A Feedback Loop Coupling 5 S rRNA Synthesis to Accumulation of a Ribosomal Protein*

(Received for publication, August 13, 1999, and in revised form, September 14, 1999)

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We have shown that elevated expression of ribosomal protein L5 in Xenopus embryos results in the ectopic activation of 5 S rRNA genes that are normally inactive. This transcriptional stimulation mimics the effect of overexpressing transcription factor III A (TFIIIA), the 5 S rRNA gene-specific transcription factor. The results support a model in which a network of nucleic acid-protein interactions involving 5 S rRNA, the 5 S rRNA gene, TFIIIA, and L5 mediates both feedback inhibition of 5 S rRNA synthesis and coupling of 5 S rRNA synthesis to accumulation of a ribosomal protein, L5. We propose that these mechanisms contribute to the homeostatic control of ribosome assembly.

Ribosome biogenesis in eukaryotic cells requires the synthesis of RNAs by all three nuclear RNA polymerases: RNA polymerase I for the 28 S, 18 S, and 5.8 S rRNAs; RNA polymerase II to produce mRNAs that encode ribosomal proteins; and RNA polymerase III to synthesize 5 S rRNA. The mechanisms responsible for coordinate accumulation of the various ribosomal components are poorly understood, especially with respect to coupling synthesis of 5 S rRNA to that of the other ribosomal RNAs and of the ribosomal proteins to the production of ribosomal proteins.

Transcription factor IIIA (TFIIIA)1 is a 5 S rRNA gene-specific transcription factor that binds to the internal control region of 5 S rRNA genes in the first step of transcription complex assembly (1, 2). Remarkably, TFIIIA also binds to 5 S rRNA, the gene product, to form a 7 S RNP complex in a fashion that is competitive and incompatible with simultaneous binding to 5 S rRNA gene (3, 4). The 7 S RNP complex accumulates to high levels in Xenopus oocytes as a prelude to subsequent assembly of ribosomes near the end of oogenesis (3, 5, 6).

Interestingly, the 7 S RNP has also been proposed to mediate a feedback regulation mechanism controlling 5 S rRNA synthesis in somatic cells (3), because 5 S rRNA can compete for the binding of a protein, TFIIIA, required for its own synthesis. The feedback regulation model has been supported by experiments showing that expression in Xenopus embryos of mutant forms of TFIIA that have reduced affinity for 5 S rRNA (7) leads to levels of 5 S rRNA synthesis that are considerably higher than is obtained with comparable expression of wild-type TFIIA (8). This result suggests that compromising the 5 S rRNA binding activity of TFIIIA leads to elevated 5 S rRNA gene transcription because of impairment of the normal feedback inhibition loop. This interpretation is further supported by the observation that 5 S rRNA synthesis in vitro is less sensitive to inhibition by 5 S rRNA when transcription is mediated by the mutant form of TFIIA that results in high level 5 S rRNA gene transcription in vivo (8).

5 S rRNA binds not only to TFIIIA but also to ribosomal protein L5. L5 and 5 S rRNA interact to form a 5 S RNP (9–11) that is believed to be a precursor for incorporation into the large subunit of the ribosome (12). Thus, 5 S rRNA can form binary complexes with either TFIIIA or L5, but binding to the two proteins is competitive, and ternary complexes containing both proteins cannot be detected.2 The interaction of 5 S rRNA with either TFIIIA or L5 to form discrete RNPs is also likely to be important in the shuttling of 5 S rRNA, TFIIIA, and L5 between the nucleus and cytoplasm (13–18).

The network of nucleic acid-protein interactions involving TFIIIA, L5, 5 S rRNA, and the 5 S rRNA gene suggests a model in which 5 S rRNA synthesis is coupled to the accumulation of a ribosomal protein (L5). In the proposed regulatory loop (Fig. 1), an increase in the concentration of L5 would result in displacement of the equilibrium between each of the relevant RNPs (5 and 7 S) and its constituent components in opposite directions, resulting in the formation of additional 5 S RNP and the release of free TFIIIA from 7 S RNPs. The TFIIIA released from 7 S RNPs would be available for binding to and nucleating transcription complex formation on additional 5 S rRNA genes. Thus, 5 S rRNA synthesis would be responsive to levels of L5 expression, even though there is no reason to believe L5 is directly involved in 5 S rRNA synthesis in any way. Brow and Geiduschek (19) have proposed a comparable model for coupling 5 S rRNA synthesis in yeast to accumulation of YL3, the yeast homolog of L5, but in vivo evidence supporting such a model for coupling 5 S rRNA synthesis to ribosomal protein accumulation in any species has been lacking.

