

SURVEY AND SUMMARY

Spatial organization of transcription by RNA polymerase III

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ABSTRACT

RNA polymerase III (pol III) transcribes many essential, small, noncoding RNAs, including the 5S rRNAs and tRNAs. While most pol III-transcribed genes are found scattered throughout the linear chromosome maps or in multiple linear clusters, there is increasing evidence that many of these genes prefer to be spatially clustered, often at or near the nucleolus. This association could create an environment that fosters the coregulation of transcription by pol III with transcription of the large ribosomal RNA repeats by RNA polymerase I (pol I) within the nucleolus. Given the high number of pol III-transcribed genes in all eukaryotic genomes, the spatial organization of these genes is likely to affect a large portion of the other genes in a genome. In this Survey and Summary we analyze the reports regarding the spatial organization of pol III genes and address the potential influence of this organization on transcriptional regulation.

LINEAR GROUPINGS OF CO-REGULATED GENES

The organization of coregulated genes is commonly found in the single dimension of a linear chromosome and the advantages for co-regulation of expression are obvious. This type of linear association is evidenced by gene clusters that are found in all three domains of life (1). In bacterial operons, coding regions for multiple genes are synthesized as a single transcript from a common promoter. The classic example is the bacterial *lac* operon, which includes the *lacZYA* genes under one promoter, in close proximity to the adjacent *lacI* regulatory gene under its own separate promoter. The direct association of this set of multiple genes, with multiple promoters, implies the utility of having related genes exist in the same subcellular environment. Archaeal genomes also exhibit this type of linear organization, such as a heat shock regulon that is found in all sequenced archaeal

genomes (2). Operons are now known to exist widely in *Caenorhabditis elegans*, making up a surprising 15% of the genome (3). Most of these operons are conserved in *Caenorhabditis briggsae* and some are found in more distant nematodes (3). Other interesting examples of clustered linear gene arrangements can be found in metazoans, including the *HOX* gene clusters, which encode multiple transcription factors that pattern embryonic development along the anterior–posterior axis. The order of the genes in these clusters demonstrates a remarkable linear correlation to the actual location of gene expression in the animal, which suggests an intriguing method of coordinating gene position and expression. The human β -globin locus on chromosome 11 is another example of linear gene organization, with the epsilon, gamma, delta and beta genes arrayed 5'–3' in the order in which they are developmentally expressed.

RIBOSOMAL DNA IS ORGANIZED LINEARLY AND SPATIALLY

Across all forms of life, the one gene family that is almost always found as an operon, typically in multiple copies, is that of the ribosomal DNA (rDNA). In prokaryotes the rDNA operon contains combinations of 16S, 23S and 5S genes, which are usually co-transcribed and then processed into individual RNAs by a series of endo- and exonucleolytic events in concert with assembly with protein subunits. In certain species, such as *Chlamydia pneumoniae* and *Mycobacterium tuberculosis*, the operon is found in only one copy (4). Other genomes contain multiple rDNA operons that are clustered at multiple sites on the chromosome, as seen in *Escherichia coli* and *Bacillus subtilis* (4). In eukaryotes, the rDNA operon includes the genes encoding 18S, 5.8S and 28S subunits, which are transcribed by RNA polymerase I (pol I). This operon is usually found in numerous tandem repeats: ~150 in *Saccharomyces cerevisiae*, ~400 in human and many thousands in various plant species (5). rDNA repeats are usually found in linear clusters at multiple chromosomal sites, although some species like *S.cerevisiae* have only one rDNA locus (5). This co-transcription of RNAs that are later processed into individual, functional

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RNAs used to be thought unusual for eukaryotic transcription units, but has now been demonstrated to be widespread for small, functional RNAs including small nucleolar (sno) RNAs and micro RNAs (6,7).

These linear arrays of eukaryotic ribosomal RNA genes serve as the organization foci of three-dimensional structures termed nucleoli, where rRNAs are transcribed, processed and assembled into pre-ribosomes. Thus, the rDNA clusters around which nucleoli form are termed Nucleolus Organizer Regions (NORs). It has now been determined that not all NORs are active [6 of 10 in human HeLa cells (8)] and while silent NORs may still be found at or near nucleoli (9), it is possible that they occupy different nucleolar regions. Different species contain varying numbers of nucleoli, which are visible by microscopy, as they are densely packed with RNA and protein. Within the nucleolus are three definable regions. The fibrillar center (FC) is surrounded by the dense fibrillar component (DFC). These substructures are surrounded by a granular component (GC). rDNA is found within the FC and rRNAs are transcribed at the interface of the FC and DFC (10–12). Modification and pre-ribosome assembly continue in the DFC and GC. Near the nucleolar periphery in transformed cells is a subnuclear structure called the perinucleolar compartment (PNC), which is full of RNAs and RNA binding proteins (13,14). Along with other specialized nuclear bodies (15), nucleoli demonstrate that it is quite advantageous to the organism to have a designated place for related nuclear functions to occur. Importantly, however, it is now known that the nucleolus is the location of much more activity than just rRNA transcription and ribosome assembly (16).

