Ribosome Components Are Associated with Sites of Transcription

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Summary

It is generally believed that eukaryotic ribosomes first associate with mRNA in the cytoplasm. However, we show with chromosomal immunostaining and in situ hybridization that ribosomal subunits are present at transcription sites of Drosophila salivary gland chromosomes. Immunostaining was carried out with antibodies specific for 27 ribosomal proteins, two translation factors and one that specifically recognizes rRNA. In situ hybridization was with several probes specific for both rRNA subunits. The kinetics of recruitment following transcription initiation suggest that the association is with newly transcribed pol II transcripts. These data indicate that ribosome components associate with nascent RNP complexes within the nucleus.

Introduction

In eukaryotic cells, nascent pre-mRNA rapidly associates with a number of proteins and snRNPs. This association is required for the complex mRNA maturation processes that occur in the nucleus. Because protein synthesis is thought to occur exclusively in the cytoplasm, it is assumed that in eukaryotes, unlike in prokaryotes, the mRNA associates with ribosomal subunits only after nuclear export.

However, several lines of evidence suggest that translation or a translation-like mechanism may also exist within the nucleus. For example, evidence for nuclear scanning of the open reading frame (ORF) comes from studies in the field of nonsense mediated mRNA decay (NMD). NMD describes the general phenomenon by which the presence of a nonsense mutation often leads to a reduction in mRNA levels (Maquat, 1995). It is generally accepted that recognition of a nonsense codon occurs during cytoplasmic translation. Premature translation termination is then thought to activate a specific protein complex—called the surveillance complex—which triggers accelerated decay of the aberrant mRNA (Culbertson, 1999; Czapinski et al., 1999; Rentze and Kulozik, 1999; Maquat and Carmichael, 2001).

Contrary to the expectation that nonsense codon recognition occurs exclusively in the cytoplasm, studies in mammalian cells indicate that NMD may take place in the nucleus by a mechanism independent of cytoplasmic translation. For example, several reports indicate that NMD of many mRNAs occurs while the mRNA is still associated with the nucleus, and that the stability of the cytoplasmic mRNA is unaffected by the presence of a nonsense mutation (Urlaub et al., 1989; Baserga and Benz, 1992; Cheng and Maquat, 1993; Lozano et al., 1994; Carter et al., 1996). Furthermore, several reports indicate that nonsense mutations can affect the splicing of the corresponding pre-mRNA (Naeger et al., 1992; Dietz et al., 1993; Dietz and Kendzior, 1994; Lozano et al., 1994; Aoufouchi et al., 1996; Gersappe et al., 1999).

It is difficult to imagine how premature translation in the cytoplasm could affect such an early event in mRNA biogenesis. In addition, splicing appears to be coupled to transcription, at least in higher organisms (Beyer and Osheim, 1988); therefore, it is conceivable that nonsense mutation recognition could occur while the transcript is still nascent. This possibility is supported by two recent studies showing that nonsense mutations can affect pre-mRNA 3' end processing (Brogna, 1999) and lead to an accumulation of pre-mRNA at the site of transcription (Muhlemann et al., 2001).

If a nuclear scanning machine exists, the most parsimonious explanation is that it is the ribosome. Indeed, the nucleus appears to contain ribosomes and other components of the translation apparatus (Ringborg et al., 1970; Lejbkowicz et al., 1992; Sanders et al., 1996; Lund and Dahlberg, 1998; Dostie et al., 2000). It is generally accepted that within the nucleus ribosomal particles are confined to the nucleolus and nonfunctional, but the isolation of nuclear polysomes and some evidence of nuclear translation was reported more than 3 decades ago (Goldstein, 1970; Allen, 1978; Gold, 1978). Recent evidence for nuclear polysomes was reported in Dictostelium discoideum (Mangiarotti, 1999). Evidence for the presence of functional ribosomes in the nucleus was provided in a recent study showing that protein synthesis can occur in a number of distinct, but not yet well defined, intranuclear foci in mammalian cells (Ibora et al., 2001).

