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Two potential *Petunia hybrida* mitochondrial DNA replication origins show structural and in vitro functional homology with the animal mitochondrial DNA heavy and light strand replication origins

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Summary. Four *Petunia hybrida* mitochondrial (mt) DNA fragments have been isolated, sequenced, localized on the physical map and analyzed for their ability to initiate specific DNA synthesis. When all four mtDNA fragments were tested as templates in an *in vitro* DNA synthesizing lysate system, developed from purified *P. hybrida* mitochondria, specific initiation of DNA synthesis could only be observed starting within two frements, oriA and oriB. When DNA synthesis incubations were performed with DNA templates consisting of both the A and B origins in the same plasmid in complementary strands, DNA synthesis first initiates in the A-origin, proceeds in the direction of the B-origin after which replication is also initiated in the B-origin. Based on these observations, a replication model for the *P. hybrida* mitochondrial genome is presented.

Key words: Mitochondrial DNA – *Petunia hybrida* – Physical map – Replication origin

Introduction

Nuclear DNA replication is a process that has been studied in various organisms. In organelles like chloroplasts and mitochondria, however, the replication process has not yet been analyzed in such detail. The best characterized mechanisms of mitochondrial (mt)DNA replication have been described for yeast (de Zamaroczy et al. 1984) and for animals (Clayton 1982).

The yeast *Saccharomyces cerevisiae* mitochondria show circular homogeneous mitochondrial genomes (70 kbp, Wallace 1982) containing at least eight replication origins. These have been identified and studied by the analysis of deleted mitochondrial genomes in yeast petite strains using biochemical techniques and by sequence homology. These origins are localized in AT-rich intergenic sequences in both DNA strands and are found all over the genome. In each functional replication origin, replication in yeast mitochondria is a bi-directional process (Baldacci et al. 1984).

The animal mitochondrial genome consists of homogeneous, closed circular molecules (±16 kbp; Wallace 1982). Replication origins have been identified and analyzed by means of electron microscopic (EM) analysis and thymidine-incorporation studies. Each mtDNA molecule contains two distinct origins of replication. One is located in the heavy (H)-strand and the other one in the light (L)-strand, each with its own conserved characteristics. Replication starts in the H-strand origin in a unidirectional, clockwise, way (Chang et al. 1985). When replication of the daughter H-strand is two-thirds completed, L-strand DNA synthesis initiates, followed by unidirectional elongation in a counter-clockwise direction (Wong and Clayton 1985).

In higher plants the replication mechanism of the mitochondrial genome has not yet been elucidated. The main reason for this is the lack of identified mtDNA replication origins because the methods, used for their localization cannot be applied in higher plants. Analysis of both yeast and mammalian mtDNA replication origins has indicated that these origins contain AT-rich regions, one or more potential stem-and-loop structures, and GC clusters. Therefore, an alternative approach for identifying plant mtDNA replication origins is the isolation and analysis of DNA regions containing these structures; such DNA regions can be considered as a pre-selection of potential replication origins (de Haas et al. 1986).

Progress in the detailed understanding of the mechanisms and control of both prokaryotic and eukaryotic DNA replication has been achieved by *in vitro* analysis of replication. Replication in such *in vitro* systems closely resembles DNA replication *in vivo*, as has been demonstrated for the eukaryotic Simian virus (SV)40 (Li and Kelly 1984), adenovirus (for a minireview see Stillman 1983), for *E. coli* (for a minireview see Zyskind and Smith 1986), and for mammalian mtDNA (Wong and Clayton

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1985). As a consequence, in addition to the study of replication mechanisms, such systems are also suitable for the identification of potential replication origins. Therefore, to identify and characterize replication origins, potential replication origin regions can be analyzed for their ability to initiate DNA replication in such *in vitro* DNA synthesizing systems.

Here we report experiments designed to test potential replication origins of *P. hybrida* mtDNA for at least three parameters; (1) the direction of DNA synthesis (2) the mutal effect of these origins on each other and (3) their localization on a physical map of the *P. hybrida* mitochondrial master genome. A mtDNA replication mechanism model of *P. hybrida* is proposed based on these parameters and on sequence data.

Materials and methods

Strains and plasmids. Cloning of *P. hybrida* mitochondrial DNA sequences was performed in vector YIp5, which is a recombinant between pBR322 and the yeast *ura3* gene, without a yeast origin of replication (Struhl et al. 1979). Testing of mtDNA inserts in YIp5 for ars activity was performed by transformation to *S. cerevisiae* strain DL1α: *αleu 2-3, leu 2-112, his 3-11, his 3-15, ura 3-251, ura 3-372* (van Loon et al. 1983). The *Escherichia coli* strain used for transformation was JA 221 (*recA, hsdR, leu136, trpE5, ΔlacY*.

