

Table of Contents

1	Abstract	1
2	Introduction	4
2.1	From a nascent transcript to a mature messenger RNA	4
2.2	Conserved sequences and the chemistry of the pre-mRNA splicing	5
2.3	Building blocks of the spliceosome	7
2.4	Molecular motors of the spliceosome	10
2.5	Step-wise assembly of the spliceosome	12
2.6	Dynamic changes of the spliceosomal RNA-RNA networks	14
2.7	Dynamic exchange of spliceosomal proteins	15
2.8	Prp2, Spp2 and step 1 catalysis by the spliceosome	16
2.9	Prp16, step 2 factors and step 2 catalysis	18
2.10	A two-state conformational model of the spliceosome	18
2.11	A kinetic proofreading mechanism ensures fidelity of splicing reaction	19
2.12	Yeast as an excellent model organism for the study of splicing	20
2.13	A biochemical approach to purify yeast spliceosomes	22
2.14	Mass spectrometry as a tool to investigate the spliceosomal proteome	23
2.15	Investigation of the structure of spliceosomes by electron microscopy	24
2.16	Goals of this study	25
3	Materials	27
3.1	Fine chemicals and media	27
3.2	Enzymes	29
3.3	Commercial reagent sets	29
3.4	Affinity chromatography resins	29
3.5	Antibodies	29
3.6	DNA oligonucleotides	30
3.7	2'-O-methylated RNA oligonucleotides	30
3.8	Plasmids	30
3.9	Strains	31
3.10	Working equipment	31
3.11	Machines	32
4	Methods	33
4.1	Standard methods for nucleic acid production and analysis	33
4.1.1	Estimation of amount and quality of RNA and DNA	33
4.1.2	Production of 5'-MS2-tagged yeast wild-type actin DNA by PCR	34
4.1.3	Denaturing polyacrylamide gel electrophoresis of RNA	34
4.1.4	Visualisation of RNA by silver staining	35
4.1.5	RNA identification by northern blotting	35
4.1.6	Removing a hybridised probe from a nylon membrane	36
4.1.7	Preparative RNA transcription <i>in vitro</i> and purification of yeast pre-mRNA	36
4.1.8	Transcription <i>in vitro</i> and purification of radioactively labelled pre-mRNA	37
4.1.9	Primer extension	38
4.2	Standard methods for protein analysis	39
4.2.1	Estimation of amount and quality of proteins	39
4.2.2	Denaturing polyacrylamide gel electrophoresis of proteins (SDS-PAGE)	39
4.2.3	Visualisation of proteins resolved by SDS-PAGE with Coomassie G250	40
4.2.4	Visualisation of proteins by silver staining	40
4.2.5	Protein identification by western blotting	40
4.2.6	Preparing nitrocellulose membrane for re-probing	41
4.3	Recombinant protein production and purification	41
4.3.1	Over-expression and purification of MBP-MS2 fusion protein	41

4.3.2	Cloning and expression strategy for recombinant spliceosomal proteins	42
4.3.3	Purification of recombinant spliceosomal proteins	42
4.3.4	Fermentation of yeast cells	43
4.4	Special methods	44
4.4.1	Preparation of yeast whole-cell extract	44
4.4.2	Small-scale splicing reaction <i>in vitro</i>	45
4.4.3	Splicing <i>in vitro</i> in the presence of a 2'-O-methylated RNA oligonucleotide....	46
4.4.4	Native PAGE analysis of spliceosomal complexes	46
4.4.5	Splicing reactions <i>in vitro</i> for preparative B ^{actΔprp2} spliceosome purification ...	47
4.4.6	Preparative purification of the spliceosome	48
4.4.7	Analytical splicing reconstitution <i>in vitro</i> with purified spliceosomes and recombinant proteins	49
4.4.8	Preparative splicing reconstitution <i>in vitro</i>	50
4.4.9	Recovery of RNA from splicing and splicing reconstitution <i>in vitro</i>	50
4.4.10	Quantification of step 1 splicing efficiency in the reconstitution <i>in vitro</i> ...	51
4.4.11	Micrococcal nuclease treatment of yeast whole-cell extracts and of affinity-purified yeast spliceosomes	51
4.4.12	Prp2 NTPase assay with colorimetric read-out	52
4.4.13	Prp2 NTPase assay with TLC read-out	52
4.5	Mass spectrometry	53
4.6	2D electrophoresis	53
4.7	Electron microscopy	53
5	Results	54
5.1	Development of the <i>in vitro</i> splicing reconstitution assay	54
5.1.1	Blocking of the spliceosome assembly before step 1 catalysis by a 2'-O-methylated oligonucleotide	54
5.1.2	Purification and characterization of the B1 'oligo'-stalled spliceosomes	57
5.1.3	Test of splicing catalysis in the presence of <i>prp2-1</i> extract	60
5.1.4	Purification and characterization of the pre-catalytic B ^{actΔprp2} spliceosomes..	61
5.1.5	Purified B ^{actΔprp2} spliceosomes are splicing-competent intermediates	64
5.1.6	Cwc25 is required for efficient catalysis of step 1 of splicing	66
5.1.7	Spp2 is required for step 1 catalysis by the purified B ^{actΔprp2} spliceosomes	68
5.1.8	Reconstitution of step 2 of splicing with recombinant proteins	70
5.1.9	Identification of proteins tightly and loosely associating with the B ^{actΔprp2} spliceosome	72
5.2	Compositional and structural remodelling of the spliceosome by Prp2	76
5.2.1	Prominent, ATP-dependent remodelling of the spliceosome by Prp2	76
5.2.2	Prp2/ATP activity leads to destabilisation of U2 SF3a/b, RES, Cwc24, and Cwc27 proteins	77
5.3	Requirements of Prp2 for catalytic activation	82
5.3.1	RNA downstream of the BPS is required for spliceosome activation by Prp2	82
5.3.2	ATPase activity of Prp2 is stimulated by RNA depending on its length	84
5.3.3	Prp2 catalytically activates the spliceosome in the presence of any NTP	86
5.3.4	Spliceosomal Prp2-dependent GTP hydrolysis is stimulated by the RNA downstream of the BPS	87
5.3.5	Length of the RNA downstream of the BPS correlates with the efficiency of step 1 catalysis	90
5.4	Characterisation of the Cwc25 activity and its binding affinity to the spliceosome	92
5.4.1	Cwc25 is a heat-resistant protein	92

