The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes

Edward B. Mougey,[1] Marina O'Reilly,[2,3] Yvonne Osheim,[4] Oscar L. Miller Jr.,[2] Ann Beyer,[4] and Barbara Sollner-Webb[1]

[1]Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205 USA; [2]Department of Biology and [4]Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908 USA

When spread chromatin is visualized by electron microscopy, active rRNA genes have a characteristic Christmas tree appearance: From a DNA "trunk" extend closely packed "branches" of nascent transcripts whose ends are decorated with terminal "balls." These terminal balls have been known for more than two decades, are shown in most biology textbooks, and are reported in hundreds of papers, yet their nature has remained elusive. Here, we show that a rRNA-processing signal in the 5′-external transcribed spacer (ETS) of the *Xenopus laevis* ribosomal primary transcript forms a large, processing-related complex with factors of the *Xenopus* oocyte, analogous to 5′ ETS processing complexes found in other vertebrate cell types. Using mutant rRNA genes, we find that the same rRNA residues are required for this biochemically defined complex formation and for terminal ball formation, analyzed electron microscopically after injection of these cloned genes into *Xenopus* oocytes. This, plus other presented evidence, implies that rRNA terminal balls in *Xenopus*, and by inference, also in the multitude of other species where they have been observed, are the ultrastructural visualization of an evolutionarily conserved 5′ ETS processing complex that forms on the nascent rRNA.

[*Key Words*: rRNA processing complex; terminal balls; eukaryotic rRNA transcription units; chromatin spreads; *X. laevis*]

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One of the earliest, yet still highly informative and much used methods of studying eukaryotic gene expression, is by direct electron microscopic visualization of the transcribing chromatin (Miller and Beatty 1969a). This technique of native chromatin visualization (forming so-called Miller spreads) has facilitated numerous advances in the analysis of gene expression. These include (1) the finding that eukaryotic rRNA genes are organized typically in tandem head-to-tail arrays separated by "nontranscribed spacers" (Miller and Beatty 1969a); (2) the demonstration that the polarity of ribosomal transcription is from 18S to 28S (Reeder et al. 1976), (3) the first analyses of the synchrony of rRNA transcriptional turnon during development (McKnight and Miller 1976; Foe 1978; Franke et al. 1979); (4) the first indication of transcription of cloned rRNA genes (Trendelenburg and Gurdon 1978); and (5) the ability to visualize spliceosome complexes on nascent RNA polymerase II-driven transcripts (Osheim et al. 1985; Beyer and Osheim 1988).

[3]Present address: Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218 USA.

Because of their ease of identification, rRNA genes are the most amenable to study in Miller spreads. In the resultant micrographs, the active rRNA-coding regions have a characteristic tree-like appearance, with a DNA "trunk" from which close-packed ribonucleoprotein "branches" of increasing length extend. What distinguishes rRNA transcription units from the multitude of RNA polymerase II-driven transcription units is not only their size and high density of nascent transcripts but also the characteristic presence of a "terminal ball" (also called terminal knob and terminal granule) at the distal ends of the ribosomal transcripts (Miller and Beatty 1969a). Such terminal balls are not typically found at the ends of polymerase II-driven transcripts (e.g., Miller and Bakken 1972; McKnight and Miller 1976). Possibly because of these terminal balls decorating the rRNA branches, active ribosomal genes in such spreads are routinely referred to as "Christmas trees," not merely as "pine trees."

First reported on ribosomal transcripts in amphibians [*Xenopus* and *Triturus* (*Notophthalmus*)], terminal balls have been observed since then on rRNA gene transcripts

across eukaryotes, in all tissues and organisms examined, including algae (*Acetabularia*), fungi (*Saccharomyces*), slime molds (*Physarum*), higher plants (*Zea mays*), insects (*Drosophila*, *Dytiscus*, *Bombyx*), birds (quail), and mammals (mouse, rat, hamster, human) (Miller and Beatty 1969a,b; Miller and Bakken 1972; Hamkalo et al. 1973; Spring et al. 1974; Trendelenburg 1974; McKnight et al. 1976; Puvion-Dutilleul et al. 1977a,b; Grainger and Ogle 1978; Jamrich et al. 1979; Greimer and Deltour 1984; Saffer and Miller 1986; Scheer and Benavente 1990). Yet, despite the characteristic presence of the terminal balls reported in well over a hundred papers over the last 23 years (for additional early references, see Franke et al. 1979), their function and biochemical nature are still unknown.

The ubiquitous presence of terminal balls suggests that they represent a feature of ribosomal gene expression that is conserved across eukaryotic evolution. This conservation is especially striking because terminal balls appear to form in the rapidly diverging 5′-external transcribed spacer (ETS) region of the rRNA. It has been suggested that the terminal balls represent a coiling up of a substantial 5′ portion of the nascent pre-ribosomal ribonucleoprotein (pre-rRNP) (Puvion-Dutilleul et al. 1977b; Franke et al. 1979; Puvion-Dutilleul 1983) or that they may be part of ribosomal particles that are forming on the nascent rRNA (Puvion-Dutilleul 1983; Osheim and Beyer 1985), possibly facilitating the formation and export of the 40S ribosomal subunit to the cytoplasm (Miller and Hamkalo 1982). Other possibilities include their being a remnant of the RNA polymerase I initiation process, components bound to the 5′ polyphosphate group that is retained on the ribosomal primary transcript, or a rRNA processing complex (Reeder et al. 1979; Sollner-Webb and Mougey 1991).

In separate studies, we have identified a processing event that takes place early in the maturation of mouse pre-rRNA and removes the first 650 nucleotides of the primary transcript. This processing is readily reproduced in vitro using mouse cell extracts (Miller and Sollner-Webb 1981) and it occurs efficiently in vivo, as ~90% of mouse pre-rRNA molecules lack the first 650 nucleotides of the primary transcript (Miller and Sollner-Webb 1981; Gurney 1985). A 120-nucleotide region immediately downstream of the processing site directs this processing (Craig et al. 1987, 1991; Fig. 1), and it forms a sizable specific complex that sediments at ~20S, with a number of distinct polypeptides of the extract (Kass and Sollner-Webb 1990). This 5′ ETS processing also requires the U3 small nuclear ribonucleoprotein (snRNP) (Kass et al. 1990). The mouse processing signal is ~85% conserved in human and rat pre-rRNA, starting at residues 422 and 793, respectively, as well as in other mammals, and these regions also direct processing (Kass et al. 1987; Stroke and Weiner 1989; Tower et al. 1989). A report that *Xenopus* oocyte pre-rRNA does not exhibit processing in its 5′ ETS (Savino and Gerbi 1991) suggested that this kind of rRNA processing might be limited to mammalian species. However, we found recently that *Xenopus laevis* pre-rRNA of cultured kidney cells undergoes a 5′