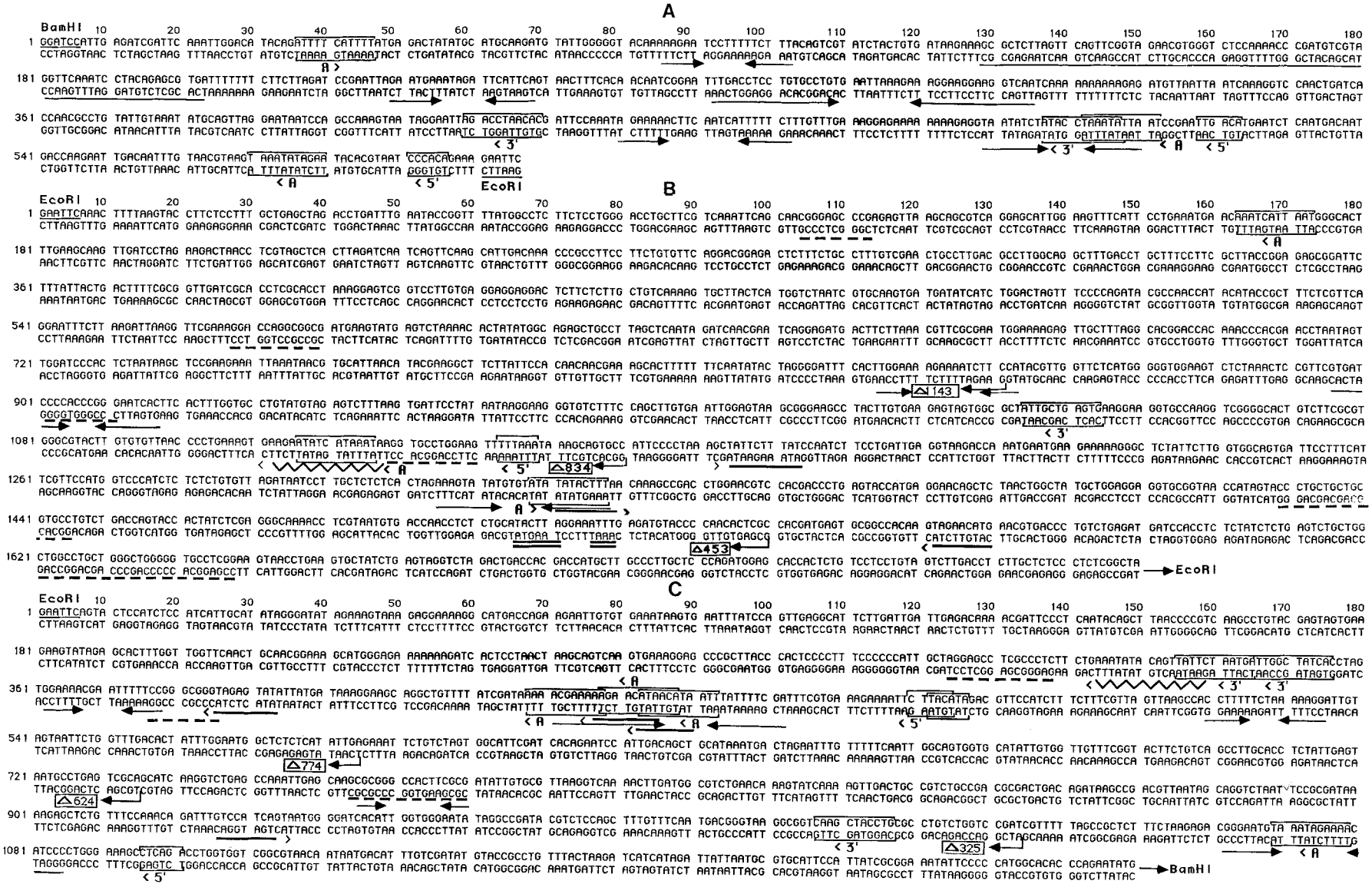
Reagents. Deoxyribonucleotides (dNTPs), ribonucleotides (rNTPs), *Micrococcus lysodeicticus* DNase, dithiothreitol (DTT), large fragment of *E. coli* DNA polymerase I (Klenow fragment), *E. coli* DNA polymerase I, T4 ligase, polyvinyl alcohol (PVA) 24000, *Bal*31 and restriction endonucleases were purchased from Boehringer (Mannheim). Bovine serum albumine (BSA), salmon sperm DNA, phenylmethanesulfonyl fluoride (PMSF), p-toluenesulfonyl fluoride (TOSF) and spermidine were purchased from Sigma Chemicals Co (St. Louis). Cytosine-β-D-arabino furanoside (araCTP) was from P. L. Biochemicals Co (USA). Radioactive nucleotides were purchased from Amersham (Amersham).

