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Steven M. Theg
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Plastid Biology

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Plastid Biology

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Preface

Photosynthesis is the process through which the energy inherent in sunlight is captured in the chemical bonds of reduced carbon compounds, thereby providing the food upon which almost all life depends. In addition, the production of oxygen as a result of the utilization of water as the ultimate electron donor to the photosynthetic electron transport chain has transformed our atmosphere, allowing for the emergence of oxygenic respiration, without which there would be no human life on Earth.

Photosynthesis is carried out in plants and algae in chloroplasts. Given their central role in energy transduction in the biosphere, chloroplasts have been the focus of attention for generations of scientists. This volume brings together many aspects of modern research into plastids relating to their biogenesis, functioning in photosynthesis and utility for biotechnology.

Plastids had their origins in free living photosynthetic bacteria and took up residence in the primitive eukaryotic cells through endosymbiosis. While they have lost most of their DNA to the nucleus, they retain a functioning genome and are capable of a limited but critical amount of semi-autonomous protein synthesis. Accordingly, we start this volume with a series of three chapters devoted to the handling of the genetic information contained within the plastid genome and crosstalk between the chloroplast and nucleus as the information encoded in both locations is decoded. Following this are five chapters that examine the biogenesis and differentiation of the plastid itself and the sub-structures found at the plastid surface and within the internal thylakoid system. Also included here is a treatment of the unusual non-photosynthetic plastids found within the Apicoplexa, a group of parasitic protists responsible for a number of important human diseases.

Despite having their own genomes, the vast majority of plastid proteins are synthesized in the cytosol and taken up into and subsequently distributed within the organelle. The next six chapters of the volume describe these processes, as well as the roles of molecular chaperones and proteases in protein homeostasis. This is followed by three chapters dedicated to critical aspects of chloroplast physiology relating to dissipation of excess light energy, control of electron transport and ion homeostasis. Finally, the book ends with two chapters discussing the emerging roles of plastids in biotechnology, one as a platform for synthesis of useful proteins, made

desirable because of the superior containment of transgenes within this organelle than when inserted in nuclear genomes, and the other as a source of hydrogen production to be used as biofuel.

Each of the chapters has been written by leading authorities in their respective research areas. Many chapters are the result of collaborations between experts in different laboratories, giving a broader than usual perspective on a given topic. In each case, readers will find well-crafted chapters containing information and insights for both novices and experts alike.

We are grateful to our many friends and scholars who contributed these outstanding chapters. The breadth of their knowledge and clarity of their writing have made for a unique and readable volume bringing together many disparate but interconnected topics relating to plastid biology. We are also indebted to those at Springer, especially Kenneth Teng and Brian Halm, who oversaw this project in its final stages of production.

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Part I
Genetic Material and its Expression

Chapter 1
Chloroplast Gene Expression—RNA Synthesis and Processing

Thomas Börner, Petya Zhelyazkova, Julia Legen and Christian Schmitz-Linneweber

Abstract Both transcription and transcript processing are more complex in chloroplasts than in bacteria. Plastid genes are transcribed by a plastid-encoded RNA polymerase (PEP) and one (monocots) or two (dicots) nuclear-encoded RNA polymerase(s) (NEP). PEP is a bacterial-type multisubunit enzyme composed of core subunits (coded for by the plastid rpoA, B, C1 and C2 genes) and additional protein factors encoded in the nuclear genome. The nuclear genome of Arabidopsis contains six genes for sigma factors required by PEP for promoter recognition. NEP activity is represented by phage-type RNA polymerases. Factors supporting NEP activity have not been identified yet. NEP and PEP use different promoters. Both types of RNA polymerase are active in proplastids and all stages of chloroplast development. PEP is the dominating transcriptase in chloroplasts.

Chloroplast RNA processing consists of hundreds of mostly independent events. In recent years, much progress has been made in identifying factors behind RNA splicing and RNA editing. Namely, pentatricopeptide repeat (PPR) proteins have come into focus as RNA binding proteins conferring specificity to individual processing events. Also, studies on chloroplast RNases have helped considerably to understand chloroplast RNA turnover. Such mechanistic insights are set in contrast to how little we know about the regulatory role of RNA processing in chloroplasts.

Keywords Chloroplast transcription · Chloroplast RNA polymerase · Chloroplast promoter · Chloroplast RNA processing · Chloroplast RNA-binding proteins · PPR proteins · Chloroplast splicing · Chloroplast editing · Chloroplast RNA degradation · Chloroplast nucleases

Abbreviations

CRS2	Chloroplast RNA splicing 2 protein
IR	Inverted repeat
NEP	Nuclear-encoded plastid RNA polymerase

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Nt	Nucleotides
PEP	Plastid-encoded plastid RNA polymerase
PPR	Pentatricopeptide repeat
TAC	Transcriptionally active chromosome
TFs	Transcription factors
TPR	Tetratricopeptide repeat
TSSs	Transcription start sites

1.1 Introduction

Chloroplasts, which have their own genomes (plastomes) and specific machineries for gene expression, evolved from a bacterium that was related to the extant cyanobacteria. During evolution, the majority of the cyanobacterial genes were lost or transferred to the nucleus; only a few genes, mainly those required for photosynthesis and gene expression, are currently retained in the plastome ([84, 321]; see Chap. 3). Despite the lower gene content, however, the transcriptional apparatus of higher-plant chloroplasts is more complex than that of bacteria. For example, bacteria use a multisubunit RNA polymerase to transcribe all of their genes. Chloroplasts in angiosperms and possibly in the moss, *Physcomitrella,* possess a homologous enzyme, but additionally require one or more single-subunit phage-type RNA polymerases for transcription. In contrast, the chloroplasts of algae and the lycophyte, *Selaginella,* have a simpler, more archaic apparatus that seems to rely solely on the bacteria-type multisubunit enzyme for transcription [320]. RNA processing is also more complex in chloroplasts than in bacteria, as virtually all chloroplast mRNAs, rRNAs and tRNAs are subjected to maturation, which involves trimming of the 5′ and/or 3′ ends. To become functional, many transcripts require additional *cis*- and/or *trans*-splicing, and (in the case of most land plants) editing of their nucleotide sequences [14]. Transcription and RNA processing seem to take place in close proximity, since components of both processes are found together with DNA in the nucleoids of chloroplasts [176]. In addition to tRNAs and rRNAs, many other non-coding RNAs (including a large number of antisense RNAs) have recently been found in plastids, partly through deep-sequencing strategies [58, 81, 109, 169, 188, 316, 338, 340]. Many of the detected non-coding RNAs are the products of transcription from own promoters [306, 340]; these non-coding RNAs could play a role in regulating gene expression, thus further increasing the complexity of plastid RNA metabolism [77, 108, 267, 316, 337]. A number of the recently described small plastid RNAs, however, are identical to the 3′ and 5′ end regions of mature mRNAs protected from degradation by RNA-binding proteins or stem-loop structures, and are therefore thought to represent by-products of RNA degradation and processing with questionable potential for regulatory functions [239, 340]. A well-investigated example of a plastid non-coding RNA is the *Chlamydomonas tscA* RNA which functions in trans-splicing [233].

