

SURVEY AND SUMMARY

The plurifunctional nucleolus

Thoru Pederson*

Worcester Foundation for Biomedical Research and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester Foundation Campus, 222 Maple Avenue, Shrewsbury, MA 01545, USA

Received May 4, 1998 Revised and Accepted June 18, 1998

ABSTRACT

The nucleolus of eukaryotic cells was first described in the early 19th century and was discovered in the 1960s to be the seat of ribosome synthesis. Although rRNA transcription, rRNA processing and ribosome assembly have been clearly established as major functions of the nucleolus, recent studies suggest that the nucleolus participates in many other aspects of gene expression as well. Thus, the nucleolus has been implicated in the processing or nuclear export of certain mRNAs. In addition, new results indicate that biosyntheses of signal recognition particle RNA and telomerase RNA involve a nucleolar stage and that the nucleolus is also involved in processing of U6 RNA, one of the spliceosomal small nuclear RNAs. Interestingly, these three nucleolus-associated small nuclear RNAs (signal recognition particle RNA, telomerase RNA and U6 RNA) are components of catalytic ribonucleoprotein machines. Finally, recent work has also suggested that some transfer RNA precursors are processed in the nucleolus. The nucleolus may have evolutionarily descended from a proto-eukaryotic minimal genome that was spatially linked to vicinal RNA processing and ribonucleoprotein assembly events involved in gene read-out. The nucleolus of today's eukaryotes, now surrounded by the chromatin of over 2 billion years of genome expansion, may still perform these ancient functions, in addition to ribosome biosynthesis. The plurifunctional nucleolus concept has a strong footing in contemporary data and adds a new perspective to our current picture of the spatial-functional design of the cell nucleus.

THE CLASSICAL ERA OF THE NUCLEOLUS

The nucleolus was one of the first intracellular structures to be described (1–5). It typically has the highest concentration of mass of any part of the cell (6) and the large difference between its refractive index and that of the surrounding nucleoplasm gives rise to the prominence of the nucleolus in phase contrast microscopy.

The discovery of the chromosomal nucleolar organizer locus established the nucleolus as a genetically determined element (7,8). Subsequently, the nucleolus was found to be the site of rRNA synthesis (9–16), a major advance in the cell biology of eukaryotic gene expression that was elegantly capped by the ultrastructural visualization of ribosomal genes in action (17–19).

CELL FUSION PROVIDES THE FIRST CLUE TO NUCLEOLAR PLURIFUNCTIONALITY

In 1965, it was observed that virus-induced cell fusion produced heterokaryons, i.e. cells with a nucleus from each of the two fused cells (20). (Like all advances, this one built on considerable previous work, which is thoughtfully reviewed in chapter 5 of 21.) This led to a series of studies on the properties of heterokaryons (22). These investigators were keen to fuse HeLa (human) cells with chicken erythrocytes. The nucleus of the avian erythrocyte contains extremely condensed chromatin, has no visible nucleolus and is transcriptionally silent. When fused with a HeLa cell, the chicken erythrocyte nucleus undergoes chromatin decondensation, transcriptional activation and formation of a nucleolus (23). The onset of chicken-specific protein synthesis in the heterokaryons was measured using immunological methods and it was found that chicken proteins did not appear until the erythrocyte nucleus had formed a nucleolus (24). It was reasoned that since the HeLa cell nucleus of the heterokaryon had a perfectly good nucleolus and since the HeLa cytoplasm of the heterokaryon contained functional ribosomes from the outset, the delayed appearance of chicken-specific proteins until formation of a nucleolus in the erythrocyte nucleus must mean that the chicken nucleolus was required for production of certain chicken mRNAs, an impressive deduction from a simple but elegant experiment. When I read this paper (24) in 1969 as a post-doctoral fellow, it made a very strong impression on me. The idea that the nucleolus might play a role in mRNA production was developed further (25), but did not influence the leading nuclear RNA investigators of that era.