Since premature translation termination appears to affect cotranscriptional events (Brogna, 1999; Muhlemann et al., 2001), we reasoned that functional ribosomes may be present at transcription sites. We therefore tested whether ribosomes are recruited to nascent transcripts using the giant polytene chromosomes of Drosophila melanogaster salivary glands. These chromosomes are composed of more than a thousand copies of intimately and precisely synapsed transcriptionally active chromatids, in which the many copies of a gene correspond to a defined region of the chromosome. This feature, as well as the detailed cytogenetic maps of these chromosomes, provides a unique system to visualize proteins and other factors associated with active genes and their nascent transcripts.

Using chromosomal immunostaining with antibodies directed against ribosomal components and in situ hybridization with probes complementary to the rRNA, we show that the translation apparatus is present at tran-
Table 1. Ribosomal Proteins Immunostaining

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>Immunogen (Antigene)</th>
<th>Fly Similarity</th>
<th>Staining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4X</td>
<td>HS4X1</td>
<td>RMRGTISREHP I....SVE DKLAAKGQSGG TH-....</td>
<td>78%</td>
<td>3 (+++)</td>
</tr>
<tr>
<td>S8</td>
<td>HS82</td>
<td>GKKRPYHKK5 (83% similar to A. mellifera gene)</td>
<td>d.n.a.</td>
<td>4 (++)</td>
</tr>
<tr>
<td>S11</td>
<td>HS111</td>
<td>DLYHTYRKNR I.....FV......</td>
<td>99%</td>
<td>1 (+++)</td>
</tr>
<tr>
<td>S15</td>
<td>HS152</td>
<td>QRRRLNGLRK (100% similar to C. elegans gene)</td>
<td>d.n.a.</td>
<td>3 (++++)</td>
</tr>
<tr>
<td>L5</td>
<td>HL52</td>
<td>HSTKRFPQYDSES R.....</td>
<td>83%</td>
<td>2 (+)</td>
</tr>
<tr>
<td>L7a</td>
<td>HL7a2</td>
<td>TNYKRFYDHIR P.F.E.HH...</td>
<td>99%</td>
<td>3 (+)</td>
</tr>
<tr>
<td>L13a2</td>
<td>HL13a2</td>
<td>KIRHKKQQLM</td>
<td>d.n.a.</td>
<td>1 (+)</td>
</tr>
<tr>
<td>L17</td>
<td>HL171</td>
<td>AKQATVTQW</td>
<td>81%</td>
<td>3 (+)</td>
</tr>
<tr>
<td>L18</td>
<td>HL182</td>
<td>RSGRKXFTSAR</td>
<td>100%</td>
<td>2 (+)</td>
</tr>
<tr>
<td>L28</td>
<td>HL281</td>
<td>KERTPTKSS</td>
<td>80%</td>
<td>2 (+++)</td>
</tr>
<tr>
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<td>HL322</td>
<td>KHRWKPRqdID</td>
<td>100%</td>
<td>4 (+)</td>
</tr>
<tr>
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<td>HL35a2</td>
<td>KRKLNRQREHT</td>
<td>d.n.a.</td>
<td>3 (+)</td>
</tr>
<tr>
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<td>HL36a2</td>
<td>KHNQHKTVQYK</td>
<td>d.n.a.</td>
<td>1 (+)</td>
</tr>
<tr>
<td>L39</td>
<td>HL392</td>
<td>EQQKQRPFPQW</td>
<td>81%</td>
<td>2 (+)</td>
</tr>
<tr>
<td>L12</td>
<td>anti-P Prot</td>
<td>KRRKKEKRSEEREDDMGFD E....</td>
<td>94%</td>
<td>2 (n.a.)</td>
</tr>
</tbody>
</table>

The second column is the names of the antibodies. The third column is the sequence of the peptides used; below is that of the Drosophila homolog; a dot denotes residues conserved. Differences are shown: in plain text are conservative changes and in bold are nonconservative substitutions. The dash denotes gaps. The percentage refers to the level of similarity. d.n.a. stands for data not available in the database. The staining levels (those in brackets refer to staining in the nucleolus) were estimated from two slides (note that for the nucleolus this was done 4 months later, during which some fading occurred).

scription sites. Moreover, the kinetics of ribosome recruitment indicate that the association occurs cotranscriptionally.

