' end formation at a level ~30% of that observed with the wild-type β WT gene (F.Geraghty and M.Antoniou, in preparation). In addition, whereas very low levels of unspliced, double splice site mutant β DM RNA reach the cytoplasm, β Py (Py tract/IVS-II substitution) transcripts are transported to the cytoplasm at 30% of β WT (F.Geraghty and M.Antoniou, in preparation).

Upon induced erythroid differentiation for 4 days, the full-length RNA probe (Figure 1A) produces focal hybridization signals in the nuclei of MEL cells transfected with both the double splice site β DM (MEL β DM; Figure 6A, a) and the Py tract/IVS-II substitution (MEL β Py; Figure 6B, c) mutant constructs. No cytoplasmic staining is detected in the MEL β DM cells (Figure 6A, a), whereas a faint labelling can be observed with MEL β Py cells (Figure 6B, c, arrows). Following treatment with actinomycin D for 5 min, the proportion of MEL β DM cells showing a nuclear signal for human β -globin RNA is not significantly altered (Figure 6A, b). In contrast, the same treatment induces a highly significant decrease in the number of MEL β Py cells that score positive for a focal signal of human β -globin RNA in the nucleus (Figure 6B, d).

These data indicate that the majority of double splice site β DM transcripts are retained at the site of transcription whereas newly synthesized Py tract/IVS-II substitution mutant β Py RNA molecules are partially released and eventually reach the cytoplasm. As the first intron is correctly spliced in all mutants analysed (Collis *et al.*, 1990; Antoniou *et al.*, 1998 and data not shown) and the efficiency of 3' end formation of β DM and β Py mutant RNA is 30% of wild-type in both cases (F.Geraghty and M.Antoniou, in preparation), this suggests that the splicing factors that are still capable of binding to the mutant second intron of β DM but fail to interact with the β Py variant are contributing to retention.

Discussion

The β LCR/MEL cell system was chosen for these studies as it affords high physiological levels of gene expression from within a natural chromatin context (Blom van Assendelft *et al.*, 1989; Talbot *et al.*, 1989; Collis *et al.*, 1990). We therefore selected clones or pools of stably transfected MEL cells with a high transgene copy number in order to maximize the sensitivity of detecting human β -globin transcripts at all stages of their synthesis, maturation and transport within the nucleus. Interestingly, despite these advantages and the use of hybridization conditions which allow virtually 100% access of the probe to the target RNA within the nucleus (Wijgerde *et al.*, 1995), we were only able to detect human β -globin RNA as a focal concentration near the site of transcription (Figure 1). In addition, the use of oligonucleotide probes that span the intron-exon boundaries of the human β -globin gene also show that the first intron is spliced while the transcript is still at the gene locus, whereas removal of the second intron takes place either immediately prior to rapid release

from the site of transcription or at some other location within the nucleus after transport from the region of the gene template (Figure 2). The failure to visualize human β -globin RNA transcripts at a location other than in the vicinity of the gene locus clearly reflects the sensitivity limits of the experimental procedure that is unable to detect single RNA molecules in transit through the nucleoplasm. These results also imply that the mature mRNA does not concentrate in any other nuclear compartment as it is being transported to the cytoplasm and therefore in all likelihood follows a broad diffuse pathway as has been described for rat β -actin gene transcripts (Femino *et al.*, 1998).

The principal discovery of this study was the demonstration that pre-mRNA mutants unable to be exported to the cytoplasm due to the inability to undergo splicing or 3' end processing, are retained within the nucleus in close proximity to the site of transcription (Figures 4–6). Our experimental approach was based on the direct *in situ* visualization of wild-type and mutant β -globin RNAs in the nucleus of cells treated with the transcription inhibitors actinomycin D, α -amanitin and DRB. A similar type of analysis utilizing transcription inhibitors in conjunction with RNA *in situ* hybridization has recently been used to determine the lifetime of α -, β - and γ -globin primary transcripts in fetal liver cells obtained from transgenic mice (Gribnau *et al.*, 1998). These data reveal that with probes complementary to the 5' end of human β -globin primary transcripts, focal signals are still visible after 15 min of DRB treatment. This is consistent with previous studies indicating that DRB does not affect initiation of transcription but aborts elongating transcripts ~400–600 bp from the initiation site. Interestingly, the signal produced by 3' end probes had completely disappeared after 7.5 min of DRB treatment. Therefore, the kinetics of release of primary transcripts in fetal liver cells from the site of transcription in single-copy human β -globin transgenic mice appears very similar to that observed in multicopy transfected MEL cells.