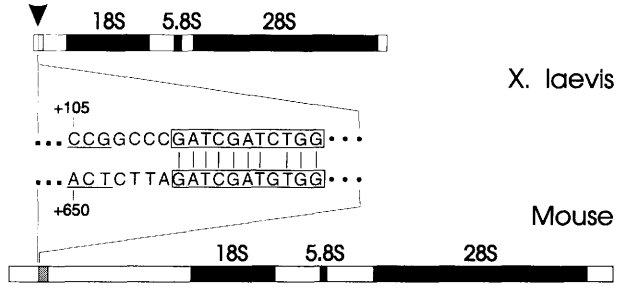

Figure 1. The 5′ ETS processing region. The entire *X. laevis* (*top*) and mouse (*bottom*) pre-rRNA and expansions of a 5′ portion are represented. The arrowhead indicates the 5′ ETS processing site. The stippled boxes (next to arrowheads) denote 120- and 200-nucleotide segments that are conserved in *X. laevis–X. boralis* rRNA and in mouse/human/rat/CHO rRNA, respectively; the first 11 nucleotides of these segments are conserved between frog and mammals (boxed residues in the inner diagram; Bourbon et al. 1988). Furlong et al. (1983) noted most of the *X. laevis/X. borealis* conserved sequence. The conserved sequence block in mouse and human rRNA was reported by Kass et al. (1987), whereas that of rat was reported by Bourbon et al. (1988). The processing sites are indicated by the underlining in the middle diagrams to the left of the boxed segment; the 18S region starts at residue 714 and 4007 in *X. laevis* and mouse, respectively.

ETS processing that is strictly analogous to that of mouse in numerous respects, including in vitro reproduction of this processing both in *Xenopus* and in mouse cell extracts, the presence of a 120-nucleotide region just downstream from the +105 processing site that is conserved between various *Xenopus* species (Fig. 1), the association of the processing region with a number of polypeptides of the cell extract to form a specific ~20S complex, and a requirement for the U3 snRNP in the processing (Mougey and Sollner-Webb, 1993). The first 11 nucleotides of the *Xenopus*-conserved signal and the mammalian-conserved signal are the same, and this element is critical both for the frog and for the mouse processing reactions.

In this study we show that the terminal balls characteristic of ribosomal transcription units are the ultrastructural visualization of the 5′ ETS rRNA processing complex that we have studied biochemically. To this end, various *Xenopus* rRNA derivatives have been analyzed for their ability to direct processing and complex formation; parallel constructs were analyzed for terminal ball formation by microinjecting plasmid templates into *Xenopus* oocyte nuclei and visualizing spreads of the resultant nucleoprotein by electron microscopy. Processing-competent rRNAs are shown to form processing complexes and terminal balls, whereas processing-incompetent RNAs do neither. These parallel data, plus several other observations, indicate that terminal balls represent the processing complex that has been analyzed biochemically. Furthermore, the fact that terminal balls have been seen in rRNA spreads all across eukaryotic species suggests that this 5′ ETS processing complex for-

mation has a conserved and evolutionarily advantageous function.

Results

The visualization of terminal balls in electron microscopic spreads of transcribing ribosomal chromatin demonstrates that these structures are large and ubiquitously present at or near the 5′ end of nascent rRNA transcripts throughout eukaryotic species. Although it was initially thought that such 5′ ETS processing involving formation of a large complex may be specific to mammals, the recent demonstration of a completely analogous 5′ ETS processing in *X. laevis* tissue culture cells (Mougey and Sollner-Webb, 1993) raises the possibility that the 5′ ETS-processing complex might be what is visualized as the terminal ball structures. Consistent with this proposal, the in vitro-processing complex is ~20S, it is located near the 5′ end of the pre-rRNA, and, at least in mouse, the processing complex forms rapidly and remains on the nascent pre-rRNA after cleavage (Kass and Sollner-Webb 1990; Mougey and Sollner-Webb, 1993). In apparent contradiction to this hypothesis, however, *Xenopus* oocytes—which were one of the original cell types in which terminal balls were observed (Miller and Beatty 1969a,b)—have been reported not to undergo 5′ ETS processing (Savino and Gerbi 1991).

To determine whether *Xenopus* oocytes exhibit 5′ ETS processing, we used S1 nuclease analysis to examine RNA from isolated *Xenopus* oocyte nuclei for 5′ ends corresponding to the approximately +105 ETS processing site (Fig. 2A). Such molecules are indeed obtained from these germinal vesicles (lane 3; G.V.), and their 5′ ends map to residues +105 to +107 of the pre-rRNA, the same positions as the processed rRNA in the cultured kidney cells (lanes 1, 2; sequence shown in Fig. 1). In the *Xenopus* oocyte, however, the abundance of the processed rRNA is low, only ~1% that of the unprocessed rRNA. We have also been unable to detect 5′ ETS processing of in vitro-made rRNA that is added to a homogenate of oocyte nuclei or microinjected into nuclei of intact oocytes (data not shown), whereas extracts of cultured *X. laevis* kidney cells are active for this 5′ ETS processing (Mougey and Sollner-Webb, 1993). Thus, *Xenopus* oocytes do contain 5′ ETS processing activity, but one of the requisite processing components may be quite limiting. This limiting component could be one that is needed for the formation of the complex on the 5′ ETS processing sequences or it could be one that is necessary for processing but does not stably associate with the substrate rRNA.

Because terminal balls are observed in *Xenopus* oocytes, both on the nascent pre-rRNAs on the $>10^6$ genomic and amplified rRNA genes (Miller and Beatty 1969a,b) and on the transcripts of microinjected rRNA genes (Trundelenberg and Gurdon 1978; Bakken et al. 1982), the components that form these structures must be rather abundant in the oocyte. We therefore examined whether *Xenopus* oocytes have an excess of the factors needed to assemble the 5′ ETS complex. This experiment (Fig. 2B) used the in vitro transcript *X293* RNA that contains *X. laevis* rRNA residues 28–293 and is processing-competent in the *Xenopus* kidney cell extract, as