THE 5S rRNA GENE IS ALSO CLUSTERED IN MOST LINEAR GENOMES

In addition to the 18S + 5.8S + 28S rRNA transcription unit, there is one rRNA subunit that is synthesized by pol III, 5S rRNA. In many bacterial and archaeal species the 5S genes are part of the rDNA operon (4), which is transcribed by a single polymerase into one long precursor RNA. In eukaryotes, however, the biosynthesis of 5S RNA is much different. While the other rRNA genes are transcribed by RNA pol I, 5S genes are transcribed by RNA polymerase III (pol III), the polymerase also responsible for synthesis of tRNAs and other small, non-translated RNAs. In addition to being transcribed by a separate polymerase, the chromosomal locations of eukaryotic 5S genes are also rarely contiguous with the large ribosomal RNA repeats. There are certain species, exemplified by *S.cerevisiae* (17) and the slime mold *Dictyostelium discoideum* (18), in which individual 5S genes are found within the large rDNA repeats, adjacent to the pol I transcription units. However, most species carry 5S genes detached from the rDNA in just one major cluster of tandem repeats, like chicken (19) or a few clusters, such as *Arabidopsis thaliana* (20) and *Drosophila virilis* (21). Only rarely, as in *Neurospora crassa* (22), *Schizosaccharomyces pombe* (23) and *Acanthamoeba castellanii* (24) are the 5S genes linearly scattered. While the transcription of the scattered 5S genes of *Acanthamoeba* can be coordinately regulated with that of the large ribosomal subunits (25), linear clustering of the heavily expressed 5S genes could derive benefits from

the organization as tandem repeat units. Physical proximity could facilitate targeting of regulatory proteins, transcription factor binding or even polymerase re-initiation. In addition, clustering of genes for which the RNA products require similar RNA processing and protein assembly would greatly facilitate spatial organization of these pathways.

Basal expression of 5S genes is very efficient, but these genes are also responsive to multiple stimuli [reviewed in (26)]. For example, 5S genes are downregulated (coordinately with ribosomal RNA genes) when *A.castellanii* enters its dormant state, called encystment (25) and during nutritional starvation in *S.cerevisiae* (27). Furthermore, there is developmental regulation of 5S clusters in *Xenopus* (28), wherein the 20 000 oocyte 5S genes are heavily expressed in oocytes but the 400 somatic 5S genes are preferred in somatic tissues (26). The distinctive regulation pattern of the oocyte-type and somatic-type 5S genes might be facilitated by the fact that the tandem clusters of these genes reside at distinct chromosomal positions (29), although this has not been tested.

One way that cells might coordinately regulate 5S transcription by pol III with pol I transcription of the rDNA repeats would be to bring the gene clusters together. Localization of 5S genes to the nucleolus could provide a platform for coordinate regulation of all ribosomal RNAs. With 5S gene clusters that are differentially regulated, it might be advantageous to co-localize only the active 5S clusters with nucleoli.

SPATIAL ORGANIZATION OF 5S GENES

As mentioned above, the *S.cerevisiae* and *Dictyostelium* 5S genes are attached to the rDNA repeats and are therefore nucleolar by definition. However, there is now extensive evidence of 5S gene clusters at chromosomal locations distant from the rDNA that preferentially associate with the nucleolus, nucleolar periphery or the surrounding nucleoplasm. The studies described in this section are all direct visualizations of 5S genes in nuclei, using *in situ* hybridization. Interpretation of data acquired by this method is fairly straightforward, because the genes themselves are detected.

The haploid genome of the garden pea plant *Pisum sativum* has three clusters of 5S genes, totaling about 5000 copies (30). Therefore, the diploid nucleus contains six clusters, which are often found in pairs presumed to be sister chromatids (31). None of these clusters is near an NOR (Nucleolus Organizer Region, see above). Nevertheless, *in situ* hybridization of root tip cell sections has revealed an association of one or more of the 5S gene cluster pairs with the nucleolar periphery (31). In fact, many cells contain two or three pairs associated with the nucleolar periphery (31). While this means that not all clusters are nucleolar, the preference was found to be significant (31). 5S transcripts were found exclusively in the nucleolus, distributed in a distinctive pattern which corresponds to the GC of the nucleolus (31,32).

Crepis capillaris is a plant species in which the single 5S cluster is found on the same chromosome arm as an NOR (33). *C.capillaris* nuclei contain one large, centrally located nucleolus and *in situ* hybridization of root sections has shown that the 5S genes have a preferred localization with respect to the nucleolar periphery (33). In a diploid interphase nucleus, the two gene clusters are both found halfway between the large nucleolus and the nuclear envelope (33).