5.4.2	Cwc25 interacts with the spliceosome after or concomitantly with the remodelling by Prp2	93
5.4.3	Cwc25 functions after Prp2 in an ATP-independent manner	94
5.4.4	Cwc25 binds to the spliceosome with picomolar affinity	96
5.5	Investigation of the kinetics and of the effect of temperature on step 1 catalysis	97
5.5.1	Kinetics of step 1 catalysis by the purified spliceosomes	97
5.5.2	Differential impact of temperature on the Prp2/ATP-mediated catalytic activation and the Cwc25-dependent step	100
5.6	Two dimensional gel analysis of the B ^{actΔprp2} proteome.....	102
5.6.1	Two-dimensional electrophoresis map of the B ^{actΔprp2} proteome and phosphoproteome	102
5.6.2	The spliceosomes purified using buffers containing 170 mM NaCl undergo catalytic activation by Prp2	108
5.7	Purification of B ^{actΔprp2} spliceosomes with improved homogeneity for high resolution structural studies by electron cryomicroscopy	109
5.7.1	Optimisation of the preparation procedure of the B ^{actΔprp2} spliceosomes for cryo-EM	109
6	MS tables.....	113
	Table 6.1 Protein composition of the <i>S. cerevisiae</i> B ^{actΔprp2} spliceosome.....	113
	Table 6.2 Identification of proteins stably and weakly bound to the B ^{actΔprp2} Spliceosome.....	117
	Table 6.3 Comparison of the association of proteins with <i>S. cerevisiae</i> B ^{act} and B* spliceosomes.....	121
	Table 6.4 Comparison of the association of proteins with the spliceosomes reconstituted <i>in vitro</i>	122
	Table 6.5 Protein composition of the B ^{actΔprp2} spliceosome assembled on the ActΔ6 pre-mRNA and reconstituted <i>in vitro</i> in the presence of Prp2, Spp2, without or with ATP.....	123
7	Discussion	125
7.1	Blocking spliceosome assembly by a 2'-O-methylated RNA oligo complementary to the region between branch site and 3' splice site	126
7.2	Characterisation of the yeast B ^{actΔprp2} spliceosome	127
7.3	Reconstitution of step 1 and step 2 catalysis <i>in vitro</i>	129
7.3.1	Cwc25 is a step 1 splicing factor.....	129
7.3.2	Minimum protein requirements for step 2.....	130
7.4	Investigation of the Prp2 activity using the reconstitution <i>in vitro</i>	132
7.4.1	Prp2 liberates the branch site for step 1	132
7.4.2	Structural remodelling of the spliceosome by Prp2	135
7.4.3	Requirements for Prp2 to catalytically activate the spliceosome.....	137
7.5	Time and temperature dependencies of the catalytic activation and step 1 catalysis.....	139
7.6	Purification of the B ^{actΔprp2} spliceosomes with improved homogeneity.....	142
7.7	Perspectives.....	143
8	References	145
9	Appendix	156
9.1	List of Figures	156
9.2	List of Tables.....	157
9.3	Abbreviations	158
9.4	Acknowledgements	159
9.5	<i>Curriculum vitae</i>	160
9.6	Publication and public presentations.....	161