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Review

Organization of (pre-)mRNA metabolism in the cell nucleus

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Abbreviations: BrUTP = 5-bromouridine 5'-triphosphate; EMARG = electron microscopic autoradiography; hnRNA = heterogeneous nuclear RNA; ICN = interchromosomal channel network; RNP = ribonucleoprotein; RPII = RNA polymerase II; snRNA = small nuclear RNA.

1. Introduction

Pre-mRNAs are synthesized and processed in the cell nucleus. Accurate pre-mRNA processing is a prerequisite for export of mature mRNAs to the cytoplasm and comprises several co- and posttranscriptional modifications, like 5'-capping, adenosine methylation, 3'-cleavage and poly(A) addition, and intron removal during splicing. These activities must take place at the site of transcription or during intranuclear transport between the gene and the nuclear envelope. Pre-mRNA processing requires many proteins and small nuclear RNAs (snRNAs), most of which are concentrated in a number of well-defined nuclear substructures, e.g., interchromatin granule clusters, perichromatin fibrils and coiled bodies. The function of these nuclear substructures is not fully understood, but must somehow be related to pre-mRNA processing. Evidently, knowledge of the dynamic interplay between sites of pre-mRNA synthesis, sites containing factors for pre-mRNA processing, and (routes of) intranuclear RNA transport is important for understanding nuclear pre-mRNA metabolism. The finding that only a fraction of the nuclear pre-mRNA population is eventually exported as mature mRNA to the cytoplasm suggests that nuclear (pre-)mRNA metabolism plays an important role in the control of gene expression.

2. Transcription of pre-mRNA genes

Synthesis of mRNAs starts with transcription by RNA polymerase II (RPII), which is one of the three nuclear DNA-dependent RNA polymerases [1–3]. Transcription by RPII takes place in the nucleoplasm and covers the majority of extranucleolar transcription in most eukaryotic cells. Where actively transcribed pre-mRNA genes are localized in the nucleus and how these transcription sites correlate to specific nuclear substructures is mainly studied by two methods: (i) high resolution autoradiography on [³H]uridine-labeled cells using electron microscopy, which was frequently done in the 1970s [4, 5], and (ii) the recently developed technique employing the UTP-analogue 5-bromouridine 5'-triphosphate (BrUTP) to label nascent RNA [6, 7]. Results obtained by these two techniques are essentially mutually compatible. A third method to be discussed here is *in situ* hybridization which is useful to visualize (potential) sites of transcription of individual genes [8]. For a more comprehensive review on the localization of RPII transcription see [9].

2.1 Electron microscopic autoradiography

The first technique that was developed to visualize sites of RNA synthesis in the nucleus is electron microscopy combined with high resolution autoradio-

graphy (EMARG) on cells labeled with [^3H]uridine [4, 5]. EMARG is often combined with the regressive-EDTA technique [10] which stains specifically ribonucleoprotein (RNP) structures in the nucleus. After incubating cells for a short time with [^3H]uridine, strong labeling is observed in the nucleolus, which corresponds to rRNA synthesis [11–13]. Extranucleolar labeling, which is the result of RNA synthesis by RPII as well as RPIII, is weaker and is scattered throughout the nucleoplasm. Transcription sites are localized at the surface of condensed chromatin domains in association with specific ultrastructural entities called perichromatin fibrils. Perichromatin fibrils have a diameter varying between 3 and 5 nm, sometimes up to 20 nm [4]. Their exact nature is not yet known, but they probably correspond to (nascent) transcripts that are associated with heterogeneous nuclear RNP (hnRNP) proteins and/or snRNPs [14, 15]. Perichromatin fibrils are sometimes located close to clusters of interchromatin granules, another kind of RNP-containing nuclear substructure. Interchromatin granules are 20–25 nm in diameter and appear in large clusters distributed randomly throughout the nucleoplasm [4]. Clusters of interchromatin granules are, even after long incubation times, only weakly labeled by [^3H]uridine, or remain unlabeled [4, 5]. It is therefore unlikely that they represent major sites of transcription.

A major drawback of EMARG is its low spatial resolution, which is less than that of the electron microscope [5]. In addition, EMARG is a complex method and often requires long exposure times. A great advantage, however, is the preservation of nuclear ultrastructure due to fixation of [^3H]uridine-labeled cells without permeabilization. EMARG has therefore contributed considerably to our knowledge about RNA synthesis in relation to nuclear ultrastructure.