The two homologous chromosomes are aligned parallel to one another, with a preferred distance between them and this orientation is still present through early/mid prophase, when the nucleolus is still intact (33). However, once the nucleolus breaks down in late prophase, the orientation becomes random (33). Another plant, *Petunia hybrida*, which also has a single 5S cluster on the same chromosome arm as an NOR and a large, central nucleolus, shows similar localization of 5S genes to a point halfway between the nucleolar periphery and the nuclear envelope (33). Study of a fourth plant, *Zea mays* (maize), found that 5S genes at pachytene and later meiotic stages are not associated with the nucleolus (34), although the positions of the nucleolus and the chromosomes in nuclei of this species are significantly altered at the pachytene stage (35). A recent study has shown the 5S genes in *A.thaliana* in several loci around the nucleus that do not necessarily correspond to the positions of the 45S ribosomal repeats, although the two regions were not counterstained directly (36).

The genome of *Drosophila melanogaster* has one cluster of 5S genes, located at position 56F on chromosome arm 2R, containing ~165 repeats (37–39). This site is not near either of the two NORs, which are on the X and Y chromosomes. The relationship between the 5S locus and the nucleolus has been examined in multiple different tissues (40–43). In the nuclei from salivary glands and Malpighian tubules (a secretory tissue), the 5S gene cluster on polytene chromosomes has been found to maintain a precise position near the nucleolus, between the nucleolar periphery and the nuclear membrane (40). Similar data was reportedly obtained for rectal and brain cells (40). Separate studies of polytene chromosomes in salivary gland cells also found that the 5S gene cluster at 56F is one of the chromosomal regions with which nucleoli preferentially associate (41,42). One report that, at first glance, appears to contradict these data is the description that in prothoracic gland nuclei, the 5S locus was never found near the nucleolus (43). However, the cutoff value used for this measurement was just 2.25 μm (43), whereas the diameter of the nucleus in this cell type is 22–25 μm (44). This cutoff may encompass insufficient volume to find a gene locus that may be nearby the nucleolus but not touching it directly. The previous characterizations of the 5S locus as being preferentially positioned near the nucleolus (41,42) or between the nucleolus and the nuclear membrane (40) were less stringent, thereby finding a slightly looser physical relationship which may still be functionally important. Another notable difference in methods is that whereas the other nuclei described in this section were fixed prior to *in situ* hybridization, the prothoracic gland tissue was sectioned and visualized without any fixative (43).

The *Xenopus laevis* genome contains ~20 000 oocyte-specific 5S genes in multiple clusters and about 400 somatic 5S genes in a single cluster (45). All of these gene clusters appear to be telomeric, with the somatic genes found at the end of chromosome 9 and the oocyte genes found at the ends of most chromosomes (46,47).

In situ hybridization of brain cells with a probe complementary to all the 5S genes—the one cluster of somatic genes that are expressed in these cells and the many clusters of oocyte genes that are not expressed—shows a distribution of 5S genes across the nucleus, with a possible preference for

the nuclear periphery (46). Observations were similar for kidney cells, although the visible nucleoli in these cells seem to have some associated 5S genes (46). However, only the somatic cluster, 2–3% of the genes, is expressed in these tissues. In the nuclei of gonads from ovary and testis, there is a clear preferential association of 5S genes with nucleoli (46). Significantly, these are the cells in which the oocyte-specific genes are expected to be expressed (48). After progression of the spermatogonia into nearly mature spermatids, when the genes are not expected to be expressed (49), they become distributed along the length of the nucleus (46). An additional study has looked at two *Xenopus* cultured cell lines, one which does not express the oocyte-specific 5S genes and one which expresses them in small amounts (50). In both cell lines, 5S genes were found scattered throughout nuclei (50). However, these cells had been treated with the microtubule depolymerizing drug nocodazole and there is recent evidence that microtubules are required for the subnuclear positioning of some pol III genes (51).

The case of the *Xenopus* 5S rRNA genes emphasizes a crucial point in studies of the positioning of pol III transcription units in nuclei. Although the developmental expression of the *Xenopus* 5S genes is well documented so that different classes or clusters of genes could theoretically be examined individually, the conditional and developmental regulation of pol III transcription units in most eukaryotes has not been characterized in any depth. Therefore, studies on the localization of whole classes of genes should be viewed with some caution, since in most cases it is not known whether individual genes are transcriptionally active.

Visualization of 5S genes in mammalian nuclei has also been performed. In the Chinese hamster haploid genome, there are an estimated 3500 5S genes (52), which seem to be organized in two major clusters (53). When Chinese hamster liver cell nuclei are probed with labeled total RNA, to label all varieties of DNA in the genome, there is a dispersion of signal throughout the nuclei (52). However, when liver or brain cell nuclei are probed with purified, labeled 5S RNA, to visualize the 5S gene positions, the signal is preferentially found at nucleoli (52). This would be consistent with a subset of genes corresponding to the transcriptionally active units being preferentially positioned at the nucleolus. Recently, 5S genes in mouse nuclei have also been found to be primarily perinucleolar (B. Sollner-Webb, personal communication).