Results

Many Ribosome Components Are Recruited to Transcriptionally Active Chromosomal Regions

In three-dimensional reconstructions, the ribosome appears as a bundle of RNA decorated with about 80 surface proteins. As these ribosomal proteins are exposed on the external surface of the ribosome, it was expected that they may be accessible. Rabbit polyclonal antibodies were previously prepared against 26 human ribosomal proteins using peptides that show no significant similarity to other proteins in the database. These reagents are highly specific and recognize a single band in immunoblots of purified ribosomes, and most of them also recognize a single band in crude cell extracts (Nadano et al., 2000). Ribosomal proteins are evolutionary well conserved, and the regions corresponding to the immunogenic peptides are present in Drosophila (Table 1).

We tested these antibodies and a previously well-characterized autoimmune serum (Elkon et al., 1986) that recognizes the large subunit protein RpL12 and found that 20 of them strongly and specifically stain actively transcribed regions of the polytene chromosomes (level 2 or higher, Table 1). These regions correspond to decondensed chromatin and are most apparent in highly transcribed loci, where the chromatin is expanded to form puffs. Figure 1 shows four examples of the staining pattern (level 3 or 4, Table 1) using antibodies specific for two small subunit proteins (RpS15 and RpS30) and two large subunit proteins (RpL7a and RpL32). Staining is localized to interbands (decon-
Figure 1. Ribosomal Proteins Are Associated with Transcription Sites

Indirect immunofluorescence using antibodies against ribosomal proteins and Cy3 conjugated secondary antibody (red): (A) anti-RpS15, (B) anti-RpS30, (C) anti-RpL7a, and (D) anti-RpL32. Indirect immunofluorescence for Pol IIo using FITC-labeled secondary antibody (green) is shown in (E) through (H). Merged images are shown in (I) through (L). Centromeric regions are indicated by a “c.” Arrows indicate the 87A and 87C heat shock puffs that are occasionally transiently induced during dissection. The line in (B) and (J) indicates a fragment of the nucleolus.

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densed regions) and is particularly apparent at major puffs. All squashes were counterstained with DAPI to visualize the chromosome banding pattern. The interbands correspond to sites of active transcription, as indicated by double labeling experiments in which the chromosomes are also stained with an antibody specific for a hyperphosphorylated isoform of RNA polymerase II (Pol IIo, panels 1E–1H, and merge in panels 1I–1L). Pol IIo is an activated form of the polymerase, engaged in transcription elongation and localized to active sites of transcription (Weeks et al., 1993; O’Brien et al., 1994; Bregman et al., 1995).

It should be noted that some puffs stain weakly if at all for ribosomal proteins despite strong staining for Pol IIo. This is most apparent in panels I and J (the puffs are indicated by arrowheads). These puffs correspond to heat shock genes, which are occasionally briefly induced during dissection. Under longer and deliberate heat shock induction, these puffs also stain for ribosomal proteins; there is, however, a temporal delay between Pol IIo and ribosomal staining (see below).

The remaining seven antibodies produced weaker signals (level 1, Table 1); they were too faint to draw strong conclusions. But even this staining intensity was above
Figure 2. A Nucleolar Protein Does Not Redistribute to Transcription Sites during Squashing and RNase Sensitivity

(A) Indirect immunofluorescence for ribosomal protein RpS15 (red) and a nucleolar protein, fibrillarin (green). RpS15 localizes to both transcription sites and to the nucleolus, while fibrillarin is exclusively nucleolar. The nucleolus is labeled “no,” “c” denotes the centromeric region in proximity of the nucleolus that often strongly stains for the r proteins. Arrows point to regions in the nucleolus where RpS15 is present and fibrillarin is absent.

(B) Indirect immunofluorescence for RpS15 and RpS30 after incubation with RNase (see Experimental Procedures). Glands were exposed to RNase for less than a minute (i.e., the time necessary to dissect the glands, which roughly takes between 15 and 60 s) or 2 min as indicated in the figure. RpL32, Rpl17, and RpL7a staining is less sensitive to RNase; at least 5 min incubation was required to achieve a drastic reduction.
Ribosomes at Transcription Sites

Figure 3. Ribosomal RNA Is Present at Transcription Sites

(A) Indirect immunofluorescence for the large rRNA subunit, using a Cy3-labeled secondary antibody (top panel, red signal), immunofluorescence for RpL32 (middle panel, green signal). The bottom panel is a merge of the two panels above. The nucleolus is labeled “no.”

