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Finding the Exosome

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Abstract

We describe the events surrounding the identification of the exosome complex and the subsequent early development of the field. Like many scientific discoveries, the initial identification and characterization of the exosome was a based on a combination of skill, good fortune - and the availability of cutting edge technology.

Introduction

The early 1990s were in some respects a frustrating time for ribosome research. On the one hand, established plasmid-based rDNA systems enabled the precise mapping and mutagenesis analysis of processing sites within the pre-rRNA sequence, and a steady flow

of processing factors were being identified by genetic, biochemical and bioinformatic approaches. However, genetic depletion of the identified trans-acting factors typically caused a common set of defects in pre-rRNA processing that led to a general loss of rRNA levels, and it was therefore impossible to identify the specific molecular function of the protein. We therefore decided to concentrate on trying to identify enzymes that participate in the pre-rRNA processing reactions. The identification of these factors promised opportunities for more insightful experiments such as in vitro biochemical analyses, targeted mutagenesis studies and studies on the regulation of the pathway.

From northerns to nucleases

We were therefore interested in screening banks of conditional yeast mutants for those showing blocks in specific steps in the pre-rRNA processing pathway, rather than just the loss of the pre-rRNAs. The problem was that generating and testing banks of mutants required, and still requires, a great deal of work. Happily, at about this time Zoe Lygerou, in the group of Bertrand Séraphin also working at EMBL, was attempting to identify mutants defective in the interaction between the U4 and U6 small nuclear RNAs. To this end they had generated a collection of around 250 temperature-sensitive (ts) lethal yeast strains, extracted RNA from each and resolved the RNA on non-denaturing gels suitable for separating low molecular weight RNAs. The gels were far from ideal for the analysis of pre-rRNA processing defects and carried RNA from only around 250 strains, not all of which had given usable separation (see Figure 1). The blots were, however, to hand and so we screened them by northern hybridization for defects in processing of the similarly sized 5.8S rRNA.

A small number of the ts mutants showed apparent alterations in 5.8S rRNA levels and/or accumulation of potential precursors and for two of these, reanalysis of the mutant by RNA extraction and separation on denaturing gels confirmed the suggested phenotype. The first mutant to be characterized was found to be defective in 5' maturation of both pre-tRNA and 5.8S rRNA. The corresponding gene was cloned by complementation of the ts growth phenotype. Subsequent analysis revealed that it encoded a protein that was a common component of the endoribonucleases RNase P and RNase MRP, the first such protein to be identified [1]. RNase P cleaves the 5' end of tRNAs (reviewed in [2]), whereas RNase MRP cleaves the pre-rRNA at site A3 both in vivo and in vitro [1, 3, 4]. The protein is essential, conserved to humans and was designated Pop1 (for processing of precursor RNAs). This finding led us to propose that RNase MRP and RNase P have evolved from a common enzyme involved in ribosome synthesis [5].

Encouraged by these results, we then turned our attention to the second ts mutation. In this case, better characterization of the pre-rRNA processing phenotype revealed a ladder of 3'-extended forms of the 5.8S rRNA. The corresponding gene was cloned and designated as *RRP4* (rRNA processing defective); like Pop1, the Rrp4 protein was found to be essential and well conserved to humans. By expressing an epitope-tagged form of Rrp4 in yeast and purifying the protein from cell lysates, we showed that Rrp4 had an associated 3' to 5' exoribonuclease activity. These observations led us to propose that the 3' end of the mature 5.8S rRNA is generated by a 3' to 5' exonuclease acting from the downstream C2 cleavage site [6].