Importantly, our results also indicate that in order to be informative on the kinetics of release of RNA from the vicinity of the gene locus, exposure to transcription inhibitors should be for short periods. After prolonged drug treatments no difference is observed between wild-type and mutant RNAs, presumably due to degradation of the arrested transcripts.

One possible explanation for the retention of human β -globin pre-mRNA processing mutants in the vicinity of the site of transcription is that the release of transcripts is blocked by the stalled processing machinery attached to the nuclear matrix (Verheijen *et al.*, 1986; Smith *et al.*, 1989; Blencowe *et al.*, 1994). As spliceosome assembly and splicing are normally cotranscriptional events, it is expected that spliceosomes attach to the nuclear matrix at the site of transcription. Therefore, stalling of a mutant pre-mRNA due to impaired splicing should occur in the vicinity of the site of transcription. The finding that both splicing and polyadenylation factors can associate with the CTD of RNA polymerase II (Mortillaro *et al.*, 1996; Yuryev *et al.*, 1996; Du and Waren, 1997; McCracken *et al.*, 1997; reviewed by Steinmetz, 1997), also implies that RNA processing mutants may be retained close to the DNA template by remaining tethered to a stalled or

abnormal processing machinery associated with the CTD of the polymerase. The range of pre-mRNA mutants we have analysed further suggests that assembly of the processing machinery for both splicing and 3' end formation is involved in the retention mechanism.

Importantly, it has been reported that a hyperphosphorylated form of the large subunit of RNA polymerase II associates with the nuclear matrix (Mortillaro *et al.*, 1996; Vincent *et al.*, 1996). It is therefore conceivable that RNA polymerase II may interact directly with the nuclear matrix via the phosphorylated CTD. Alternatively, RNA polymerase II may be indirectly associated with the nuclear matrix through the association of RNA processing components with both the matrix (Verheijen *et al.*, 1986; Smith *et al.*, 1989; Blencowe *et al.*, 1994) and the CTD (see Steinmetz, 1997). In either case, retention of pre-mRNA processing mutants near the site of transcription could be explained by stalled processing machinery that is tethered to the nuclear matrix via the CTD.

A prediction of the model in which both the splicing and the 3' end processing machinery are associated with the CTD of RNA polymerase II (Steinmetz, 1997) is that a normal mRNA would be released from the CTD as it is processed, whereas a pre-mRNA processing mutant would remain bound to the processing machinery and therefore to the CTD. Consequently, the polymerase may not be released from the gene at the termination of transcription of a mutated RNA. This would result in oncoming RNA polymerase molecules stalling on the template and therefore reducing the overall rate of synthesis. Furthermore, if RNA processing mutants fail to be released from the site of transcription, this should result in a local accumulation and consequent increase in intensity of the foci produced by *in situ* hybridization. However, a careful examination of the wild-type MEL β WT and 5' splice site mutant MEL β SM cells that harbour a similar transgene copy number (data not shown), shows that there is no increase in signal intensity (compare Figures 1B, d and 4A, a). This observation is consistent with previously reported biochemical analysis indicating that in transfected cells the steady-state levels of this mutant RNA within the nucleus is the same as that observed with the wild-type normal transcript (Antonioni *et al.*, 1998). This is consistent with the notion that mutated RNAs are stalled on the template with a concurrent feedback mechanism to the transcription machinery. Without such a feedback the cells with mutant genes should have accumulated a significantly higher amount of RNA at the site of transcription than those containing the normal β -globin gene.

