The RNA 3′ cleavage factors CstF 64 kDa and CPSF 100 kDa are concentrated in nuclear domains closely associated with coiled bodies and newly synthesized RNA

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The cleavage stimulation factor (CstF), and the cleavage and polyadenylation specificity factor (CPSF) are necessary for 3′-terminal processing of polyadenylated mRNAs. To study the distribution of 3′ cleavage factors in the nuclei of human T24 cells, monoclonal antibodies against the CstF 64 kDa subunit and against the CPSF 100 kDa subunit were used for immunofluorescent labelling. CstF 64 kDa and CPSF 100 kDa were distributed in a fibrogranular pattern in the nucleoplasm and, in addition, were concentrated in 1-4 bright foci. Double immunofluorescence labelling experiments revealed that the foci either overlapped with, or resided next to, a coiled body. Inhibition of transcription with α-amanitin or 5,6-dichloro-β-D-ribofuranosyl-benzimidazole (DRB) resulted in the complete co-localization of coiled bodies and foci containing 3′ cleavage factors. Electron microscopy on immunogold double-labelled cells revealed that the foci represent compact spherical fibrous structures, we named ‘cleavage bodies’, intimately associated with coiled bodies. We found that ~20% of the cleavage bodies contained a high concentration of newly synthesized RNA, whereas coiled bodies were devoid of nascent RNA. Our results suggest that the cleavage bodies that contain RNA are those that are adjacent to a coiled body. These findings reveal a dynamic and transcription-dependent interaction between different subnuclear domains, and suggest a relationship between coiled bodies and specific transcripts.

Keywords: cleavage factors/coiled bodies/nuclear bodies/PML

Introduction

The nucleus is compartmentalized. By light microscopy and electron microscopy, many different domains inside the nucleus can be visualized, which have distinct morphologies, compositions and probably distinct functions (for reviews, see Spector, 1993; van Driel et al., 1995). The most conspicuous example of a nuclear compartment is the nucleolus (for reviews, see Hernandez-Verdun, 1991; Raška and Dundr, 1993; Scheer et al., 1993). In this structure, synthesis and processing of rRNA and assembly of pre-ribosomal particles takes place. The function of many other nuclear domains is less clear. Coiled bodies (Raška et al., 1991), nuclear bodies containing the protein PML (PML bodies) (Szostecki et al., 1990; Ascoli and Maul, 1991; Stuurman et al., 1992; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994) and clusters of interchromatin granules (Puvion and Moyne, 1981; Raška et al., 1990; Spector et al., 1991) have been identified in nuclei of many cell types. Coiled bodies and clusters of interchromatin granules contain high concentrations of splicing factors, suggesting that these domains play some role in the processing of pre-mRNA.

In addition to splicing, nearly all mammalian mRNAs are co-transcriptionally or post-transcriptionally modified by 3′ cleavage and polyadenylation (for reviews, see Wahle, 1992; Wahle and Keller, 1992; Sachs and Wahle, 1993). A number of protein factors are involved in these reactions. For the cleavage reaction, the cleavage and polyadenylation specificity factor (CPSF), the cleavage stimulation factor (CstF), poly(A) polymerase and cleavage factors I and II are required (Christofori and Keller, 1988; Gilmartin and Nevins, 1989; Takagaki et al., 1989). CPSF and poly(A) polymerase are also essential for polyadenylation. Little is known about the spatial distribution of the factors involved in these 3′ modifications of mRNA.

CPSF consists of four subunits of 160, 100, 73 and 30 kDa. CstF consists of three subunits of 77, 64 and 50 kDa. The CstF 64 kDa subunit contains a ribonucleoprotein (RNP)-type RNA binding domain (Takagaki et al., 1992), and can be UV cross-linked to RNA (Takagaki et al., 1990; Gilmartin and Nevins, 1991). Also the CPSF 160 and 30 kDa subunits can be UV cross-linked to RNA (Gilmartin and Nevins, 1989; Keller et al., 1991; Jenny et al., 1994). Monoclonal antibodies (mAbs) against the 50 and 64 kDa subunits of CstF (Takagaki et al., 1990), and against the 100 and 160 kDa subunits of CPSF (Jenny et al., 1994) have been used to immunofluorescently label CstF and CPSF in human cells. These labelling experiments showed that both factors are localized in the cell nucleus.