2.2 Labeling nascent RNA with BrUTP

A new method to visualize sites of transcription in the nucleus was recently developed [6, 7, 16]. This method is based on the incorporation of BrUTP *in situ* into nascent RNA. Sites of BrUTP incorporation are visualized by a mAb that specifically recognizes bromouridine, followed by immunofluorescence microscopy. We observe a punctated nuclear staining, composed of many hundreds of domains scattered throughout the nucleus, except the nucleolus. No staining is observed when the transcription reaction is done under conditions that inhibit RPII (i.e., 1 $\mu\text{g/ml}$ α -amanitin). As expected, the staining is sensitive to RNase. Obviously,

ly, the staining represents RNA synthesized by RPII. Similar punctated patterns are observed (i) after BrUTP incorporation *in vitro* during run-on transcription in permeabilized cells, and (ii) after short-term labeling *in vivo* after microinjecting BrUTP directly into the cell. In both assays most BrUTP-labeled RNA is still nascent, which demonstrates that nuclear domains that contain BrUTP-labeled RNA represent genuine sites of transcription.

We initially suggested that the immunofluorescent BrUTP patterns, consisting of many hundreds of transcription domains per nucleus, might indicate that each domain contains several actively transcribed genes grouped closely together during transcription [7]. Recent electron microscopic studies on BrUTP-labeled nuclei indicate, however, that this is probably not the case (Wansink *et al.*, in preparation). In electron microscopic preparations of BrUTP-labeled nuclei we observe as many as several thousands of gold-labeled transcription sites per nucleus. These transcription sites are distributed more or less uniformly throughout the nucleoplasm, particularly at the surface of lumps of electron-dense nuclear material, most likely chromatin (Fig. 1). The apparent discrepancy in the number of transcription sites between immunofluorescence labeling and immunogold labeling is probably just the result of the difference in resolution between light microscopy and electron microscopy. We assume that most light-microscopically defined transcription domains correspond to several gold-labeled transcription sites, and therefore to at least several actively transcribed genes.

What kind of RPII transcripts are visualized in the nucleoplasm? In principle, all nascent class II transcripts are labeled during BrUTP incubation. These comprise mainly pre-mRNAs, but also transcripts from repetitive DNA elements, and most snRNAs [2, 17]. Whether or not the location of an actively transcribed gene is visualized in the microscope depends on the number of nascent transcripts, on the elongation rate of the engaged transcription complexes, on the size of the gene, and on the efficiency of immunolabeling. It can therefore not be excluded that, for example, genes which are transcribed at a low rate are not detected after labeling with BrUTP (Note that practically the same arguments hold for labeling RNA with [^3H]uridine).

Labeling nascent RNA with BrUTP is the first technique that visualizes transcription at high resolution in the light microscope. It provides unique possibilities in double-labeling experiments to study the localization

Fig. 1. Ultrastructural localization of transcription sites in a nucleus of the human epidermoid carcinoma cell line A431. Nascent RNA was labeled with BrUTP during run-on transcription. Incorporated BrUTP was stained with ultra-small gold-conjugated antibodies followed by silver enhancement. Samples were embedded in Epon. Sections of $0.25\ \mu\text{m}$ were cut, and inspected in the electron microscope. Shown is a detail of a nucleus at high magnification. Nascent RNA is preferentially localized on the border of electron-dense material (CH), probably condensed chromatin. CP = cytoplasm; Bar = $0.2\ \mu\text{m}$. (Courtesy of D.G. Wansink and O.C.M. Sibon.)

of transcription sites in relation to other nuclear activities and components [6, 7, 18, 19]. Unfortunately, the use of BrUTP in long-term *in vivo* labeling experiments is limited, because the presence of BrU in pre-mRNA inhibits splicing [20, 21]. Extension of the BrUTP-technique to the electron microscopical level has been successful and offers new possibilities to study RNA synthesis in relation to nuclear ultrastructure [22, 23, Wansink *et al.*, in preparation].

2.3 *In situ* hybridization

In situ hybridization is very useful to visualize (potential) sites of transcription of individual pre-mRNA genes [8]. In principle, the position of any gene can be localized if a specific probe is available. The Epstein Barr virus genome in Namalwa cells is integrated preferentially in the inner 50% of the nuclear volume [24]. Also the neu oncogene is found in the nuclear interior [8]. In contrast, the dystrophin gene is usually localized near the nuclear envelope [8]. It has been suggested that each gene occupies a specific position in the interphase nucleus [25]. Thus far, however, a

clear correlation between the location of a gene in the nucleus and its transcriptional activity has not been recognized.