The human genome contains at least one major cluster of 5S genes on chromosome 1, containing 100–150 repeats, one smaller cluster of 5–10 repeats and 200–300 dispersed 5S genes (54–56). *In situ* hybridization with a probe complementary to the major 5S cluster on chromosome 1 has shown that this cluster often localizes to the nucleolar periphery (57). This result has been confirmed by another study, in which it was estimated that one third to one half of 5S foci are perinucleolar (B. Sollner-Webb, personal communication). Correspondingly, the 5S RNA in the nucleus is found exclusively in the nucleolus (57). In a separate report, the conclusion was given that 5S genes are not associated with the nucleolus in human lymphocyte nuclei (58). However, the published micrographs indicate that 5S genes may in fact overlap nucleoli in a subset of cases (58). Furthermore, cells in this study were stimulated with phytohemagglutinin, a compound that appears to alter the normal nuclear architecture by

causing the formation of additional nucleoli with unusual morphology (59).

These many observations of 5S transcription units in a wide range of species suggest that the nucleolus can be an organizing site for 5S gene localization. Logically, this may contribute to the co-regulation of 5S genes with the other ribosomal RNAs. However, 5S is not the only small RNA that should share this coordinate expression. tRNAs are also necessary for translation and are the other major class of RNAs transcribed by pol III.

tRNA GENES RARELY FORM LINEAR CLUSTERS IN EUKARYOTIC CHROMOSOMES

Similar to the way bacteria have 5S genes conveniently located within the rDNA operon, bacterial tRNA genes are often found in cistrons. These units often contain multiple tRNA genes and some contain protein-coding regions. Furthermore, many prokaryotic rDNA repeats contain one or more tRNA genes (4,60). Eukaryotic tRNA genes are organized much differently, with single tRNA genes having developed into individual transcription units through the creation of specialized RNA polymerase transcription factors (TFIIIC) that recognize 'promoters' within the conserved elements of the tRNA coding sequences [reviewed in (26)]. In rare cases, there is pronounced linear clustering of tRNA genes. For example, some oocyte-specific tRNA genes in the *Xenopus* genome are found in a few main clusters of repeats (61,62), each of which encodes multiple different isoaccepting tRNAs, although there are also dispersed units (63–65). *S.pombe* also has a unique arrangement with multiple clusters of tRNA genes present within the centromeric heterochromatin (66,67). Interestingly, these *S.pombe* centromeric tRNA genes act as heterochromatin barriers (68), a function of tRNA genes which has also been demonstrated at the silent mating-type locus in *S.cerevisiae* (69). Other examples of tRNA gene clusters are mostly isolated cases within genomes that have predominantly scattered tRNA genes. Examples include salmon (70), brown trout (70), salamander (71), rat (72), mouse (73) and human (74).

Because tRNA genes are primarily found scattered throughout linear genomes, it seems reasonable that they would be physically distributed throughout nuclei. However, as with linear clustering of 5S genes, spatial clustering of tRNA genes with each other might support their high-level transcription and localization of the clustering to the site of rRNA synthesis may aid in coordinate regulation. Indeed, most pre-tRNAs are nucleolar in *S.cerevisiae* (75,76), although one particularly long-lived pre-tRNA has been shown to be dispersed in the nucleoplasm (77). Moreover, early processing enzymes that are required for the biosynthesis of mature tRNAs show localization to nucleoli. RNase P, responsible for 5' end maturation of tRNAs, is nucleolar in *S.cerevisiae* (75,78). Subunits of this enzyme have also been localized to the human PNC (57) and the nucleolus (79), although this association has been suggested to be transient (79). Another tRNA processing enzyme, Mod5, which isopentenylates A₃₇ of some tRNAs, is also found pooled in the nucleolus (80). With these maturation steps occurring in the nucleolus, it may be beneficial to physically connect tRNA transcription to this

subnuclear region. An observation in support of this is that mutation or deletion of various yeast nucleolar proteins (Cbf5 and pol I transcription machinery) influences transcriptional events at tRNA loci (78,81). Specifically, a local form of transcriptional silencing (tRNA gene mediated or 'tgm' silencing) caused by proximity to an active tRNA gene is released by disruption of nucleolar organization (78,82).

SPATIAL CLUSTERING OF tRNA GENES

The relationship of tgm silencing to nucleolar components led to the hypothesis that tRNA genes were nucleolar in yeast, despite the fact that the 275 genes are dispersed throughout the linear maps of all 16 chromosomes. Using probes complementary to several multi-copy tRNA gene families, *in situ* hybridization to cells that were fixed during growth to prevent chromosomal rearrangement has shown this hypothesis to be true (Figure 1) (83). tRNA gene localization to the nucleolus requires active transcription of the gene (83), reminiscent of the same requirement for tgm silencing (82). Therefore, the role of nucleolar positioning in tgm silencing was examined by disrupting normal nucleolar function with several pol I proteins and Cbf5 (81). It was found that multiple nucleolar disruptions are correlated to a release of silencing and a dispersion of both pre-tRNAs and tRNA genes throughout the nucleoplasm (81). These observations suggest that tRNA gene localization to the nucleolus is a mechanistic component of silencing near tRNA genes. The effects of this silencing can be seen in the fact that pol II genes are underrepresented near tRNA genes in the yeast genome (84), with the exception of the preferential integration of Ty retrotransposons at pol III transcription units (85). The nucleolar antigens involved in rRNA biosynthesis are not the only aspects of nuclear architecture that play a role in tRNA gene positioning; it has now been shown that microtubules are required for tRNA gene clusters to localize to the nucleolus (51).