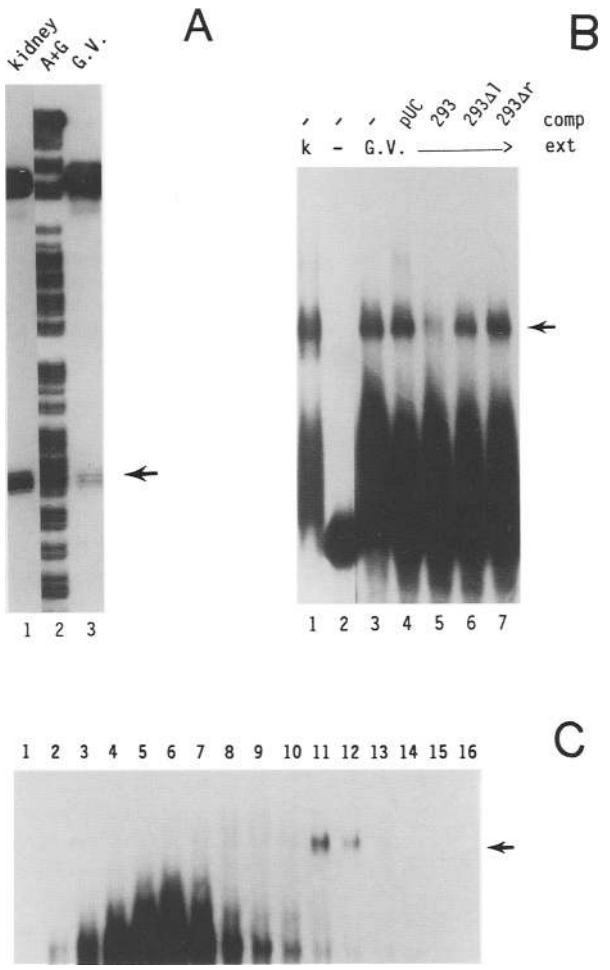

Figure 2. Processing and specific complex formation in the *X. laevis* RNA 5′ ETS in oocytes. (*A*) The 5′ ends of cellular RNA isolated from cultured kidney cells (lane *1*) or germinal vesicles [(lane *3*); GVs or oocyte nuclei] were mapped by S1 nuclease analysis using a 5′-end-labeled probe. The dark upper band represents RNA that starts at residue +1; the bands indicated by the arrow represent RNAs whose 5′ ends map to residues +105, +106, and +107. Lane *2* is an A+G sequence analysis of the probe DNA (Maxam and Gilbert 1977). (*B*) *X293* RNA (lane *2*) was incubated in a reaction with cultured kidney cell extract (lane *1*) or with GVs (oocyte nuclei) that had been preincubated with no competitor (lane *3*) or with the indicated processing-competent (lane *5*) and processing-incompetent (lanes *4*, *6*, *7*) competitor RNAs. The resultant nucleoprotein was resolved by native polyacrylamide gel electrophoresis. The arrow indicates the specific retarded complex. (*C*) *X293* RNA was incubated with oocyte nuclear contents, and the reaction was fractionated by velocity centrifugation. Aliquots of the indicated fraction (lane *1*, top of gradient) were analyzed on a nondenaturing polyacrylamide gel. The arrow indicates the specific retarded complex, which comigrates with the complexes in *B*. Sedimentation markers of catalase, thyroglobulin, and 18S and 28S *Xenopus* rRNA (with S_w^{20} values of 11S, 19S, 18S, and 28S, respectively) were centered in fractions 7, 11, 11.3, and 16.8, respectively.

well as two processing-incompetent derivatives that lack the left and right portions of the essential 11-nucleotide conserved element of the processing signal, the *X293Δl* and *X293Δr* RNAs (see Materials and methods, Fig. 1; Mougey and Sollner-Webb, 1993). In a homogenate of *Xenopus* oocyte nuclei, processing-competent *X293* RNA assembles a slowly migrating complex (Fig. 2B, lane 3; position indicated by the arrow). Using extracts that were preincubated with unlabeled competitor RNAs, this complex is seen to be specific for the rRNA 5′ ETS processing signal. Preincubation with the *X293* RNA inhibits complex formation on the radiolabeled rRNA added subsequently (Fig. 2B, lane 5), whereas preincubation with processing-incompetent *X293Δl* or *X293Δr* RNA or with pUC RNA does not (lanes 4,6,7). Furthermore, this oocyte-formed complex comigrates with the specific complex that forms on *X293* RNA in an extract of *Xenopus* kidney tissue culture cells (Fig. 2B, lane 1; Mougey and Sollner-Webb, 1993). This indicates that *Xenopus* oocyte nuclear factors assemble a specific complex on exogenously added copies of the 5′ ETS processing signal.

From the amount of 5′ ETS complex formed by the oocyte nuclear components (Fig. 2B, data not shown) and the number of pre-rRNA molecules per oocyte (Sollner-Webb and McKnight 1982), we calculate that the oocyte has a capacity for complex formation that is ~10% in excess over its endogenous content of pre-rRNA molecules. This is approximately the same as the capacity for complex formation by the tissue culture cell extract, calculated relative to that cell's pre-rRNA content (data not shown). Thus, although oocytes exhibit only a limited extent of 5′ ETS processing (Fig. 2A), they appear to have an excess of the components needed to form the specific 5′ ETS complex.

The size of the 5′ ETS complex formed by the *Xenopus* oocyte factors was assessed using velocity centrifugation. The complex on *X293* RNA has a sedimentation coefficient of ~20S (Fig. 2C). This is the same S value as for the 5′ ETS processing complexes formed on mouse pre-rRNA in the mouse cell extract (Kass and Sollner-Webb 1990) and that formed on *Xenopus* pre-RNA in the kidney cell extract (Mougey and Sollner-Webb, 1993). It is also approximately the sedimentation coefficient of four nucleosomes. The 5′ ETS processing complex, therefore, must be a sizable structure, presumably >100 Å in diameter, that should be visible in electron microscopic spreads.

Although the biochemical analysis shows that *Xenopus* oocytes have sufficient capacity for the formation of a processing-related complex of the appropriate size to be a terminal ball structure, there are statements in the literature indicating that terminal balls only form on pre-rRNA molecules that are several kilobases in length (e.g., Puvion-Dutilleul et al. 1977b; Franke et al. 1979; based on observations in *Triturus* and rat), posing a potential counterargument to this hypothesis. These observations were interpreted to indicate that terminal ball formation involves rRNA sequences located kilobases beyond the 5′ ETS processing segment, but alternatively they could reflect the kinetics of terminal ball formation in these organisms. In fact, re-examination of electron microscopic spreads from *Xenopus* oocytes showing nascent transcripts of cellular rRNA genes (Miller and Beatty 1969a; Fig. 3A) and showing transcripts on microinjected full-length rRNA genes (Trendelenburg and Gurdon 1978; Bakken et al. 1982; Fig. 3B) demonstrates terminal balls already on short RNAs near the 5′ end of the transcription unit (Fig. 3A,B). They can be seen on the transcripts by the third or fourth polymerase, which is the electron-dense bead at the base of each transcript, located every 80–100 nucleotides along the gene. Therefore, terminal balls can form on *Xenopus* pre-rRNAs that are ~300–400 nucleotides in length. This coincides well with the region shown above for direct formation of the processing complex.

