Multiple copies of the coding regions for the light-harvesting B800-850 α - and β -polypeptides are present in the *Rhodopseudomonas palustris* genome

Monier Habib Tadros^{1,2,*}
and Karin Waterkamp¹

¹*Institute of Biology 2, Microbiology, Schaezlestrasse 1, D-7800 Freiburg and ²European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, D-6900 Heidelberg, FRG

*Corresponding author.

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A reverse-phase HPLC System for isolation of the water insoluble α - and β -polypeptides of the light-harvesting complex II (LH II) of *Rhodopseudomonas (Rps.) palustris* without employment of any detergent was developed. The material obtained was of high purity and suitable for direct microsequence analysis. Chromatographic analysis could resolve at least two major β -polypeptides, β_a and β_b , two major α -polypeptides, α_a and α_b , and two additional minor polypeptides. N-terminal amino acid sequencing shows that the resolved peaks correspond to different polypeptide species and that the minor species have an N-terminal sequence identical to that of the α_b polypeptide. An oligonucleotide derived from the amino terminal sequence of the α_a polypeptide was utilized to screen a genomic library from *Rps.palustris*. Several independent clones have been characterized by Southern blot and nucleotide sequence analysis. We show that *Rps.palustris* contains at least four different clusters of β and α genes. Two clones contain sequences potentially coding for β_a – α_a and β_b – α_b polypeptides; and two additional clones potentially coding for β and α peptides which we named β_c – α_c and β_d – α_d , which did not correspond to the major purified polypeptides. In addition to the protein chemistry data, the conservation at the amino acid level and the presence of canonical ribosomal binding sites upstream of each of the identified genes strongly suggest that all four coding regions are expressed.

Key words: B800-850 antenna complex (LH II)/light-harvesting polypeptide/multigene family

Introduction

Rhodopseudomonas (Rps.) palustris is a rod-shaped facultative photoheterotrophic bacterium which reproduces by budding (Whittenbury and McLee, 1967). Energy is provided by a respiratory metabolism under aerobic conditions in the dark, or by a photosynthetic metabolism under anaerobic light conditions (Drews, 1978, 1985; Kaplan, 1978; Pfennig, 1978). In the phototrophic mode, the cells exhibit infoldings of the cytoplasmic membrane which extend into the cell forming characteristic stacks of flattened membranes (Whittenbury and McLee, 1967; Tauschel and Drews, 1967). The synthesis of intracytoplasmic membranes (ICM) including the components of the photosynthetic apparatus is controlled by the oxygen partial

pressure in the growth medium. Low oxygen levels induce the formation of the photosynthetic membranes (Firsow and Drews, 1977). The ICM of *Rps.palustris* contain at least three major pigment–protein complexes: the reaction centre (RC), which is in close contact with the light-harvesting bacteriochlorophyll complex LH I (B875, B870, B880 or 1020 in other species). This antenna system, together with a second complex termed LH II (B800-850), absorbs visible and near-infrared radiation and transfers this energy with high efficiency to the photochemical RC. In the RC the light energy is transduced into a charge separation state that, in conjugation with bc_1 -oxidoreductase, generates a transmembrane proton gradient.

The B800-850 complex is apparently associated with two proteins, β and α , with mol. wts of ~ 9000 and $11\,000$ respectively (Firsow and Drews, 1977). It has been reported that under different light intensities additional polypeptides are present, which migrate close to the positions of the β - and α -polypeptides of B800-850 on SDS–PAGE (Hayashi *et al.*, 1982a). It is possible that those additional polypeptides belong to a light-harvesting complex (Hayashi *et al.*, 1982a; Varga and Staehlin, 1985). Previous workers concluded that the β - and α -polypeptides were coded by two adjacent genes forming a single $\beta\alpha$ cluster in *Rhodobacter (Rb.) capsulatus* (Youvan and Ismail, 1985) and *Rb.sphaeroides* (Kiley and Kaplan, 1987).