To understand how the nucleus is functionally organized, we are studying the subnuclear organization and spatial relationship of the synthesis, 3′-terminal cleavage, polyadenylation and splicing of RNA. Here we investigate how factors involved in 3′-processing are related spatially to sites of RNA synthesis, and previously identified nuclear compartments enriched in other RNA processing factors.
found that the foci. These foci (Figure 2884) pattern fibrogranular both factors described for Ascence labelling et al., 1994; Jenny et al., 1990; Matera and Ward, 1993), fibrillarin (Raška et al., 1991), and Nopp140 and NAP57.

To this end, immunofluorescence and immunogold labelling experiments were performed using an anti-CstF 64 kDa mAb and an anti-CPSF 100 kDa mAb, combined with confocal laser scanning microscopy, three-dimensional image analysis and electron microscopy. We found that CstF 64 kDa and CPSF 100 kDa are present throughout the nucleoplasm, except in the nucleolus. In addition, both factors were concentrated in a few nuclear foci in close association with two other types of domains, known as coiled bodies and PML-containing nuclear bodies.

Results

Immunofluorescent labelling of CstF 64 kDa and CPSF 100 kDa

To study the distribution of the RNA 3'-processing factors, we used a mAb which specifically recognizes the 64 kDa subunit of human CstF (Takagaki et al., 1990, 1992) and a mAb which specifically recognizes the 100 kDa subunit of human CPSF (Jenny et al., 1994), for immunofluorescence labelling of T24 human bladder carcinoma cells. As described for HeLa cells (Takagaki et al., 1990; Jenny et al., 1994; Krause et al., 1994), we found labelling of both factors throughout the nucleus, except for the nucleoli. The majority of both labels was found in a homogeneous fibrogranular pattern in the nucleoplasm. In addition, we found that CstF 64 kDa (Figure 1A) and CPSF 100 kDa (Figure 1B) were concentrated in a few brightly labelled foci. These foci appeared as small subnuclear domains with a distinctly higher fluorescent signal than the surrounding nucleoplasm. They appeared to be roughly spherical with a diameter of 0.3-1 μm. Typically, 1-4 CstF foci or CPSF foci were present per nucleus.

The foci were not observed during mitosis; both cleavage factors were found homogeneously distributed in the cell, excluding the chromosomes (Figure 2A-D). In late telophase, when the nuclear membrane has formed and the chromosomes start to decondense, CstF 64 kDa accumulated again in the nucleus (Figure 2E and F). Apparently, the foci disintegrate upon entering mitosis and are reassembled after cell division.

Foci of CstF and CPSF are associated with coiled bodies

Coiled bodies are spherical subnuclear structures, found in nuclei of plant and animal cells and owe their name to their distinct appearance in the electron microscope (Raška et al., 1991). Coiled bodies are highly enriched in the protein p80-coilin (Andrade et al., 1991) and also contain high concentrations of splicing factors (Carmo-Fonseca et al., 1991; Raška et al., 1991; Matera and Ward, 1993), fibrillarin (Raška et al., 1991), and Nopp140 and NAP57.

Fig. 1. Immunofluorescent labelling of human T24 bladder carcinoma cells with mAbs against the CstF 64 kDa subunit (A) and against the CPSF 100 kDa subunit (B). Note the overall diffuse labelling in the nucleoplasm, plus a few bright foci.

Fig. 2. Optical sections of T24 cells stained for DNA with propidium iodine (A, C and E) and labelled with anti-CstF 64 kDa (B, D and F). Cells in metaphase (A and B) or anaphase (C and D) show an overall CstF 64 kDa labelling in the cell, excluding the chromosomes. No bright foci, as found in interphase cells, were observed. CstF 64 kDa begins to accumulate in the nucleus during late telophase when the nuclear membrane has reformed and the chromosomes start to decondense (E and F). Bar represents 2 μm.
(Meier and Blobel, 1994). They have proved to be dynamic structures in the nucleus (Carmo-Fonseca et al., 1992; Antoniou et al., 1993; Malatesta et al., 1994; for a review, see Lamond and Carmo-Fonseca, 1993). In the fluorescence microscope, coiled bodies are visible as bright nuclear dots, their number, in most cell types, ranging from one to five per nucleus.

The size, shape and subnuclear distribution of the CstF and CPSF foci resembled that of coiled bodies. To investigate whether there is any relationship between these domains, T24 cells were double labelled with anti-CstF 64 kDa or anti-CPSF 100 kDa, in combination with a polyclonal antibody against p80-coilin. We used confocal laser scanning microscopy to compare the two nuclear domains. Strikingly, the double labelling did not show a simple co-localization but revealed a complex relationship between coiled bodies and foci. The foci labelled by anti-CstF 64 kDa and by anti-CPSF 100 kDa were found to overlap either partially or completely with coiled bodies or were situated adjacent to coiled bodies (Figure 3). The foci, labelled by the two antibodies against the 3' cleavage factors, showed an identical distribution relative to coiled bodies, which made it very likely that both antibodies labelled the same nuclear structure.