A number of *in situ* hybridization studies suggest that interchromatin granule clusters are involved in transcription. The *c-fos* gene [26] and the fibronectin gene [27] are usually found close to domains highly enriched in the splicing factor SC-35. These domains correspond to clusters of interchromatin granules [28]. *In situ* hybridization with a poly(dT) probe has shown that interchromatin granule clusters contain high concentrations of poly(A)⁺ RNA [29–31]. This does not mean, however, that these domains are sites of transcription, as was suggested by Carter *et al.* [29], because (i) probably the entire poly(A)⁺ RNA population is detected with a poly(dT) probe, i.e., newly synthesized poly(A)⁺ hnRNA, poly(A)⁺ hnRNA en route to the cytoplasm, and poly(A)⁺ hnRNA that is stable or stored in the nucleus [e.g., 32, 33], and (ii) it is not yet certain that polyadenylation occurs at the site of transcription, or elsewhere in the nucleus. Moreover, (iii) earlier EMARG studies on [³H]thymidine-labeled [34] and [³H]uridine-labeled cells [11, 12] have

already shown that interchromatin granule clusters do not contain DNA and are not transcriptionally active. As for the *c-fos* and fibronectin genes, we too observe many transcription sites close to interchromatin granule clusters after labeling nascent RNA with BrUTP [7, Wansink *et al.*, in preparation]. However, in our preparations also many RPII transcription sites are seen that are not localized near interchromatin granule clusters. Although the genes that are transcribed at those sites are not known, this observation shows that the localization of *c-fos* and fibronectin transcription is not representative for all class II transcription sites. Transcription sites close to clusters of interchromatin granules may represent a specific subset of genes.

The combination of *in situ* hybridization and electron microscopy – i.e., high specificity and high resolution – offers interesting opportunities to investigate the localization of specific active and inactive genes at the ultrastructural level [26, 35]. Thus far, no single copy genes have been visualized by *in situ* hybridization in the electron microscope. Electron microscopic *in situ* hybridization will also be valuable in the ultrastructural localization of RNA. Intron- and exon-specific probes will give insight in the localization of successive steps in pre-mRNA processing and the route of (pre-)mRNA transport from the site of transcription to the nuclear envelope.

3. Capping

Soon after initiation of transcription the 5'-end of the growing pre-mRNA is supplied with a 7-methylguanosine cap [36, 37]. Capping is carried out by a capping enzyme. The function of the 5'-m⁷G cap is not fully understood, but it is probably involved in splicing, export of mature mRNA out of the nucleus, mRNA stability, and initiation of protein synthesis in the cytoplasm [38, 39, and references therein]. To our knowledge, the localization of the capping enzyme in the nucleus has not yet been achieved. However, since capping and initiation of transcription are tightly coupled events, one may assume that the capping enzyme is localized at or closely near sites of pre-mRNA synthesis.

4. Methylation

(Pre-)mRNAs can become methylated at the 2'-hydroxyl group of the second nucleoside and at the

N⁶-position of internal adenosine residues [40, and references therein]. The function of these methylations is largely unknown, but it is speculated that they, like the 5'-cap, are involved in (pre-)mRNA processing and stability, and translational efficiency. A 2'-O-methyltransferase was characterized in HeLa cell nuclei [41]. In addition, N⁶-adenosine methyltransferase was partially purified from HeLa cell nuclei [40, 42]. Evidence is presented that N⁶-methyladenosine residues are conserved during pre-mRNA processing [43, 44]. This suggests that adenosine methylation precedes splicing and possibly occurs cotranscriptionally (i.e., at the transcribed gene).