*Tel: +1 508 842 8921; Fax: +1 508 842 7762; Email: thoru@sci.wfbr.edu

THE NUCLEOLUS AND mRNA PRODUCTION

In the past few years several studies have rekindled the idea (24,25) of a possible connection between the nucleolus and mRNA production. Spliced *c-myc* mRNA has been localized in the nucleolus of mammalian cells (26) and two human retroviral proteins involved in mRNA export, namely the Rex protein of HTLV-I and the Rev protein of HIV, have a predominantly nucleolar localization (27–29). In yeast there is a correlation between aberrant nucleolar morphology and mutations in mRNA processing or export (30–32) and poly(A)⁺ RNA accumulates in the nucleolus in some of these mutants and during heat shock (33). In addition, the majority of the abundant trimethylguanosine-capped spliceosomal small nuclear RNAs (snRNAs) are localized in the nucleolus of both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (34).

A possible link between non-rRNA processing or traffic and the nucleolus is also suggested by its dynamic relationship to juxtannucleolar structures known as coiled bodies, described at the turn of the century as ‘nucleolar accessory bodies’ (35). Coiled bodies have been found to contain spliceosomal snRNPs (36–39), as well as several (predominantly nucleolar) proteins, including fibrillarin, nucleolin and B23/NO38 (40,41). In addition, coiled bodies contain at least two proteins not normally found in nucleoli, namely p80 coilin (42) and the EWS family oncoprotein homolog pigpen (43). Additional evidence for an intimate connection between coiled bodies and the nucleolus comes from recent p80 coilin mutational studies, which have defined coilin domains that are required for its localization within coiled bodies (44). Interestingly, in some of the coiled body non-localizing p80 coilin mutants p80 localizes in a dispersed, circumferential perinucleolar pattern and causes nucleolar disorganization (44). Moreover, inhibition of serine/threonine protein phosphatase activity by treatment of cells with okadaic acid leads to nucleolar accumulation of p80 coilin and spliceosomal snRNPs and mutation of p80 coilin amino acid 202 from serine to a non-phosphorylatable amino acid (aspartate) also causes both p80 coilin and snRNPs to localize in nucleoli (45). With the caveat that some of these studies (44,45) employed transfected cells that were massively overexpressing p80 coilin relative to its normal levels, these results suggest a dynamic relationship among coiled bodies, spliceosomal snRNPs and nucleoli.

In addition to coiled bodies, a second example of a nucleolus-associated structure that contains spliceosomal snRNPs is the perinucleolar compartment (PNC). This structure consists of a specialized region on or very near the surface of the nucleolus in transformed mammalian cells that was initially detected by virtue of its content of a pre-mRNA intron polypyrimidine tract-binding protein, also known as hnRNP protein I (46,47), and also by the presence of certain small RNAs transcribed by RNA polymerase III, including the Ro antigen-associated RNAs hY1, hY3 and hY5, RNase MRP RNA and RNase P RNA (47). A recent investigation has revealed that PNCs also contain trimethylguanosine-containing RNAs, two pre-mRNA splicing factors, poly(A)⁺ RNA and the poly(A)-binding protein PAB II (S.Huang, T.J.Deernick, M.H.Ellisman and D.L.Spector, submitted for publication). This investigation also employed high resolution electron microscopy to reveal that PNCs are intimately connected to the nucleolar surface, rather than constituting a juxtannucleolar organelle. The finding of pol II transcripts, poly(A)⁺ RNA, trimethylguanosine-capped RNAs and other pre-mRNA proces-

sing factors in PNCs is extremely interesting in relation to the evidence for the role of the nucleolus in mRNA production discussed earlier.