We found coiled bodies and foci labelled by anti-CstF 64 kDa or anti-CPSF 100 kDa paired with a varying degree of overlap. As shown in Table I, 42% of the foci labelled by anti-CstF 64 kDa precisely co-localized with a coiled body. In addition, 51% of the CstF foci were associated with a coiled body but did not co-localize with it; 18% could be clearly distinguished as separate round structures adjacent to coiled bodies (Figure 3A and B). We found a few single coiled bodies but rarely observed foci enriched in CstF 64 kDa or CPSF 100 kDa unassociated with a coiled body.

The association and degree of overlap between foci of 3' cleavage factors and coiled bodies was analysed further quantitatively by measuring red and green signal intensities along an axis through the foci (Figure 4). The distances between the peaks in the two signals, representing a coiled body and a CstF focus, range from zero (Figure 4B) to ~0.5 μm (Figure 4A). Our findings show that foci of cleavage factors and coiled bodies are distinct nuclear domains with a strong spatial and, therefore, possibly functional relationship.

**Foci of 3' cleavage factors appear as distinct structures in the electron microscope**

We used electron microscopy to investigate the structure of the foci enriched in cleavage factors and their spatial relationship to coiled bodies in closer detail. Unfortunately, a post-embedment labelling approach did not provide sufficient signal for CstF (see Materials and methods). Pre-embedment labelling, performed according to several fixation, permeabilization and labelling protocols, only gave specific CstF 64 kDa labelling under particular conditions (described in Materials and methods). Coiled bodies were labelled specifically with p80-coilin, and often CstF 64 kDa labelling was also found in these structures. Distinct structures, containing a significantly stronger CstF labelling than the surrounding material, could be found in close proximity to a coiled body (Figure 5A and B). These spherical structures had a compact and fibrous appearance, which differed slightly between experiments, but was always distinct from a coiled body. All the available evidence points to the fact that these structures represent the light microscopically defined foci of cleavage factors, which we will refer to from now on as 'cleavage bodies'.

In some cases, the coiled body appeared to contain a stronger CstF labelling than the adjacent cleavage body. Interestingly, the coiled body and cleavage body were not always found in direct contact but also somewhat separated from each other (Figure 5B). These electron microscopical data confirm that cleavage bodies are distinct nuclear structures that have an intimate spatial relationship with coiled bodies.

**Cleavage bodies in various cell types**

Foci of RNA 3' cleavage factors were also found in other cell types, although sometimes they were less frequent or less distinct than in T24 cells (Figure 6A and B). Human fibroblasts rarely contained coiled bodies; however, the few coiled bodies that were found overlapped with a brightly labelled CstF 64 kDa focus (Figure 6E and F). HeLa cells often contained a domain associated with coiled bodies that showed a slightly higher concentration of CstF 64 kDa (data not shown). CaCo cells were found to have up to 15 coiled bodies per nucleus. Most coiled bodies were not associated with a focus of cleavage factors. However, in 1–5% of the nuclei, bright foci could be found in or adjacent to coiled bodies (Figure 6C and D).

We have found that foci of RNA 3' cleavage factors are most prominent in T24 cells and that equivalent structures can be found in other human cell types.

**Some cleavage bodies contain newly synthesized RNA**

To study the relationship between the distribution of the 3' cleavage factors and sites of RNA synthesis, intact cells were microinjected with 5-bromo-UTP (BrUTP). By this method, newly synthesized RNA becomes labelled with 5-bromouridine, which can be detected immunofluorescently, thus visualizing sites of RNA synthesis in the nucleus (Wansink et al., 1993, 1994). Microinjected cells double labelled with anti-CstF 64 kDa showed that the numerous bright spots of newly synthesized RNA did contain CstF 64 kDa, but that the cleavage factor was not concentrated specifically at these sites. Cleavage bodies immunofluorescently labelled with anti-CstF 64 kDa did sometimes contain a high concentration of newly synthesized RNA (Figure 7); ~20% of the cleavage bodies co-localized with a bright spot of labelled RNA. The remaining cleavage bodies did not appear to contain newly synthesized RNA. Microinjected cells double labelled with anti-p80-coilin showed newly synthesized RNA to be absent from coiled bodies (data not shown). This implies that only the cleavage bodies that do not overlap with a coiled body contain newly synthesized RNA.