In this communication we report the isolation and purification of the water insoluble β - and α -polypeptides on reverse-phase HPLC (RP-HPLC). We show that the isolated native LH II complex contains at least two major β - and two major α -polypeptides and additional minor species. We also

Fig. 1. Absorption spectrum of the isolated light-harvesting bacteriochlorophyll–carotenoid–protein complex (LH II) from intracytoplasmic membranes of *Rhodopseudomonas palustris*. For details see Results and Materials and methods.

show that these are coded by different genes. The data suggest for the first time that there might be several types of LH II complexes in *Rps.palustris*, a fact not previously observed in anoxygenic phototrophic bacteria.

Results

Isolation of the polypeptides from the LH II complex

LH II (B800-850) has been isolated as described in Materials and methods. The isolated complex has two absorption bands

Fig. 2. Protein pattern in an SDS-PAGE slab gel stained with Coomassie brilliant blue. (A) Purified intracytoplasmic membrane LH I. (B) Isolated LH II. For details see Materials and methods. Samples 1, 2, 3, 4 and 5 correspond to the protein fragments obtained at peaks 1, 2, 3, 4 and 5 (Figure 3) which were eluted from RP-HPLC treated LH II complex (Figure 3). All samples were heated at 60°C for 30 min and applied onto an 11.5–16.5% continuous gradient gel. All the tracks are taken from a single slab gel, but for convenience only the relevant tracks have been cut and presented here.

at 800 and 850 nm (Figure 1). The purified native complex contains two types of polypeptides, β and α , with mol. wts of ~ 9000 and $11\,000$ respectively (Figure 2, lane B). These polypeptides are highly hydrophobic and insoluble in water. Formic acid (50%) was necessary to dissolve the B800-850 polypeptide components (LH II polypeptides) which were then separated by RP-HPLC (Figure 3). Five peaks were resolved (Figure 3) whose polypeptide content was analysed by SDS-PAGE and is shown in Figure 2 (lanes 1–5). Judging from the M_r , we conclude that peaks 1 and 2 contain two β -like polypeptides, whereas peaks 3, 4 and 5 contain α -like polypeptides. It is also clear from a close inspection of lane 4 that the α -like polypeptide contains two distinct, although closely migrating, species. In order to obtain sufficiently pure material the content of peaks 1 and 2 was rechromatographed (see Materials and methods), whereas the material present in peaks 3, 4 and 5 was sufficiently pure as judged by N-terminal sequence analysis.

The first 10 amino acid residues of each polypeptide were determined and the sequences are shown in Figure 4. The data indicate that there are two β -type polypeptides, which we named β_a and β_b , and two major α -type polypeptides, which we named α_a and α_b . The material from peak 4 contains a homogeneous amino terminal sequence, identical to that of polypeptide α_b . At this stage of the work it was not possible to establish whether the minor polypeptides present in peak 4 were distinct gene products or post-synthetic variants of the α_b species.

Multiple light-harvesting α - and β -polypeptides are coded by a multigene family

The observation of multiple β - and α -polypeptides was not anticipated. The relationship of these polypeptides to each other or to the LH II (B800-850) complex is not clear. In order to clarify this issue, we decided to analyse the corresponding genes. For this purpose, a genomic library from *Rps.palustris* was screened with oligonucleotides (see Materials and methods) derived from the sequence of the NH_2 terminus of the α_a species. Six positive phages were identified and isolated (named phages 1 to 6). In this work

Fig. 3. Elution pattern of isolated LH II complex, separated by RP-HPLC. Freeze-dried LH II complex (4 mg) was dissolved in $150\ \mu\text{l}$ of 50% formic acid. Octyl-LiChrosob RP-column (C_8) was used. Elution was performed with 50% formic acid as solvent A and 50% 2-propanol in formic acid as solvent B. The gradient is indicated by a dotted line. After peak 5, no other protein peaks were detected. However, the gradient was continued to 100% B for 10 min, before re-equilibration of the column. The flow-rate was $1.5\ \text{ml/min}$ at room temperature. Polypeptides present in peaks 1 to 5 correspond to the bands in lanes 1 to 5 (Figure 2) respectively.