A glimpse of the exosome

Glycerol gradient ultracentrifugation analyses indicated that Rrp4 is a component of a moderately sized complex with an estimated mass of approximately 300-400kDa. To identify other protein components of this complex, the epitope-tagged Rrp4 was affinity purified from peak gradient fractions. These studies predated the establishment of the TAP tagging procedure [7] and the purifications were performed using a noncleavable protein A tag (see Figure 2). After binding to IgG beads, retained proteins were nonspecifically eluted with acetic acid and resolved by SDS-PAGE. A significant problem with early analyses was the predominance of IgG molecules on the protein gels due to acid leaching from the beads. This problem was partially minimized by using gel loading buffers lacking mercaptoethanol. At this time, the identification of proteins from excised gel bands was far from routine but, fortunately, Mathias Mann was then developing this approach and agreed to analyze the samples. Despite the large amount of IgG present in the samples, Andrej Schevchenko in Matthias Mann's lab identified four other proteins from the first gels, along with Rrp4. For consistency, we designated these proteins Rrp41, Rrp42, Rrp43, and Rrp44. The band containing Rrp41 was much stronger than expected in the initial gels, but 6 peptides sequenced by mass spectrometry (a lot for that time) all corresponded to Rrp41, leading us to conclude (incorrectly as it turned out) that it was present in multiple copies in the complex [8]. Subsequent analyses were to reveal the presence of multiple, similarly sized proteins in the complex. Reassuringly, genetic depletion of each of the identified proteins elicited the same 5.8S rRNA processing phenotype observed in the original *rrp4-1* ts mutant. To our delight, one of the proteins identified, Rrp44, was clearly homologous to bacterial RNase R, a member of the RNase II family of processive, hydrolytic exonucleases. We obtained a recombinant expression construct for Rrp44 from the lab of Takeharu Nishimoto, who had previously identified the *S.pombe* homologue of Rrp44 (Dis3) through interactions with the Ran GTPase. Using this, we were able to show that Rrp44 had the predicted processive,

exoribonuclease activity. We now had a candidate enzyme for the observed 5.8S rRNA processing activity.

However, we noted that the processive activity of recombinant Rrp44 did not correlate with the distributive activity that was associated with Rrp4 pull-downs from yeast cell extracts, suggesting that either the activity of Rrp44 was altered upon assembly into the complex or that at least one other component of the complex also has catalytic activity. Both explanations eventually turned out to be correct. Before analyzing the Rrp4 complex, we had expressed Rrp4 in *E. coli* and tested the recombinant protein for exonuclease activity. We observed a distributive activity very similar to that seen with the affinity purified yeast complex, which convinced us that Rrp4 was a distributive exonuclease. This result proved to be irreproducible and presumably reflected the presence of a contaminant activity. Studies by Andrej Dziembowski and Bertrand Séraphin [9] later showed that the distributive in vitro activity of the exosome was probably actually due to Rrp6, which was subsequently identified as a component of the complex.

The complete complex

Bioinformatics analyses also played an important part in the characterization of the exosome complex. Initial BLAST analyses revealed that Rrp41 was homologous to another *E. coli* nuclease, RNase PH. BLAST searches failed to identify homologues for Rrp42 and Rrp43, but Sara Mian identified a family of yeast proteins related to RNase PH including Rrp41, Rrp42 and Rrp43 [8, 10]. This family was also predicted to include the uncharacterized products of the *YDR280w* and *YGR095c* genes. Around this time Roy Parker and Reed Wickner discovered that Rrp41 was identical to the product of the previously identified but uncloned *SKI6* gene, which the Parker lab then showed to function cytoplasmic mRNA turnover [11, 12]. They also realized that yet another yeast protein, Mtr3, was homologous Rrp41/Ski6 and RNase PH.

Based on this sequence homology, Christine Allmang made conditional mutants of the two uncharacterized genes and showed that depletion of the encoded proteins also led to the accumulation of 3' extended forms of 5.8S rRNA [13]. We named these genes *RRP45* and *RRP46*, respectively. Analysis of the *mtr3-1* ts mutant, obtained from the lab of Alan Tartakoff, revealed a similar defect in 5.8S rRNA maturation. Exosome complexes from Archeae and plants exhibit exonuclease activity mediated by RNase PH related subunits [14-16], but this does not appear to be the case for other eukaryotic systems tested.