Evidence of tRNA gene positions in other systems is scarce and we are aware of only one other report of *in situ* hybridization of tRNA genes. In this study, Chinese hamster liver and brain sections were hybridized with radiolabeled, purified tRNAs ('4S' RNA) in order to probe the subnuclear positions of active genes. The images show tRNA genes preferentially associated with nucleoli (52), although the possibility exists that the 4S RNA probes may have also contained other RNA species (e.g. 5S rRNA).

OTHER POL III GENES

5S and tRNA genes, while encoding the most abundant small RNAs, are not the only pol III transcribed genes; in fact, in many genomes they are not even the most abundant DNA elements containing potential pol III promoters. Other pol III transcripts include the U6 snRNA, the RNA components of RNase P and MRP (although the MRP RNA is not pol III transcribed in *S.cerevisiae*), as well as 7SL and 7SK RNAs [for review, see Ref. (26)]. Pol III also transcribes some viral genes during infection, such as the VA RNAs of adenovirus. However, in many organisms the most highly repeated potential promoters for pol III are found within the Short Interspersed Nuclear Elements (SINES). For example, human Alu elements arising originally from 7SL transcripts

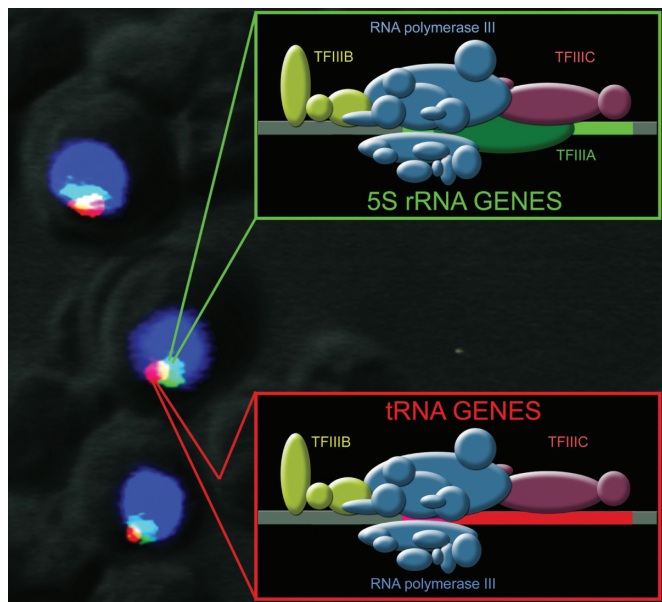


Figure 1. *In situ* hybridization using short, oligonucleotide probes reveals that tRNA genes (red) overlap 5S genes (green) at the nucleolus. DAPI staining of chromatin is shown in blue. tRNA genes and 5S genes are both transcribed by RNA polymerase III and they also share the same transcription factors, although 5S genes require one additional factor, TFIIIA. Gene colors in the line drawings correspond to image pseudocoloring, while protein colors are arbitrary. Co-localization of tRNA and 5S genes may provide a basis for coregulation of their transcription with one another. Further, nucleolar co-localization of these genes might facilitate allows coordination of transcription with another heavily expressed component of the protein synthesis machinery, the large ribosomal RNA gene repeats that are by RNA polymerase I. With the current resolution it is not possible to conclusively interpret whether signals from tRNA and 5S genes are coincident or adjacent. Figure courtesy of Martin Thompson.

reportedly constitute at least 10% of the genome (86). Mouse SINES, including the 7SL-derived B1 elements and tRNA-derived B2 elements, make up over 7% of the genome (87).

While the low-copy genes of these additional pol III RNAs (e.g. U6, 7SL) would be very difficult to visualize using *in situ* hybridization, SINE elements are present in enough copies to easily detect. Preliminary attempts to broadly localize the positions of Alu elements in human HeLa cells by *in situ* hybridization showed that they formed a granular pattern throughout the nucleoplasm, with no particular association with the nucleolus [(58), C.P. Paul and D.R. Engelke, unpublished data]. It is difficult to interpret this result, however, since few of the Alu elements are transcribed under normal conditions and it is not known which elements are activated in response to inducing conditions, such as cellular stresses. This is an extremely exaggerated case of the problem with probing tRNA genes in mammalian nuclei. Although most or all of the yeast tRNA loci are known to be transcribed, the transcriptional activity of predicted tRNA genes in mammalian nuclei [(88), D. Coughlin and D.R. Engelke, unpublished results] is largely unknown.