The DNA fragments hybridizing to the oligonucleotides were subcloned and sequenced. The nucleotide sequences of the 1.9 kb *SalI* fragment and of the 1.7 kb *EcoRI* fragment from phage 1, the 2.3 kb *SalI*–*EcoRI* fragment from phage 4 and the 1.4 kb *BamHI*–*EcoRI* fragment from phage 6 (Figure 5) were determined (data not shown). The analysis of these sequences revealed in each case the presence of open reading frames (ORFs) potentially coding for α - and β -polypeptides with an organization similar to that

Fig. 4. The N-terminal sequence of polypeptides obtained from peaks 1, 2, 3, 4 and 5 (Figure 3) isolated by RP-HPLC of the purified LH II pigment-protein complex.

Fig. 5. Restriction map of phages 1, 4 and 6 (1 cm equals 1 kbp) isolated from the λ genomic library in EMBL 3 arms vector bearing the α -oligoprobe hybridizing fragments. The detailed restriction map obtained by DNA sequencing of the expanded fragments (1 cm equals 100 bp) displays the positions of the β and α ORFs ($\beta_a, \beta_b, \beta_c, \beta_d$ and $\alpha_a, \alpha_b, \alpha_c, \alpha_d$).

A ATGGCTGAC.....AAGACGCTACCGGCTGACGGTCGAGGA
B ATGCGAGACGATCCGAACAAGGTCTGGCCGACCGGTCTGACGATCGCGGA
C ATGGTCGACGATTCCGAAAAGGTCTGGCCGACGGGCTGACGATTGCGGA
D ATGGTAGACGATCCGAACAAGGTCTGGCCGACTGGGCTGACCATCGCGGA

A ACGTCGGC.....TAA
B CGGCGCCGGCTGCTCCGCCGCCAAGAAGTAA
C CGGCACCGGCTGCGCCGGCT...AAGAAGTAA
D AGGCCGTC.....TAA

(A)

(B)

LH II β_a MADKTL---TGLTVEESELHKHVIDGTRIFGAIAVAHFLAYVYSPWLH
 LH II β_b MADDPNKVMPTGLTIAESELHKHVIDGTRIFGAIAVAHFLAYVYSPWLH
 LH II β_c MVDDSKKVWPTGLTIAESEEIHKHVIDGARIFVAIAVAHFLAYVYSPWLH
 LH II β_d MVDDPNKVMPTGLTIAESELHKHVIDGSRIFVAIAVAHFLAYVYSPWLH
 ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++

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are two such clusters in the same orientation, separated by ~1400 bp. The amino terminal sequence of the β -like coding regions does not correspond to any of the β -polypeptides previously characterized (see Figure 4). In contrast the N-terminal sequence of the two α -like coding regions corresponds to that determined for the α_b -polypeptide (see Figure 4), and therefore also to that of the material present in the minor peak 4 (Figures 3 and 4). For reasons that will be explained below, we named these coding regions β_c , β_d , α_c and α_d . The $\beta\alpha$ cluster present in phage 4 contains β and α coding regions compatible with the N-terminal sequence of the β_a - and α_a -polypeptides previously determined (Figure 4); for these reasons we named these coding regions β_a and α_a . Correspondingly in the case of phage 6, the β and α coding regions were compatible with those of the β_b and α_b previously determined.

Discussion

In all isolated and analysed light-harvesting complexes of *Rhodospirillaceae*, two bacteriochlorophyll polypeptides, named α and β , have been found. The isolation procedure used to separate these polypeptides has involved gel filtration and ionic exchange (Shiozawa *et al.*, 1980; Brunisholz *et al.*, 1981; Tadros *et al.*, 1982; Theiler *et al.*, 1984) or gel filtration, ionic exchange and FPLC (Wechsler *et al.*, 1987). Furthermore, extraction of the lyophilized membrane or complex with organic solvent (Shiozawa *et al.*, 1980; Brunisholz *et al.*, 1981) could fail to extract all the different polypeptide components belonging to the complex under investigation. This problem is avoided by direct application of the isolated complex on HPLC. The previous methods are time-consuming and uncertain, and therefore inconvenient.

In this study the polypeptides were separated directly from purified native antenna complex with RP-HPLC without employment of any detergent. The solvent used is volatile and easily removed by evaporation. Dialysis was not required. For separation, 70 min is required compared with several days for previous purification methods. The material was highly pure (Figures 2 and 3) and suitable for direct microsequence analysis (Figure 4). Several different polypeptides were isolated from the native, purified LH II (B800-850) complex, which we named β_a , β_b , α_a and α_b (peaks 1, 2, 3 and 5, Figure 3) present in the ratio 1:1:1:1, plus additional minor species. The gene coding for LH II (B800-850) polypeptides (Youvan and Ismail, 1985) and the genes coding for LH I (B870) polypeptides (Youvan *et al.*, 1984a,b) have already been cloned and characterized in *Rb. capsulatus*.