To investigate the correlation of terminal balls with the 5′ ETS processing complex, we first prepared a derivative of a full-length rRNA gene from which was deleted a 2-kb segment including the 5′ ETS-processing signal (the segment beginning at residue +13 and encompassing the rest of the ETS and most of the 18S region; diagramed at the bottom of Fig. 4). Upon microinjection into *Xenopus* oocyte nuclei, this *EA·3′* gene is actively transcribed; Figure 4 is a montage of resultant micrographs and their tracings. Rather than forming Christmas trees, however, the *EA·3′* construct forms pine trees, with nascent transcripts lacking the usual terminal decorations. Terminal balls can be assessed easily by comparing the ends of the transcripts with the granules representing the RNA polymerase molecules along the DNA axis (Fig. 3 and 4). The endogenous cellular rRNA genes present in these same spreads displayed the usual terminal ball structures (data not shown). Thus, terminal ball formation requires rRNA sequences within the 2-kb segment downstream from residue +13, including the 5′ ETS processing site. This result also indicates that terminal ball formation is not inherent in the RNA polymerase I transcriptional process per se or in the 5′ polyphosphate terminus of the resultant transcripts.

To determine whether the rRNA sequences of the *X293* construct that direct formation of the processing complex (Fig. 2B; Mougey and Sollner-Webb, 1993) also support terminal ball formation, the 266-bp rDNA segment from the X293 gene was inserted into the 5′ portion of the transcribed region of the *EA·3′* gene, yielding *EA293·3′* (rRNA residues 28–293; see Materials and methods). The processing-competent *X293* segment is now present at the 5′ end of each transcript. After microinjection into *Xenopus* oocytes and electron microscopic visualization, *EA293·3′* is seen to form transcription complexes of the Christmas tree variety, with transcripts bearing terminal balls. Representative resultant micrographs are shown in Figure 5. Thus, the same small rRNA segment that directs assembly of the biochemically detected processing complex also directs terminal ball formation.

The data in Figure 6 demonstrate that the same 11-nucleotide conserved region is critical for terminal ball

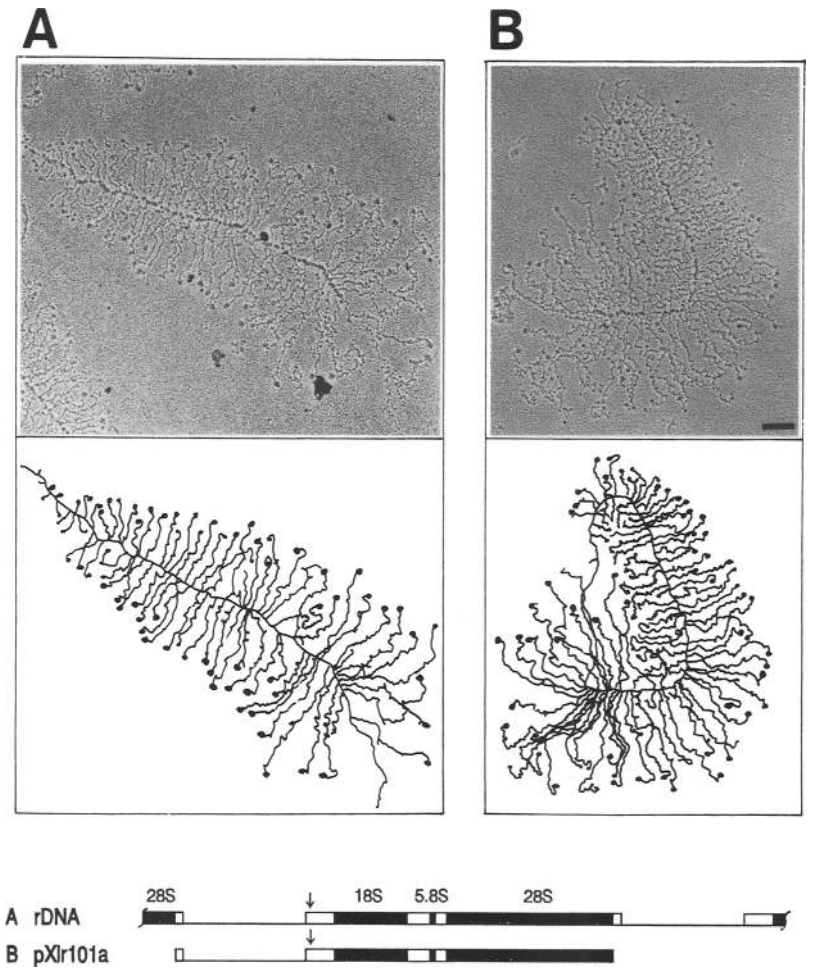

Figure 3. Electron microscopic visualizations of transcription units on intact rRNA genes. (*A*) *X. laevis* oocyte nuclei were isolated, and their contents dispersed and deposited on grids by the chromatin spreading and electron microscopic visualization technique of Miller and Beatty (1969a; Miller and Bakken 1972). A transcription unit from an amplified genomic rRNA gene array is shown, as well as a tracing of the micrograph. The beads representing the polymerase at the base of each transcript are not shown in the tracings. (*B*) pXlr101a, a complete repeating unit of *X. laevis* rDNA cloned into pBR322, was microinjected into the nuclei of *X. laevis* oocytes, which were subsequently spread and visualized as in *A*. Bar, 0.2 μm. Below is a diagram of a segment of the genomic rDNA repeat and the cloned rDNA repeating unit present in pXlr101a. Arrows indicate the 5′ ETS processing site.

formation and for rRNA processing complex formation. To this end, we prepared a construct identical to *EA293·3′*, except that the 11-nucleotide critical segment of the processing signal was inactivated by the Δl mutation. This mutation makes the RNA incompetent for processing complex formation (see Fig. 2B). Transcription complexes visualized from this *EA293Δl·3′* construct, after its microinjection into *Xenopus* oocytes, are again of the pine tree variety—densely packed transcript branches lacking terminal ball structures (Fig. 6). Because the processing complex assayed biochemically by gel retardation, velocity centrifugation, and competition ability is a large structure that forms on the same small rRNA region and requires the same critical residues as the terminal ball structures detected by electron microscopy, we conclude that the latter structure is the ultrastructural visualization of the former.