Studies performed on Balbiani ring pre-mRNAs expressed in the salivary gland cells of *Chironomus tentans*, suggest that splicing may occur either during or after transcription, depending on the position of the intron in the gene. In this particular system, introns located near the 5' end of the gene are excised cotranscriptionally while introns closer to the 3' end are more frequently spliced after release of the RNA into the nucleoplasm (Bauren and Wieslander, 1994; Wetterberg *et al.*, 1996). An immediate question raised by these results is whether introns spliced post-transcriptionally assemble the spliceosome at the site of transcription or after release of the pre-mRNA into the nucleoplasm. If the spliceosome assembles cotranscriptionally and excision of the intron

can occur in the nucleoplasm, this would imply that completion of the splicing reaction is not required for release from the site of transcription. In fact, as the assembly of a functional spliceosome involves a dynamic and timely rearrangement of its components (reviewed by Madhani and Guthrie, 1994; Ares and Weiser, 1995), it is feasible to anticipate that splicing factors may detach from the CTD prior to the final catalytic steps of splicing. On the other hand, a post-transcriptional assembly of the spliceosome would argue that interaction of splicing factors with the CTD is not essential for loading the spliceosome on a pre-mRNA.

A striking exception to the rule that mRNAs can be exported only after completion of processing occurs in retroviruses, which have evolved a mechanism that allows the nuclear export of unspliced forms of viral RNAs. This mechanism is best characterized in human immunodeficiency virus type 1 (HIV-1) and involves the virally encoded protein Rev (for a recent review see Stutz and Rosbash, 1998). Upon binding of Rev to the Rev response element (RRE) present in the intron of immature viral mRNA, the complex is transported to the cytoplasm by virtue of interacting with CRM1/exportin 1 through a leucine-rich nuclear export signal present at the Rev C-terminal end. Thus, the association of Rev with an RRE promotes the interaction of the RRE-containing mRNA with exportin and consequently its export from the nucleus. In addition, it is possible that binding of Rev to the intronic RRE interferes with spliceosome assembly, thereby contributing to its premature release from the processing machinery. Consistent with this idea, there is evidence that Rev specifically blocks assembly of U4/U6 and U5 snRNPs into the spliceosome (Kjems and Sharp, 1993). Our observations indicating that unprocessed β -globin RNAs are retained at the site of transcription suggest that Rev may have a dual function in promoting export of unspliced viral RNAs. In addition to its well-established interaction with the exportin pathway, Rev may contribute directly to release of unspliced RNA from the spliceosome and hence from the site of transcription. Clearly, further experiments are needed to determine the spatial and temporal relationships between sites of transcription, spliceosome assembly and splicing in the nucleus.

Irrespective of the mechanism(s) responsible for the observed retention of mutant pre-mRNAs, a major conclusion from this study is that mechanisms which prevent export of pre-mRNA processing mutants to the cytoplasm, operate in close proximity to the site of transcription. Therefore, the efficiency of splicing and 3' end formation appears to be rate limiting for the release of mRNAs pre-assembled with processing factors at the site of transcription.

Materials and methods

Gene constructs

The wild-type (BWT) and mutant β SM, β DM, β Py and β IVSI human β -globin genes were cloned in the microlocus LCR expression vector (Collis *et al.*, 1990), and are described in detail elsewhere (Antoniou *et al.*, 1998). Briefly, a β -globin gene harbouring a fully functional 89 bp deletion mutant of the second intron was used as the starting point for generating the β SM, β DM and β Py constructs (Antoniou *et al.*, 1998). The β VSI gene is as described previously (Collis *et al.*, 1990). The

BWT, β SM, β DM and β Py genes extend to +1800 bp past the poly(A)-addition site, whereas the β IVSI construct terminates at +45 bp. This difference in the extent of 3' flanking sequences does not in itself compromise the efficiency of 3' end formation (Antoniou *et al.*, 1998). All these genes begin at a *Sna*BI site at -265 bp from the transcriptional start point and were cloned between the *Cla*I and *Asp*718 sites of the microlocus LCR expression vector (Collis *et al.*, 1990).