The LH I, but not the LH II, genes are co-transcribed with the single copies of L and M genes. Therefore, the LH I genes are probably arranged in a single cluster. In contrast we show in this report that the LH II genes of *Rps. palustris* are arranged in at least four different clusters coding for different β and α couples. The previous report that only one LH I gene cluster was present in *Rb. capsulatus* was based on Southern blots using oligonucleotides corresponding to parts of the α and β gene sequences. Since the different genes have different sequences in *Rps. palustris*, the oligonucleotides used could have failed to detect additional α and β gene copies. In fact the α oligonucleotide hybridized to several bands in *Rb. capsulatus* genomic DNA (Youvan

and Ismail, 1985). The question of the gene number in *Rb. capsulatus* should therefore be reinvestigated. A comparison of the various amino acid sequences suggests that the isolated genes account for all the characterized α - and β -polypeptides. The amino-terminal sequence of the β_a - and β_b -polypeptides is different (Figure 4) and corresponds to that of the coding regions present in phages 4 and 6 respectively. The amino terminal sequence of the α_a -polypeptide (Figure 4) corresponds to that of the coding region present in phage 4 which is clustered with the gene for β_a . Thus it is likely that the phage 4 cluster codes for the two major polypeptides, β_a and α_a . All the other α -like coding regions have identical amino terminal sequences corresponding to that of the α_b -polypeptide and to that of the minor α -polypeptides of the LH II complex contained in peak 4 (see Figures 3 and 4). By analogy with the DNA cloned in phage 4, it is likely that the gene for the major α_b species is clustered with the gene for the major β_b species as the ratio of these two polypeptides in the LH II complex is 1:1. The two minor α -polypeptides are most likely coded by the two clusters present in phage 1, coupled with two β coding regions whose corresponding polypeptides were not found in the purified LH II complex. It is most likely that minor β -polypeptides, whose hydrophobicity is identical to that of the major β -polypeptides (this emerges from a computer assisted analysis of the various amino acid sequences, and will be published elsewhere), could not be resolved by RP-HPLC. It is perhaps significant that the gene clusters coding for the minor species are rather close on the *Rps. palustris* genome (see Figure 5). We propose the name β_c , β_d , α_c and α_d for these minor species.

It is known that for a stable assembly of the light-harvesting complex, the LH II α - and β -polypeptides and the pigments must be present. However, little is known about how the β - and α -polypeptides assemble in the membrane to form the functional antenna complex.

Our observations indicate that there might be several different LH II complexes formed by the association of different α and β subunits. This may explain difficulties experienced by other workers in the field who have attempted to obtain high resolution X-ray diffraction data using crystals of the light-harvesting complex. It remains to be established whether any α and β combination is possible or whether there are restrictions to the compability of the various α - and β -polypeptides. It is attractive to think that the polypeptides coded by the same cluster are actually preferentially associated. For the sake of simplicity we refer to light-harvesting complex II (LH II)-A, LH II-B, LH II-C and LH II-D for the putative complexes formed by the association of the β - and α -polypeptides from clusters A to D respectively. The isolated complex has absorption maxima at 800 and 850 nm (Figure 1). It is not clear whether LH II-A, LH II-B or both complexes are responsible for the absorption at 800 and 850 nm. However, if only one of these pairs of α - and β -polypeptides is responsible for the B800-850 absorption, the other complex must have very similar absorption maxima.

The significance of the multigene copies is not fully known and is under investigation. In the case of *Rps. acidophila* strain 7050 it has been shown that cells grown at 400 lux show additional maxima at 800, 830 and 850 nm, while cells grown at 2000 lux have only absorption bands at 800 and 850 nm (Cogdell *et al.*, 1983). Similar observations are

reported from *Rps. palustris* and *Chromatium vinosum* (Hayashi and Morita, 1980; Hayashi *et al.*, 1982a,b; Varga and Staehlin, 1985).