5. Cleavage and poly(A) addition

Processing of the 3'-end of a pre-mRNA consists of endonucleolytic cleavage of the nascent transcript followed by addition of a poly(A) tail to the resulting 3'-end [45]. Histone (pre-)mRNAs form an exception to this rule because they are not polyadenylated. A poly(A) tail is generally 100–200 residues long and is thought to be involved in mRNA export, stability, and translation in the cytoplasm [46]. The poly(A) signal (AAUAAA), located 10–30 bases upstream of the cleavage and polyadenylation site, and some surrounding sequences direct accurate 3'-end processing. Specific cleavage factors, a poly(A) polymerase, and a nuclear poly(A) binding protein (PABII), which are involved in this process, have been characterized [45]. PABII is found in interchromatin granules and perichromatin fibrils [47], where also poly(A)⁺ RNA is localized [31]. Poly(A) polymerase has not yet been localized in the nucleus. Since cleavage occurs while the RNA is still nascent, this must take place at the site of pre-mRNA synthesis. Polyadenylation occurs soon after pre-mRNA synthesis [48], but its location is not yet known. It is becoming evident that polyadenylation is tightly coupled to splicing [49, 50, and references therein]. The order in which polyadenylation and removal of introns takes place is not clear, except that the 3'-terminal intron appears to play a critical role in 3'-end formation [51–53]. This suggests that polyadenylation and splicing are spatially correlated, and may even colocalize.

6. Splicing

6.1 *Splicing factors are concentrated in distinct nuclear substructures*

Splicing consists of the removal of introns and subsequent ligation of exons from a pre-mRNA, which then results in the formation of a full-length mRNA [54]. Many proteins and snRNAs are involved in this process. The temporal and spatial organization of splicing is not fully understood. In contrast, the localization of splicing factors has been thoroughly investigated in the last two decades. Splicing factors are concentrated essentially in three different types of domains in the nucleus: (i) clusters of interchromatin granules, (ii) clusters of perichromatin fibrils, and (iii) coiled bodies [4, 5, 55]. Evidently, these domains must somehow be involved in splicing.

6.1.1 *Interchromatin granule clusters*

Clusters of interchromatin granules do probably not contain DNA and are not major sites of transcription, since they are not labeled by [³H]thymidine [34] and only slowly by [³H]uridine [5, 11, 12], respectively. Interchromatin granule clusters are highly enriched in snRNAs, snRNP proteins, and non-snRNP splicing factors, e.g., SC-35 [28, 56, 57]. In contrast, they contain only relatively few hnRNP proteins [56]. Obviously, poly(A)⁺ RNAs, which are present in high concentration in interchromatin granule clusters [29–31, 33], bind little or no hnRNP proteins, unlike probably most hnRNAs in perichromatin fibrils. This suggests that poly(A)⁺ RNA in interchromatin granule clusters either consists of other RNA species than poly(A)⁺ RNA in perichromatin fibrils, or it is in a different stage of maturation than that in perichromatin fibrils. Together, these results indicate that interchromatin granule clusters do not represent major sites of transcription. Evidence is in favour of the hypothesis that interchromatin granule clusters are also not major sites of splicing (see below).

6.1.2 *Perichromatin fibrils*

Perichromatin fibrils do represent sites of transcription, since they become labeled immediately after a short pulse of [³H]uridine [11, 12]. SnRNAs, snRNP proteins, and non-snRNP splicing factors are associated with perichromatin fibrils [28, 56, 57]. They also contain poly(A)⁺ RNA [29–31]. Perichromatin fibrils are the major location of hnRNP proteins in the nucle-

us [56]. Therefore, it is likely that perichromatin fibrils represent (or contain) nascent transcripts [15].

6.1.3 *Coiled bodies*

Coiled bodies are spherical structures with a diameter of 0.5–1 μ m [58]. Their number varies from cell to cell and ranges between zero and six per nucleus. Coiled bodies are highly enriched in snRNAs, snRNP proteins, and p80-coilin [56, 59–61]. The non-snRNP splicing factor U2AF is also present in coiled bodies [62], whereas SC-35, also a non-snRNP splicing factor, is not [59]. Poly(A)⁺ RNA is not detected in coiled bodies [31, 33]. It is not known whether transcription occurs in coiled bodies. Coiled bodies are normally only observed in transformed or immortal cell lines and not in cell lines of defined passage [61]. This suggests that coiled bodies are not essential to the cell and do not play a general role in RNA processing. Their number may reflect the physiological state of the cell. Coiled bodies may, however, be involved in processing of specific transcripts. It has also been suggested that coiled bodies are involved in post-splicing events, e.g., recycling of snRNPs or degradation of introns [58]. Coiled bodies were recently also detected in nucleoli, suggesting a nucleolar function or origin [63, 64].