THE NUCLEOLUS AND SIGNAL RECOGNITION PARTICLE BIOSYNTHESIS

The signal recognition particle (SRP) is a translational arrest machine that binds to the N-terminal signal peptide sequence of nascent secretory and membrane proteins, blocks the peptidyl transferase activity of 28S rRNA and then docks onto a receptor on the cytoplasmic face of the endoplasmic reticulum, followed by release of the translational elongation block (48). The SRP of higher eukaryotes contains an essential, 300 nt RNA component and six proteins (49,50). A recent study has shown that microinjection of fluorescent SRP RNA into the nucleus of mammalian cells leads to a very rapid localization in nucleoli (51; see also cover). After its initial nucleolar localization, SRP RNA is observed to exit the nucleolus and enter the cytoplasm (51). Microinjection of fluorescent SRP RNA mutants into the nucleus identifies two regions of the molecule that are important for nucleolar localization, namely a major portion of the *Alu* domain and helix 8 (51). These results, together with the observation that one of the SRP proteins, SRP68, contains a pentapeptide (RRQRR) that has been implicated as part of a nucleolar protein targeting element (52), point to the nucleolus as the site of SRP RNA processing and/or ribonucleoprotein assembly. This is reinforced by the finding that a portion of nuclear SRP RNA biochemically fractionates with purified nucleoli (53; J.R.Mitchell, J.Cheng and K.A.Collins, submitted for publication). These new results (51) point to the nucleolus as a site of SRP RNA processing and/or ribonucleoprotein assembly. They also raise the possibility that the SRP exits from the nucleolus in association with ribosomal subunits, compatible with the known affinity of the SRP for ribosomes during the SRP functional cycle (48,54).

TELOMERASE AND THE NUCLEOLUS

Telomerase is a reverse transcriptase ribonucleoprotein (RNP) enzyme that synthesizes telomeric DNA repeats at the ends of eukaryotic chromosomes (55). Although there has been substantial progress recently in the structure and functional characteristics of telomerase, less is known about where and how in the cell telomerase is assembled into a functional RNP enzyme. In the macronucleus of the ciliate protozoa *Oxytricha* and *Euplotes*, telomerase RNA is localized both at sites of replicating DNA as well as in spherical foci throughout the nucleoplasm (56). These nucleoplasmic foci also contain rRNA, but not rDNA (T.R.Cech, personal communication), suggesting that they may be sites of post-transcriptional rRNA processing and nascent ribosome assembly. The fact that these foci contain telomerase RNA, as well as trimethylguanosine-capped RNAs (56), raises the possibility that they may be centers for processing, RNP assembly and export of diverse RNAs in the macronuclei of these hypotrichous ciliates.

These findings in protozoa take on added momentum and breadth in the light of recent studies in mammalian cells, which have revealed that a portion of telomerase RNA co-fractionates with highly purified HeLa cell nucleoli, consistent with the shared homology of this RNA with the H/ACA box family of snRNAs (J.R.Mitchell, J.Cheng and K.A.Collins, submitted for publication), and that telomerase RNA has a predominantly nucleolar

localization in proliferating mouse embryo fibroblasts (D.Brocchi and T.De Lange, personal communication).

An additional suggestion of a connection between the telomerase-associated machinery and the nucleolus comes from recent studies of the family of *S.cerevisiae* transcriptional suppressors known as SIR proteins (for silent information regulator). SIR proteins are responsible for maintaining transcriptional inactivation of certain yeast genes, including the silent mating type alleles *HML* and *HMR* (57,58), as well as telomere-proximal genes. In a long lifespan mutant of *S.cerevisiae* one of the SIR genes (*SIR4*) was found to be truncated, resulting in a gain of function mutation (*sir4-42*) that confers a long lifespan phenotype (59). A putative *AGE* locus has been postulated, which normally limits lifespan but which is silenced in the *sir4-42* mutant (59). The most pertinent finding in terms of the topic of the present review is that the SIR3 and SIR4 proteins become redistributed from telomeres to nucleoli in the *sir4-42* mutant (60). This is accompanied by excision of blocks of rDNA repeats as extranucleolar (and extrachromosomal) cassettes, which has been directly linked to senescence in *S.cerevisiae* (61), and concomitant nucleolar fragmentation (62). It is possible that the redistribution of SIR proteins to nucleoli is causally related to excision of rDNA repeats and that there is no particular meaning to the telomere → nucleolus relocation of SIR proteins as regards the plurifunctional nucleolus hypothesis being presented here. On the other hand, given that telomerase RNA is associated with nucleoli in mammalian cells and with putative mini-nucleoli in ciliate protozoa (*vide supra*), the yeast SIR proteins may, in addition to their demonstrated role in lifespan determination, reflect a telomere–nucleolus link more subtle than has been envisaged so far.