Discussion

When visualized by electron microscopy in spread chromatin preparations, transcribing rRNA genes are known to form Christmas tree-like structures with terminal balls characteristically decorating the 5′ ends of the nascent rRNA branches (Miller and Beatty 1969a). The identity and role of these terminal balls has remained unknown, despite their observation in scores of species, reported in well over a hundred publications (representative references are listed in the introductory section). In a separate study, a site of processing complex formation has been identified in the *X. laevis* precursor rRNA at residue +105 of the 5′ ETS, 600 nucleotides upstream of the 18S region (Fig. 2; Mougey and Sollner-Webb, 1993; Fig. 1). This processing in the frog is the analog of 5′ ETS processing in the mouse (Miller and Sollner-Webb 1981), and it directs formation of a specific complex in *Xenopus* oocytes as well as in *Xenopus* tissue culture cells. The large structures that the processing region of both frog and mouse assemble with factors of the cell extract sediment at ~20S (Fig. 2B,C; Kass and Sollner-Webb 1990). The *Xenopus* and mouse 5′ ETS processing signals also share a common 11-nucleotide sequence just beyond the processing site that is required both for the processing and for specific complex assembly, in both the frog and mouse systems (Fig. 1 and 2B; Mougey and Sollner-Webb, 1993). We will now summarize the considerable evidence that this 5′ ETS processing complex is the ultrastuctural basis for the terminal balls.

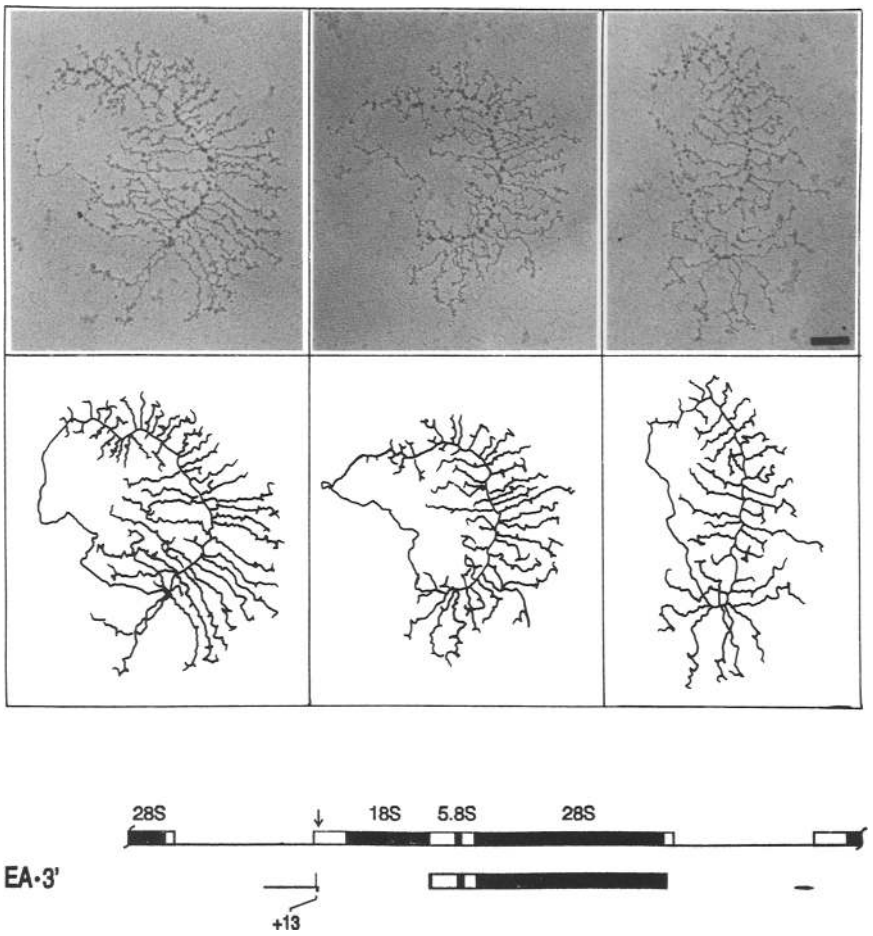

Figure 4. Electron microscopic visualization of active *EA·3′* genes. The plasmid *EA·3′* was microinjected, spread, and visualized as in Fig. 3B. Three examples with tracings are shown. Note that terminal balls, which in Fig. 3 were larger than the polymerases, are not observed on the *EA·3′* plasmid transcripts. In these same spreads, the chromatin of the endogenous cellular rRNA genes showed terminal balls, as in Fig. 3A. For all plasmids studied, at least 15 well-spread molecules were examined, and all showed patterns typified by the presented micrographs. Bar, 0.2 μm. In the diagram aligned below the rDNA repeat are shown those rDNA segments plus the marker segment (the small box below the line) that are cloned to form *EA·3′*. The *EA·3′* transcript lacks a 2-kb segment present in the genomic rRNA transcript, starting at residue +28.

Terminal balls cannot be the result of condensation of several kilobases of the primary ribosomal transcript, as had been proposed (Puvion-Dutilleul et al. 1977b; Franke et al. 1979), as they are already present on *Xenopus* pre-rRNA transcripts of ~300-nucleotide length (Fig. 3). This can also be concluded from re-examining published micrographs of *X. laevis* spreads (e.g., McKnight and Miller 1976; Trendelenburg 1981) and from transcript mapping (Osheim and Beyer 1985; see also Discussion in Miller and Bakken 1972). The terminal balls also cannot be inherent to RNA polymerase I-catalyzed transcription units, as they are not observed on spreads of transcribing plasmid *EA·3′*, which uses the rDNA promoter (Fig. 4). Re-examination of published micrographs shows that terminal balls were also absent from the RNA polymerase I-catalyzed transcription units that are occasionally observed in ribosomal "nontranscribed spacer" regions (Franke et al. 1979; Trendelenburg 1981, 1982).

When rRNA residues through +293 are included on an RNA polymerase I-catalyzed transcription unit, terminal balls are observed (Fig. 5). Because this region is substantially upstream of the 18S/5.8S/28S coding regions, terminal balls cannot involve rRNA sequences that will end up in the mature ribosome, as had been inherent in various suggested models for the nature of the terminal balls (Puvion-Dutilleul et al. 1977b; Franke et al. 1979; Puvion-Dutilleul 1983; Osheim and Beyer 1985).

Although terminal ball formation requires only rRNA sequences in the 5′ portion of the 5′ ETS, these structures do not form on the transcripts of a plasmid containing the subcloned rDNA promoter/initiation region extending through RNA residue +113, as revealed by re-examination of published micrographs (pX1r14D; Bakken et al. 1982). These data point to the importance of the frog sequence between residue +113 and +293 in terminal ball formation. This region corresponds closely with the frog-conserved 5′ ETS processing sequence, residues +112 through ~+233 (Mougey and Sollner-Webb, 1993).