MEL cell transfections

The generation, maintenance and induced differentiation of stably transfected, G418 resistant MEL cell clones was as described previously (Antoniou, 1991). The MEL β WT and MEL β SM clones harbour ~14 copies of the transgene as a tandem array whereas MEL β DM, MEL β Py and MEL β IVSI are large populations of stably transfected cells with an average transgene copy number of five (data not shown). Transgenes were confirmed to have integrated at a single chromosome site by fluorescence *in situ* hybridization of cells in metaphase (data not shown). Immunofluorescence was performed using an antibody specific for human globin (Immuno-rx, Augusta, GA, USA), as described (Fraser *et al.*, 1993). Actinomycin D (5 μ g/ml), α -amanitin (100 μ g/ml) and DRB (75 μ M) were added to cells that had been induced to undergo erythroid differentiation for 2 days.

Probes used for *in situ* hybridization

Genomic cloned probes (see Figure 1A) were labelled with either digoxigenin-11-dUTP (Boehringer Mannheim) or dinitrophenyl-11-dUTP (DNP; Molecular Probes) by nick-translation (Lichter *et al.*, 1991). The full-length RNA probe extends over the entire transcribed region of the human β -globin gene and consists of a 3.7 kb fragment extending from the *Sna*BI site at -265 bp from the transcriptional start point to a *Bgl*III site at +1816 bp past the poly(A)-addition site. The 3' RNA probe is a 771 bp fragment that extends from a position 212 bp upstream and 559 bp downstream of the human β -globin gene poly(A)-addition site. Therefore, the 3' RNA probe by *in situ* hybridization detects nascent transcripts that have been transcribed past 212 nucleotides upstream of the polyadenylation site as well as those that have undergone termination and 3' cleavage. As a result both 'terminating/nearly terminated' and 'terminated' transcripts will be detected. The DNA probe is the LCR expression vector (Collis *et al.*, 1990) into which the human β -globin genes under analysis were cloned.

Splice junction (SJ) oligonucleotide probes were purchased from Cruchem (UK):

Exon I-II spanning, SJ I/II, 5'-ACCACCAGCAGC/CTGCCCAGG-GCC-3';

Exon II-III spanning, SJ II/III, 5'-GTTGCCCAGGAG/CCTGAAGTT-CTC-3'.

The forward slash mark indicates the exon boundaries. In addition to these 24 nucleotides complementary to the human β -globin sequence, the following stretch of non-specific sequence was added to increase the intensity of the hybridization signal and therefore sensitivity of the assay (Zhang *et al.*, 1994):

5' end, 5'-TTTTGCTTGCTTGCTT-3';

3' end, 5'-TTGCTTGCTTGCTT-3'.

The underlined bases show the positions of the nucleotides bearing an adjunct of DNP.

In situ hybridization

Cells were allowed to adhere onto poly-L-lysine coated coverslips and washed with phosphate buffered saline (PBS). The cells were then fixed with either 3.7% formaldehyde in PBS for 10 min, and permeabilized in 0.5% Triton X-100, 0.5% saponin (Zirbel *et al.*, 1993), or in 4% formaldehyde/5% acetic acid/0.9% NaCl and digested with 0.01% pepsin in 0.01 M HCl (Wijgerde *et al.*, 1995). Cloned probes were hybridized for 16 h at 37°C in 50% formamide/2 \times SSC/10% dextran sulfate/50 mM sodium phosphate pH 7.0. Post-hybridization washes were in 50% formamide/2 \times SSC (3 \times 5 min at 45°C) and either 2 \times SSC (3 \times 5 min at 45°C) for the full-length and 3' RNA probes, or 0.5 \times SSC (3 \times 5 min at 45°C) for the DNA probe. Hybridization with oligonucleotides was performed in 20% formamide/2 \times SSC/10% dextran sulphate/0.2% BSA/1 μ g/ μ l tRNA, at 37°C for 3 h. Post-hybridization washes were in 20% formamide/2 \times SSC (3 \times 5 min at 42°C) and 2 \times SSC (3 \times 5 min at 42°C). The sites of hybridization were visualized using antibodies directed against either digoxigenin (Boehringer Mannheim) or DNP (Molecular Probes) and appropriate secondary antibodies coupled to fluorescein, rhodamine or Texas Red (Vector Laboratories; Jackson