VISUALIZING THE SYNTHESIS OF POL III TRANSCRIPTS

One way of circumventing the question of which genes are actually transcribed is to directly analyze the subnuclear

location of nascent pol III transcripts. Early studies of tRNA modification within nucleoli of the chironomid *Smittia parthenogenetica* (89,90) suggested that tRNAs were synthesized in nucleoli, but the site of modification is not necessarily the site of transcription. Recent work has looked more directly at the subnuclear location of RNA synthesis (transcription) by first using polymerase-specific transcription inhibitors and subsequently pulsing cells with labeled nucleotides to visualize sites of incorporation. Theoretically this technique could allow the observation of all transcription by a given polymerase if contribution by other RNA polymerases could be eliminated.

There are two commonly used compounds for this purpose. Actinomycin D is an antibiotic known to inhibit pol I at low doses ($\sim 0.04 \mu\text{g/ml}$) and all transcription at higher doses ($\sim 300 \mu\text{g/ml}$). Another commonly used inhibitor, α -amanitin, is a mushroom toxin that inhibits pol II transcription at low doses ($\leq 2 \mu\text{g/ml}$) and both pols II and III from vertebrates at higher doses ($\sim 250 \mu\text{g/ml}$). While these drugs may be useful for limited transcription applications, they might not be ideal for analyzing subnuclear organization. Actinomycin D and α -amanitin, even at low concentrations, can cause significant redistribution of nucleolar proteins, changes in rDNA condensation and even diffusion of chromosome domains (91–94). These distortions may contribute to unknown effects regarding the normal subnuclear distribution of genes. As an example, tRNA genes have been shown to disperse when ribosomal RNA transcription is compromised by specific mutations in the pol I machinery (81). With this knowledge in mind, studies that use these compounds should be interpreted cautiously.

In an investigation of pol III transcription in HeLa cells, treatment of permeabilized cells with various concentrations of α -amanitin was followed by Br-UTP incorporation and immunolabelling (95). With no drug treatment, Br-UTP is incorporated into nascent transcripts throughout the nucleoplasm and nucleolus. Using $2 \mu\text{g/ml}$ α -amanitin to inhibit pol II, much of the nucleoplasmic signal disappears, with signal remaining at the nucleoli and at a few sites in the nucleoplasm (1/5 the number at 1/3 the intensity, as compared to the untreated cells) (95). After this α -amanitin treatment, the authors characterize the nucleoplasmic sites as being clusters of multiple (~ 6) nascent transcripts and multiple pol III complexes. In the nucleoplasm alone, there were an estimated $0.35 \text{ pol III clusters}/\mu\text{m}^2$ or 1800 sites per nucleus (95). The authors did not identify single, dense clusters of nascent pol III transcripts that would be expected from the 5S locus on chromosome 1, although thin sections might not have exposed such masses. The rare instances of ‘complex clusters’ that were found in some cells might represent the 5S transcripts. Analysis of the nucleolar foci was not given and transcription by pol III at or near nucleoli was not ruled out by the published data.

A second study, using cultured human fibroblast cells, did essentially the same experiment as listed above (96). Cells were treated with $2 \mu\text{g/ml}$ α -amanitin to inhibit pol II, then permeabilized and pulsed with Br-UTP. The resulting images show significant transcription in the nucleoplasm (96)—lower than in the untreated cells, but probably not the reduction to 1/15 of the untreated level, as the previous paper calculated (95). Again, the drug treatment did not eliminate nucleolar

signal, leaving open the possibility of pol III transcription at or near nucleoli. Consistent with the earlier work, this study also found no spot of substantially thicker labeling that would correspond to the array of 5S genes.

In a third analysis, Actinomycin D was used to inhibit pol I transcription in HeLa cells before pulsing with labeled nucleotides (97). The resulting transcripts, presumably synthesized by pols II and III, were found throughout the nucleoplasm, at a prominent focus within the PNC and somewhat in the nucleoli (97). This result seems to indicate transcription by pol II or III in the PNC, except that the addition of 300 µg/ml α -amanitin, which should also inhibit pols II and III, does not diminish the signal in the PNC (97). The interpretation of this result is therefore unclear.

A fourth study of this type looked at transcription of VA RNA genes by pol III in adenovirus-infected HeLa cells (98). After inhibition of pol II with 1 µg/ml α -amanitin, bromo-UTP incorporation is found in the nucleolus and limited nucleoplasmic foci (98). These sites are suggested to reflect the transcription of VA RNAs (98), although it should logically include all cellular pol I and pol III transcription as well. Once again, this experiment does not rule out pol III transcription at or near the nucleolus.

These observations may indicate that pol III synthesizes RNAs from multiple sites in the nucleus, including the nucleoplasm and possibly the nucleolus and PNC.

SUBNUCLEAR DISTRIBUTION OF POL III TRANSCRIPTS AND PROTEINS

So far, this survey has probed the spatial organization of pol III transcription by considering evidence of where the genes are located and where the transcription occurs. The final major category of research into this question is the subnuclear distributions of both pol III transcripts and proteins that bind to the genes. These observations are likely to be secondary verification of transcription sites, since neither RNAs nor proteins are stationary and might accumulate at discrete sites when not actively engaged. This has been demonstrated for pol II transcription components (99). Nevertheless, the organization of these components may help provide clues to the organization of the biosynthetic pathways and even their methods of regulation.