THE NUCLEOLUS AND PROCESSING OF OTHER SMALL RNAs, INCLUDING tRNA

In addition to the recent evidence for a role of the nucleolus in the biosynthesis of SRP and telomerase RNAs or RNPs summarized above, there are other recent findings that bear on possible non-rRNA processing events in the nucleolus. Two vertebrate small RNAs that are associated with fibrillarins have been found to direct 2'-*O*-ribose methylation of the U6 snRNA and one of these RNAs co-fractionates with purified nucleoli (K.Tycowski and J.A.Steitz, personal communication). Although U6 RNA is, in the steady-state, localized mainly in nucleoplasmic foci ('speckles') and coiled bodies (63,64), it has recently been demonstrated that newly transcribed U6 RNA transits through the nucleolus for 2'-*O*-ribose methylation (P.Ganot, M.-L.Bortolin and T.Kiss, personal communication). Also noteworthy in this context is the observation that fluorescent U6 RNA, but not U1 or U2 RNAs, rapidly localizes in the fibrillar regions of nucleoli when injected into the *Xenopus* oocyte nucleus (A.Narayanan, R.M.Terns and M.P.Terns, personal communication).

Another recent study has revealed that in *S.cerevisiae* an isoform of the enzyme that encodes isopentenyl-6-adenosine synthetase, a tRNA modification enzyme, is localized in nucleoli (L.A.Hunter, A.L.Benko, J.P.Aris, D.R.Stanford, N.C.Martin and A.K.Hopper, submitted for publication). This suggests either that this aspect of tRNA processing occurs in nucleoli or that the nucleolar enzyme modifies other, non-tRNAs. To date, isopentenyl-6-adenosine has been reported only in tRNAs however (65).

RNase P RNA, the co-catalytic component of the eukaryotic pre-tRNA processing enzyme RNase P, has been localized in both

the nucleolus and nucleoplasm of mammalian cells (66). Initially, this observation of RNase P RNA in the nucleolus (66) was regarded as reflecting concurrent reports that RNase P plays a role in pre-rRNA processing (66,67 and references therein). However, recent *in situ* hybridization studies in *S.cerevisiae* indicate that some tRNA precursor molecules, including both intron-containing and intronless ones, are also present in the nucleolus (E.Bertrand, F.Houser-Scott, A.Kendall, R.H.Singer and D.R.Engelke, submitted for publication). Thus, the nucleolar localization of RNase P RNA (66) may reflect its roles in both pre-rRNA and pre-tRNA processing. Another intron-containing pre-tRNA has been localized in the nucleoplasm of *S.cerevisiae* (S.Sarkar and A.K.Hopper, submitted for publication; D.R.Engelke, personal communication), suggesting that some pre-tRNAs may be processed at extranucleolar sites, consistent with the presence of RNase P in both the nucleolus and nucleoplasm (66). The finding that some pre-tRNAs are processed in the nucleolus adds yet another aspect of gene read-out to the nucleolar repertoire.

Finally, another recent investigation bears on an apparent role of common RNA processing factors in the nucleolus and nucleoplasm. The 5'-end processing of small nucleolar RNAs (snoRNAs) in the nucleoplasm, excised from pre-mRNA introns or other snoRNA precursor molecules, has been found to be mediated by two exonucleases that also function in the 5'-end processing of 5.8S rRNA in the nucleolus (68). While these latter results do not directly address the plurifunctionality of the nucleolus, they do bear on the intriguing issue of how the nucleolus and nucleoplasm may have co-evolved during the descent of eukaryotes, to be discussed shortly.