In the present study it has been shown that cells of *Rps. palustris* grown at 2000 lux synthesize a LH II complex absorbing at 800 and 850 nm (Figure 1). The four polypeptides β_a , β_b , α_a and α_b which have been isolated from this complex in equal molar amounts were coded and expressed from gene clusters cloned in phages 4 and 6 (Figure 5). The *in vivo* gene expression of the other β and α coding regions is under investigation.

Multigene families have been described in cyanobacteria. The *psbA* gene which encodes a photosystem II component, the QB protein, has been found in several copies of cyanobacteria (Curtis and Haselkorn, 1984; Mulligan *et al.*, 1984; Golden *et al.*, 1986). Each of the three *psbA* gene copies in *Anacystis nidulans* R2 is capable of producing enough functional QB protein on its own to support normal photoautotrophic growth (Golden *et al.*, 1986). The chlorophyll a/b proteins in *Petunia* (Dunsmuir *et al.*, 1983) and *Lemna* (Kohorn *et al.*, 1986) of the major light-harvesting complexes are known to be specified by multiple nuclear genes. Most studies on the regulation of B800-850 complex synthesis have been performed by examining cells shifted from one environmental condition to another (Kaplan, 1981; Chory *et al.*, 1984; Donohue and Kaplan, 1986). Furthermore it has been shown (Kiley and Kaplan, 1987; Donohue and Kaplan, 1986) that the LH I and LH II complexes can be regulated both coordinately (downshift in light intensity) or non-coordinately (increase in light intensity). In the present study we have shown that the 5' flanking region of the four copies coding for α - and β -polypeptides have different DNA sequences suggesting that each of them is transcribed from a different promoter, which could be differently regulated. The level of the LH II complex in the cell could then be the result of a complex regulatory network acting on the different genes for the α - and β -polypeptides under different environmental conditions.

The first report on multiple gene clusters of the bacterial photosynthetic apparatus in this work is a challenge to investigate the role of these gene clusters in the adaptation of cells to different growth conditions, in the regulation of expression of these different genes and in the capability of the various β - and α -polypeptides to assemble in a stable LH complex.

Materials and methods

Organism and culture conditions

Rps. palustris, strain 1e5 (strain collection, Lehrstuhl für Mikrobiologie, Freiburg, FRG), was cultivated phototrophically in malate medium RÄH at 30°C (Drews, 1965).

Isolation of the LH II complex from intracytoplasmic membranes

The LH II complex was isolated as previously described (Firsow and Drews, 1977) with the following modifications. Cells in the exponential growth phase were harvested, washed two times with 10 mM Na-phosphate buffer, pH 8.0, and homogenized in a French pressure cell in the presence of DNase and PMSF. The preparation was centrifuged for 20 min at 15 000 r.p.m. in a Sorvall SS34 rotor. The supernatant was further centrifuged for 60 min at 45 000 r.p.m. in a Ti60 rotor and the pellet was washed in buffer. The membranes were resuspended in phosphate buffer plus EDTA (5 mM) and treated twice with ultrasonics for 30 s (0°C, nitrogen atmosphere). The sonicated membranes were pelleted by ultracentrifugation. The membranes, resuspended in phosphate buffer to a concentration of ~400 µg bacterio-

chlorophyll/ml, were further diluted 1:1 with phosphate buffer containing 1% lauryldimethylamineoxide (LDAO). After solubilization by stirring at room temperature in the dark for 30 min, the suspension was diluted with an equal volume of sodium phosphate buffer and layered onto a sucrose step gradient (0.3/0.6/1.2 M sucrose in phosphate buffer containing 0.5% LDAO). After centrifugation (38 000 r.p.m. in a Ti60 rotor for 18 h), the three pigmented bands were collected. The upper band contained LH II (B800-850, Figure 1), the lower band contained the RC plus LH I (B875). The upper band containing LH II was dialysed for 2 days against phosphate buffer. The dialysed material was applied to a DEAE-Sephacose CL-LB column for chromatography and equilibrated with 10 mM phosphate buffer plus 0.05% LDAO. The B800-850 complex was eluted with 40 mM phosphate buffer. Column chromatography was repeated once.