5S RNAs might be expected to be nucleolar at some point in order to associate with pre-ribosomes. As noted above, 5S nascent transcripts are nucleolar by definition in yeast, because the 5S genes are within the large ribosomal repeats. 5S RNA is also nucleolar in at least two other species where 5S genes are associated with the nucleolar periphery [*P.sativum*, (31)] or PNC [human, (57)]. Further studies have also found these transcripts in the nucleoli of other human cell types (100,101) and in monkey (100). Analysis of various developmental stages in *Xenopus* oocytes has shown that 5S RNA accumulates in nucleoli during the vitellogenic stage, which correlates to the timing of rRNA synthesis and ribosome assembly (102).

Early tRNA transcripts in *S.cerevisiae* are mostly nucleolar, where they are processed by RNase P (75). Currently there is not sufficient data for the localization of pre-tRNAs in other systems. A human pre-tRNA^{Ser} microinjected into

rat nuclei was not found to be concentrated anywhere particular within a short time course (103), but endogenous pre-tRNAs in organisms other than yeast have not been localized. In higher eukaryotes, this may be due to the severe lack of information about tRNA genes. It is currently not known how many tRNA genes exist in the human genome, nor which of the predicted ones are expressed. While the mammalian genome projects have published predictions of tRNA genes, which of these are actually expressed has not been extensively tested. Recent analysis of the mouse genome has identified as many as 1500 potential tRNA genes; transcripts of ~1000 of these can be detected by northern blot (D. Coughlin, personal communication).

Other pol III transcripts, many of which are subunits of RNPs, have also been localized in various organisms. Both mature RNase P RNA and its precursor form are mostly nucleolar in *S.cerevisiae* (75) and it has been found to be in human nucleoplasm, nucleoli and the PNC (57,79,104), where different localizations have been suggested to represent different levels of maturation. The RNA subunit of RNase MRP is found mostly in the nucleolus and slightly in the PNC in human cells (57,104,105). Y RNAs are components of Ro RNPs, which are cytoplasmic RNPs of unclear function found in higher eukaryotes. There are four Y RNAs encoded in the human genome, three of which are found in PNCs in addition to the cytoplasm (57). The signal recognition particle (SRP) RNA (also known as 7SL RNA) is found in human nucleoli and PNC and rat and *Xenopus* nucleoli (101,105–107). Microinjected SRP RNA is nucleolar in *Xenopus* nuclei and transiently nucleolar in rat nuclei (103,107). Human Alu transcripts are mostly nucleoplasmic, but also concentrated in the PNC (105). U6 RNA has been found in multiple areas of the nucleus. In *S.cerevisiae* it is nucleoplasmic (75). In human cells it is found in the nucleoplasm as well as Cajal bodies, which are known sites of small nuclear RNP biogenesis [for review, see Ref. (15)] (101,108,109). In *Xenopus* oocytes, U6 RNA has been found to be concentrated in Cajal bodies (110) as well as nucleoli (111,112), which may reflect a trafficking of the RNA through Cajal bodies to nucleoli (113).

It is important to note that in addition to the subnuclear positions described above, many endogenous pol III transcripts are also found in the cytoplasm. 5S and 7SL are predominantly cytoplasmic (101,105,106), where they function in translation. The Y RNA components of Ro RNPs, which also function in the cytoplasm, are also mostly cytoplasmic (57). RNase MRP is active in both the nucleus and mitochondria, so the localization of its RNA component to both nucleus and cytoplasm is expected (57,104,105). RNase P RNA is primarily in the nucleolus in *S.cerevisiae*, where the enzyme functions (75). In human cells, however, RNase P RNA is found in the cytoplasm in addition to the nucleus (57,104). For the RNAs that are functional only in the cytoplasm, the nuclear forms are presumed to be newly synthesized RNAs before export, although retrograde transport from cytoplasm to nucleus (114) has not been ruled out.

Artificial constructs with various pol III promoters driving expression of small RNAs (ribozymes, aptamers, siRNAs) show transcripts localized similarly to the corresponding endogenous RNAs (101). 5S and 7SL promoters target siRNA transcripts to the nucleolus and cytoplasm, similar

to the endogenous 5S and 7SL transcripts (101), although it is important to note that these promoter regions are primarily internal to the genes and fusion transcripts include targeting signals from the endogenous RNA sequences. In contrast, the U6 gene utilizes an upstream promoter region and the fusion of this endogenous promoter onto small RNA constructs was found to target transcripts to the nucleoplasm and nuclear speckles (101,109), indistinguishable from the sites of the endogenous U6 transcripts. These studies of artificial transcripts suggested that it was not necessarily properties of the RNA sequences that were trapping U6 transcripts in the nucleus and directing them to speckles, but the very fact that they were being transcribed by RNA pol III and not directed to any other specific cellular destination. Transcripts from a tRNA promoter-driven small RNA construct were found throughout the nucleoplasm and possibly in punctate foci (109).