**Cleavage bodies and coiled bodies completely co-localize after inhibition of RNA polymerase II transcription**

5,6-Dichloro-β-D-ribofuranosyl-benzimidazole (DRB) and α-amanitin can selectively inhibit transcription of premRNA by RNA polymerase II (RPⅡ). Upon treatment
Coiled bodies and foci of 3' cleavage factors are tightly associated dynamic structures. Cells were double labelled with anti-CstF 64 kDa (A, C and D) (green) or anti-CPSF 100 kDa (B) (green) and anti-p80-coilin (red), and analysed by confocal laser scanning microscopy. Single optical sections are shown. The cleavage factors are found in a homogeneous fibrogranular pattern throughout the nucleoplasm and concentrated in a few brightly labelled foci (arrowheads). Coiled bodies and foci of cleavage factors are sometimes found next to each other as separate nuclear domains (A and B). Treatment of cells with α-amanitin for 2 h, to inhibit RNA polymerase II transcription, resulted in a complete co-localization (indicated by yellow) of coiled bodies and foci (C). After 6 h α-amanitin treatment, CstF 64 kDa was found in large rounded-up speckles often associated with remnants of coiled bodies (D). Foci of 3' cleavage factors, coiled bodies and some of the PML bodies are found clustered in ultrastructural trios in the nucleus (E) (see also magnified area). An optical section is shown of a triple labelled cell, labelled with anti-CstF 64 kDa (green), anti-p80-coilin (red) and an auto-immune serum recognizing PML bodies (blue). Bars represent 2 μm.

With these drugs, splicing factors like snRNPs and U2AF no longer concentrate in coiled bodies and redistribute in the nucleus to structures called speckles (Spector et al., 1991; Carmo-Fonseca et al., 1992). To investigate whether the association between cleavage bodies and coiled bodies is similarly transcription dependent, we cultured T24 cells in the presence of either α-amanitin or DRB for 2 h. The CstF 64 kDa (Figure 3C) and CPSF 100 kDa (not shown)
Table I. Effect of inhibition of RNA polymerase II on the spatial relationship between coiled bodies and foci of 3′ cleavage factors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of nuclei</th>
<th>No. of CstF foci</th>
<th>Co-localizing</th>
<th>Partially overlapping</th>
<th>Adjacent</th>
<th>Single</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>28</td>
<td>57</td>
<td>24 (42%)</td>
<td>19 (33%)</td>
<td>10 (18%)</td>
<td>4 (7%)</td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>11</td>
<td>18</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DRB</td>
<td>6</td>
<td>11</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

The number of CstF 64 kDa labelled foci that are co-localizing, partially overlapping and adjacent to coiled bodies is shown. The cells were treated with α-amanitin or DRB for 3 h to inhibit RNA polymerase II transcription. Percentages in parentheses indicate the number of CstF-foci relative to the total amount of CstF-foci.

labelling in DRB-treated cells and in the α-amanitin-treated cells showed the same distribution as found in uninhibited control cells. We still observed coiled bodies and cleavage bodies, although the numbers of both had somewhat decreased (0–3 per nucleus).

There was a significant change, however, in the relationship between coiled bodies and the foci of cleavage factors after α-amanitin or DRB treatment. Both domains showed an almost complete co-localization (Figure 3C and Table I). Foci next to bodies or with partial overlap, often seen in uninhibited cells, were never observed in inhibitor-treated cells. Control experiments showed that drug-treated cells had staining patterns typical for transcription-inhibited nuclei (Carmo-Fonseca et al., 1992), i.e. coiled bodies no longer contained the U2 snRNP splicing factor, which almost completely concentrated in 10–20 large rounded-up speckles, together with the non-snRNP splicing factor SC-35 (data not shown).

Clearly, CstF 64 kDa and CPSF 100 kDa behaved differently from the splicing factors mentioned above. They did not dissociate from coiled bodies but instead appeared to accumulate in them. These findings show that transcription levels affect the spatial relationship between coiled bodies and cleavage bodies.

**CstF 64 kDa and CPSF 100 kDa redistribute to rounded-up speckles after prolonged α-amanitin treatment**

After incubation with α-amanitin for 6 h, almost all nuclei showed a different CstF 64 kDa and CPSF 100 kDa labelling pattern compared with uninhibited cells or cells that were inhibited for up to 2 h. After 6 h, the labelling was concentrated in 10–20 large round structures in the nucleoplasm (Figure 3D), similar to observations by Krause et al. (1994) of α-amanitin-treated HeLa cells.