(B) In situ hybridization with a probe corresponding to the second half of the 18S rRNA. The probe was synthesized from a PCR fragment by in vitro transcription using T7, which transcribes the antisense strand. The PCR product was amplified with the two following primers: Sp6-18S; 5'-GGGTATTTAGGTGACACTATAGCATTAATCAAGAACGAAAGT-3'; T7-18S; 5'-GGTATTTAGGTGACACTATAGCATTAATCAAGAACGAAAGT-3'. The nucleolus is indicated by the white lines and by the “no” label. The gray panel shows the DAPI staining.

(C) In situ hybridization with the sense probe (Sp6).

pears more RNase resistant: staining is unaffected by very brief incubations with RNase but reduced after longer incubation times (Figure 2B, compare staining at <1 min and after 2 min incubation). Large subunit proteins are somewhat more resistant to RNase, in that incubations of 5 min or longer were required for a drastic reduction in signal (data not shown and Figure 2B legend). The RNase sensitivity could reflect nascent mRNA association or an effect on the ribosome itself. The latter may be particularly relevant for the large subunit antibodies, where longer incubation times were required to drastically reduce the signal (see also the Figure 2B legend). In addition to affecting staining of the chromosomes and the nucleolus, RNase digestion also affects the “background” staining around the chromosomes, which is probably due to ribosomes in the cellular debris.

Ribosomal RNA Is Also Present at Sites of Transcription

The presence of so many ribosomal proteins suggests that complete ribosomal subunits are present at transcription sites. To address this possibility we used two different techniques: immunolocalization with an antibody directed against the rRNA and in situ hybridization with probes complementary to several regions of the 18S and 28S rRNA.

A monoclonal anti-rRNA antibody was previously isolated from mice with a lupus erythematosus-like syndrome, and it was demonstrated that this antibody recognizes an antigenic determinant on the large rRNA subunit rather than a protein component (Lerner et al., 1981). Staining of the polytene chromosomes with this anti-rRNA antibody shows the presence of rRNA at most transcriptionally active loci (Figure 3A, red signal). The same chromosomes were also stained with the anti-RpL32 antibody (Figure 3A, green signal) and the two signals are extensively overlapping (Figure 3A, merge in yellow). As expected, staining is also apparent in and around the nucleolus (labeled “no”).

Because rRNA is in a highly folded conformation and may not be readily accessible to hybridization probes, we made riboprobes corresponding to five different segments of the rRNA spanning essentially the entire 18S and 28S rRNA. From each fragment, we made sense and antisense hapten-labeled riboprobes. Both were used in a highly sensitive two-step in situ hybridization
Molecular Cell

Figure 4. Translation Factors Are Associated with Transcription Sites

Indirect immunofluorescence for initiation factor eIF2 (A) and release factor eRF3 (D and G) using Cy3-labeled secondary antibody (red). Pol IIo is shown in (B), (E), and (H) (green). Merged images are shown in (C) and (F). (G) eRF3 localization to transcription sites is RNA dependent. Indirect immunofluorescence for eRF3 after 1 min of treatment with RNase. (H) Same nucleus as in (G), showing Pol IIo signal.

procedure, in which the primary in situ signal was amplified by TSA (tyramide signal amplification, see Experimental Procedures). An example of these in situ hybridizations, with a probe corresponding to a region of the 18S rRNA, is shown in Figure 3 (Figure 3B, antisense; Figure 3C, sense). The antisense probe clearly stains the interbands of the chromosomes (gray panels, interbands are the segments weakly stained by DAPI). This is particularly visible in the two magnified areas (Figure 3B, insets to the right of the main red panel). Note that the probe strongly stains the nucleolus, which is fragmented (indicated by the lines). The other probes, one corresponding to the remaining portion of the 18S and three other corresponding to the 28S, gave comparable results (data not shown and Figure 3B legend).

Translation Factors Are Also Associated with Sites of Active Transcription

The presence of ribosomes at active transcription sites is an indication that translation might occur at that location. To further address this possibility, we tested whether translation factors are also present at this location.