THE PLURIFUNCTIONAL NUCLEOLUS

If it is true that the nucleolus, beyond producing ribosomes, also plays a role in processing or export of some mRNAs, SRP RNA and tRNA and also in processing or RNP assembly of telomerase RNA and U6 snRNA this would, to say the least, expand our view of this intranuclear organelle. But first we must ask whether there is anything that contradicts the plurifunctional nucleolus hypothesis. The most immediate issue that comes to mind is the anucleolate condition.

A naturally occurring mutation in *Xenopus laevis* has a deletion of the entire nucleolar organizer and embryos homozygous for this mutation arrest at the tadpole stage (69). Anucleolate *Xenopus* embryos do not synthesize rRNA (15) and the interpretation of developmental arrest of these embryos as reflecting a lack of sufficient (new) ribosomes for post-metamorphosis development certainly was, and remains, the most plausible one. However, in the light of the plurifunctional nucleolus idea, other phenotypic consequences of the anucleolate condition can be contemplated. The lack of zygotic SRP production in anucleolate embryos, postulated by the plurifunctional nucleolus hypothesis, raises the question of how far into development maternal SRP would suffice and whether an impaired level of membrane or secreted protein synthesis would be developmentally rate limiting at metamorphosis in the amphibian embryo. Similarly, the number of chromosome replications that take place between amphibian fertilization and metamorphosis and the expected extent of telomere shortening might, or might not, impair development beyond the tadpole stage in the absence of the nucleolus and its postulated role in telomerase production. Maternal stores of telomerase, U6 RNA

and tRNA might be sufficient to support development of anucleolate embryos up to (and beyond) the tadpole stage.

There are biological situations intermediate between the anucleolate and nucleate condition and these must also be reckoned with as regards the plurifunctional nucleolus hypothesis. The typical nucleolus likely reflects, cytologically, the presence of multiple active rDNA genes, together with the elaborate enzymatic and RNP machinery that directs rRNA processing as well as the presence of nascent ribosomal subunits. Single copies of *Drosophila* rDNA genes placed at chromosomal locations outside the nucleolar organizer are actively transcribed and miniature nucleoli appear (70). In *S.cerevisiae* deletion mutants missing an essential subunit of RNA polymerase I (pol I), rRNA can be transcribed by RNA pol II from an rDNA-containing plasmid, leading to formation of numerous intranuclear granules that, although much smaller than *S.cerevisiae* nucleoli, contain known nucleolar proteins and are thus termed mini-nucleolar bodies (71). The intranuclear location of the rDNA transcription apparatus in *S.cerevisiae*, including its organization into a nucleolus, is determined by the deployed RNA pol I versus pol II rDNA promoter and flanking DNA elements (M.Nomura, personal communication). Similarly, when single copy extra-chromosomal (plasmid) rDNA genes are placed into yeast cells lacking nucleolar organizers, nucleus-wide rRNA production occurs from the plasmid rDNA genes and is sufficient to support cell growth (72). The plurifunctional nucleolus hypothesis presented here does not distinguish between the requirement for a full-blown nucleolus as an essential workbench for the other envisaged functions (processing or export of some mRNAs, SRP RNA and tRNA and processing or RNP assembly of telomerase RNA and U6 RNA) versus the possibility that these other functions would still occur at non-nucleolar or mini-nucleolar nucleoplasmic sites at levels sufficient to support cell growth, as in the aforementioned ectopic rDNA expression experiments. Finally, it is reasonable to suppose that the relative nucleolar mass devoted to each of its (postulated) several roles would be proportional to the levels of each biosynthetic function. It is thus not surprising that the landscape of typical nucleoli, i.e. ones that contain multiple rRNA genes, largely reflects rRNA transcription, processing and ribosome assembly (73).