EXPERIMENTAL PROCEDURES

Synthetic mRNAs—Plasmids pT3myc/L1-N41+5’-UTR-DS and pT3myc/L5AN16 (gifts of W. Michael Wormington, University of Virginia Charlottesville, VA) were linearized with BamHI and used as templates for synthesis of L1 and L5 mRNAs. For L5, the 9E10 Myc epitope tag replaced 16 amino acids normally found at the N terminus of the protein; for L1, the 41 N-terminal amino acids were deleted and replaced with the Myc tag: pT3myc/L5AN16 was constructed by cloning the KpnI/BamHI fragment of pSP65AT-L5b (11) between the PstI and BamHI sites of a plasmid derived from Bluescript KS+ and referred to as the “Myc vector” by Peculis and Gall (20). KpnI and PstI ends were blunted with T4 DNA polymerase in each case prior to subcloning.

pT3myc/L1-N41+5’-UTR-DS was constructed by subcloning a HindIII/BamHI fragment of pL1–1.3 (21) between the blunted PstI and BamHI

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* This work was supported by grants from the National Institutes of Health (NIH) (GM48035 to D. R. S. and HD24673 to M. T. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported during a portion of these studies as a post-doctoral fellow of NIH Training Grant HD07194.

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1 The abbreviations used are: TFIIIA, transcription factor III A; RNP, ribonucleoprotein; UTR, untranslated region.

2 B. Scripture and P. Huber, personal communication.
L5 as an Indirect Regulator of 5 S rRNA Synthesis

Fig. 1. A model for feedback regulation of 5 S rRNA synthesis and coupling of 5 S rRNA synthesis to accumulation of ribosomal protein L5.

sites of the Myc vector. The normal 5'-untranslated region (UTR) of L1 was then reconstituted upstream of the Myc-L1 fusion by the insertion of synthetic oligonucleotides containing the L1 5'-UTR between KpnI and NcoI sites.

RNAs were synthesized in vitro with T3 RNA polymerase using MegaScript reagents from Ambion and were capped by inclusion of m7G(5')ppp(5')G (Ambion) in the transcription reaction. Synthetic RNAs were purified by DNase I treatment, extraction with phenol/chloroform, and chromatography on Sephadex G-100. The recovered RNA was then concentrated by precipitation with alcohol and quantified spectrophotometrically.

Other Methods—Synthetic mRNAs were tested prior to injection by in vitro translation in rabbit reticulocyte lysate using materials and procedures obtained from Promega. RNA polymerase III from Xenopus ovaries was purified through the DEAE-Sephadex step according to Cozzarelli et al. (22). Embryo injection, chromatin/nuclei preparation, and labeled RNA isolation and analysis were performed as described by Rollins et al. (8). Western blots on total embryonic extracts were performed using a monoclonal antibody to the Myc tag incorporated at the N terminus of L1 and L5, goat anti-mouse IgG:horseradish peroxidase conjugate, and chemiluminescent detection with LumioLO (obtained from Kirkegaard and Perry Laboratories, Gaithersburg, MD). Expression levels of Myc-tagged proteins were estimated from autoradiographic exposures adjusted to ensure that signals obtained were in the linear response range of the film.

RESULTS AND DISCUSSION

We have tested the model of Fig. 1 by overexpressing L5 in Xenopus embryos and measuring the effect of elevating the in vivo L5 concentration on the transcription of 5 S RNA genes. We have chosen this approach because previous work has shown that injection of synthetic mRNA into fertilized eggs results in the elevated expression of encoded proteins during subsequent embryonic development (23) and because our previous work has also shown that TFIHIIA overexpression during embryonic development results in the ectopic activation of 5 S rRNA genes that are normally inactive (8). The model of Fig. 1 suggests that elevating L5 expression would mimic the effect of TFIHIIA overexpression, at least to a limit imposed by the intracellular concentration of 7 S RNP.

Synthetic, capped L5 mRNA was prepared by in vitro synthesis from an appropriate template using T3 RNA polymerase. As a control, mRNA for ribosomal protein L1 was prepared in parallel. In both cases, an N-terminal Myc epitope tag was present so that we could confirm in vivo translation of the synthetic mRNAs following injection into embryos. Prior to injection, synthetic mRNAs were tested by in vitro translation in rabbit reticulocyte lysates to ensure they were active as substrates for protein synthesis (data not shown). Sibling groups of fertilized Xenopus eggs were then injected with a mixture of [α-32P]UTP and either L5, L1, or no synthetic mRNA. Embryos were allowed to develop and were collected for further analysis at about stage 10–10.5 (early gastrula) (24). Total RNA was prepared from each group of embryos and analyzed on a denaturing polyacrylamide gel (Fig. 2) to determine how much 5 S rRNA had been synthesized during early embryonic development following injection of the synthetic mRNAs. The results were analyzed and quantified by normalizing the amount of labeled 5 S rRNA to the amount of labeled tRNA. Although there was some quantitative variability from one injection experiment to the next, normalized 5 S RNA synthesis in L5-injected embryos was almost always elevated relative to the no-RNA control (average fold stimulation of 2.7 ± 1.7 (S.D.) in four independent experiments), whereas 5 S RNA synthesis in L1-injected embryos was indistinguishable from that in the control embryos. Western blots with an anti-Myc monoclonal antibody were used to measure L1 or L5 synthesis in vivo following injection of the synthetic mRNAs. These experiments demonstrated that both Myc-tagged L1 and L5 were readily detectable and expressed at roughly equivalent levels (data not shown). Thus, the lack of any effect on 5 S RNA synthesis in L1-injected embryos was not due to a failure to express and accumulate L1 protein.