This chapter focuses on recent studies dealing with the function of RNA polymerases in plastid gene expression and the role of RNA-binding proteins in the processing of chloroplast transcripts. For more information, a number of recent reviews provide more details on the evolution and regulation of chloroplast transcription, the function of plastid sigma factors, and on plastid RNA processing [14, 155, 160, 262, 320].

1.2 RNA Synthesis

1.2.1 The Plastid-Encoded Plastid RNA Polymerase (PEP) is a Bacteria-Type Multisubunit RNA Polymerase

Homologs of the cyanobacterial RNA polymerase subunits α, β, β′ and β″ are encoded by the plastid *rpoA, B, C* and *C1* genes; together, these form the core of the *p*lastid-*e*ncoded plastid RNA *p*olymerase (PEP; [111, 198, 269, 272]). Similar to the gene organization in bacteria, *rpoA*, which encodes the α subunit of PEP, is found in a gene cluster with several genes encoding ribosomal proteins [223], while *rpoB*, *rpoC* and *rpoC1,* encoding the β, β′ and β″ subunits, respectively, together form an operon [127, 269]. The PEP β and β′ subunits can serve as functional substitutes for the homologous subunits of the *E. coli* RNA polymerase [265]. PEP is sensitive to tagetitoxin, an inhibitor of bacterial transcription [178], further demonstrating the high degree of conservation between the plastid-encoded and eubacterial RNA polymerases. However, the PEP α subunit does not substitute for the *E. coli* homolog in transplastomic tobacco plants [285]. As the bacterial polymerase, the chloroplast core enzyme requires a sigma (σ) factor for promoter recognition and initiation of transcription [162]. While *Chlamydomonas reinhardtii* has only one nuclear gene encoding a sigma factor [26], land plants and the red algae, *Cyanidioschyzon merolae* and *Cyanidium caldarium*, possess several sigma factor genes ([154, 165, 180], for reviews on higher plant sigma factors see [262, 290, 291]). It is not yet known whether the less complex organization of the transcriptional apparatus in algae (PEP alone and fewer sigma factors) is causally related to the lower degree of transcriptional regulation in algal chloroplasts versus those of higher plants [62, 76].

PEP can be isolated from plastids as a soluble enzyme or an insoluble form, also known as *t*ranscriptionally *a*ctive *c*hromosome (TAC), which contains DNA, RNA, the PEP subunits, and a large number of other proteins [37, 89, 144, 164, 215, 230]. Similar to isolated nucleoids [241], TAC exhibits *in vitro* transcriptional activity. The soluble PEP fraction isolated from mustard (*Sinapis alba*) etioplasts, referred to as PEP-B, consists of only the core subunits (Fig. 1.1a; [217, 276]. However, the existence of transcription factors in very low amounts and/or only loosely associated with PEP-B cannot be completely ruled out. Soluble PEP preparations from photosynthetically active plastids, called PEP-A, contain the

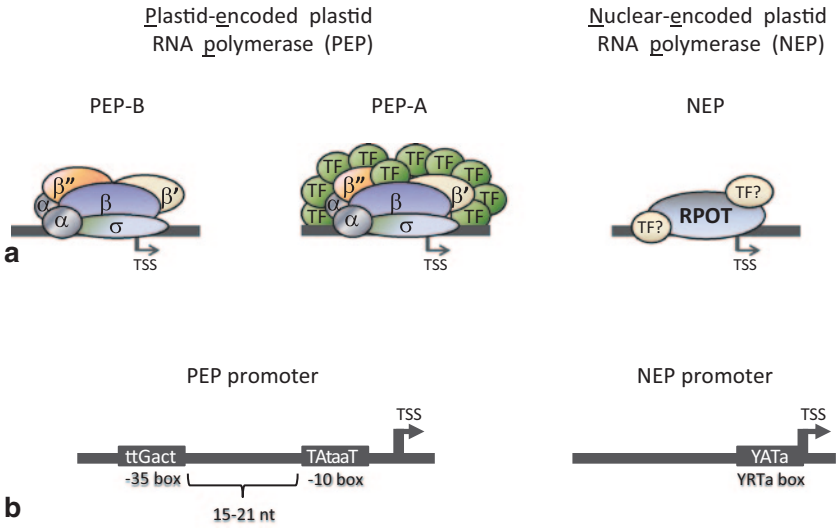

Fig. 1.1 Plastid RNA polymerases and their promoters. **a** PEP-A and PEP-B represent the soluble forms of PEP isolated from chloroplasts and etioplasts, respectively. PEP-B comprises the core subunits 2 α, 1 β, 1 β′ and 1 β″. For promoter recognition and transcription initiation, a σ factor is needed. PEP-A has a more complex structure and consists of the core subunits, the σ factor, and auxiliary factors such as transcription factors (TFs) like the PAPs (see text). For RNA synthesis, the nuclear-encoded plastid RNA polymerase (NEP) requires only the catalytic subunit, RPOT. Unknown TFs support promoter recognition and regulation. **b** Structures of the PEP and NEP promoters, with consensus sequences as found in the barley plastome. Typical PEP promoters resemble bacterial promoters with −10 and −35 consensus sequences, while typical NEP promoters have a YRT core motif. Note, however, that many PEP and NEP promoters do not conform to the depicted structures. The transcription start sites (TSSs) are indicated by *arrows*