DNA techniques. Large scale preparations of plasmid DNA were obtained by the cleared lysate procedure, followed by CsCl ethidium bromide isopycnic centrifugation (Maniatis et al. 1982). Small preparations of plasmid DNA were prepared as described by Birnboim and Doly (1979). Cloning of DNA, analysis of DNA by restriction endonuclease digestions, agarose gel electrophoresis, Southern blot analysis and hybridization with [α-^{32}P]-labelled DNA probes were performed as described (Maniatis et al. 1982). For DNA sequence analysis cloned DNA fragments of *P. hybrida* mitochondrial DNA were subcloned in phage M13mp18 and/or 19 (Messing and Vierra 1982) and transferred to *E. coli* JM103 cells by transformation (Maniatis et al. 1982). DNA was sequenced in both directions with a 17 bp synthetic oligonucleotide primer by the method of Sanger et al. (1980).

Isolation, characterization and sequencing of P. hybrida mtDNA potential replication origin regions. In order to isolate *P. hybrida* mtDNA replication origins, purified *P. hybrida* mtDNA (Kool et al. 1985) was digested with *Bam*HI or *Bam*HI/*Eco*RI, ligated in the appropriate linearized yeast-*E. coli* shuttle vector YIp5 and analyzed for autonomous replication in yeast by transforming the mtDNA bank to the *ura3* yeast strain DL1α. Transformants were selected for uracil-independent growth; because the YIp5 vector itself cannot replicate in yeast, the observed high frequency transformation should be the result of recombinant plasmids carrying mtDNA fragments which promote autonomous replication in yeast (so-called mtDNA ARS fragments). Four different mtDNA potential replication origins inserts were isolated: a 605 bp *Bam*HI/*Eco*RI insert (pPMY I), a 666 bp *Bam*HI/*Eco*RI insert (pPMYII), a 1 994 bp *Eco*RI insert (pPMY III) and a 1 359 bp *Bam*HI/EcoRI insert (pPMY IV). Southern blot analysis showed that plasmid pPMY I contains a fragment homologous to both mtDNA and cpDNA. This was confirmed by the sequence data of this fragment which showed that pPMY I contained a transfer RNA gene coding for tryptophan (see Fig. 1 A). Plasmids pPMY II, III and IV contain authentic mtDNA fragments which originate from the higher molecular weight *P. hybrida* mtDNA. After *Bal*31 treatment and cloning in M13 these potential origin regions have been sequenced. None of the regions show complete open reading frames coding for more than 60 amino acids. Noteworthy, pPMY III contains a sequence which is analogous to the proposed promoter consensus of the *P. hybrida* ATPase 9 gene, which has also been found in mtDNA genes of other higher plant species (Young et al. 1986). It is shown that they all contain at least one yeast ars core consensus sequence 5 A/T TTTATPuTTTA/T (Stinchcomb et al. 1980; Broach et al. 1982) and two additional (semi-conserved) sequences which are necessary for autonomous maintenance in yeast; one within 25 bp 5′ upstream of the ars core (5′*T*NTG/*A*AA), Marunouchi et al. 1987) and one within 50–100 bp 3′ downstream of the ars core (5′cTtTT*AGCA*/*TA*/TA/T, Polzkill et al. 1986). It is noteworthy that in all these regions (except in pPMY II) at least one core consensus is localized in a potential stem-and-loop structure (see Fig. 1). The sequence of pPMY II is not presented because it does not contain any other elements (than the ars consensus sequence) or the conserved sequences usually found in DNA replication origins.

Preparation of in vitro DNA synthesizing systems. The *P. hybrida* liquid cell suspenison culture AK5000 was grown as described (Kool et al. (1985) with the exception that the homogenization buffer H consisted of 330 mM sorbitol, 30 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. The final mitochondrial pellet was resuspended in 1.5 ml of H-buffer and lysed by adding two volumes of lysis buffer L [0.75 M NaCl, 50 mM Tris-HCl pH 8.0, 2 mM DTT, 20% (v/v) glycerol, 50 μg/ml PMSF and 50 μg/ml TOSF; the latter two components were dissolved at 10 mg/ml in iso-propanol]. After incubation for 30′ at 4 °C (with continuously shaking on a whirl mixer) the suspension was centrifuged for 30′ at 20000 g. Under these specific conditions the