These initial results suggested that 5 S RNA synthesis in Xenopus embryos is coupled to L5 accumulation, but it was possible that the L5-mediated stimulation of labeled 5 S RNA levels resulted not from transcriptional activation of 5 S RNA genes but rather from some unexpected effect on 5 S RNA stability. We therefore chose to focus on a more direct assay to measure activation of 5 S RNA genes. In this assay, nuclei were prepared from stage 10–10.5 embryos that had been previously injected with synthetic L5, L1, or no mRNA, and transcriptional activity was measured following reconstitution with purified RNA polymerase III. Earlier work had shown that stable transcription complexes formed on 5 S RNA genes in vivo are recovered in chromatin or nuclei preparations and can be detected by adding purified RNA polymerase III and measuring 5 S RNA synthesis in vitro (8, 23, 25). Thus, the state of transcriptional programming of 5 S RNA genes in vivo can be assessed quantitatively in an in vitro transcription assay. Representative results from a single injection experiment are shown in Fig. 3 along with a quantitative summary of the results of several such experiments in Fig. 4. The data reveal an average increase of 4.7-fold in 5 S RNA synthesis from chromatin templates prepared from embryos injected with L5 mRNA when compared with control embryos injected with an equivalent volume of water only. Furthermore, L1 overexpression has essentially no effect on 5 S RNA gene activity (Figs. 3–4). One may note that there is substantial
and the results were analyzed quantitatively using a PhosphorImager. Multiple experiments of the kind represented in Fig. 3 were performed, consequence of overexpression of ribosomal proteins L1 and L5.

possibility that the enhanced transcriptional activity we ob-
activation of 5 S rRNA genes. Although we cannot exclude the
when L5 is overexpressed is a consequence of transcriptional
imbryos, the difference between these embryos and those
on a single lane was determined. This ratio was then normalized to the same ratio obtained in a control reaction using nuclei prepared from water-injected embryos. Individual data points from a series of such experiments using synthetic L1 or L5 mRNA in the original injection are plotted. n = 9 for L5, and n = 5 for L1.

quantitative variability in the extent to which 5 S rRNA syn-
these results are very similar to those obtained when TFIIIA was overexpressed during early embryogenesis using similar methods (8, 23). Because it is extremely unlikely that L5 is directly involved in the formation of 5 S rRNA transcription complexes (and there is certainly no evidence to support such an hypothesis), the most plausible interpretation of our current results is that L5 acts indirectly to increase the pool of free TFIIIA, which in turn nucleates transcription complex formation on 5 S rRNA genes that are normally inactive. This effect is a prediction of the model of Fig. 1 and thus supports the proposal that competitive binding of L5 and TFIIIA to 5 S rRNA on the one hand, and of TFIIIA to 5 S rRNA and the 5 S rRNA gene on the other, serves to couple 5 S rRNA synthesis to the accumulation of ribosomal protein L5. Furthermore, the results suggest that a pool of 7 S RNP exists in these embryonic cells that can be tapped as a source of additional TFIIIA for activation of 5 S rRNA genes. The extent to which 5 S rRNA gene expression can be activated by L5 would be limited by the concentrations of either L5, 7 S RNP, or both. Assuming the quantitative determination of 5 S rRNA synthesized in isolated nuclei reflects the number of 5 S rRNA genes that are active, a minimum estimate of the ratio of TFIIIA in 7 S RNP relative to that already bound to 5 S rRNA genes in active transcription complexes would be about 3.7. It should be noted that the current data also provide additional support for the feedback inhibition of 5 S rRNA synthesis by competitive binding of TFIIIA to 5 S rRNA and to the 5 S rRNA gene (3, 8). Competition of L5 and TFIIIA for binding to 5 S rRNA would result in activation of 5 S rRNA synthesis only if 5 S rRNA synthesis were limited by TFIIIA availability and if additional TFIIIA could be mobilized for transcription complex formation from the pool of 7 S RNPs.

The ultimate assembly of the 60 S subunit of the ribosome from three different RNAs and dozens of proteins presents a complex problem of coordinate synthesis or at least of coordinate accumulation. The data reported here provide strong support for one model by which homeostatic regulation of 5 S rRNA and a ribosomal protein to which it binds can be achieved.

Acknowledgments—We thank Martha Rollins for expert assistance and invaluable assistance in carrying out the research, and helpful discussions with Dr. Sandra K. Lemmon for providing the Myc monoclonal antibody used in Western blotting. We are grateful to Tomas Pieler, Paul Huber, Paul Romanuk, and particularly Mike Wormington for plasmids, advice, and helpful discussions.

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