PEP core subunits associated with ~10 nuclear-encoded proteins (Fig. 1.1a). PEP complexes have been assessed in etioplasts and chloroplasts; other plastid types have not yet been analyzed in terms of their protein compositions. The proteins associated with the core subunits of PEP (the *P*EP-*a*ssociated *p*roteins, or PAPs) in PEP-A preparations [276] are also observed as components of TAC (the pTACs). Experimental data support the view that the PAPs/pTACs are required for transcription and its regulation under light conditions [122, 197, 215, 217, 218]. Additional factors involved in transcription and the regulation of gene expression can be found in nucleoid preparations [138, 176, 228]. The combination of PEP with its accessory proteins may help establish nuclear control over plastid transcription and adapt transcription to endogenous and exogenous cues [276]. This is also true for the sigma factors, which confer promoter recognition to PEP. The PEP sigma factors of higher plants belong to the eubacterial σ70 family [173]. *Arabidopsis* has six different sigma factors [74, 154, 260, 262]. Sigma factors do not co-purify with PEP, perhaps because they are not needed for the elongation phase of RNA synthesis [276]. In addition, highly purified PEP complexes do not contain the plastid transcription kinase, cpCK2, or the *c*hloroplast *s*ensor *k*inase, CSK [276],

even though these enzymes are believed to regulate transcription by phosphorylating PEP subunits and sigma factors in a photosynthesis/redox-dependent manner [10, 11, 36, 126, 163, 197, 224, 225, 302]. Experimental data support the involvement of sigma factors in the regulation of plastid transcription during development and in response to changing environmental conditions (reviewed in [154, 155, 260, 262]). Transcription of plastid genes is also controlled by hormones, but future studies will be needed to identify the factors responsible for mediating the effects of hormones on plastid transcription [160, 344, 345].

1.2.2 PEP Promoters

Given the bacterial origin of PEP, it is unsurprising that many of the promoters utilized by PEP resemble the *E. coli* σ70 promoter architecture, which harbors both −35 and −10 consensus sequence elements [75, 85, 282]. The *E. coli* RNA polymerase can accurately transcribe from such PEP promoters [34, 35]. In *Chlamydomonas* chloroplasts, however, most promoters lack a conserved −35 sequence element; instead, extended −10 boxes and/or more remote sequences confer full promoter strength [24, 116, 133, 140, 141]. Furthermore, neither the −10 nor the −35 box seem to be essential for a functional PEP promoter in higher plants. According to a plastome-wide search for conserved PEP promoter motifs, the −10 element "TAtaaT" (upper-case letters indicate overrepresented nucleotides >1 bit) is located 3–9 nucleotides (nt) upstream of the transcription start site of 89% of all primary (unprocessed) transcripts in the chloroplasts of mature barley leaves, and the −35 element "ttGact" can be found 15–21 nt upstream of 70% of the PEP promoters harboring this −10 motif (Fig. 1.1b; [340]). Comparable whole-genome analyses are not yet available for algae and dicots. The −10 and −35 boxes can be complemented or replaced by other sequences, most of which have not yet been identified. For instance, the mustard *psbA* promoter harbors a regulatory element (TATATA) between the −10 and −35 promoter elements; *in vitro*, this regulatory element promotes a basal level of transcription in the absence of the −35 region in plastid extracts from dark- and light-grown plants. However, the −35 element is essential for the full promoter activity required during active photosynthesis [64, 161], and it is needed for *in vitro* transcription in barley chloroplasts [137]. In the case of the wheat *psbA* promoter, an extended −10 sequence (TGnTATAAT) is utilized as the sole *psbA* promoter element by PEP in mature chloroplasts. PEP obtained from developing chloroplasts in the leaf base, however, requires both the −10 and −35 boxes, suggesting that different transcription factors may participate during chloroplast development [248]. Several *cis*-elements required for the binding of regulatory proteins in the context of PEP promoters have been described. A 22-bp sequence, known as the AAG box, plays an important role in regulating the blue light-responsive promoter of *psbD* (which encodes the photosystem II reaction center chlorophyll protein, D2) by providing a binding site for the AAG-binding factor, PTF1, which acts as a positive regulator [7, 137]. The blue-light dependent

activation of the *psbA* and *psbD* promoters in *Arabidopsis* chloroplasts depends on the sigma factor, SIG5, whose expression is stimulated by blue light [204]. SIG5 is also responsible for the enhanced transcription of *psbD* and several other genes under various stress conditions ([193]; Yamburenko et al., unpubl. data). Similarly, a transcription factor binds to a sequence −3 to −32 nt upstream of the *rbcL* transcription start site and enhances transcription [136]. *In silico* analyses suggest that there are many more, yet-uncharacterized nuclear-encoded plastid transcription factors [258, 312].

Similar to most protein-encoding genes/operons and the rRNA gene cluster, the majority of tRNA genes are transcribed by PEP from typical σ^{70}-like promoters upstream of the transcription start site [155]. In addition, some reports suggest that several tRNAs are transcribed from gene-internal promoters; these include the spinach *trnS*, *trnR* and *trnT* [53, 86, 323], the mustard *trnS*, *trnH* and *trnR* [156, 195, 196], and the *Chlamydomonas trnE* [119]. However, the exact tRNA-related internal promoter elements and the polymerase(s) capable of recognizing them have not yet been elucidated.

1.2.3 The Nuclear-Encoded Plastid RNA Polymerase (NEP) is Represented by Phage-Type RNA Polymerases

In stark contrast to the bacterial RNA polymerase, PEP is not sufficient to transcribe all plastid genes in higher plants. Instead, a *n*uclear-*e*ncoded plastid RNA *p*olymerase (NEP) activity participates in and is essential for plastid transcription [1, 102, 271]. The first evidence for the existence of one or more NEP enzymes came from studies on the effect of translation inhibitors on cytoplasmic and plastid ribosomes [65]. Active RNA synthesis occurs in ribosome-deficient plastids, suggesting a nuclear location for the gene(s) responsible for this activity [39, 95, 102, 271]. Moreover, transcription takes place in plastids of the parasitic plant, *Epifagus virginiana,* even though its plastome lacks genes encoding the core subunits of PEP [68, 189]. Similarly, plastid genes are transcribed in PEP-knockout transplastomic tobacco plants, but these plants have an albino phenotype, suggesting that NEP alone cannot provide for photosynthetically active chloroplasts [1, 88, 151].