In the meantime, we had made two further modifications to the exosome purification procedures. Firstly, we resolved the cell lysates by ion exchange chromatography prior to SDS-PAGE analysis. This enabled a significant scale-up of the experiments and provided a purification step that was considerably more rapid and as equally effective as resolving lysates through glycerol gradients. We subsequently adopted the technique, developed by Dirk Görlich, for eluting proteins retained on IgG beads with a gradient of increasing $MgCl_2$ concentration [17]. Together, these two approaches produced gels that allowed the identification of Rrp45, Rrp46, Mtr3 and two newly identified proteins, Rrp40 and Csl4, in addition to Rrp4 and Rrp41-44. Genetic depletion of Rrp40 and Csl4 also led to the same 5.8S rRNA processing phenotype. The gels of the gradient eluate fractions also revealed that Rrp44 was not as stably bound to the complex as the other identified components, demonstrating the existence of a stable nonomeric "core". We were also able to biochemically resolve two distinct forms of the complex that differed by the presence or absence of the nuclear protein Rrp6, another exoribonuclease that had been shown to function in 5.8S rRNA processing by the group of Scott Butler [18]. Subsequently, we and others identified three additional substoichiometric components of the complex, the cytoplasmic GTPase Ski7 and the nuclear RNA binding proteins Rrp47/Lrp1 and Mpp6 [19-24].

A broader perspective

We generated cDNA expression constructs of the gene encoding the human homologue of Rrp4 from a HeLa cDNA library that was provided by Karsten Weis, who was then in the lab of Angus Lamond at EMBL, and were able to show that overexpression of the encoded protein complemented the ts growth phenotype of the *rrp4-1* mutant. Coimmunoprecipitation studies subsequently showed that a complex closely related to the yeast exosome was present in human cells, where it had initially been identified as the PM-Scl complex, a target of autoimmune antibodies in patients suffering from the unfortunate sounding polymyositis-scleroderma overlap syndrome (PM-Scl - now termed Scleromyositis) [13, 25]. The human exosome complex was then purified by expressing epitope-tagged hRrp4 and shown to act in ARE-mediated mRNA decay [26].

The barrel shape of the exosome was first predicted by Ambro van Hoof and Roy Parker [27], who insightfully suggested that the structure might be analogous to the proteasome. Support for this model was provided by structural analysis of the Bacterial PNPase, which was shown to have a domain composition strikingly similar to that of the exosome [28, 29]. Finally, the crystal structures of the archaeal and then the human exosome confirmed the exosome structure as a barrel with a central cavity through which substrates pass [16, 30-32].

The distributive activity of yeast exosome complexes observed in vitro did not correlate well with genetic and biochemical data that indicated potent activity in vivo on highly structured RNA-protein complexes. The obvious hypothesis was that the exosome was largely dependent upon additional cofactors for substrate identification and activation in vivo, and numerous cofactors have since been identified; reviewed in [33]. Recent studies have also revealed an endoribonuclease activity of Rrp44 [34-36].

Although the exosome was initially characterized as a pre-rRNA processing complex, it was immediately clear from the growth characteristics of the ts *rrp4-1* mutant that it had additional functions that are essential for normal cell growth. In addition to the studies linking the exosome to cytoplasmic mRNA turnover [12], work in our lab and others soon revealed functions in the processing of small stable RNAs [37] and subsequently showed it to function in surveillance pathways that eliminate aberrantly processed or assembled transcripts [38-41]. Loss of these functions would nevertheless not be expected to trigger the rapid growth inhibition that can be seen in conditional exosome mutants. This may now have been largely explained by recent analyses of the plethora of cryptic noncoding RNAs; see for example [42] and reviews by [33, 43]. These have revealed a fundamental function of the exosome and its cofactors as key gatekeepers of the transcriptome.

Conclusion

Despite many publications, the identification of a vast number of substrates and the characterization of numerous cofactors, key question concerning the exosome remain unanswered. In particular, how are the distinct catalytic activities of the complex coordinated and how do the cofactors modulate the activity of the exosome complex at the molecular level? Resolving these questions will require a range of approaches - and should continue to provide a fruitful field of study for some time to come.

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Figure Legends:

Figure 1: Identification of strain At187 harboring the *rrp4-1* mutation.
A: RNA was extracted under non-denaturing conditions from a collection of temperature-sensitive mutant yeast strains, separated by native gel electrophoresis and analyzed by northern hybridization. Due to the native gel conditions 5.8S rRNA largely remains associated with the 25S rRNA, but the ITS2 probe in strain At187 lit bands above the 5.8S rRNA. B: Subsequent analyses on denaturing gels revealed that these represented the accumulation of 3' extended species. A sequencing ladder was run on the same gel and transferred to the northern filter as a size marker. Following back-crossing, the *RRP4* gene was cloned by complementation of the ts-phenotype of the *rrp4-1* mutation that is carried by this strain.