When the 11-nucleotide conserved element in the 5′ ETS processing signal is inactivated with the Δl deletion, neither terminal ball formation nor the biochemically detected complex formation is observed (Fig. 2B and 6). This demonstrates that the evolutionarily conserved sequence element that is necessary for 5′ ETS processing and complex formation is also essential for terminal ball formation.

Our conclusion that the terminal ball is the 5′ ETS processing complex is also supported by high-resolution

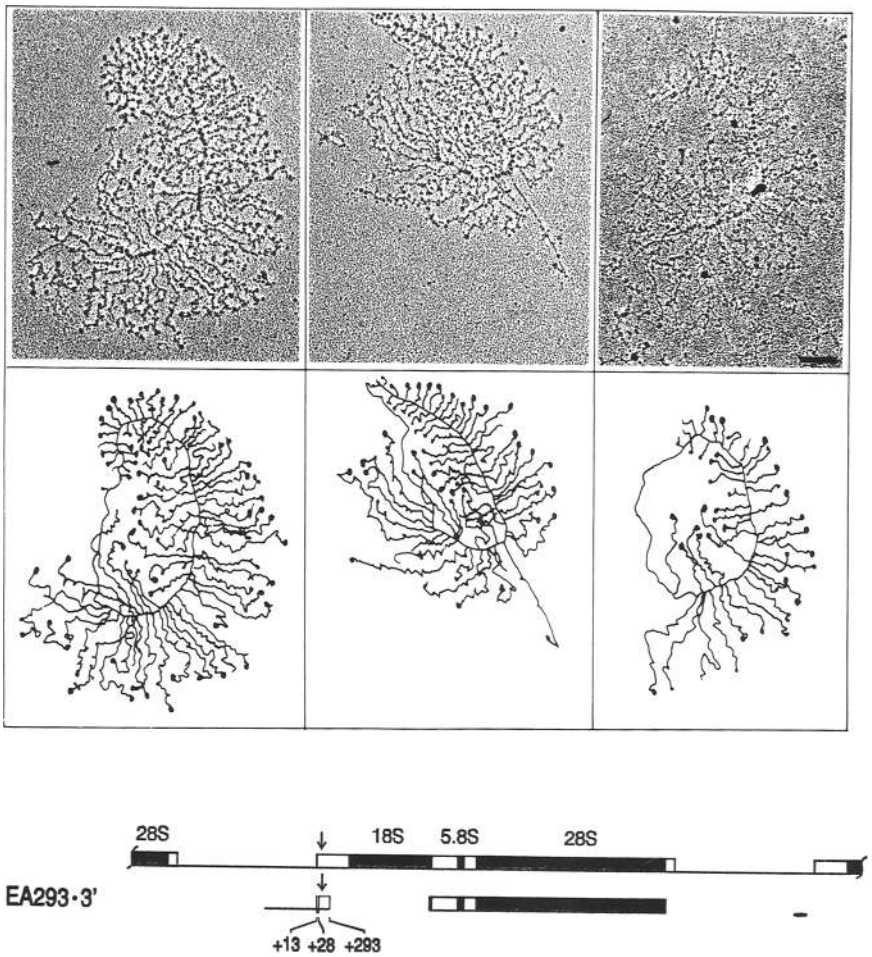

Figure 5. Electron microscopic visualization of active *EA293·3′* genes. The plasmid *EA293·3′* was microinjected, spread, and visualized as in Fig. 3B. Three examples with tracings are shown. Terminal balls are observed. Bar, 0.2 μm. In the diagram, the segments cloned to form *EA293·3′* are shown below the rDNA repeat. *EA293·3′* is like *EA·3′* but with the 28–293 rDNA segment inserted near the 5′ end of the transcribed region.

micrographs of CHO ribosomal transcription units (Puvion-Dutilleul et al. 1977a). These micrographs show that the "terminal" balls in this species are not truly terminal but frequently have a short RNA whisker protruding 5′ from the ball. Such 5′ terminal whiskers might be expected from the ~650 nucleotides of rRNA upstream of the CHO 5′ ETS processing complex. We predict that high resolution spreads of other rodent rRNA transcripts would show similar terminal whiskers, corresponding to the ~650- to 800-nucleotide segments upstream of their 5′ ETS-processing signal. Indeed, re-examination of a published micrograph of a mouse ribosomal transcription unit (Scheer and Benavente 1990) also appears to show such whiskers 5′ of the terminal balls. In contrast, any potential 5′ whisker in spreads from frog would be only ≤100 nucleotides in length and therefore would not be expected to be obvious in chromatin spreads.

Fibrillarin is a polypeptide that can be found associated with numerous nucleolar snRNAs [U3, U8, U13, U14, X, and Y (for review, see Fournier and Maxwell 1993; Sollner-Webb et al. 1993)]. In situ localization using an anti-fibrillarin antibody has demonstrated the presence of this antigen in the terminal balls of ribosomal transcription units (Scheer and Benavente 1990). This result also supports our concept of the terminal balls, because the U3 snRNP (which contains fibrillarin) has been shown to bind to this 5′ ETS processing complex in vitro (Kass et al. 1990) and to be present on the 5′ ETS in the vicinity of this processing site in vivo (Maser and Calvet 1989; Stroke and Weiner 1989).

Transcription units visualized on genes transcribed by RNA polymerase II do not typically show terminal balls (e.g., Miller and Bakken 1972; McKnight and Miller 1976; Puvion-Dutilleul et al. 1977b), as noted above. However, balls (or "granules") that are generally internal (but occasionally near the 5′ end of the RNA) have been observed, and these are concluded to be the spliceosome complexes involved in pre-mRNA processing (Osheim et al. 1985; Beyer and Osheim 1988). These pre-mRNA internal balls frequently first arise as pairs of smaller balls, arguably the 5′ and the 3′ splice site complexes, which later (further down the transcription unit) appear to aggregate and can reveal a looped-out intron. Still later, this intron loop may no longer be visualized, indicating that the complete splicing reaction has taken place. Thus, there is precedent for granules seen on nascent RNA in electron microscopic chromatin spreads to be processing complexes.