SUMMARY: A 'NEW-CLEOLUS'

How the cell nucleus is spatially organized to support its functions and how the gene read-out machinery itself contributes to nuclear architecture on the one hand and moves about within the nuclear milieu on the other have been long-standing issues in eukaryotic cell biology (74–76). The nucleolus has been one of the most well-defined and least sullied intranuclear components. Yet, as reviewed in this article, there is now a rather compelling body of evidence that the nucleolus may perform gene read-out functions beyond ribosome synthesis. To summarize, there is evidence that the nucleolus plays a role in the processing or export of a subset of mRNAs. Moreover, the RNA component of the SRP, part of the translational machinery, appears to also transit through the nucleolus. In addition, the RNA subunit of another RNP, telomerase, is associated with the nucleolus in mammalian cells and with rRNA-containing nucleoplasmic sites in ciliate protozoa. Furthermore, studies of cell senescence in yeast have uncovered a link between telomere-associated proteins and the nucleolus. In addition, U6 RNA, thought to be a catalytic

component of the spliceosome, also transits through the nucleolus in its maturation. It is interesting to note that all three of these RNAs, i.e. SRP RNA, telomerase RNA and U6 RNA, are parts of catalytic RNP enzymes (SRP is a GTPase, telomerase is an RNA-directed DNA polymerase and U6 RNA is a catalytic component of the spliceosome). Finally, it appears that some tRNA processing also occurs in the nucleolus.

These findings suggest that, in addition to its classically defined function, namely ribosome biosynthesis, the nucleolus also supports production of other parts of the gene expression machinery, including tRNA and another component of the translational apparatus, the SRP, as well as a RNP enzyme involved in maintenance of chromosome integrity (telomerase) and a catalytic RNA of the spliceosome (U6 RNA). There may have been a stage in proto-eukaryotic evolution in which the translational machinery and the chromosome end maintenance apparatus operated in the immediate vicinity of a minimal genome, kinetically facilitating or reducing the dimension of key reactions that were essential. This envisaged reaction center may have been a chemically efficient locus and therefore a selectively advantageous gene read-out design on the evolutionary path to eukaryotes. The nucleolus of today's eukaryotic cells may represent the descendant of such a previous minimal nucleoid, now surrounded by the chromatin of over 2 billion years of genomic expansion. The postulated ancient congression of rRNA transcription, processing and ribosome assembly with tRNA, U6 RNA and mRNA processing and SRP and telomerase RNA processing and RNP assembly would have presumably evolved when the proto-eukaryotic ancestor had only a single copy ribosomal rRNA gene (or a low copy number). The plurifunctional nucleolus hypothesis invokes the supposition that the chemical affinities that initially collated the processing and assembly of the various parts of the gene read-out equipment at the sites of ribosome synthesis remain operative today in the nucleoli of extant eukaryotes.

ACKNOWLEDGEMENTS

For generously communicating unpublished results, I thank Tom Cech (Howard Hughes Medical Institute, University of Colorado), Kathleen Collins (University of California, Berkeley), Titia De Lange (Rockefeller University), David Engelke (University of Michigan), Anita Hopper (Pennsylvania State University College of Medicine), Sui Huang (Northwestern University Medical School), Tamás Kiss (Université Paul Sabatier, Toulouse, France), Nancy Martin (University of Louisville School of Medicine), Masayasu Nomura (University of California, Irvine), David Spector (Cold Spring Harbor Laboratory), Michael Terns (University of Georgia) and Kazimierz Tycowski and Joan Steitz (Howard Hughes Medical Institute, Yale University School of Medicine). I am grateful to Joseph Gall (Department of Embryology, Carnegie Institution of Washington, Baltimore), Marty Jacobson (Xenometrix, Inc., Boulder), Lynn Margulis (University of Massachusetts, Amherst), Gary Olsen, Claudia Reich and Carl Woese (University of Illinois), Norman Pace (University of California, Berkeley) and Joan Politz in my laboratory for helpful comments on a draft of the manuscript. I also thank Susan Kilroy (my laboratory) for preparing the cover figure. Research cited from the author's laboratory was supported by NIH grant GM-21595-22 and by the G. Harold and Leila Y. Mathers Charitable Foundation.

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