Proteins that bind to pol III genes have been localized in some systems, but not many. In *S.cerevisiae*, most of the subunits of pol III and its transcription factors have been fused to GFP and imaged as a part of a global localization study of yeast proteins (115). Each of the tested proteins were scored as either nuclear or nucleolar (115). High-resolution immunogold localization of the C53 subunit of pol III found the protein to be throughout the nucleoplasm (116). However, the GFP and immunogold results are not easily interpreted, since each of the pol III proteins and transcription factors must be nucleolar to transcribe the 5S genes, which are among the rDNA repeats and nucleolar by definition.

Potentially more informative data are from genomes where pol III genes are not found linearly associated with the rDNA, although most of these analyses have not been able to distinguish which proteins are actively binding and transcribing pol III genes. In *S.pombe*, pol III transcription factor TFIIC has been localized to the nuclear periphery and the nucleolus (117). These sites have been suggested as anchor points for genome organization, wherein the TFIIC complex binds to (and anchors) pol III-transcribed genes along with multiple newly discovered DNA loci that are enriched for TFIIC but not pol III (117). In human HeLa cells, primary fibroblasts and myogenic cells, transcription factor TFIIB has been localized to concentrated foci throughout nuclei (118). In *Xenopus* oocyte nuclei (germinal vesicles), many pol III subunits and transcription factors have been detected in Cajal bodies (110,119). However, the Cajal bodies are thought to be the location where components are assembled into their respective complexes (110,119) and not necessarily the site of transcription.

A final protein to be discussed is the La protein, which has long been known to bind to pol III transcripts (120). More recently, two different forms of human La have been identified, where the phosphorylated form is nucleoplasmic and the nonphosphorylated form is in the nucleolus (specifically the DFC) (96,121). The phosphorylated, nucleoplasmic form binds to pol III transcripts (96,121). The nonphosphorylated, nucleolar form has now been found preferentially associated with pol III genes (122). The ability to distinguish different forms of a protein such as La, where only one form binds directly to pol III genes, may provide the most relevant method to relate protein localization to gene localization.

POL III GENES AS ORGANIZERS OF GENETIC INFORMATION

If the hundreds of pol III genes in a given genome are spatially organized inside the nucleus, then the positioning of the rest of the genome is likely to be affected. Thus, pol III genes can be considered organizers of genetic information, joining several other chromosomal entities that are thought to act as subnuclear anchors. In *S.cerevisiae*, not only are most of the tRNA genes positioned at the nucleolus, but the telomeres and centromeres also have preferred positions around the nuclear periphery (123,124). These elements have been suggested to act as tethers to hold chromosomes in place (125). The mechanisms of structural organization for both centromeres and tRNA genes rely, at least in part, on microtubules (51,126,127). In addition to the spatial organization of large families, there are also pol II-transcribed genes in yeast that appear to be dynamically repositioned upon activation (128,129). These types of spatially mobile DNA elements demonstrate the ability of a cell to use three-dimensional organization as a mechanism for transcriptional regulation.

In higher eukaryotes, there is also widespread structural organization of DNA. Individual chromosomes are thought to form chromosome 'territories' within the nucleus, with preferential localization of heterochromatin to the nuclear periphery [reviewed in (130)]. Within this general architecture there are many individual examples of genes that prefer certain areas of the nucleus (131–133), genes that spatially associate with distant enhancer elements (134–137) and coregulated genes that cluster together, such as the 5S genes described here and a family of globin genes (138–140). The sites of clustering may not be random, as mammalian cells have been found to have pol II 'transcription factories' that house actively transcribed DNA regions, with inactive regions suggested to form loops outside these foci (99,141,142).

The evidence so far is preliminary, but the expression-dependent positioning of genes in the nucleus strongly suggests that spatial organization is likely to be a factor influencing the regulation and evolution of genomes. We know, for example, that four of the five classes of Ty retrotransposon in *S.cerevisiae* preferentially integrate near tRNA genes (85), which puts them in a defined subnuclear position (83) under silencing conditions (82). A similar situation has been observed to the DRE elements in *Dictyostelium* (143). The fifth and final type of yeast Ty retrotransposon, Ty5, preferentially integrates at telomeres and silent mating type loci (144), which puts them also at defined subnuclear positions (123) under silencing conditions (145). This suggests that there was selective pressure for the Ty retrotransposons to develop integration strategies that placed them in particular types of environments. This particular symbiotic relationship between retrotransposon elements and pol III transcription units does not seem to hold true in metazoans, but other forces influencing genome expression and organization might well be causing an alternative architecture to be favored. There is not yet evidence that the linearly dispersed pol III-transcription units or the highly repetitive SINE elements influence subnuclear organization of DNA, but their influence on local and long-distance chromatin organization has not been systematically explored.

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