The rRNA terminal balls are ~250 Å in diameter

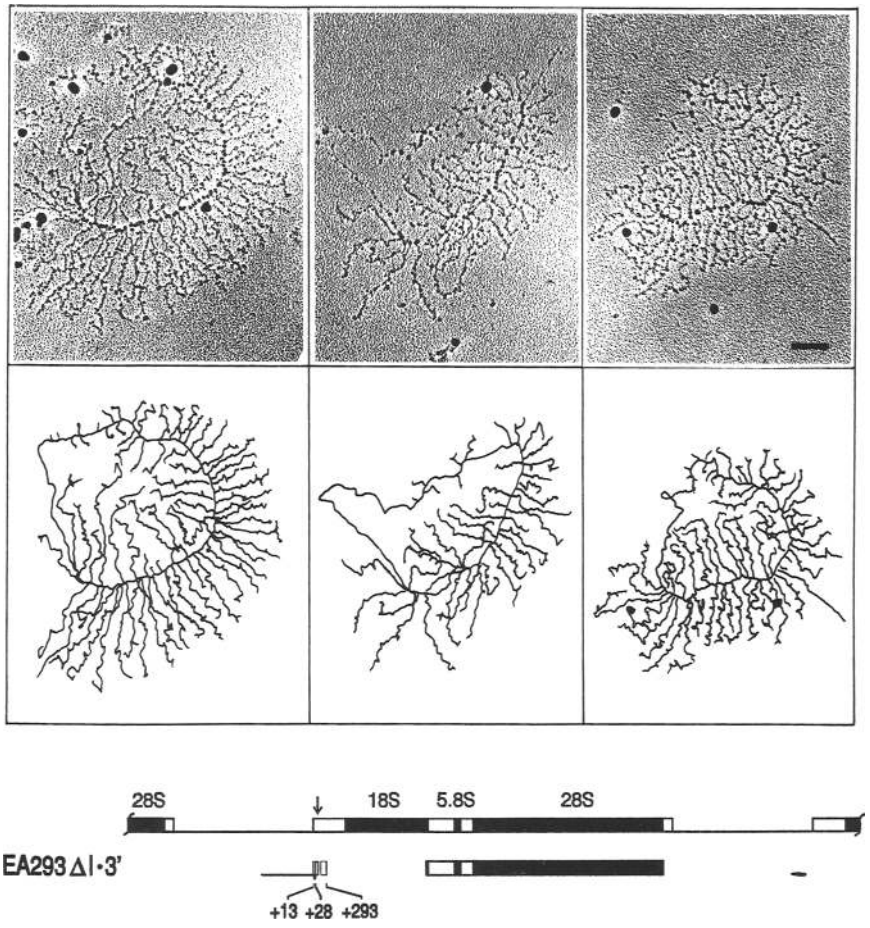

Figure 6. Electron microscopic visualization of active *EA293Δl·3′* genes. The plasmid *EA293Δl·3′* was microinjected, spread, and visualized as in Fig. 3B. Three examples with tracings are shown. Terminal balls are not observed on *EA293Δl·3′* plasmids, but they were present on the chromatin of the endogenous cellular rRNA genes. Bar, 0.2 μm. The diagram shows the segments cloned to form *EA293Δl·3′*, which is the same as *EA293·3′* except for bearing the Δl deletion of the critical 11-nucleotide segment just beyond the 5′ ETS processing site at +105.

(Miller and Bakken 1972; Puvion-Dutilleul et al. 1977b), approximately the size of the complexes on the 5′ and the 3′ splice sites of the pre-mRNAs (Osheim et al. 1985; Beyer and Osheim 1988). This suggests that the ETS complex on the intact rRNA gene is a sizable entity, most likely containing components in addition to the four polypeptides identified as binding to this region by UV cross-linking (Mougey and Sollner-Webb, 1993) plus the U3 snRNP. Nonetheless, the ETS processing complex is considerably smaller than the complete spliceosome complex (~400 Å in diam.; Osheim et al. 1985; Beyer and Osheim 1988), indicating that it consists of considerably fewer species than the ~100 polypeptides plus ~5 snRNAs of the complete spliceosome.

Because the terminal balls seen in ribosomal spreads from amphibians and mammals are concluded to be the ETS processing complex, we argue that this is also the case in the vast number of other eukaryotes whose rRNA gene transcripts all showed terminal balls when examined by electron microscopy (representative references in the introductory section; Franke et al. 1979). Consistent with this hypothesis that all eukaryotes form a 5′ ETS processing complex on their nascent rRNA, molecular analysis of the pre-rRNA of numerous species has revealed more than one 5′ end location, with the downstream end shown to result from rRNA processing [*Neurospora* (Tyler and Giles 1985); yeast (Hughes and Ares 1991)] or inferred to derive in this manner [*Physarum* (Blum et al. 1986); *Tetrahymena* (Sutiphong et al. 1984); *Bombyx* (Fujiwara and Ishikawa 1987); maize (McMullen et al. 1986); wheat (Barker et al. 1988); pea (Piller et al. 1990)]. The 5′ processing in yeast is also known to require the U3 snRNP (Hughes and Ares 1991). It now seems likely that all of these species will have a 5′ ETS processing that involves formation of a large terminal ball complex and will be U3-requiring and otherwise analogous to the processing events of mouse and frog. Other cell types might show little or no actual 5′ ETS processing, yet will assemble a terminal ball complex, akin to the situation in *Xenopus* oocytes.

It is notable that the efficiency of ETS processing varies widely. The processed species comprises ~90% of precursor rRNA in mouse cells (Miller and Sollner-Webb 1981), ~50% in human cells (Kass et al. 1987), ~30% in *Xenopus* kidney cells (Mougey and Sollner-Webb, 1993) and in *Xenopus* follicle cells (data not shown), and ~1% in *Xenopus* oocytes (Fig. 2A). Nonetheless, processing complex formation evidently occurs efficiently on the nascent ribosomal transcripts in all eukaryotic cell types, as shown by electron microscopic visualization of rRNA terminal balls (references above). This suggests that what has been conserved in evolution may not be

the act of 5′ ETS processing per se, but rather the ability to form a processing complex in the 5′ ETS and its assembly relatively early during the transcription process.

The ubiquitious presence of 5′ ETS processing complex formation across eukaryotes (unicellular and multicellular, plants and animals) indicates that it offers an evolutionarily advantageous function. Because this processing complex forms in the 5′ ETS region of the pre-rRNA, which ultimately will be destroyed and not become part of the ribosome, it is tempting to hypothesize that it functions to aid in later processing steps of the pre-rRNA. During pre-rRNA transcription and its concurrent assembly into RNP (Chooi and Leiby 1981), the large nascent transcripts are packed very densely, and this mass could shield later processing sites from rapid processing complex formation. Processing factors present in *cis* on the rRNA, assembled when the nascent transcript was short and a binding site was more available, could serve to facilitate downstream cleavages. Consistent with this proposal, a site needed for U3 binding (and presumably for a complex formation) in the 5′ ETS of yeast is required for the subsequent processing of the 18S segment in vivo (Beltrame and Tollervey 1992).