NEP is represented by one or more phage-type RNA polymerases in higher plants [97, 98, 153], encoded by the *RpoT* (*R*NA *po*lymerase of the phage *T3*/*T7* type) genes [97]. In contrast to the multi-subunit PEP, these phage-type enzymes are composed of only a single catalytic subunit, possibly associated with only one or a few auxiliary factor(s) (see below; Fig. 1.1a; [146]). While monocots and the basal angiosperm, *Nuphar*, contain only one plastid phage-type RNA polymerase (RPOTp; [46, 66, 148, 332]), eudicots have two of these enzymes, RPOTp and RPOTmp, the latter of which is targeted to both plastids and mitochondria [98, 99, 142, 147]. Knocking out the *RpoTp* or *RpoTmp* genes in *Arabidopsis* yields plants with delayed chloroplast biogenesis and slightly altered leaf morphogenesis, while *RpoTp/RpoTmp* double mutants exhibit a more severe phenotype characterized by extreme growth retardation [110]. Transgenic tobacco and *Arabidopsis* plants

overexpressing RPOTp show increased transcription from a set of NEP promoters [159], and RPOTp recognizes distinct NEP promoters *in vitro* [146]. Even though RPOTmp fails to drive transcription from NEP promoters *in vitro* [146], the enzyme plays a distinct role in plastid transcription during the early developmental stages of *Arabidopsis* [54].

Specific antibodies detect both RPOTp and RPOTmp in the stroma and membrane fractions of plastids (J. Sobanski et al., unpublished data, [5, 46]) and the two phage-type polymerases can be prepared from plastids in both soluble and membrane-bound forms (J. Sobanski et al., unpublished data, [5, 6]). The RING H2-protein mediates the binding of RPOTmp to the stromal side of the thylakoid membrane in spinach [6]. RPOTp and RPOTmp are not detected in purified PEP fractions, PEP-containing TAC preparations, or the proteome of plastid nucleoids [176, 199, 215, 276], most likely because the phage-type polymerases are much less abundant than the PEP subunits in chloroplasts.

The phage T7 RNA polymerase is a genuine single-subunit enzyme; the complete process of transcription (including promoter recognition, initiation, elongation and termination) is performed by a single protein, regardless of whether the DNA template is linear, circular or supercoiled [277]. Similarly, the *Arabidopsis* RPOTp polymerase is able to correctly recognize promoters, transcribe the gene, and stop at a (bacterial) terminator without additional factors in *in vitro* assays, provided that the DNA templates are in the supercoiled conformation [146]. However, *Arabidopsis* RPOT polymerases are also capable of correctly initiating transcription *in vitro* on linear double-stranded DNA templates if the base sequence of the promoter is altered to prevent base pairing (i.e., if the promoter region is already in a partially open state; A. Bohne and T. Börner, unpublished data). This finding suggests that, similar to the related phage-type RNA polymerases in yeast and human mitochondria [59, 179, 232, 284], RPOT polymerases need additional factors to melt the DNA duplex at promoter regions *in organello*. However, such factors have not yet been identified in plants [231]. As shown for PEP (see above), transcription by NEP is also affected by developmental and environmental cues (reviewed in [155, 160]). In the case of the Type II Pc promoter of spinach chloroplasts, a specific transcription factor, CDF2, is involved in the development-dependent decision on whether to use the NEP promoter or the PEP promoter for transcription of the *rrn* genes [23]. Future work is warranted to identify additional NEP-interacting factors and the signaling pathways responsible for regulating NEP activity.

1.2.4 NEP Promoters

In green chloroplasts, PEP transcripts are overrepresented, while most of the transcripts generated by NEP are of low abundance and not easily detectable [101, 158]. Therefore, the NEP transcription start sites have been identified in plants lacking PEP activity [1, 112, 264, 273, 287, 340]. Based on their architectures, the NEP promoters can be grouped into three types: Type-Ia, Type-Ib, and Type-II [158, 319]. The majority of the analyzed NEP promoters belong to

the Type-I NEP promoters, which are characterized by a conserved YRTa core motif located a few nucleotides upstream of the transcription start site (Fig. 1.1b; [340]). The plastid promoters share the YRTa motif with many plant mitochondrial promoters [112]. The similarity of the NEP and mitochondrial promoters is not surprising, since the NEP-encoding genes originated from duplication(s) of the gene encoding the mitochondrial RNA polymerase [320]. NEP accurately initiates transcription at the *Oenothera berteriana* mitochondrial *atpA* promoter when integrated into the tobacco plastome, suggesting that there are relationships not only between the promoters and RNA polymerases of plant mitochondria and chloroplasts, but also among the factor(s) involved in promoter recognition [27]. The Type-I promoters are further divided into two subclasses, Type-Ia and -Ib. Type-Ia promoters have only the YRTa box as a conserved sequence motif. No sequence elements outside of this core motif have significant influence on *in vitro* transcription from the tobacco *rpoB* Type-Ia promoter [157]. However, deletion analysis of the 5′-flanking region of the *Arabidopsis rpoB* fused to GUS and transiently expressed in the chloroplasts of cultured tobacco cells suggests the existence of additional regulatory elements upstream of the YRTa sequence [113]. The Type-Ib NEP promoters carry an additional conserved sequence motif ($ATAN_{0-1}GAA$), called the "GAA box", located approximately 18–20 nt upstream of the YRTa motif [319]. Deletion analysis of the tobacco Type-Ib P*atpB*-289 promoter reveals that the GAA box plays a functional role in promoter recognition both *in vivo* and *in vitro* [129, 325]. There is no Type-Ib promoter in the barley chloroplast genome, suggesting that this promoter type may not be used by NEP in the plastids of Poaceae and perhaps other monocots [340].