We observed that even after 6 h of α-amanitin treatment some T24 nuclei still showed small foci rich in CstF 64 kDa or CPSF 100 kDa, which completely co-localized with foci containing p80-coilin (data not shown). Most
p80-coilin labelling, however, was from disrupted coiled bodies showing small curved structures in the nucleoplasm as reported for HeLa cells (Carmo-Fonseca et al., 1992).

These remnants of coiled bodies (red structures in Figure 3D) no longer contained high concentrations of CstF 64 kDa or CPSF 100 kDa, and were sometimes found associated with nucleoli but could also often be found in close contact with the large rounded-up speckles. In uninhibited cells, coiled bodies and cleavage bodies never overlapped with speckles, but no further specific spatial relationship between the two types of domains was found.

Apparently, initial inhibition of RPII transcription makes CstF 64 kDa and CPSF 100 kDa become concentrated in coiled bodies, but prolonged inhibition causes these 3' cleavage factors and p80-coilin to redistribute into separate structures. This further demonstrates the dynamic relationship between cleavage bodies, coiled bodies and RPII activity.

Coiled bodies, cleavage bodies and PML bodies occur as trios in the nucleus

Besides coiled bodies, many other small subnuclear domains, often referred to as nuclear bodies, have been visualized by electron microscopy (de Thé et al., 1960; for review, see Brasch and Ochs, 1992). Characterization of some of these structures has been based on immunolabelling experiments, using a variety of human autoantibodies (Szostecki et al., 1987; Ascoli and Maul, 1991; Fusconi et al., 1991; Saunders et al., 1991). The use of mAbs has shown that the proteins PML and Sp100 are highly concentrated in a specific type of nuclear body (Szostecki et al., 1990; Ascoli and Maul, 1991; Stuurman et al., 1992; Koken et al., 1994). We will refer to these structures as PML bodies. Typically, 10–30 PML bodies are found in the nucleus of a mammalian cell. Their function is unknown. Experiments have indicated that coiled bodies very often reside right next to PML bodies, but do not overlap (Grande et al., submitted).

We have compared the distribution of cleavage bodies and PML bodies, using the anti-CstF 64 kDa mAb and the human autoimmune serum SUN3 which recognizes PML bodies in the nucleus (Szostecki et al., 1987). Cleavage bodies were often found adjacent to PML bodies, but they never overlapped (data not shown). Because the number of PML bodies greatly exceeds the number of cleavage bodies per nucleus, many PML bodies were not associated with a cleavage body.

Fig. 6. Double labelling experiments with anti-p80 coilin (A, C and E) and anti-CstF 64 kDa (B, D and F), showed that foci of 3' cleavage factors associated with coiled bodies occur in various cell types, although often not as distinct or frequent as in T24 cells (A and B). In 1–5% of the CaCo cells, foci could be found completely or partially overlapping with a coiled body (C and D). F3035 fibroblasts rarely contained coiled bodies, but the few that were found always co-localized with a focus enriched with CstF 64 kDa (E and F). Bar represents 2 μm.

Fig. 7. Some cleavage bodies contained newly synthesized RNA. T24 cells were microinjected with 5-bromo-UTP which is incorporated in RNA. Sites of newly synthesized RNA are immunofluorescently visualized (A). When double labelled with anti-CstF 64 kDa (B), ~20% of the cleavage bodies contained a high concentration of newly transcribed RNA (compare magnified areas in both images). Single confocal sections are shown. Bar represents 2 μm.
From this, it seemed likely that coiled bodies, cleavage bodies and PML bodies could occur grouped together as trios in the nucleus. To test this, we performed triple labelling experiments using anti-CstF 64 kDa, anti-p80-collin and SUN3 in combination with three-channel confocal laser scanning microscopy to visualize the three types of domains simultaneously in one nucleus. As expected, there was a tight association between coiled bodies, PML bodies and cleavage bodies in all analysed nuclei. In cases where the coiled body and cleavage body did not overlap, three tightly clustered dots could be observed (Figure 3E). We rarely found a pair of coiled body and cleavage body that was not associated with a PML body. The three nuclear domains seemed to form a tight trio in the nucleoplasm.

Although coiled bodies, PML bodies and cleavage bodies may be involved in different processes, their obvious spatial relationship suggests that their functions are linked.

Discussion

We used specific mAbs against two RNA 3' cleavage factors to study the distribution of these proteins in the nucleus of human cells. CPSF comprises four subunits (160, 100, 73 and 30 kDa) and CstF comprises three subunits (77, 64 and 50 kDa). Both factors form stable functional complexes which can withstand various purification procedures (Takagaki et al., 1990; Bienroth et al., 1991; Jenny et al., 1994). It is, therefore, likely that the CstF 64 kDa and CPSF 100 kDa labelling represents the distribution of intact CstF and CPSF, although it cannot be excluded that some CstF 64 kDa and CPSF 100 kDa labelling comes from uncomplexed subunits.