6.2 *Where does splicing take place?*

In which of these three nuclear structures does splicing take place? A crucial observation is that many, if not all, splicing factors are present at the site of pre-mRNA synthesis. We found, for example, SC-35 [65] in low concentrations at many transcription sites [7]. So, in principle splicing can occur at the same site where transcription takes place. Several observations indicate that splicing is initiated cotranscriptionally [66–69]. This agrees with the finding that hnRNP proteins and snRNPs become associated with pre-mRNAs already during transcription [70–72]. It is therefore likely that splicing starts cotranscriptionally, i.e., in association with perichromatin fibrils. Since splicing of the 3'-terminal intron and 3'-end processing are coregulated [52, 53], it is not clear whether splicing is also completed at the site of pre-mRNA synthesis.

The dynamic distribution of splicing factors in the nucleus has been studied in a number of recent papers. First, virus-infected cells provide an interesting model system, since virus infection causes a dramatic redistribution of RNPs in the nucleus. During herpes virus infection splicing factors accumulate in interchromatin

granule clusters [73, 74]. This is probably due to the reduction of host mRNA transcription and the promotion of expression of viral RNAs, most of which do not contain introns. Jiménez-García and Spector [75] used adenovirus-infected HeLa cells and report that soon after virus infection snRNPs and SC-35 are recruited from interchromatin granule clusters to the sites of viral RNA synthesis, where splicing is thought to take place. Bridge *et al.* [76], however, claim that this finding only reflects the situation in a minority of the population of infected cells. A newly discovered serine kinase, which is specific for a particular class of splicing factors called SR proteins, may be involved in the (re)distribution of splicing factors [77]. Second, it was shown in HeLa cells, that upon inhibition of splicing interchromatin granule clusters round up, decrease in number, and increase in size and content of splicing factors [78]. In addition, the physical connections that are normally observed between interchromatin granule clusters (i.e., probably perichromatin fibrils) disappear. Third, the distribution of snRNPs was investigated in differentiating murine erythroleukemia cells [79]. It was shown that snRNPs aggregate in large clusters of interchromatin granules when transcription ceases at later stages of cell differentiation. Taken together, inhibition of transcription (either by herpes virus infection, α -amanitin, or terminal differentiation) causes an accumulation of splicing factors in interchromatin granule clusters. These observations suggest that interchromatin granule clusters function primarily as storage or assembly sites for splicing factors, rather than sites where splicing is concentrated. This corresponds with the idea that yeast nuclei contain 10–100 times more U1 snRNPs than is required for normal growth [80]. Splicing is concentrated outside interchromatin granule clusters, although it cannot be fully excluded that some splicing, which was initiated at the site of pre-mRNA synthesis, may be completed in or associated with an interchromatin granule cluster. Interchromatin granule clusters may in addition play a role in regeneration of spliceosomes and degradation of introns.

Suggestive evidence for the direct involvement of interchromatin granule clusters in splicing came from *in situ* hybridization studies. Nascent c-fos transcripts [26] and fibronectin transcripts [27] are found in a track closely associated, but mostly not coinciding with interchromatin granule clusters. In the case of fibronectin transcripts, a spatial separation of intron-containing and spliced transcripts is observed in the track [27]. This was interpreted to indicate that RNA is transported along the track while splicing occurs close

to or in the interchromatin granule cluster [81]. However, it cannot be excluded that the introns are already excised at the beginning of the track (i.e., at the site of synthesis) and that they are transported with the spliced transcript along the track. The interchromatin granule cluster would, in that case, not be directly involved in the splicing process, but may function in the sorting of mRNAs before export to the cytoplasm.

The paper by Wang *et al.* [82], concerning the accumulation of microinjected rhodamine-labeled pre-mRNA in interchromatin granule clusters, is often taken as evidence that interchromatin granule clusters are active in splicing. However, although rhodamine-labeled pre-mRNA was spliced accurately in an *in vitro* splicing assay [82], it cannot be excluded that due to the presence of the rhodamine groups a specific subset of transcripts was spliced incompletely *in vivo* (compare the absence of splicing of pre-mRNA containing BrU [20]). These splicing intermediates may have been transported to interchromatin granule clusters giving rise to the observed fluorescent pattern, whereas faithfully spliced mRNAs may have been exported to the cytoplasm.

In conclusion, splicing most likely initiates cotranscriptionally, i.e., the first introns are removed at the site of pre-mRNA synthesis. Then, splicing may be completed there, but may also be finished during transport of the pre-mRNA to the nuclear envelope. It is conceivable that the timing as well as the location of splicing events on a pre-mRNA containing multiple introns is strongly transcript- and cell type-specific.