ImmunoResearch). The staining of total DNA was performed after *in situ* hybridization by incubating cells for 10 min with 0.5 μ M TO-PRO-3 (Molecular Probes).

Double hybridization experiments were performed sequentially. After the first hybridization and detection steps as described above, cells were fixed with 3.7% formaldehyde in PBS for 10 min and then hybridized again. As controls for the double-labelling experiments, the complete double-hybridization procedure was carried out omitting either the DNA or the RNA probe. Under these conditions no DNA or RNA signal was detected, respectively, confirming the specificity of each labelling reaction. In addition, cells were hybridized with both DNA and RNA probes under non-denaturing conditions. In these experiments the DNA probe produces a very faint fluorescent signal in some nuclei (data not shown). These faint signals produced by the DNA probe and which colocalize with the foci produced by hybridization with the RNA probe, are likely to represent a combination of transcripts from the neomycin-resistance (TK *neo^r*) gene and those arising from within the LCR (Collis *et al.*, 1990; Ashe *et al.*, 1997) which are present on the plasmid expression cassette. Digestion with RNase A before hybridization completely abolished labelling (data not shown), confirming that the observed signals correspond to RNA hybridization. Moreover, no labelling was observed in untransfected MEL cells after 4 days of erythroid differentiation, indicating that the hybridization signal is specific for human globin RNA (data not shown).

Microscopy

Samples were examined with a Zeiss LSM 410 microscope. Confocal microscopy was performed using argon ion (488 nm) and HeNe (543 nm) lasers to excite FITC and TxRed/rhodamine fluorescence, respectively.

Acknowledgements

The excellent technical support of Jacky Hurst is gratefully acknowledged. This study was supported by grants from the Junta Nacional de Investigação Científica e Tecnológica /Programme PRAXIS XXI, Portugal, the Medical Research Council, UK, the Human Capital and Mobility Programme and Biomed 2 programmes of the European Union. N.C. and S.P. were supported by PRAXIS XXI fellowships.