The majority of the two 3' cleavage factors was found distributed in a homogeneous fibrogranular pattern throughout the nucleoplasm, and not in a distinct punctated pattern as found for sites of newly synthesized RNA (compare Figure 7A and B). Nevertheless, our double labelling experiments showed that CstF 64 kDa was present at sites of newly synthesized RNA, albeit not in elevated amounts. This is in good agreement with its extensively documented role in the processing of pre-mRNA. The CstF 64 kDa labelling outside the sites of RNA synthesis suggests that only a fraction of the CstF proteins is actually engaged in the RNA 3' cleavage process in the nucleus.

The two 3' cleavage factors were also found highly concentrated in a few foci in the nucleoplasm. Although we could not compare the CstF foci directly with the CPSF foci, the identical association with coiled bodies strongly indicated that CstF 64 kDa and CPSF 100 kDa are both concentrated in the same nuclear domain. This nuclear domain was visible in the electron microscope as a compact spherical fibrous structure, distinct from a coiled body in appearance and in protein composition. Based on these observations, we named this novel nuclear domain 'cleavage body'.

**Cleavage bodies, coiled bodies and PML bodies form a dynamic trio**

Our results indicate a tight spatial relationship between coiled bodies and cleavage bodies, which may indicate a link between their functions. A cleavage body was sometimes found next to a coiled body, but could also partially or completely overlap with it. It should be noted that the observed overlap between coiled and cleavage bodies could be caused partly by the limited resolution of the confocal microscope. Two domains next to each other could often clearly be distinguished; however, two separate bodies very close together would appear to overlap in the light microscope. Indeed, electron microscopical observations have indicated that some coiled bodies and cleavage bodies are so close together that they could not be seen as separate domains at the light microscopical level. Moreover, the resolution between subsequent focal planes in the confocal microscope is much lower than the lateral resolution and barely above the size of a coiled body and cleavage body. Therefore, we cannot rule out the possibility that at least some apparently co-localizing dots were actually two separate domains above or very close next to each other, so the percentage of co-localizing and partially overlapping bodies (Table I) may be an overestimation at the expense of the number of adjacent bodies.

The relationship between coiled bodies and cleavage bodies is dynamic, since the level of transcription affects the degree of overlap between the two types of domains: cleavage bodies and coiled bodies completely co-localized after inhibition of RIP II transcription. Although the light microscopical data suggest that both structures fuse to form one domain, there is also the possibility that the cleavage factors are transferred from the cleavage body to the coiled body. There are some indications for this latter possibility from electron microscopical observations, in which a cleavage body contained less CstF labelling than the coiled body with which it was associated. However, the precise mechanism by which both bodies interact is still unknown. We conclude that cleavage bodies, like coiled bodies, are dynamic structures that are possibly involved in the processing of pre-mRNA.

A third nuclear body, rich in the protein PML, was often found complexed with the pair of coiled body and cleavage body. The function of PML bodies is unclear. They never overlap with either coiled bodies or cleavage bodies and do not seem affected by transcription inhibition (Grande et al., submitted), but their constitutive presence in the ultrastructural trios does indicate a role for these enigmatic nuclear entities in collaboration with coiled bodies and cleavage bodies.

**The role of cleavage bodies in RNA processing**

It has been proposed that coiled bodies are compartments for the assembly or disassembly of multi-snRNP complexes, as part of the splicing cycle (discussed in Lamond and Carro-Fonseca, 1993). It is possible that CstF, CPSF and perhaps other 3'-processing factors similarly undergo transcription-dependent recycling, assembly or disassembly in cleavage bodies. Parts of the machinery in coiled bodies for the recycling of splicing factors may also be used for the recycling of 3' cleavage factors. This could explain the close association between coiled bodies and cleavage bodies.

Alternatively or additionally, coiled bodies and cleavage bodies could play a role in the processing of specific transcripts. It seems clear that coiled bodies do not contain newly synthesized RNA, since they become slowly
labelled with tritiated uridine (Moreno Diaz de la Espina et al., 1982), do not appear to contain DNA (Thiry, 1994) and do not contain nascent RNA when labelled with BrUTP (data not shown; Raška, 1995).