Transcription from Type-II NEP promoters is YRTa-independent, and is instead controlled by "non-consensus" promoter elements [160]. The best investigated example is the tobacco *clpP* NEP promoter, whose core sequence comprises the region −5 to +25 with respect to the transcription initiation site [275]. Interestingly, the *clpP* NEP promoter sequence is conserved among monocots, dicots and *C. reinhardtii*, but is not required to drive transcription in rice and *Chlamydomonas*. However, when introduced into tobacco, the rice sequence is efficiently utilized as a promoter. This promoter sequence might therefore be recognized by a distinct transcription factor or NEP enzyme that is present in dicots but not monocots, such as PROTmp [159, 275]. The Pc promoter of the *rrn* operon in spinach chloroplasts represents another non-YRTa NEP promoter [155]. The promoter region of the *rrn* operon is highly conserved in plants and contains the −10 and −35 PEP promoter elements, which drive PEP-mediated transcription of the operon in barley, tobacco, maize, and later in the development of *Arabidopsis* chloroplasts [1, 54, 112, 282, 307]. However, in spinach, as well as during the early developmental stages of *Arabidopsis* chloroplasts, NEP initiates at the Pc promoter located between the conserved PEP promoter elements [9, 54, 114, 115, 287]. Approximately 70% of the more than 200 NEP promoters used in the PEP-deficient plastids of *albostrians* barley have a YRTa box as the only conserved promoter element, and thus belong to Type-Ia. The remaining 30% of the NEP promoters lack YRTa, as well as any other consensus motif in

the region −50 downstream to +25 upstream of the transcription start sites [340]. Thus, the Type-II promoters may be regarded as a group of apparently unrelated promoters defined by the lack of YRTa.

1.2.5 Division of Labor among Different Plastid RNA Polymerases

The algae investigated to date and the lycophyte, *Selaginella moellendorffii*, do not show NEP activity; instead, PEP transcribes all of their chloroplast genes (reviewed in [320]). Angiosperms and most likely also the moss, *Physcomitrella patens*, rely on NEP in addition to PEP for plastid transcription, although the advantage of this is a matter of some debate. The establishment of NEP activity is believed to have evolved in land plants to offset elevated levels of point mutations in PEP promoters, which may have occurred due to enhanced UV irradiation after the water-to-land transition [175]. This view is supported by two observations: in the absence of PEP, numerous NEP promoters are activated in barley plastids [340]; and a NEP promoter that is inactive in wild-type *Arabidopsis*, compensates when transcription is abolished from the *atpB* PEP promoter in a sigma factor-6 knockout line [261]. An additional or alternative advantage of a second RNA polymerase activity in plastids might be stronger control of organellar transcription by the nuclear genome.

A division of labor between PEP- and NEP- mediated transcription was first proposed by Hess et al. [102] and further elaborated by Mullet [192] and Hajdukiewicz et al. [88]. Initial studies suggested that NEP plays a role in transcribing housekeeping genes, while PEP is responsible for transcribing the photosynthetic genes [1, 88, 102, 112, 130, 308]. However, later studies showed that there is no strict division of labor between the two polymerases with respect to the functional classes of plastid genes they transcribe (housekeeping/non-photosynthetic vs. photosynthetic). Many housekeeping genes have both PEP and NEP promoters, and certain non-photosynthetic genes are transcribed only by PEP in green leaves (e.g., [88, 307, 340]). A few potential NEP promoters may exist upstream of photosynthetic genes in normal green chloroplasts (Fig. 1.2; [340]), and more than 200 new NEP promoters are activated in the leaf plastids of a barley mutant lacking PEP activity, resulting in the NEP-mediated transcription of virtually all plastid genes ([339]; see also [151]).

The transcriptional activity of plastid genes massively increases with the onset of chloroplast development (reviewed in [155]). In addition, the transcription of the *rpoB-C1-C2* genes is NEP-dependent [102] and precedes the strong transcription of photosynthetic genes during chloroplast development in barley [18] and pea leaves [61]. These data, together with the detection of NEP promoters upstream of housekeeping genes (see above), led researchers to suggest that NEP might be responsible for the basal transcriptional activity in the plastids of non-green cells. With the onset of chloroplast development from non-green proplastids, increased NEP activity would transcribe the genes encoding the core subunits of PEP. Then,

PEP would take over transcription and provide the high transcriptional activity needed for further chloroplast development, including the assembly of the photosynthetic apparatus [88, 192]. Indeed, NEP promoters are more active in early leaf development, while the transcriptional activity of PEP increases during chloroplast maturation [18, 54, 58, 66, 130, 288, 342]. However, these roles of NEP and PEP in chloroplast development have not yet been directly demonstrated. More recent data show that both PEP and NEP are present and active in all investigated green and non-green tissues during all developmental stages of the leaf [38, 42, 57, 58, 125, 288, 305, 342]. Nevertheless, PEP is clearly the predominating RNA polymerase in photosynthetically active chloroplasts (Fig. 1.2; [340]). PEP transcribes the vast majority of plastid genes, including all photosynthetic genes. In mature barley chloroplasts, active NEP promoters (but no PEP promoters) were mapped within 750 nt upstream of the *rpl23* and *rpoB* coding sequences. However, *rpl23* is part of a PEP-controlled gene cluster [128, 174], leaving *rpoB-C-C1* as the only known example of an exclusively NEP-dependent transcript in monocots [340]. Although chloroplast genes can be transcribed from promoters located even further upstream of the coding region [308], no PEP-dependent transcription start sites is seen in the 2 kb region upstream of the annotated *rpoB* gene in the barley plastome (Fig. 1.2). Given that multiple promoters are very common in plastids and a large percentage of genes/operons have both NEP and PEP promoters [155, 340], it is remarkable that the expression of the genes encoding the ß, ß′ and ß″ PEP subunits is entirely dependent on NEP in both monocots and dicots [157, 287, 340].