References

- Antoniou, M. (1991) Induction of erythroid-specific expression in murine erythroleukemia (MEL) cells. In Murray, E.J. (ed.), *Methods in Molecular Biology vol. 7: Gene Transfer and Expression Protocols*. The Humana Press Inc., Clifton, NJ, pp. 421–434.
- Antoniou, M., Carmo-Fonseca, M., Ferreira, J. and Lamond, A.I.J. (1993) Nuclear organisation of splicing snRNPs during differentiation of murine erythroleukemia cells *in vitro*. *J. Cell Biol.*, **123**, 1055–1068.
- Antoniou, M., Geraghty, F., Hurst, J. and Grosveld, F. (1998) Efficient 3'-end formation of human β -globin mRNA *in vivo* requires sequences within the last intron but occurs independently of the splicing reaction. *Nucleic Acids Res.*, **26**, 721–729.
- Ares, M. and Weiser, B. (1995) Rearrangement of snRNA structure during assembly and function of the spliceosome. *Prog. Nucleic Acid Res. Mol. Biol.*, **50**, 131–159.
- Ashe, H.L., Monks, J., Wijgerde, M., Fraser, P. and Proudfoot, N.J. (1997) Intergenic transcription and transinduction of the human β -globin locus. *Genes Dev.*, **11**, 2494–2509.
- Bauren, G. and Wieslander, L. (1994) Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell*, **76**, 183–192.
- Bauren, G., Jiang, W.-Q., Bernholm, K., Gu, F. and Wieslander, L. (1996) Demonstration of a dynamic, transcription-dependent organization of pre-mRNA splicing factors in polytene nuclei. *J. Cell Biol.*, **133**, 929–942.
- Bentley, D.L. (1995) Regulation of transcriptional elongation by RNA polymerase II. *Curr. Opin. Genet. Dev.*, **5**, 210–216.
- Beyer, A.L. and Osheim, Y.N. (1988) Splice site selection, rate of splicing and alternative splicing on nascent transcripts. *Genes Dev.*, **2**, 754–765.
- Blencowe, B.J., Nickerson, J.A., Issner, R., Penman, S. and Sharp, P.A. (1994) Association of nuclear matrix antigens with exon-containing splicing complexes. *J. Cell Biol.*, **127**, 593–607.
- Blom van Assendelft, G., Hanscombe, O., Grosveld, F. and Greaves, D.R. (1989) The β -globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. *Cell*, **56**, 969–977.
- Chang, D.D. and Sharp, P.A. (1989) Regulation by HIV Rev depends upon recognition of splice sites. *Cell*, **59**, 789–795.
- Cochet-Meilhac, M. and Chambon, P. (1974) Animal DNA-dependent RNA polymerases. 11. Mechanism of the inhibition of RNA polymerases B by amatoxins. *Biochim. Biophys. Acta*, **353**, 160–184.
- Collis, P., Antoniou, M. and Grosveld, F. (1990) Definition of the minimal requirements within the human β -globin gene and the dominant control region for high level expression. *EMBO J.*, **9**, 233–240.
- Darnell, J.E., Philipson, L., Wall, R. and Adesnik, M. (1971) Polyadenylic acid sequences: role in conversion of nuclear RNA into messenger RNA. *Science*, **174**, 507–510.
- Du, L. and Warren, S. (1997) A functional interaction between the carboxy-terminal domain of RNA polymerase II and pre-mRNA splicing. *J. Cell Biol.*, **136**, 5–18.
- Eckner, R., Ellmeier, W. and Birnstiel, M.L. (1991) Mature mRNA 3' end formation stimulates RNA export from the nucleus. *EMBO J.*, **10**, 3513–3522.
- Elliot, D.J., Stutz, F., Lescure, A. and Rosbash, M. (1994) mRNA export. *Curr. Opin. Genet. Dev.*, **4**, 305–309.