In contrast, we have observed that ~20% of the cleavage bodies do contain newly synthesized RNA. These RNA-containing cleavage bodies could not be overlapping with a coiled body, because coiled bodies never contain newly synthesized RNA. Thus RNA-containing cleavage bodies most probably represent those that are located adjacent to coiled bodies. Remarkably, the percentage of cleavage bodies found next to a coiled body (18%) is about the same as the percentage of cleavage bodies found to contain newly transcribed RNA (~20%). These findings lead us to propose that this subset of cleavage bodies is associated with specific sites of RNA synthesis in the nucleus. Closely associated coiled bodies and PML bodies, although themselves outside the sites of RNA synthesis, may be involved in the processing of these specific RNAs (Figure 8).

Interestingly, recent observations by Frey and Matera (1995) and Smith et al. (1995) point to a preferential localization of coiled bodies in the close vicinity of specific gene loci. They reported that coiled bodies in HeLa cells are often found associated with the U1 and U2 snRNA gene clusters and sometimes with the histone gene cluster. A similar spatial organization has been described for a specific organelle in the nuclei of amphibian oocytes that seems closely related to the coiled body: the C-snurposome (Wu et al., 1991; Wu and Gall, 1993; Gall et al., 1995; Roth, 1995). C-Snurposomes contain the protein SPH-1, which is related to p80-collin (Tuma et al., 1993), and, like coiled bodies, are found closely associated with the histone gene loci (Gall et al., 1981; Callan et al., 1991). Interestingly, both coiled bodies and C-snurposomes are enriched in U7 snRNA (Wu and Gall, 1993; Frey and Matera, 1995), necessary for histone 3' maturation (Marzluff, 1992). We have shown here that coiled bodies are associated with two other factors involved in the 3'-processing of mRNA, namely CstF and CPSF. It is somewhat puzzling, however, that these last two factors do not appear to be involved in the 3'-processing of either the snRNA transcripts, or the histone transcripts. Apparently, coiled bodies contain elements of different 3'-processing machineries, which could indicate their involvement in the 3' formation of certain polyadenylated and non-polyadenylated transcripts.

We have shown that, after inhibition of RPII transcription, coiled bodies always completely overlap with cleavage bodies. In the proposed model, this may be explained by the assumption that cleavage body-associated genes are not transcribed continuously, causing cleavage bodies and coiled bodies to fuse during the inactive period and to divide into separate domains when transcription recommences and the services of the 3' cleavage factors are required. A cleavage body overlapping with a coiled body could be associated with an inactive gene, or an active gene which encodes a non-polyadenylated RNA, or it could be unassociated with a gene locus at that time.

Our studies have revealed new interesting relationships between different nuclear domains. Although the precise role of coiled bodies, PML bodies, cleavage bodies and other nuclear structures remains unclear, the associations and interactions we have demonstrated may lead to new insights into their nuclear function.

Materials and methods

Cell culture

T24 cells (from human bladder carcinoma), HeLa cells (from human cervical carcinoma) and CaCo cells (from human colon carcinoma) were grown on circular glass coverslips at 37°C under a 10% CO2 atmosphere in DMEM (Gibco) supplemented with 1% glutamine (Gibco), 10% fetal calf serum (FCS; Boehringer) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco)).

Human skin fibroblasts (Heng89AD and F3035) were grown at 37°C under a 5% CO2 atmosphere in a 1:1 mixture of Ham's F-10 medium (Gibco) and DMEM containing the same supplements as for T24, HeLa and CaCo cells. All cells were used at 50–70% confluency.

For drug treatment, cells were placed in fresh medium containing 50 µg/ml α-amanitin (Sigma) or 50 µM DRB (Sigma) and grown for another 2 or 6 h before fixation.

Immunofluorescence labelling

Coverslips with attached cells were rinsed once in phosphate-buffered saline (PBS) and incubated with 2% paraformaldehyde in PBS for 15 min at room temperature. After fixation, cells were rinsed twice with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells subsequently were rinsed twice in PBS, incubated in PBS containing 100 mM glycine (Sigma) for 10 min and incubated for 10 min in PBS [PBS containing 0.5% bovine serum albumin (Sigma) and 0.05% gelatine from cold water fish skin (Sigma)].

For immunolabelling, the following antibodies were used: anti-CstF 64 kDa mAb from mouse (Takagaki et al., 1990), anti-CPSF 100 kDa mAb from mouse (Jenny et al., 1994), anti-p80-collin polyclonal antibody from rabbit (kindly provided by Dr A. L.Lamond), SUN3 human autoimmune serum (kindly provided by Dr L. Bautz) and anti-Brdu (Seralab).