The nuclear genomes of the eudicots harbor two genes for NEP activity, *RPOTp* and *RPOTmp* [98], suggesting that there is also a division of labor between the two NEP polymerases. Indeed, several studies suggest that RPOTp and RPOTmp display their major activities in different tissues and developmental stages. In *Arabidopsis*, *RPOTmp* promoter activity is detected in young, non-green cells of different organs, whereas *RPOTp* expression is mainly observed in green, photosynthetically active tissues [67]. In agreement with this observation, Courtois et al. [54] found that RPOTmp is needed for the synthesis of rRNAs from the Pc promoter in *Arabidopsis* seeds during imbibition, while at later stages, PEP becomes the principle polymerase responsible for *rrn* transcription [54]. Furthermore, lack of RPOTmp activity resulted in lower accumulation of several chloroplast transcripts in young *Arabidopsis* seedlings upon illumination [8, cf. 147]. However, several lines of evidence suggest that RPOTp is also present and required early in development, and that RPOTmp may also play a role in mature chloroplasts. The activity of RPOTmp in mature chloroplasts can be deduced from the use of NEP promoters in *Arabidopsis* mutants lacking RPOTp. However, the strong NEP promoter that drives transcription of the essential *ycf1* gene in wild-type dicot chloroplasts is not used in very young RPOTp mutant seedlings, hinting that RPOTp may play a role at this early stage of development [288]. In addition, knocking out or knocking down RPOTp decreases the levels of transcripts originating from NEP promoters in both mature and developing *Arabidopsis* chloroplasts (the effect is more pronounced in the latter; [288]). RPOTp appears to prefer Type-I promoters, while RPOTmp

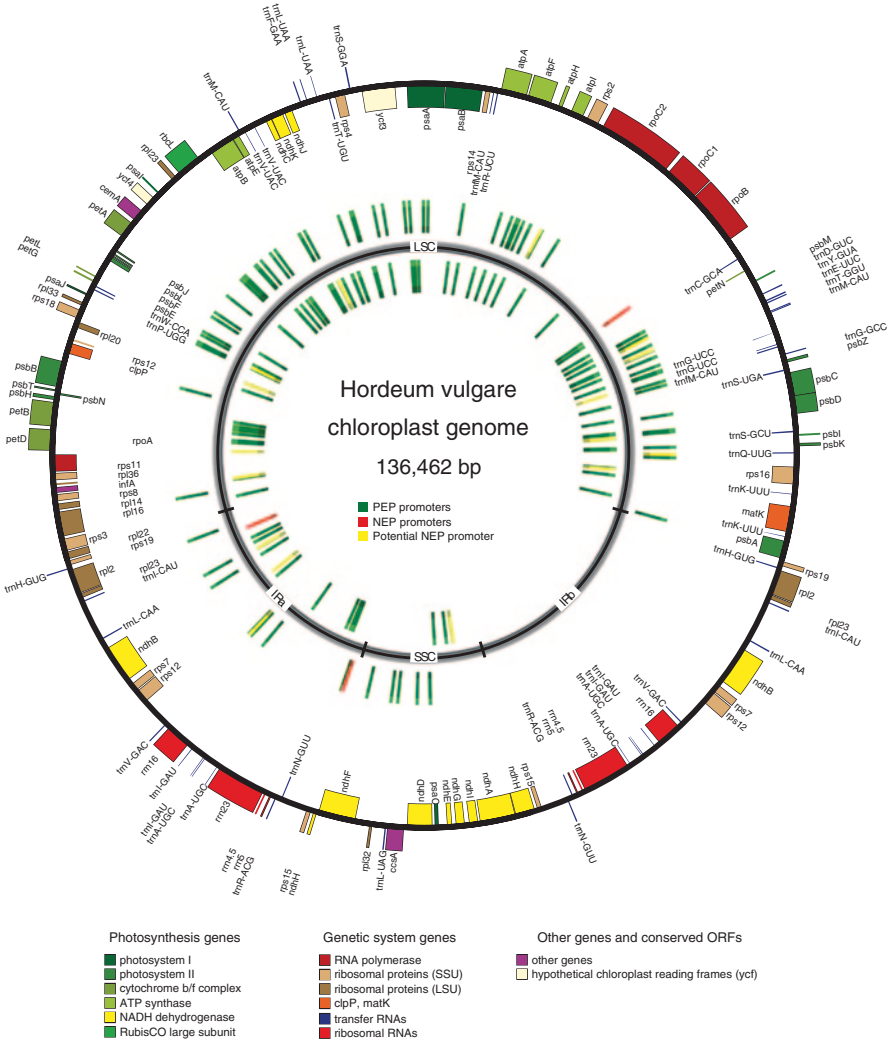

Fig. 1.2 Distribution of PEP- and NEP-dependent transcription start sites (TSSs) in mature barley chloroplasts. The *outer circle* depicts the gene organization of the barley chloroplast genome (NC_008590). The graphical representation was created using OGDraw (OrganellarGenomeDRAW; http://ogdraw.mpimp-golm.mpg.de/; [166]) and further modified. Genes at the inside and outside of the circle are transcribed clockwise and counterclockwise, respectively. Genes are color coded based on the function of the proteins they encode (see the legend *below* the circle). The *inner circle* depicts the genomic distribution of the TSSs mapped in mature barley chloroplasts as follows: *green*—PEP-dependent TSSs; *red*—NEP-dependent TSSs; *yellow*—potential NEP-dependent TSSs. TSSs mapped to the inverted repeat (IR) are shown only within IRa. The image was generated using CGView (Circular Genome Viewer; http://wishart.biology.ualberta.ca/cgview/; [281])

prefers Type-II promoters. Overexpression of RPOTp enhances the usage of Type-I promoters [159]. Similarly, usage of the non-consensus Type-II promoters of the *clpP* gene and the *rrn* operon is unaffected and enhanced, respectively, by the lack of RPOTp activity. However, most of the Type-I NEP promoters are still active in the absence of RPOTp, suggesting that RPOTmp can recognize Type-I promoters [288].

1.3 RNA Processing

Early on, transcription was recognized as a major point of gene regulation in bacteria, epitomized by the operon model of Jacob and Monod [118]. In addition to the core transcriptional machinery, a number of factors (repressors or activators of transcription) are known to determine the usage of bacterial promoters. Such modulators of transcription initiation are DNA-binding proteins, and include the famous *trp* repressor [250]. Bacterial RNAs are translated as they are transcribed, so there is very little posttranscriptional RNA processing. Splicing, RNA editing and intercistronic processing are rare events in bacteria; thus, transcription initiation and RNA degradation largely determine mRNA expression and eventual protein production [83]. Although non-coding RNAs have lately come into focus as regulators of gene expression in bacteria, prokaryotes undergo relatively little regulated RNA processing.