IF2 is an evolutionary conserved protein that functions in general translation initiation by promoting in yeast and bacteria the binding of the initiator methionyl tRNA to the small ribosomal subunit (Choi et al., 1998). In higher eukaryotes the IF2 orthologous gene, eIF5B, is essential for the joining of the 40S and 60S subunits and for generating a translation competent ribosome (Pestova et al., 2000). The Drosophila homolog of IF2/eIF5B was recently cloned, and an antibody has been generated and characterized (Carrera et al., 2000). The IF2 antibody stained the chromosomes and colocalized with Pol IIo (Figures 4A–4C), similar to what was observed with the ribosomal protein antibodies and by in situ hybridization. Staining is also present around and in the nucleolus (Figure 4A, indicated by the arrow).

We also localized eRF3, essential for polypeptide release from the ribosome (Zhouravleva et al., 1995). The
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Drosophila homolog of eRF3, also known as dSUP35, was recently characterized, and a specific antibody generated (Basu et al., 1998). eRF3 is also associated with transcriptionally active loci (Figures 4D–4F) and is also apparent in the nucleolus. This can be clearly seen in Figure 6P. eRF3 staining was also sensitive to RNase (Figures 4G–4H).

Incorporation of Amino Acid in Polytene Chromosomes

The presence of both ribosomal subunits and translational components suggests that translation may be occurring at transcription sites. To test directly for translation, salivary gland cultures were starved for methionine and then incubated in the presence of 35S methionine/cysteine for different lengths of time. The glands were immediately fixed and squashed and the sites of amino acid incorporation detected by autoradiography. Under these conditions, radioactive amino acids are readily incorporated at the chromosomes. With an incubation period as short as 5 min, we observed very strong amino acid incorporation over the chromosomes and the nucleolus (Figure 5A, the nucleolus is indicated by the arrow). Importantly, the radioactivity appears to be preferentially localized to transcriptionally active regions (Figures 5B–5D). This is visible when the radioactive signal is viewed with the underlying DAPI banding pattern; the weakly stained regions correspond to actively transcribed loci (Figure 5C, note that the radioactive signal and the chromosomes are in two slightly different planes of focus). A high level of radioactivity is also apparent in correspondence with the puff (indicated by the arrow). The level of amino acid incorporation and the pattern at the chromosomes does not vary with incubation times from 5 to 15 min (data not shown).

Amino acid incorporation is not affected by treating the cell with thapsigargin 30 min prior to and during the metabolic labeling (not shown). Thapsigargin is a potent, cell-permeable, IP3-independent intracellular calcium releaser (widely used, from flies to human) that has been shown to rapidly and potently inhibit active and passive transport of proteins into the nucleus (Greber and Gerace, 1995). In contrast, treatment with the translational inhibitor cycloheximide completely blocks incorporation (Figure 5E).

Recruitment of Ribosomal Subunits to Heat Shock Loci

To visualize specific genes and to address the temporal relationship between transcription initiation and factor recruitment, we analyzed heat shock genes. Following a heat shock, these genes rapidly stain for hyperphosphorylated Pol II (Pol IIo; Figures 6B and 6E). Under our experimental conditions, heat shock puffs are apparent at six sites. Five of these sites correspond to genes encoding hs proteins. The largest puffs are at 87A and 87C, which correspond to two loci encoding Hsp70 proteins (Lis et al., 1981). The one at 93D corresponds to a gene that is frequently described as noncoding. However, at least one of its transcripts is polysome associated and engaged in translation (Fini et al., 1989).

Following a 5 min heat shock, strong staining for Rpl32 is also observed at 93D (which is also expressed at lower levels at room temperature). Weaker staining is also apparent at 87A/C (Figure 6A). After a 45 min heat shock, Rpl32 staining was strong at all 6 puffs, and there was a clear correlation with the strength of the Pol IIo signal (Figures 6D–6F). A similar staining pattern was also observed after a 15 and 30 min heat shock, and with anti-RpS15 or anti-RpS30 reagents (data not shown). Figures 6G, 6J, and 6M show the portion of the right arm of chromosome III that spans the region between 93D and 87A/C. After a 45 min heat shock, there is a strong staining for Rpl32 at 87A/C, and a rapid decrease in staining intensity is observed after a 5 min recovery from heat shock (compare figures 6J and 6M).
Figure 6. Heat Shock Induces the Recruitment of Ribosomal Proteins to Sites of Transcription