Fixed cells were incubated overnight at 4°C with primary antibodies diluted in PBS. Subsequently, cells were washed four times for 5 min each time in PBS and incubated with secondary antibodies diluted in PBS for 1.5 h at room temperature.

For single and double labelling, donkey anti-mouse IgG coupled to DTAF (Jackson ImmunoResearch Laboratories) and donkey anti-rabbit IgG coupled to TRITC (Jackson) were used. After labelling, cells were washed twice for 5 min in PBS and twice for 5 min in PBS, followed by incubation in PBS containing 0.4 µg/ml Hoechst 33258 (Sigma) for 5 min.

For triple labelling, donkey anti-mouse IgG coupled to DTAF (Jackson) and donkey anti-rabbit IgG coupled to Texas red (Jackson) were used for 1 h. After this labelling, cells were washed four times for 5 min in PBS and incubated with mouse anti-human IgG coupled to Cy3 (Jackson) for 1 h at room temperature. Finally, cells were washed twice for 5 min in PBS and twice for 5 min in PBS.

All coverslips were mounted in PBS containing 90% glycerol and 1 mg/ml p-phenylenediamine (Sigma).
Microinjection

T24 cells were microinjected with BrUTP as described by Wansink et al. (1993, 1994). After microinjection, cells were cultured for 10 min at 37°C and subsequently fixed and labelled as described above.

Light microscopy

Images of single-labelled cells were produced on a Leitz Aristoplan microscope equipped with epifluorescence optics. Pictures were photographed on Kodak Tri-X 400 ASA film. Images of double-labelled cells were produced on a Leica confocal laser scanning microscope with a 100×/1.35 oil immersion lens. A dual wavelength laser was used to excite DTAF and TRITC simultaneously at 488 and 514 nm respectively. The fluorescence signals from both fluorochromes were recorded simultaneously. Optical cross-talk was quantified and subtracted as described previously (Manders et al., 1992). Image analysis was performed using SCIL-IMAGE software.

Three-dimensional images of triple-stained nuclei were recorded with a Sarastro 'Phobos 1000' confocal microscope (Molecular Dynamics Inc., Sunnyvale, CA). A 100×/1.3 Planapo oil immersion Zeiss objective was used. This confocal microscope has been modified for the pixel-simultaneous recording of three different fluorophores. The specimen was simultaneously illuminated with light of 488, 568 and 648 nm from an Ar ion and a Kr ion laser. Three photomultipliers were used to record the different signals. To improve channel separation, the set-up has been modified for the application of a new technique: intensity-modulated multiple-beam scanning (IMS) microfluorometry (Carlsson et al., 1994; Manders et al., 1995). With this technique, optical cross-talk between different fluorescence signals was reduced substantially (<0.5%).

Immunogold labelling and electron microscopy

Post-embedding immunogold labelling on thin cryosections and thin low-cryosections of T24 and HeLa cells was carried out as previously described (Raška et al., 1995). Whereas p80-collin labelling was found satisfactory, this approach did not yield significant labelling for CstF, despite very gentle fixation conditions such as 30 min fixation in 2% paraformaldehyde. Only a pre-embedding approach gave a positive result.

Cells grown on coverslips were washed in PBS, fixed in 2% paraformaldehyde in PBS for 10 s, washed again and permeabilized for 3 min in 1% Triton X-100 in PBS containing 4.5% polyvinylpyrrolidone and 0.2 M sucrose at room temperature. Subsequently, the cells were washed and fixed for 30 min with 2% paraformaldehyde in PBS, washed in PBS and incubated in 10% FCS in PBS for 10 min. The cells were incubated with primary antibody anti-CstF 64 kDa in 5% FCS for 3 h, washed in PBS, and incubated with anti-mouse 1 nm gold adducts (Aurion, Belgium) in 5% FCS in PBS, overnight at 4°C. The cells were further fixed for 20 min in 2% paraformaldehyde in PBS. Silver intensification was carried out with the Aurion intensification kit in two consecutive 30 min intervals. For immunogold double labelling, the cells additionally were exposed to anti-p80-collin (polyclonal antibody from rabbit, kindly provided by Dr E.Chan) in 5% FCS for 3 h, washed in PBS, and incubated with anti-rabbit 1 nm gold adducts in 5% FCS in PBS and a second round of intensification (2 × 30 min) was performed. After fixation, the cells were embedded in epon. A diamond knife from Diatome (Suisse) and a Reichert (Leica) Ultracut E ultramicrotome were used for sectioning. The sections were stained with 5% uranyl acetate in water for 60 min. The sections were observed in an Option 109 electron microscope equipped with transilite optics.

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