7. Intranuclear RNA transport and mRNA export

When pre-mRNA processing is completed and a mature mRNA is formed, in principle two possibilities exist: (i) the mRNA is transported to a nuclear pore complex in the nuclear envelope and is exported to the cytoplasm, or (ii) the mRNA is retained in the nucleus and is either degraded or stored.

7.1 RNA tracks

With regard to intranuclear RNA transport, two models currently exist [see also 80, 81, 83]. The first model is inspired by the intriguing observation that specific viral transcripts are concentrated in a curvilinear track in nuclei of Epstein-Barr virus (EBV)-infected cells [84]. This has been interpreted to mean that these transcripts

move from their site of synthesis along a defined pathway to the nuclear envelope. These EBV-transcripts are associated with the nuclear matrix, which suggests that the matrix provides a structural basis for intranuclear RNA transport [85]. Tracks have also been observed for a few other viral transcripts and for endogenous RNAs, albeit that these tracks were often less pronounced than in the case of the EBV transcript [26, 27, 84, 86]. Two proteins are localized in tracks in the nucleus, i.e. the viral protein Nef [87] and the nucleolar protein Nopp140 [88]. Taken together, these observations gave rise to the hypothesis that transcripts (and proteins) do not diffuse freely throughout the nucleus, but move (or are actively transported) along a defined route to the nuclear envelope.

A few questions concerning this model need to be solved. First, RNA tracks are not a general phenomenon for nuclear transcripts, because a number of transcripts are described which are concentrated in foci [27, 84, 86], rather than tracks. These foci probably correspond to sites of RNA synthesis. It cannot be excluded that also an RNA track corresponds to a site of RNA synthesis and reflects an elongated “Christmas tree” of nascent transcripts [80, 83], although this is not likely considering the principles of chromatin condensation in the nucleus. Still, to investigate this possibility, *in situ* hybridization experiments using probes specific for the 5'-end and the 3'-end of a large gene should be done to determine precisely the localization of the gene relative to the RNA track. Second, tracks often stop at a considerable distance from the nuclear envelope [81]. How does RNA transport go from there? Third, since it is not likely that all molecules of a specific kind of transcript are visualized by *in situ* hybridization, an additional diffuse distribution of transcripts cannot be excluded. The latter two points may be explained by the second model for intranuclear RNA transport, based on simple diffusion.

7.2 Diffusion of RNA through interchromosomal channels

A second, completely different model for intranuclear RNA transport was recently proposed by the group of Bingham [83, 89]. They have analyzed the localization of specific transcripts in polytene nuclei of *Drosophila*. Transcripts are found in a high concentration at their site of synthesis, but also in a somewhat lower concentration in a fine network of channels throughout the nucleus. These observations are explained by proposing that pre-mRNA processing takes place in the inter-

chromosomal channel network (ICN), in which RNA transport occurs by diffusion. During transport (pre-)mRNAs must be bound to specific components in the ICN, since (pre-)mRNAs are not easily extractable. The ICN is defined by exclusion from condensed chromatin and the nucleolus, and contains splicing factors and bulk poly(A)⁺ RNA [89]. Zirbel *et al.* [90] have described a similar nuclear compartment. Although the idea of intranuclear RNA transport based on conventional diffusion is appealing, it remains to be proven whether the observations in *Drosophila* polytene nuclei containing an over-expressed gene construct are representative for normal diploid interphase nuclei.

7.3 mRNA export to the cytoplasm

Like nucleocytoplasmic transport of proteins, export of mRNAs occurs through pores in the nuclear envelope [91–93]. Translocation through a nuclear pore complex has been visualized for poly(A)-coated gold particles [94], the Balbiani ring RNP particle in polytene nuclei [95], and recently also for bulk poly(A)⁺ RNA in mammalian cells [33]. In the latter case, poly(A)⁺ RNA was associated with all pore complexes observed, suggesting that all are able to export poly(A)⁺ RNA [33]. This does, nevertheless, not exclude the possibility that some nuclear pore complexes are specialized in export of specific mRNAs. It is suggested that in *Drosophila* blastoderm embryos particular transcripts are exported through a subset of nuclear pores located on one side of the nucleus [96]. This assumed mechanism, termed vectorial nuclear export, may explain the sorting of particular mRNAs to either the apical or basal cytoplasm in these embryos.