- Femino, A.M., Fay, F.S., Fogarty, K. and Singer, R.H. (1998) Visualisation of single RNA transcripts *in situ*. *Science*, **280**, 585–590.
- Fraser, P., Pruzina, S., Antoniou, M. and Grosveld, F. (1993) Each hypersensitive site of the human β -globin locus control region confers a different developmental pattern of expression on the globin genes. *Genes Dev.*, **7**, 106–113.
- Goldberg, I.H., Rabinowitz, M. and Reich, E. (1962) Basis of actinomycin action. 1. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. *Proc. Natl Acad. Sci. USA*, **48**, 2094–2101.
- Gribnau, J., Boer, E., Trimborn, T., Wijgerde, M., Milot, E., Grosveld, F. and Fraser, P. (1998) Chromatin interaction mechanism of transcriptional control *in vivo*. *EMBO J.*, **17**, 6020–6027.
- Hamm, J. and Mattaj, I.W. (1990) Monomethylated cap structures facilitate RNA export from the nucleus. *Cell*, **63**, 109–118.
- Huang, S. and Spector, D.L. (1996) Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.*, **133**, 719–732.
- Izaurrealde, E. and Mattaj, I.W. (1992) Transport of RNA between nucleus and cytoplasm. *Semin. Cell Biol.*, **3**, 279–288.
- Jarmolowski, A., Boelens, W.C., Izaurrealde, E. and Mattaj, I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.*, **124**, 627–635.
- Kedinger, C., Gniazdowski, M., Mandel, J.L., Jr, Gissinger, F. and Chambon, P. (1970) Alpha-amanitin: a specific inhibitor of one of two DNA-dependent RNA polymerase activities from calf thymus. *Biochem. Biophys. Res. Commun.*, **38**, 165–171.
- Kersten, W., Kersten, H. and Rauen, H.M. (1960) Action of nucleic acids on the inhibition of growth by actinomycin of *Neurospora crassa*. *Nature*, **187**, 60–61.
- Kjems, J. and Sharp, P.A. (1993) The basic domain of Rev from human immunodeficiency virus type 1 specifically blocks the entry of U4/U6 and U5 small nuclear ribonucleoprotein in spliceosome assembly. *J. Virol.*, **67**, 4769–4776.
- Kramer, A. (1996) The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.*, **65**, 367–409.
- Lamond, A.I., Konarska, M.M. and Sharp, P. (1987) A mutational analysis of spliceosome assembly: evidence for splice site collaboration during spliceosome formation. *Genes Dev.*, **1**, 532–543.
- Lee, M.S. and Silver, P.A. (1997) RNA movement between the nucleus and the cytoplasm. *Curr. Opin. Genet. Dev.*, **7**, 212–219.
- Legrain, P. and Rosbash, M. (1989) Some *cis*- and *trans*-acting mutants for splicing target pre-mRNA to the cytoplasm. *Cell*, **57**, 573–583.
- LeMaire, M.F. and Thummel, C.S. (1990) Splicing precedes polyadenylation during *Drosophila* E74A transcription. *Mol. Cell Biol.*, **10**, 6059–6063.
- Lichter, P., Boyle, A.L., Cremer, T. and Ward, D.C. (1991) Analysis of genes and chromosomes by non-isotopic *in situ* hybridisation. *Genet. Anal. Tech. Appl.*, **8**, 24–35.
- Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G. and Rutter, W.J. (1970) Specific inhibition of nuclear RNA polymerase II by α -amanitin. *Science*, **170**, 447–449.
- Lutter, L.C. (1982) Photoreactivation of amanitin-inhibited RNA polymerase II. *J. Biol. Chem.*, **257**, 1577–1578.
- Madhani, H.D. and Guthrie, C. (1994) Dynamic RNA-RNA interactions in the spliceosome. *Annu. Rev. Genet.*, **28**, 1–26.