Figure 2: First analysis of the exosome complex
Analytical gel of the first exosome preparation that was purified over a glycerol gradient. Western analyses of the gradient fractions identified two peaks of Rrp4, which were then purified by affinity chromatography on IgG sepharose beads, eluted with acetic acid and the proteins resolved by SDS-PAGE. Lane 1, pooled gradient fractions from peak I. Lane 2, supernatant from peak I. Lane 3, acid eluate from peak I. Lane 4, 10-fold enriched eluate fraction. Lanes 5-8, equivalent samples from peak II. The sizes (kDa) of molecular weight markers are indicated. Protein bands in the peak II eluate fraction that were subsequently identified by mass spectrometry are labeled.

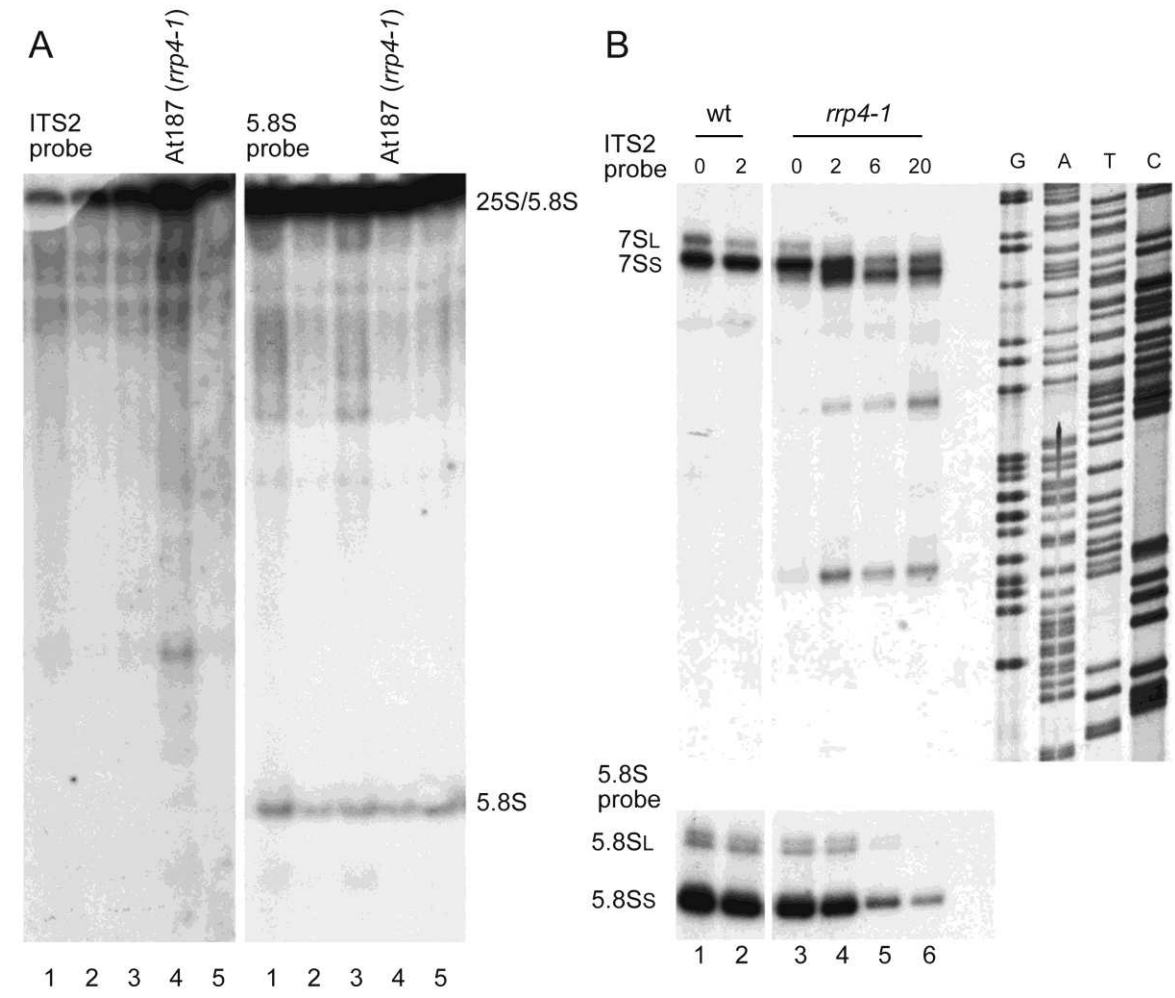
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Figure 1