The molecular mechanism of mRNA export is not yet known. The RNA export process is saturable and energy dependent, and it involves common factors as well as specific factors for different classes of RNA [39, and references therein]. Accurate pre-mRNA processing is a prerequisite for export. The presence of a 5'-m⁷G cap and a mature 3'-end are important [38, 97–99], and splicing or splicing complex formation delays or inhibits export [97, 100]. It is therefore hypothesized that (pre-)mRNAs are retained in the nucleus by virtue of their association with specific intranuclear components. For example, snRNPs associate with pre-mRNAs during splicing and may thereby inhibit export of unspliced pre-mRNAs. When splicing is completed, the mature mRNA may be released from the splicing complexes and is then ready for export. The influenza virus NS1 protein inhibits export of mRNAs, probably

by binding to their poly(A) tail [101–103]. In contrast, the human immunodeficiency virus type I protein Rev is responsible for the export of specific viral transcripts which are yet unspliced or only partially spliced [101]. Since Rev shuttles between nucleus and cytoplasm, this may be achieved by direct binding of Rev to the RNA [104, 105]. A final step in the control of mRNA export may be exerted by specific RNA binding components of the nuclear pore complex [93]. Evidently, since the regulation of mRNA export prevents the presence of immature, unstable, and unwanted mRNAs in the cytoplasm, and thus the synthesis of erroneous polypeptides, mRNA export is likely to constitute an important level in the control of gene expression.

8. (Pre-)mRNA degradation and storage in the nucleus

There is a growing consensus that part of the nuclear (pre-)mRNA population is retained in the nucleus and never exported to the cytoplasm. Experimental evidence for this is limited, however [106–109]. Nuclear retention may be due to incomplete or incorrect pre-mRNA processing, which is then followed by RNA degradation in the nucleus. RNA retention may also indicate that (pre-)mRNA is stored in the nucleus or has a structural role.

RNA degradation in the nucleus is a relatively unexplored field, compared to that in the cytoplasm. Some nuclear exo- and endoribonucleases have been characterized [110–113]. Also RNA debranching enzymes, which are essential for the degradation of introns, have been characterized [114–116]. Introns are thought to be rapidly degraded after excision, although some cases of stable introns have been reported [68, 117, 118, and references therein]. It is reasonable to assume that pre-mRNAs are usually protected from the action of RNases. This may be achieved by packing (pre-)mRNA in specific RNP complexes, for example in combination with hnRNP proteins [119]. Another possibility is that RNases reside exclusively in specific subnuclear compartments dedicated to RNA degradation. Experiments addressing the subnuclear localization of nuclear RNases, e.g., by using specific antibodies, will give clues to where in the nucleus RNA degradation takes place.

Storage of mRNA in the form of mRNPs is a common principle in the cytoplasm, especially in germ cells. Whether storage of mRNA occurs also in the nucleus is not clear. Nuclear ultrastructures called

Fig. 2. Schematic representation of the proposed model of the organization of pre-mRNA metabolism in the cell nucleus (see also text). A detail of a section through a nucleus is shown. Pre-mRNAs (represented by small black dots) are synthesized on the surface of chromosome domains (CH; large grey blobs). In the electron microscope, these pre-mRNAs can be recognized as perichromatin fibrils when regressive EDTA staining is used. After cleavage, pre-mRNAs are released in the space between chromosomes, termed interchromosomal channel network (ICN). Splicing starts with the association of splicing factors to nascent transcripts. These factors are probably recruited from large aggregates of interchromatin granules (IG), which may also be involved in regeneration of splicing factors (dashed arrows). After 3'-end formation, splicing may be completed at the site of transcription, followed by export of mature mRNA through a nuclear pore complex (NP) to the cytoplasm (CP; straight arrow, route 1). An alternative possibility is that splicing is completed during transport to the nuclear envelope. This may occur in association with an IG, since these contain a high concentration of poly(A)⁺ RNA (straight arrows, route 2). A conspicuous subnuclear domain in the ICN is the coiled body (C), which also contains splicing factors. The coiled body is somehow involved in pre-mRNA processing. At this time, however, its precise role remains unclear. (Comparable schemes have been presented by others [15, 123].)

perichromatin granules have been implicated in storage of RNA, but their exact nature and function remains to be elucidated [120]. *In situ* hybridization is valuable in studying the nuclear distribution of specific transcripts and has shown that most transcripts are concentrated at their site of synthesis. In addition, it has been shown that poly(A)⁺ RNA is present in high concentration in interchromatin granule clusters [29–31]. As was pointed out earlier, this poly(A)⁺ RNA can correspond only partly to newly synthesized RNA. So, interchromatin granule clusters contain probably old and stable poly(A)⁺ RNA, or structural poly(A)⁺ RNA [33]. An interesting finding is the concentration of large amounts of specific transcripts near or in the nucleolus [121, 122]. It is not clear whether these accu-

mulations reflect sites of pre-mRNA processing prior to transport via the nucleolus, long-term storage sites, or have yet another nuclear function.