Indirect immunofluorescence for RpL32 (A and D) and Pol IIo (B and E) after 5 and 45 min heat shock as indicated. Merged images are shown in (C) and (F) (similar staining was obtained after 15 and 30 min heat shock—not shown). Staining for RpL32 and pol IIo of the 93D and 87A/C region of chromosome III at room temperature (G–I), after 45 min (J–L), and after 5 min recovery from heat shock (M–O). Immunostaining with anti-eRF3 (red) and anti-Pol IIo (green) after 5 min heat shock (P and Q) and after 45 min (R and S). Note that with the exception of 93D, eRF3 staining is drastically reduced all over the chromosomes.

eRF3 staining is also observed after a 5 min heat shock (Figure 6P). Unlike RpL32, however, most of the signal at 87A/C is gone after 45 min, whereas the signal at 93D remains strong (Figure 6R). It should also be noted that after a 45 min heat shock the staining for eRF3 is drastically reduced all over the chromosomes except at 93D (compare Figures 6P and 6R).

In summary, these data show that transcriptional induction by heat shock is rapidly followed by the recruitment of ribosomal proteins and eRF3. A prolonged heat shock causes a drastic reduction in global eRF3 staining, whereas the RpL32 staining remains strong. Perhaps there is some translation inhibition in response to a persistent heat shock.

Ribosome Recruitment Is Cotranscriptional

RNA associated with chromosomes is traditionally considered to be nascent, meaning that it still contacts pol
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Figure 7. Ribosomal Protein Recruitment Can Occur Cotranscriptionally

(A) Indirect immunofluorescence for RpL32 (red) and Pol II o (green) in chromosome squashes from glands incubated with 1 μM Ecstasy for varying lengths of time, as indicated. Staining in the absence of ecysone is shown in the top panels. DAPI staining is shown in white. White lines indicate the positions of Eip74EF (E74) and Eip75B (E75). Blue lines indicate a locus (arbitrarily named 74) that is not induced by ecysone. Merged images are shown to the right. The arrow indicates the border between division 74 and 75.

(B) eRF3 is recruited to sites of transcription cotranscriptionally. Immunostaining with anti-eRF3 (red) and anti Pol II o (green) of squashes from glands incubated with 1 μM Ecstasy for varying lengths of time as indicated.

II and is not yet cleaved and polyadenylated. However, studies in S. cerevisiae indicate that cleaved and polyadenylated transcripts may remain associated with their sites of transcription (Jensen et al., 2001). In addition, some preliminary observations in our laboratory indicate that polyadenylated transcripts accumulate at heat shock puffs (data not shown). Given these results and considering that the poly(A) tail plays an important role in ribosomal recruitment and translation initiation in the cytoplasm, it seemed possible that the ribosomal protein staining only reflects an association with nonnascent RNAs.

To address this possibility, we extended the analysis to two well-studied ecysone inducible loci, Ecysone-induced protein 74EF (Eip74EF) and Ecysone-induced protein 75B (Eip75B). These genes are organized into large transcription units: Eip74EF spans 60 kb of DNA, and Eip75B spans 108 kb (Burtis et al., 1990; Segraves and Hogness, 1990). Both genes can be directly induced by ecysone in salivary glands cultured in vitro (Ashburner, 1972, and Experimental Procedures). It has been estimated that under these conditions the rate of trans- 

cription, for Eip74EF at least, is 1.1 kb/min and the mature mRNA first appears after 1 hr (LeMaire and Thummel, 1990). Under similar conditions, completion of Eip75B transcription would take at least 90 min. We found that ribosomal protein recruitment to Eip74EF and Eip75B is apparent as early as 15 min after ecysone induction (Figure 7A). A similar experiment with eRF3 shows that eRF3 association also occurs as early as 15 min after induction (Figure 7B). Surprisingly, the recruitment of eRF3 starts well before transcription of the translation termination codon in the main ORF, for both Eip74EF and Eip75B. For Eip74EF, this may be explained by the fact that the mRNA contains an unusually long 5’ UTR (1891 nucleotides) with 17 short upstream ORFs (Burtis et al., 1990). The 5’-UTR of Eip75B has not yet been analyzed.