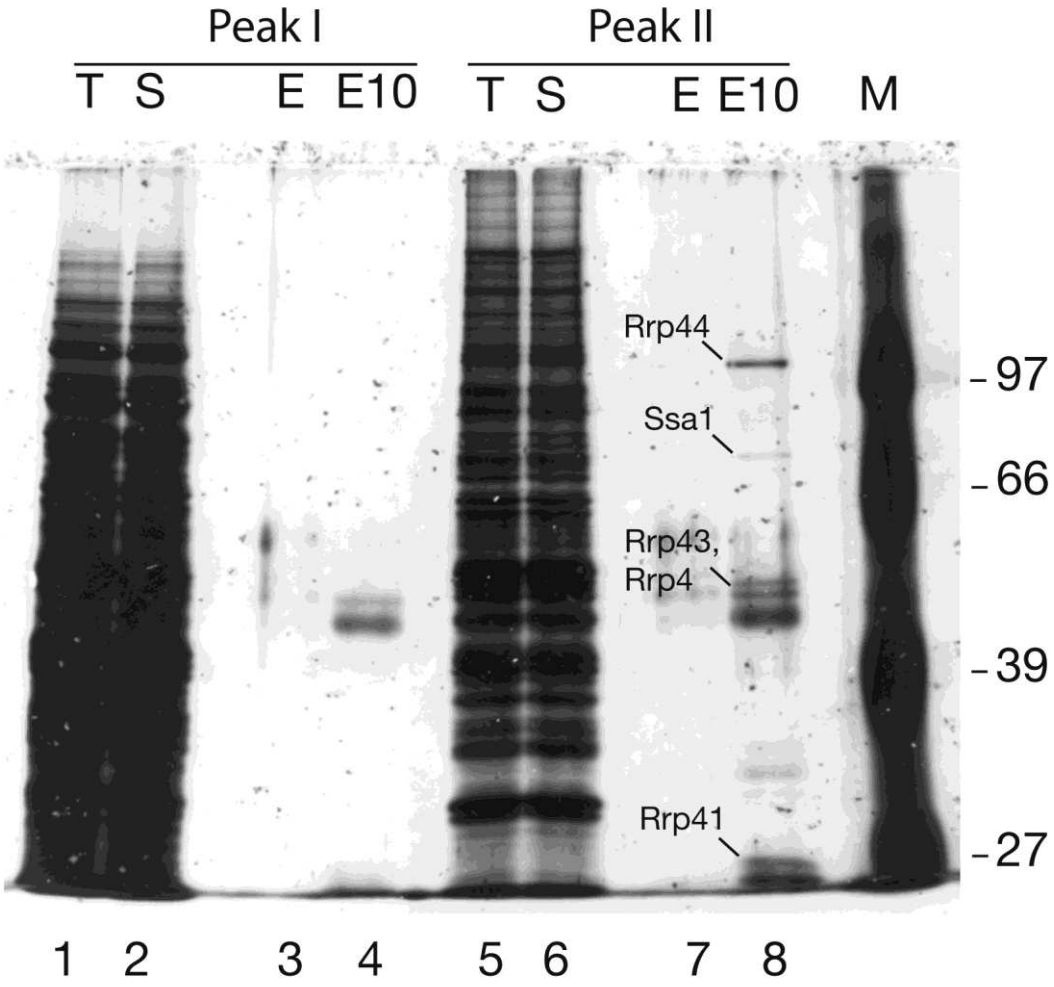
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Figure 2