- McCracken,S., Fong,N., Yankulov,K., Ballantyne,S., Pan,G., Greenblatt,J., Patterson,S., Wickens,M. and Bentley,D.L. (1997) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature*, **385**, 357–361.
- Milot,E. *et al.* (1996) Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell*, **87**, 105–114.
- Moore,M.J., Query,C.C. and Sharp,P. (1993) Splicing of precursors to mRNA by the spliceosome. In Gesteland,R. and Atkins,J. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 303–357.
- Mortillaro,M.J., Blencowe,B.J., Wei,X., Nakayasu,H., Du,L., Warren, S.L., Sharp,P.A. and Berezney,R. (1996) A hyperphosphorylated form of the large subunit of RNA polymerase II is associated with splicing complexes and the nuclear matrix. *Proc. Natl Acad. Sci. USA*, **93**, 8253–8257.
- Nakielnny,S., Fischer,U., Michael,W.M. and Dreyfuss,G. (1997) RNA transport. *Annu. Rev. Neurosci.*, **30**, 269–301.
- Neugebauer,K.M. and Roth,M.B. (1997) Distribution of pre-mRNA splicing factors at sites of RNA polymerase II transcription. *Genes Dev.*, **11**, 1148–1159.
- Nguyen,V.T., Giannoni,F., Dubois,M.F., Seo,S.J., Vigneron,M., Kedinger,C. and Bensaude,O. (1996) *In vivo* degradation of RNA polymerase II largest subunit triggered by α -amanitin. *Nucleic Acids Res.*, **24**, 2924–2929.
- Nigg,E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature*, **386**, 779–787.
- SAS Institute Inc. (1990) *SAS User's Guide to Statistics, Version 6*. 4th edn. SAS Institute Inc., Cary, NC.
- Smith,H.C., Harris,S.G., Zillmann,M. and Berget,S.M. (1989) Evidence that a nuclear matrix protein participates in pre-messenger RNA splicing. *Exp. Cell Res.*, **182**, 521–533.
- Steinmetz,E.J. (1997) Pre-mRNA processing and the CTD of RNA polymerase II: the tail that wags the dog? *Cell*, **89**, 491–494.
- Stutz,F. and Rosbash,M. (1998) Nuclear RNA export. *Genes Dev.*, **12**, 3303–3319.
- Talbot,D., Collis,P., Antoniou,M., Vidal,M., Grosveld,F. and Greaves, D.R. (1989) A dominant control region from the human β -globin locus conferring integration site-independent gene expression. *Nature*, **338**, 352–355.
- Tennyson,C., Klamut,H.J. and Worton,R.G. (1995) The human dystrophin gene requires 16 h to be transcribed and is cotranscriptionally spliced. *Nature Genet.*, **9**, 184–190.
- Verheijen,R., Kuijpers,H., Vooijs,P., VanVenrooij,W. and Ramaekers,F. (1986) Distribution of the 70k U1 RNA-associated protein during interphase and mitosis. Correlation with other U RNP particles and proteins of the nuclear matrix. *J. Cell Sci.*, **86**, 173–190.
- Vincent,M., Lauriault,P., Dubois,M.-F., Lavoie,S., Bensaude,O. and Chabot,B. (1996) The nuclear matrix protein p255 is a highly phosphorylated form of RNA polymerase II largest subunit which associates with spliceosomes. *Nucleic Acids Res.*, **24**, 4649–4652.
- Visa,N., Alzhanova-Ericsson,A.T., Sun,X., Kiseleva,E., Bjorkroth,B., Wurtz,T. and Daneholt,B. (1996a) A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell*, **84**, 253–264.
- Visa,N., Izaurralde,E., Ferreira,J., Daneholt,B. and Mattaj,I. (1996b) A nuclear cap binding complex binds Balbiani ring pre-mRNA co-transcriptionally and accompanies the ribonucleoprotein particle during nuclear export. *J. Cell Biol.*, **133**, 5–14.
- Wetterberg,I., Bauren,G. and Wieslander,L. (1996) The intranuclear site of excision of each intron in Balbiani ring 3 pre-mRNA is influenced by the time remaining to transcription termination and different excision efficiencies for the various introns. *RNA*, **2**, 641–651.
- Wijgerde,M., Grosveld,F. and Fraser,P. (1995) Transcription complex stability and chromatin dynamics *in vivo*. *Nature*, **377**, 209–213.
- Wu,Z., Murphy,C., Callan,H.G. and Gall,J.G. (1991) Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres and snurposomes. *J. Cell Biol.*, **113**, 465–483.
- Xing,Y., Johnson,C.V., Dobner,P.R. and Lawrence,J.B. (1993) Higher level organisation of individual gene transcription and RNA splicing. *Science*, **259**, 1326–1330.
- Yuryev,A., Patturajan,M., Litingtung,Y., Joshi,R.V., Gentile,C., Gebara,M. and Corden,J.L. (1996) The CTD of RNA polymerase II interacts with a novel set of SR-like proteins. *Proc. Natl Acad. Sci. USA*, **93**, 6975–6980.
- Zhang,G., Taneja,K.L., Singer,R.H. and Green,M.R. (1994) Localisation of pre-mRNA splicing in mammalian nuclei. *Nature*, **372**, 809–812.
- Zhang,G., Zapp,M.L., Yan,G. and Green,M. (1996) Localisation of HIV-1 RNA in mammalian nuclei. *J. Cell Biol.*, **135**, 9–18.
- Zirbel,R.M., Mathieu,U.R., Kurz,A., Cremer,T. and Lichter,P. (1993) Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. *Chromosome Res.*, **1**, 93–106.

Received January 12, 1999; revised and accepted March 18, 1999