Materials and methods

Plasmid constructs

X293 was generated by inserting *X. laevis* rDNA residues +28 to +293 [a *Nar*I fragment from rDNA clone *pXlr14a* (Sollner-Webb and Reeder 1979)] into the *Acc*I site of pGEM3, downstream from the T7 promoter. The 11-nucleotide block, conserved between frog and mammals (Fig. 2A), extends from residue +112 to +122. *X293Δl* is a derivative of X293 in which rDNA residues +105 to +119 were deleted by Bal 31/S1 digestion, starting from the *Pvu*I site at residue +113 (partial digest), followed by recircularization of the plasmid. *X293Δr* is a derivative of *X293* in which residues +113 to +134 were deleted by inserting a *Sma*I–*Not*I fragment (residues +135 to +177) in place of a *Pvu*I (blunted)–*Not*I fragment (residues +113 to +177). All deletion end points were determined by sequence analysis (Maxam and Gilbert 1977). *pXlr101a* contains the entire *X. laevis* rDNA repeating unit (Bakken et al. 1982) inserted into pBR322 by *Hin*dIII cleavage and ligation. *EA · 3′* contains the *X. laevis* rDNA enhancer/promoter/marker region from plasmid EA (Pape et al. 1989; rDNA residues −1147 to +13) joined upstream of the last two-thirds of the rDNA-coding region (the 5.15-kb *Xba*I–*Hin*dIII fragment from *pXlr101a* that starts 60 nucleotides before the end of the 18S region) and then the T3 fail-safe termination region (the *Hae*III fragment from residue −243 to −188 upstream of the promoter), cloned in *Sal*I–*Eco*RI-cut pBR322. *EA293 · 3′* is like EA · 3′except that the +28 to +293 region of *X293* has been added between the EA region and the 3′ rRNA region. *EA293Δl · 3′* is the same as *EA293 · 3′* except that the +28 to +293 region bears the +105 to +119 deletion from *X293Δl*.

S1 nuclease analysis

RNA, prepared from logarithmically growing cultures of *X. laevis* kidney cells (~12 μg), from manually isolated *X. laevis* oocyte nuclei (two germinal vesicles, GVs), and from follicle cells (Henderson and Sollner-Webb 1986; Windle and Sollner-Webb 1986) was hybridized to 40 fmoles of strand-separated probe and digested with S1 nuclease (Sollner-Webb and McKnight 1982). The *X. laevis* ETS probe corresponds to the 5′ portion of the *X293* transcript, an *Eco*RI–*Not*I fragment, 5′ labeled at the *Not*I site at +179. The products were analyzed on a 9 M urea/8% polyacrylamide gel. The marker (Maxam and Gilbert 1977) sequencing track of the probe DNA migrates 1½ nucleotides faster than the corresponding fragment generated by S1 nuclease (Sollner-Webb and Reeder 1979).

In vitro reaction and complex formation

X. laevis oocyte nuclei were isolated manually (Wilkinson and Sollner-Webb 1982), homogenized by pipetting, and clarified by centrifugation at 12,000 *g* for 30 sec at 4°C. Where indicated, S-100 extract of log phase *X. laevis* kidney cells (line Xl–K2) propagated in tissue culture was used (McStay and Reeder 1990). The substrate and competitor RNAs were transcribed from the T7 promoter of the appropriate plasmid, linearized at the *Hin*dIII site in the polylinker downstream of the rDNA region, and gel isolated as described (Kass and Sollner-Webb 1990). The substrate RNAs were labeled with ^{32}P and the competitor RNAs with ^{3}H, the latter for quantitation.

The 25-μl in vitro reactions were at a final concentration of 20 mM HEPES (pH 7.9), 120 mM KCl, 2 mM $MgCl_2$, 9% glycerol, 2 mM DTT, 0.14 mM EDTA, and 1.5 mM ATP and contained 40 units of RNAsin (Promega). They also contained four GVs worth of oocyte nuclear homogenate and 8.0 fmoles of labeled RNA substrate. The extract was preincubated for 60 min with a 40-fold molar excess of the indicated competitor RNA, and the substrate RNA was then added and incubation continued for an additional 90 min. The reactions were then brought to 260 μg/ml of heparin and incubated for an additional 10 min at 20°C. A 10-μl aliquot was directly analyzed on a native 4% polyacrylamide gel (65 : 1 acrylamide/*bis*), as described (Kass and Sollner-Webb 1990). Sucrose gradient analysis of the assembled nucleoprotein uses a scaled-up processing reaction of 75 μl containing 36 GVs and 72 fmoles of *X293* RNA. The reaction was treated as for mobility shift, except that the heparin was brought to 400 μg/ml and the reaction was then brought to 100 μl by addition of gradient buffer (reaction buffer lacking glycerol and ATP but containing 0.5 mM $MgCl_2$). It was layered onto a 5-ml 5–20% sucrose gradient in gradient buffer and sedimented at 50,000 rpm, 20°C in the SW55 rotor for ~2.5 hr. Twenty-three fractions of 225 μl were collected from the top, and 100 μl of each was analyzed by mobility shift.

Microinjection and spreading of oocyte nuclei and electron microscopic visualization of the ribosomal chromatin

Clumps of excised *X. laevis* ovary were defolliculated in cold 0.2% collagenase (Type II, Sigma) in Ca^{2+}-free modified Barth's solution (MBS) (Gurdon 1976) with shaking for 3 hr, washed extensively in cold complete MBS, and incubated in MBS overnight at 18°C (Coleman 1984). For injections, oocytes were placed on a 1-mm Nitex mesh screen covered with 5% Ficoll (Sigma) in MBS. Approximately a 40 nl volume was injected into each germinal vesicle. The plasmid DNA for injection was at 0.5 mg/ml and was dissolved in 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, and 200 μg/ml of α-amanitin. After injection, oocytes were allowed to recover in 5% Ficoll in MBS for 1–2 hr, rinsed extensively with MBS, and incubated in MBS for 4–24 hr at 18°C. Chromatin dispersal and electron microscopic visualizations were as described previously (Miller and Bakken 1972). More than 15 examples of each transcribing plasmid were examined, and in each case, all showed the same patterns as the

examples in the figures shown for each plasmid. The ribosomal transcription units of the plasmids are readily distinguished from those on the endogenous rRNA genes by their presence on a small circular molecule of the appropriate size, rather than in long tandem arrays.

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The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes.

E B Mougey, M O'Reilly, Y Osheim, et al.

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