In chloroplasts, however, every primary RNA is subject to some form of modification after transcription [278]. As in bacteria, chloroplasts express the majority of their genes as polycistronic RNAs. However, the bacterial concept of the operon as a cluster of co-regulated genes does not fully apply to plastids. Instead of being directly translated, numerous polycistronic transcripts function as precursors that are cleaved into smaller polycistronic or monocistronic RNAs, many of which still require splicing and/or RNA editing to become functional [14, 278]. Thus, RNA maturation further increases the complexity of RNA populations arising from most genes. Major events in plastid RNA maturation (*e.g.,* 5′- and 3′-end processing and intercistronic processing) involve the action of ribonucleases that have low sequence specificity, and the extent of processing is often determined by barriers such as RNA-binding protein and the presence of secondary structures [14, 278].

This part of our review focuses on the poorly understood complexity of posttranscriptional processes in chloroplasts. We will summarize the most important findings on the central processes of RNA splicing, editing and end maturation, and then focus on studies that point to the potential regulatory functions of these RNA processing steps. In contrast to translational regulation, which is discussed in the accompanying article by Nickelsen et al. (Chap. 3), only a few studies demonstrate that RNA processing has a true rate-limiting effect on chloroplast gene expression. We will not attempt a detailed discussion of the large body of work on the mechanistic aspects of RNA processing. For this, we direct the reader to recent reviews

on the individual RNA processing steps of splicing, editing and RNA degradation [50, 117, 279].

1.3.1 Chloroplast RNA Splicing

1.3.1.1 Chloroplast Introns and Factors

The two dominant classes of introns found in the chloroplast genes are the group I and group II introns, which are archaic introns believed to be the precursors of the eukaryotic spliceosomal introns [45, 104, 247, 270, 310]. Group I and group II introns are structurally different, and harbor subdomains that have specific functions in the splicing reaction [242]. For example, the group II introns share six secondary domains that fold into a structure that is held together by tertiary interactions within the intron and with exonic sequences [185]. This structure brings together the splice sites, intron-internal guiding sequences, and the branch point. The number of introns and their positions within the genome are relatively stable; the chloroplast genes of land plants usually contain around 20 introns, all but one of which fall into group II (for example: 17 intron in maize chloroplasts, 21 in Arabidopsis thaliana chloroplasts, [252]). These introns disrupt protein-encoding genes as well as those for tRNAs. In chlorophyte algae, group I introns are far more dominant, and the overall intron number per genome is more variable than that in land plants (e.g. 7 introns in *C. reinhardtii*, 27 in *Pseudendoclonium*; [181, 219]). In addition, some chlorophytes also have introns in their rRNA-encoding genes [235]. These introns are all ribozymes by definition, and bacterial group I and group II introns can be made to self-splice *in vitro* [242]. However the chloroplast introns require *trans*-acting factors for excision [252]. A large and growing set of nuclear-encoded proteins important for chloroplast splicing have been identified over the past 15 years. These factors are not related to the nuclear spliceosomal machinery, but instead have been evolutionarily recruited from very different sources. For example, the maize chloroplast RNA splicing 2 protein (CRS2) is a modified peptidyl-tRNA-hydrolase [120], while the *Chlamydomonas Raa2* is derived from pseudouridine synthase [213]. Other known splicing factors contain various RNA binding domains, including the CRM domain found in ribosome-assembly factors [16], the abundant RRM domain [257], the mTERF domain [92], and the organelle-specific PPR domain [19, 52, 55, 135]. In accordance with their diverse origins, the target ranges of these factors differ somewhat, although they overlap. The known factors and their target introns are listed in Table 1.1.

In terms of molecular functions, these factors are believed to help mold the intron into a structure that allows splicing to occur. Intron folding could, for example, be promoted by high-affinity, sequence-specific interactions that stabilize otherwise transient RNA-internal interactions [208]. Proteins could also block competing non-productive folding pathways, or act as helicases to actively resolve misfolded RNA structures [90, 100]. Finally, the proteins may help juxtapose the 5′-splice site

Table 1.1 Chloroplast RNA Splicing Factors

Name	Type[a]	Spec[b]	Loc[c]	Target site(s)	Evidence	Mutant phenotype	Reference
APO1	Zinc-finger-like	A.t	cp	*psaA, psaB, ycf3 int.2, clpP int1, petD, ndhA, ndhB*	Genetic	Albino, pale	Watkins et al. [318]
CAF1	CRM	Z.m; A.t	cp	*pet Dint., trnG int., rps16 int., rpl16 int., ycf3 int.1, clpP int.1, rpoC1 int., ndhA int.*	Genetic	Albino	Asakura and Barkan [2], Ostheimer et al. [207]
CAF2	CRM	Z.m; A.t	cp	*rps12 int.1; petB int., ndhB int., ndhA int., ycf3 int.1*	Genetic	Albino	Asakura and Barkan [2], Ostheimer et al. [206]
CFM2	CRM	A.t	cp	*trnL int., ndhA int., ycf3 int.1, clpP int.2*	Genetic	Pale green	Asakura and Barkan 2007
CFM3a	CRM	A.t	cp/mt	*ndhB int., rpl16 int., rps16 int.,trnG int., petB int., petD int.*	Genetic	Pale green, stunted growth	Asakura et al. [3]
cNAPL	Nucleosome assembly-like	C.r	cp	*tscA*	Biochemical	–	Glanz et al. [79]
CPN60	Heat shock protein	C.r	cp	*psaA*	Biochemical	–	Balczun et al. [12]
CRS1	CRM	Z.m; A.t	cp	*atpF*	Genetic	Pale	Asakura and Barkan [2], Jenkins et al. [120, 121], Ostersetzer et al. [208]
CRS2	CRM	Z.m	cp	*rps12 int.1, ycf3 int.1, clpP int.1*	Genetic	Ivory	Asakura and Barkan [2], Ostheimer et al. [207]
HCF107	HAT		cp	*psbH*	Genetic	hcf	Hammani et al. [94]
HCF152	PPR	A.t	cp	*petB int.*	Genetic	hcf	Meierhoff et al. [165]
MatK		N.t	cp	*atpF int., trnK int., trnA int., trnI int., trnV int., rpl2 int., rps12 int.2*	Biochemical	Lethal	Zoschke et al. [306]
OTP51	PPR-LAGLIDADG		cp	*ycf3 int.2*	Genetic	Albino	de Longevialle et al. [55]
PPR4	PPR	Z.m	cp	*rps12 int.1*	Genetic	Albino	Schmitz-Linneweber et al. [257]
PPR5	PPR	Z.m	cp	*trnG-UCC int.*	Genetic	Albino, pale	Beick et al. [19]
Raa1	OPR	C.r	cp	*psaA int1 and int2*	Genetic	No phototrophic growth	Merendino et al. [184]