9. The (pre-)mRNA pathway between gene and nuclear pore: a model

Taken together, the different transcripts and the various steps in pre-mRNA processing that were discussed in the preceding sections suggest that nuclear pre-mRNA metabolism is highly transcript-specific and cell type-specific. Nevertheless, the following generalized picture of how pre-mRNA metabolism is organized in the cell nucleus can be drawn (Fig. 2).

Synthesis of an mRNA starts somewhere in the nucleoplasm with a gene that loops out of an inactive, condensed chromatin domain to be transcribed at its surface. RPII and transcription factors bind to specific DNA elements in the gene and transcription is initiated. Soon after initiation, an m⁷G cap is added to the 5'-end of the nascent transcript. During elongation of transcription, several hnRNP proteins and, in case introns are present, snRNPs and non-snRNP splicing factors associate with the transcript, which can then be recognized as a perichromatin fibril in the electron microscope. In this model, splicing factors are recruited from nearby interchromatin granule clusters. The growing pre-mRNA is methylated at specific adenosine residues and splicing is initiated. Arriving at the 3'-end of the gene, RPII transcribes the polyadenylation signal and the nascent transcript is cleaved shortly thereafter. Probably still at the transcription site, a poly(A)-tail is added to the 3'-end of the transcript. In the meantime, splicing has progressed and in some cases will have finished already shortly after cleavage or poly(A) addition. Splicing is probably completed at the site of transcription although it cannot be excluded that it continues (together with polyadenylation) during intranuclear transport to the nuclear envelope, possibly near a cluster of interchromatin granules. The mature mRNA moves (this is either an active or passive process) to the nuclear envelope. We suggest that this may occur first in a track along a defined pathway but probably soon by conventional diffusion through the ICN. When the nuclear envelope is reached, the mature mRNA associates with a nuclear pore complex and is translocated into the cytoplasm where it can be translated.

How is this sequence of processing steps integrated in the overall organization of the nucleus? In principle,

two main compartments can be discerned in interphase nuclei, i.e., the chromatin compartment, and the interchromatin space (Fig. 2) [123, and references therein]. Each chromosome occupies its own, distinct territory in the nucleus. The transcriptionally active periphery of a chromosome, which is probably less condensed, is in contact with the interchromatin space. The interchromatin space thus connects all chromosomes and forms a channeled network (the ICN) throughout the nucleus. It is supposed that nuclear pre-mRNA metabolism, as described above, occurs in the ICN. Newly synthesized pre-mRNAs are released from the surface of chromosome domains into the ICN. Splicing factors are present at transcription sites, but are concentrated in local aggregates in the ICN, like clusters of interchromatin granules and coiled bodies. Finally, the ICN is involved in transport of mature RNA to the nuclear envelope [83].

10. Perspectives

Our knowledge of the organization of pre-mRNA metabolism in the nucleus has increased dramatically during the last decade. This is due to the combined effort of biochemical, cell biological, as well as ultrastructural techniques. A similar approach will again be fruitful to solve the many questions that are still ahead.

To get insight into the function of the different subnuclear domains involved in pre-mRNA metabolism, e.g., perichromatin fibrils, perichromatin granules, interchromatin granules, and coiled bodies, it is important to study their molecular composition. For example, what is the nature of the poly(A)⁺ RNAs that are present in interchromosomal granules? Specific antibodies in combination with light and electron microscopy will reveal the localization of yet unlocalized nuclear components, like poly(A) polymerase, adenosine methyltransferase, and RNase. Very little is known about the dynamics of pre-mRNA processing. Kinetic studies concerning the timing of transcription, splicing, intranuclear transport, and export of specific transcripts may give an answer to this problem. This may be combined with ultrastructural studies using *in situ* hybridization and highly specific intron and exon probes to investigate the route by which the (pre-)mRNA moves between the gene and the nuclear envelope. The real dynamics of nuclear pre-mRNA metabolism, however, can in principle only be studied in living cells. This requires the combined effort of

many scientific disciplines in order to develop (i) sensitive, vital probes, (ii) sensitive detection methods, and (iii) powerful software and computers.

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