Inefficient processing impairs release of RNA from the site of transcription

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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 transcripts of both wild-type and mutant RNAs are defective in splicing or 3’ end formation and are retained in the nucleus (Collis et al., 1991; Xing 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 pore. Also, there is currently no information available on the kinetics of processing and 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; Nakaenglish et al., 1997; Nigg, 1997). Although much recent information has begun to define the pathways that mediate and control the nucleocyttoplasmic 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 (Nakaenglish 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 pore. 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 transected
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 (βWT) 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, MELβWT, 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 transacted 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. MELβWT 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
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Fig. 1. (A) Schematic illustration of the human wild-type β-globin construct. The wild-type human β-globin gene (βWT) 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<sup>+</sup> 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 βWT construct (MELβWT) were fixed either in formaldehyde and permeabilized with Triton X-100/saponin (a–e) 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/µl) 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 (2 ng/µl) 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). (d)–(f) depict the simultaneous detection of human β-globin RNA and the transfected gene locus. MELβWT cells were induced for 2 days, and hybridized with the RNA probe labelled with digoxigenin (4 ng/µl) (e, green staining). The cells were then fixed in formaldehyde, denatured and hybridized with the DNA probe labelled with dinitrophenyl (2 ng/µl) (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/µl) labelled with digoxigenin (revealed with fluorescein, g), and the full-length RNA probe (4 ng/µl) labelled with DNP (detected with Texas Red, h). (i) depicts a superimposition of the two images. Cells were induced for 4 days. Bar, 10 µm.
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) MELβWT 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/µl 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/µl) labelled with digoxigenin and revealed with rhodamine (b). (c) depicts a superimposition of the two images. In (d)–(g) MELβWT 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, MELβIVSI 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: \[ \frac{N_{cy+n}}{N_{cy}} \]. (h) depicts means ± SE (three separate experiments were performed for each probe and a total of 100–200 cells were analysed). Bar, 10 µm.

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 MELβIVSI 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). MELβIVSI 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 MELβIVSI 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 MELβWT 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 MELβWT 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, MELβWT). 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) MELβWT 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 μm. (c) Kinetics of release of β-globin RNA from the site of transcription in the presence of actinomycin D. MELβWT 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 ± 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(5,27) = 8.87, 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’, βSM), 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 βSM (MELβSM) was used for in situ hybridization. After 4 days of induced erythroid differentiation, hybridization of MELβSM 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) MELβSM cells transfected with the construct βSM 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-labeling 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) MELβSM 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 MELβIVSI cells, which contain a β-globin construct that lacks completely the second intron (IVS-II), possessing only IVS-I (βIVSI; 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 µm. 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 βSM RNA, colocalize with the gene template (Figure 4A, b).

In contrast with the results obtained with the wild-type βWT construct (Figure 3), focal signals of 5′ splice site mutant βSM RNA remain visible in the nuclei of cells treated with actinomycin D for 5 min (Figure 4A, c and d; Figure 5A, MELβSM). Furthermore, the βSM 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 (βIVSI; Figure 4B, upper panel). Despite possessing normal 3′ 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 βIVSI 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 βIVSI mutant transcripts that colocalize with
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the gene locus remain visible following treatment with actinomycin D for 5 min (Figures 4B, c and d, and 5A, MELβIVSI).

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 βSM and 3’ end formation βIVSI processing mutant RNA molecules are retained near the gene locus. Importantly, after 30 min of actinomycin D treatment the number of MELβIVSI or MELβSM 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, MELβWT). The results show that after 1 h of treatment the proportion of cells with intranuclear gene 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 (MELβSM) or the mutant lacking IVS-II and defective in 3’ end formation (MELβIVSI), the proportion of cells with visible nuclear foci remained unaltered during the first 2 h of α-amanitin treatment (Figure 5B, MELβSM and MELβIVSI). However, after 2.5 h there is a significant decrease in the proportion of labelled MELβIVSI cells, whereas no change is detected in MELβSM cells. After 3 h of treatment the proportion of labelled MELβIVSI cells remains unchanged, while a significant decrease is detected for the first time in MELβSM 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 βSM and βIVSI gene constructs than in those cells harbouring the βWT transgene. Similarly, treatment of MELβWT, MELβSM and MELβIVSI 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 βDM construct which contains a 5′ splice donor (GT→AC) and a 3′ splice acceptor (AG→CT) double mutation of IVS-II. These MELβDM 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(18) = 2.04, p = 0.05]. (B) (c) MELβPy cells harbour the βPy construct, which bears a 21 bp polypyrimidine tract substitution of IVS-II. After 4 days of erythroid differentiation these cells were constructed, which bears a 21 bp polypyrimidine tract substitution of IVS-II. These MELβDM 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. (d) Quantitative analysis shows that actinomycin D induces a highly significant decrease in the mean proportion of MELβPy cells with visible intranuclear foci [Student’s t-test, t(18.6) = 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).

Discussion

The βLCT/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 multicycopy 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 MELβWT and 5′ splice site mutant MELβSM 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 (Antoniou 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 Roshbash, 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 (Kjemps 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 (βWT) and mutant βSM, βDM, βPy and βIVSI human β-globin genes were cloned in the microcruc 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 βSM, βDM and βPy constructs (Antoniou et al., 1998). The βIVSI gene is as described previously (Collis et al., 1990). The βWT, βSM, βDM and βPy genes extend to +1800 bp past the poly(A)-addition site, whereas the βIVSI 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 Snr81 site at –265 bp from the transcriptional start point and were cloned between the ClaI and Asp718 sites of the microcruc 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 MELβWT and MELβSM clones harbour ~14 copies of the transgene as a tandem array whereas MELβDM, MELβPy and MELβIVSI 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 chromosomal 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 µg/ml), α-amanitin (100 µg/ml) and DRB (75 µM) 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 Snr81 site at –265 bp from the transcriptional start point to a BgII 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 Cruachem (UK):

- Exon I–II spanning, SJ II/III, 5′-ACCAGCCAGC/CTGCCCAGG-GCC-3′;
- Exon II–III spanning, SJ I/II, 5′-ACCAGCCACGGTGCCCCAGG-GCC-3′;
- Exon III–IV spanning, SJ II/III, 5′-GTGGCCGAGAGCCTGAATTTCTC-3′;
- Exon IV–V spanning, SJ III/IV, 5′-TTTTGCGTTCGTTGCTTF-3′;
- Exon V–VI spanning, SJ IV/V, 5′-TTGCGGCGTTCGTTGC-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 (Zheng et al., 1994):

- 5′ end: 5′-TTTTGCGTTCGTTGCTTF-3′;
- 3′ end: 5′-TTGCGGCGTTCGTTGC-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× SSC/10% dextran sulfate/50 mM sodium phosphate pH 7.0. Post-hybridization washes were in 50% formamide/2× SSC (3× 5 min at 45°C) and either 2× SSC (3× 5 min at 45°C) for the full-length and 3′ RNA probes, or 0.5× SSC (3× 5 min at 45°C) for the DNA probe. Hybridization with oligonucleotide probes was performed in 20% formamide/2× SSC/10% dextran sulphate/0.2% BSA/1 µg/ml tRNA, at 37°C for 3 h. Post-hybridization washes were in 20% formamide/2× SSC (3× 5 min at 42°C) and 2× SSC (3× 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
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ImmunoResearch). The staining of total DNA was performed after in situ hybridization by incubating cells for 10 min with 0.5 μM 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) 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 TexasRed/rhodamine fluorescence, respectively.

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