Table 1.1 (continued)

Name	Type[a]	Spec[b]	Loc[c]	Target site(s)	Evidence	Mutant phenotype	Reference
Raa2	Pseudouridine synthase	C.r	cp (membrane of cps)	*psaA int. 1*	Genetic	Light sensitive (ML and HL), no phototrophic growth	Perron et al. [213]
Raa3		C.r	cp (stroma of cps)	*psaA int. 2*	Genetic	Light sensitive (ML and HL), no phototrophic growth	Rivier et al. [233]
RAA4	Pseudo uridine synthase	C.r	cp	*tscA, psaA int.,*	Genetic	Pale, lethal	Glanz et al. [80]
Rat1 and 2		C.r	cp	*psaA int.1 (tscA)*	Genetic	PSI deficiency	Balczun et al. 2005
RH3	RRM	Z.m, A.t	cp	*trnI, trnA, rps12 int.1, rps12 int.2, rpl2, 23Srrn, ycf3 int.1 and 2*	Genetic	Pale	Asakura et al. [4]
RNC1	Nuclease III	Z.m	cp	*trnV int., trnK int., trnI int., trnA int., rps12 int.2, ndhBint.,petB int., pet Dint., trnG int.*	Genetic	Albino, pale	Watkins et al. [317]
THA8	PPR	Z.m	cp	*Ycf3 int.2, trnA*	Genetic	Embryo lethal,	Khrouchtchova et al. [135]
WHY	whirly	Z.m	cp	*atpF int.*	Genetic	Albino, pale	Prikryl et al. [220]
WSL	PPR	O.s.	cp	*rpl2 int.*	Genetic	Albino, striped	Tan et al. [293]
WTF1	PORR	Z.m	cp	*petB int., ndhB int., petD int., trnG int., rpl16, rps12 int.2, atpF int., trnA int., trnI int., trnK int., trnV int.*	Genetic		Kroeger et al. 2009
Zm-mTERF4	mTERF	Z.m.	cp	*trnI int., trnA int., rpl2 int., atpF int., ycf3-intron 2*	Genetic	Albino	Hammani and Barkan [92]

[a] Type of domains encoded by the respective gene; *CRM* chloroplast RNA maturation; *PPR* pentatricopeptide repeat; *HAT* Half A TPR repeat; *PORR* Plant Organellar RNA Recognition domain; OPR Octotricopeptide repeat; mTERF mitochondrial transcription terminator domain

[b] Species: *At Arabidopsis thaliana*; *Cr Chlamydomonas reinhardtii*; *Os Oryza sativa*; So *Spinacia oleracea*; *Zm Zea amys*

[c] Localization of the protein: *cp* chloroplast; *mt* mitochondria

with the internal branch point, allowing an intron-internal phosphodiester bridge to form and freeing the 3′-OH group of the 5′-exon. The latter is brought into proximity with the 3′-splice site, the two exons are fused, and the intron is released as a circular structure known as the lariat. It is not yet clear how chloroplast factors fulfill this role at an atomic level; few biochemical or structural studies have addressed the exact binding sites of splice factors on their target introns and how these factors change the conformation of their intron ligands. For the maize factor, CRS1, we know that binding to its single target, the *atpF* intron, triggers structural changes in a particular intronic domain [208]. Footprinting analyses have demonstrated that CRS1 facilitates the internalization of intronic elements required for the core of the functional ribozyme [208]. In the future, it will be important to understand how chloroplast splicing factors act on and affect the structures of their target introns.

In addition to the nuclear-encoded splicing factors, there is also one chloroplast-encoded protein essential for splicing a set of introns: MatK. Canonical bacterial group II introns harbor reading frames for maturase proteins that specifically support the splicing of their own introns and are required for the mobility of group II introns (bacterial introns can reverse-splice into novel genomic locations, a process not happening in chloroplasts and thus not further discussed here, [149]). With one exception, the introns of the land plant chloroplasts have lost their maturase reading frames. The sole maturase left in the chloroplast, MatK, resides in the *trnK* gene and has been implicated in splicing a subset of introns characterized by specific structural elements [103, 311]. MatK was recently demonstrated to associate *in vivo* with these introns [343], but we need further structural insights into how, where and why MatK attaches to its target introns in chloroplasts.

1.3.1.2 Regulation of Chloroplast RNA Splicing

RNA splicing is an essential process, making it an ideal step for switching on or off the gene expression of intron-containing reading frames. Unspliced chloroplast RNAs accumulate to high levels, and changes in the ratio of spliced to unspliced mRNAs in different tissues have been described in maize (for the *atpF*, *petD*, *petB*, *rpl16*, and *ycf3* introns, [13, 182]), potato (*atpF*, *ndhB*, [305]), for the mustard *trn*G intron , and the tomato *ndhB* intron [125]. The latter is believed to involve inhibition of the first splicing reaction [125], but we do not yet fully understand how these shifts in splicing efficiency occur. The existing studies largely agree, however, that splicing is most effective in chloroplasts, whereas non-photosynthetic tissues show relative over-accumulations of unspliced precursor RNAs. Unexpectedly, light does not seem to generally activate splicing in land plants [13, 156]. However, it does appear to have a positive effect on the splicing of the *psbA* group I introns in *C. reinhardtii* chloroplasts [60]. At present, it is unclear if these findings reflect an active change in splicing efficiency, or if there are changes in the stability of spliced versus unspliced transcripts. It is even less clear whether the observed changes impact the amount of proteins produced from these mRNAs, *i.e.*, whether splicing can indeed be rate-limiting for gene expression. In *Chlamydomonas*, a mutation in a group