Both genes have several introns. Eip74EF has two large introns, one of 15 kb at the beginning of the ORF and a second of about 30 kb between exons 5 and 6. Eip75B has several smaller introns, a large 22 kb intron between exons 5 and 6 and a 60 kb intron between exons 7 and 8. The kinetics of translation factor recruitment therefore suggest that ribosomes associate with nascent transcripts before all splicing is completed.

Discussion

Here we provide evidence that the translation machinery is associated with sites of active Pol II transcription. The evidence is based on results from two independent techniques, immunostaining and in situ hybridization. Using immunofluorescence, we have localized 20 different ribosomal proteins, two translation factors, and 28S rRNA to the sites of transcription. The antibodies used also strongly stain the nucleolus, and with few excep-
tions there is a good correlation between chromosomal and nucleolar staining intensity (Table 1). The fact that most of these antibodies have little or no crossreactivity on Western blots (Nadano et al., 2000) as well as the shared nucleolar staining makes it unlikely that the similar staining patterns with so many antibodies are due to signals from crossreacting proteins. Moreover, the presence of ribosomal subunits is also indicated by the situ hybridization, which identifies both 18S and 28S rRNA at these sites. The association is not limited to a few loci but is a global phenomenon in which essentially all transcriptionally active regions of the chromosomes are richly decorated with ribosomal subunits and translation factors. The concurrent presence of the ribosomal proteins and the two large ribosomal RNAs strongly indicates the presence of complete ribosomal subunits rather than individual components with some nonribosomal function (Eng and Warner, 1991).

Staining for both ribosomal proteins and translation factors is sensitive to RNase, suggesting that ribosomal subunits are associated with nascent transcripts (Figure 2B). However, the RNase treatment should also affect rRNA, which would release proteins. In any case, the kinetics of recruitment following transcriptional initiation indicates that association occurs cotranscriptionally. The data therefore suggest that ribosomes are loaded onto nascent transcripts in salivary gland nuclei.

The presence of ribosomes and translation factors makes it feasible that translation is occurring at these sites. Indeed, we find that amino acids are readily incorporated into proteins at these chromosomal locations in 5 min (Figure 5A). Although we cannot at present rule out the possibility that newly synthesized proteins enter the nucleus during the 5 min incubation with radioactive amino acids, the short time as well as use of the protein import inhibitor suggests that some of the incorporation is due to chromosomal translation.

However, an alternative or parallel scenario is that these ribosomes have some function other than protein synthesis. For example, they may be preloaded onto nascent mRNA prior to function in the cytoplasm (reviewed by Lykke-Andersen, 2001). Ribosomal subunits may therefore be components of the mRNP in transit to the cytoplasm, and a significant portion of ribosomal subunits may be exported via mRNP complexes.

Consistent with the idea that some fraction of the ribosomal subunits are not active, neither recruitment nor retention is detectably affected by treating the glands with the translation inhibitors puromycin or cycloheximide for 20 min prior to squashing (data not shown). Although some reduction for small subunit proteins was apparent under longer incubation times with puromycin (>1 hr), this may be an indirect effect of the drug. One possibility is that ribosomal subunits are recruited and retained at transcription sites without protein synthesis. For example, the situation may be similar to that of ER bound ribosomes, where puromycin treatment does not release the large ribosomal subunit from the membrane (Seiser and Nicchitta, 2000). With regard to recruitment, ribosomal subunits could be recruited by the transcription machinery. However, the delay between heat shock transcriptional initiation and ribosome recruitment argues against this possibility, at least for heat shock genes. It is also possible that puromycin is not effective in reaching the nuclear interior, or that translation in the nucleus is somehow differentially sensitive to puromycin. Indeed, in vivo protein labeling and PAGE analysis indicate that there is significant residual synthesis of full-length proteins in the presence of puromycin in salivary glands (data not shown).

In conclusion, we have provided evidence that ribosomes are a component of nascent RNPs. Further work, biochemical as well as ultrastructural, will be required to understand the function of these ribosomes and to define the connections between ribosomes, pre-mRNA processing, and NMD at sites of transcription.