Purification of the LH II polypeptides

The separation of the LH II polypeptides was performed with a Gynkotek (model 250 B) Liquid Chromatography System consisting of two HPLC pumps (model 300c), a gradient former (type 250 B) with a mixing chamber and a variable wavelength monitor (type SP4), set to 278 nm, and a chart recorder; octyl-LiChrosorb RP columns (C_8) (250 × 4.6 mm, 5 µm) were procured from Dr Herbert Knauer, KG (Berlin). Elution was performed with formic acid:H₂O (1:1, v/v) (solvent A) and formic acid:2-propanol (1:1, v/v) (solvent B). The eluants (solvent A and solvent B) were filtered through a 0.2 µm Millipore filter (pure cellulose) and degassed under vacuum. Freeze-dried LH II complex (2 or 4 mg) was dissolved in 80 or 150 µl of 50% formic acid respectively. This solution was immediately injected and separated on a RP-column (C_8). The gradient was started 5 min after sample injection. The flow-rate was 1.5 ml/min at room temperature. Fractions (800–1200 µl) were collected manually on the basis of the detector signal and put in a desiccator (connected to a freezing trap) containing potassium hydroxide pellets. By cautious evacuation with an oil pump to 0.1 mbar, the solvent was evaporated. Before the fractions were completely dry, 500 µl of water was added and drying was continued. This procedure was repeated three times. The samples were then suitable for rechromatography, electrophoresis or N-terminal sequence analysis. The material present in peaks 1 and 2 (Figure 3) was not homogeneous as revealed by N-terminal sequence analysis. Peaks 1 and 2 were rechromatographed under the same conditions as used before except that the flow rate was 1.7 instead of 1.5 ml/min. The N-terminal sequence of the rechromatographed peaks 1 and 2, and of peaks 3, 4 and 5 (Figure 3) is presented in Figure 4. The eluates were stored immediately at –20°C for later use or dried directly in a desiccator as described before. The elution profiles of the gradients are shown in Figure 3. The acidic solvents with a high concentration of formic acid could induce hydrolytic cleavage of the polypeptide backbone, in particular, a cleavage of the acid-sensitive Asp–Pro bond. The protein eluted in peak 2 (Figures 3 and 5) has one Asp–Pro pair. To determine the sensitivity to acid we stored this polypeptide for 48 h in 50% formic acid at 4°C, 1 week at –20°C and for 2 h at room temperature. The N-terminal sequence analysis showed that the Asp⁴–Pro⁵ bond (Figure 5) remained unaffected.

Analytical measurements

Bchl concentrations were calculated from absorption measurements in acetone-methanol (7:2, v/v) extracts at 770 nm by using the absorption coefficient of 76 cm^{–1} mM^{–1} (Clayton, 1966). Purified protein or membrane proteins were separated by SDS–PAGE on 1.0 mM slab gels (Laemmli, 1970) with a 11.5–16.5% continuous gradient of acrylamide. Gels were stained with Coomassie brilliant blue.

Amino acid sequence analysis

Automated Edman degradation was performed in a gas–liquid phase microsequencer (Tadros *et al.*, 1987). The sequencer was equipped with an on-line HPLC system and a Triector chromatography data system which allowed the direct identification and quantitative determination of the PTH amino acids.

Enzymes, vectors and probes

Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase were purchased from either Boehringer (Mannheim) or Pharmacia (Freiburg) and used according to the recommendation of the supplier.

The information obtained from the sequence of the amino terminal region of the α_a -polypeptide was used to synthesize the three oligonucleotides.

Construction of a *Rps. palustris* genomic library

Genomic DNA was extracted as described in Klug and Drews (1984). High mol. wt DNA was digested with suboptimal amounts of endonuclease *Mbo*I and cloned in the λ EMBL 3 arms vector as described (Bensi *et al.*, 1985).

Commercially available packaging extracts (ProOmega, USA), were used to plate ~ 10 000 plaques.

Radioactively labelled oligo-1, -2 and -3 were used as hybridization probes as described (Paonessa *et al.*, 1988) in three replicas of the library.

DNA sequencing

Various DNA segments were subcloned in the M13mp18 or M13mp19 vectors and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Information obtained from sequencing was exploited to synthesize new primer oligonucleotides in order to obtain additional sequence information.

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