Experimental Procedures

Stocks and Culture Methods
All of the work was done using the Canton-S strain of D. melanogaster. The larvae were cultured at 18°C in bottles containing yeast glucose food and Bromphenol Blue (Sigma). Salivary glands were dissected from wandering third instar larvae with a blue stained gut, and incubated in about puffs at stage 1 (Ashburner, 1967). Glands were cultured in supplemented Grace medium (Gibco) diluted 5:1 with a 10% solution of ethanol (Ashburner, 1972). The glands were cultured in about 50 μl of the same medium in a humid chamber at room temperature. Larvae were heat shocked at 37°C in a 1.5 ml plastic microfuge tube containing a small strip of wet tissue paper in a water bath.

Chromosome Squashing and Immunostaining
Salivary glands were dissected in 15 mM Hepes (pH 7.4), 60 mM KCl, 15 mM NaCl, 1.5 mM Spermine, 1.5 mM Spermidine, and 10% Triton. If required, Ribonuclease A was added at 1 mg/ml to the dissection buffer and incubated at room temperature. Immediately after dissection, the glands were fixed for approximately 1 min in the same buffer as above plus 4% formaldehyde. The protocol for chromosome squashing and staining is a modification of one previously published (Shopland and Lis, 1996). Slides were usually stored at –20°C in absolute ethanol and, prior to immunostaining, the chromosome spreads were rehydrated in TBS (150 mM NaCl and 10 mM Tris [pH 7.5]) and blocked in 10% FBS (fetal bovine serum) in TBS supplemented with 200 μg/ml tRNA (weaker staining seems to be enhanced by this addition). All chromosome spreads were stained with DAPI (10 min with a 100 ng/ml solution). The preps were mounted with Vectashield (Vector) and viewed using a Zeiss Axioshot epifluorescence microscope; images were captured with a digital CCD camera and manipulated using the Openlab software (Improvision).

Antibodies

The antibodies were typically diluted in blocking buffer and used at dilutions between 1:50–1:200. The antibodies against ribosomal proteins were protein-A purified and have previously been characterized (Nadano et al., 2000). The serum reacting against the P ribosomal proteins (Rpl12) was provided by Keith Elkon (Elkon et al., 1986). The anti-DSUP35 (eRF3) antibody was a gift from Michael Goldberg (Basu et al., 1998). The anti-H2 antibody was provided by Paul Lasko and affinity purified (Carrera et al., 2000). The antibody against the hyperphosphorylated Pol II (H5; purchased from Babco) was used at a 1:1000 dilution. The antibody against fibrillarin (Nop1p) was provided by John Aris (Aris and Blobel, 1988).

All secondary antibodies were purchased from Jackson Immuno Research Technologies and used as recommended. In the double labeling experiments, only preabsorbed antibodies (multiple labeling grade) were used to prevent interspecific crossreaction. In the absence of primary antibody, none of the secondary antibodies used in this study gave a signal.

In Situ Hybridization and rRNA Probes

Gland treatment and chromosome squashing were done as described above and then dehydrated in ethanol. The chromosomes were rehydrated in PBS, fixed in 4% formaldehyde/PBS for another
Hybridization was performed overnight in a hybridization chamber (Corning Inc.) at 42°C in 15 µl of hybridization buffer plus 1 µl of denatured riboprobe (see below) under a coverslip. Slides were washed first in 2× SSC/50% formamide and then in 2× SSC at 42°C. The detection was performed by using a HRP-conjugated anti-Dig antibody (Roche Molecular Biochemicals) and the signal visualized by TSA amplification (Nen Life Science Products).

DIG-labeled riboprobes were directly synthesized from PCR amplified DNA fragments (about 100 ng) using either T7 or Sp6 RNA polymerase (DIG RNA labeling kit, Roche Molecular Biochemicals).

Metabolic Labeling and Autoradiography

Salivary glands were dissected in Grace’s insect medium (GIBCO-BRL, without methionine), transferred to 40 µl of the same medium, and methionine starved for 1–2 hr. Labeling was with 10 µCi of TRAN35S-label (ICN) for 5 min or longer. Chromosome squashes were prepared as described above. To detect the signal, slides were coated with autoradiographic emulsion (Hypercoat LM-1, Amer sham), exposed overnight, and developed (developer and fixer were purchased from Polysciences Inc.).

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