Fig. 1 A–C. Nucleotide sequence of *P. hybrida* mtDNA regions pPMY I (*A*), pPMY III (*B*) and pPMY IV (*C*). The nucleotide sequence of *P. hybrida* mtDNA region pPMY II is not presented as it does not contain any relevant sequences or elements related to replication origin characteristics. *Thin underlined regions* (in panel *A*) indicate a tRNA tryptophan gene (nucleotides 129–202). The *boxed regions* (marked "A") indicate sequences representing yeast autonomously replicating (ars) consensus sequence 5′^{A}/$_{T}$TTTATPuTTT^{A}/$_{T}$. *Boxed regions* (marked "5′ or 3′") represent sequences (surrounding the ars core) related to the conserved 5′-box (5′TNT^{G}/$_{A}$AA) and 3′-box (5′cTtTTAGC^{A}/$_{T}$^{A}/$_{T}$^{A}/$_{T}$) found in *D. melanogaster* and *S. cerevisiae* chromosomal and mitochondrial ars fragments. *Underlined* nucleotides are 100% conserved in the original organisms, small nucleotides are 33% conserved. *Long arrows* represent stem-and-loop structures whereas *small arrows* (< >) mark the 5′-3′ orientation. *Thick dashed underlined* nucleotides indicate GC clusters whereas *solid underlined* nucleotides represent possible yeast/*E. coli* gyrase-like recognitions sites TPuTGPyTPyTPu. *Zig-zag underlined* sequences indicate sequences related to yeast mtDNA transcription initiation sites (5′TATTACTTATATATTT). The *solid double underlined* nucleotides (panel *B*) represent a promoter region found in *P. hybrida* mitochondrial genes. The *boxed numbers* (in panels *B* and *C*) refer to the number of nucleotides which were deleted in the corresponding deleted pPMY-clones. The last nucleotides of the sequence of pPMY III (in panel *B*) and IV (in panel *C*) are not presented as they do not contain any relevant elements. EMBL accesion numbers: X15105 pMY1, X15106 pMY III and X15107 pMY IV for pPMY I, pPMY III and pPMY IV respectively


A
BamHI
EcoRI
B
EcoRI
C
EcoRI
BamHI

resulting supernatant contained the soluble DNA synthesizing activity (lysate system). The fractions were then immediately frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined by the method of Lowry et al. (1951).

Specific initiation of DNA synthesis on externally added (plasmid)DNA templates. For testing DNA synthesis on externally added DNA templates the co-isolated endogenous mtDNA in the lysate system was removed by incubating 500 µg of lysate protein with 0.5 µg *M. lysodeicticus* nuclease and 6 mM $CaCl_2$ for 10' at 37 °C. The DNase was then inactivated by the addition of 50 mM EGTA. Immediately after this inactivation the DNase-treated lysate was used for testing the DNA synthesizing activity of added synthetic template DNAs or for replication studies on added chimaeric DNA templates. This was performed as described previously for the chloroplast lysate DNA synthesis system (de Haas et al. 1987) with the exception that the incubations were performed at 27 °C with 4–5 µg/ml plasmid DNA template (for the major part covalently closed circular; 250 ng of a 7 kbp plasmid = ±1.3 nmol = ±750 pmol nucleotides in 50 µl) and with the addition of 6% (v/v) PVA 24000 in some experiments, as indicated. Analysis of the *in vitro* synthesized DNA was performed by agarose gel electrophoresis followed by autoradiography, or by using the labeled DNA as probe for Southern blots.

Results

Homology of the P. hybrida mtDNA potential replication origin regions with the yeast and mammalian mtDNA replication origin regions

Four mtDNA potential replication origin regions (inserted in plasmids pPMY I, pPMY II, pPMY III and pPMY IV) have been isolated and sequenced (see Material and methods and **Fig. 1**).

Sequence homology studies did not result in the detection of primary sequence homology with the *P. hybrida* putative cpDNA replication origin region (de Haas et al. 1986, 1987), neither with known or characterized replication origins. However, structural homology could be found in pPMY III and IV with the yeast and mammalian mitochondrial replication origins (Fig. 2). These structural features are potential target sites for enzymes involved in replication (see Kornberg 1980; Sinha et al. 1986; de Zamaroczy et al. 1984a, b; Clayton, 1982); for example, both potential mitochondrial yeast gyrase (TPuTGPyTPyTPu) and yeast transcription (5'TATTACTTATATATTT) recognition/initiation sites are present in pPMY III and IV (see Fig. 1 B and C). Furthermore, the sequence of the bottom strand of pPMY IV (Fig. 1 C) shows a majority of structural characteristics which are also present in the yeast mitochondrial replication origins as presented in Fig. 2 A.

Moreover, a T-rich secondary structure similar to that of pPMY IV (nucleotides 431–468 in Fig. 1 C) is also present in the close vicinity of the replication origin regions of two broad bean mtDNA plasmids (Wahleithner and Wolstenholme 1988) and in the mammalian mtDNA replication origin of the light strand (Wong and Clayton 1985). In these mitochondria the T-rich stem-and-loop acts as a primase recognition site involved in RNA-primed initiation of DNA replication (see blow-out in Fig. 2 A).

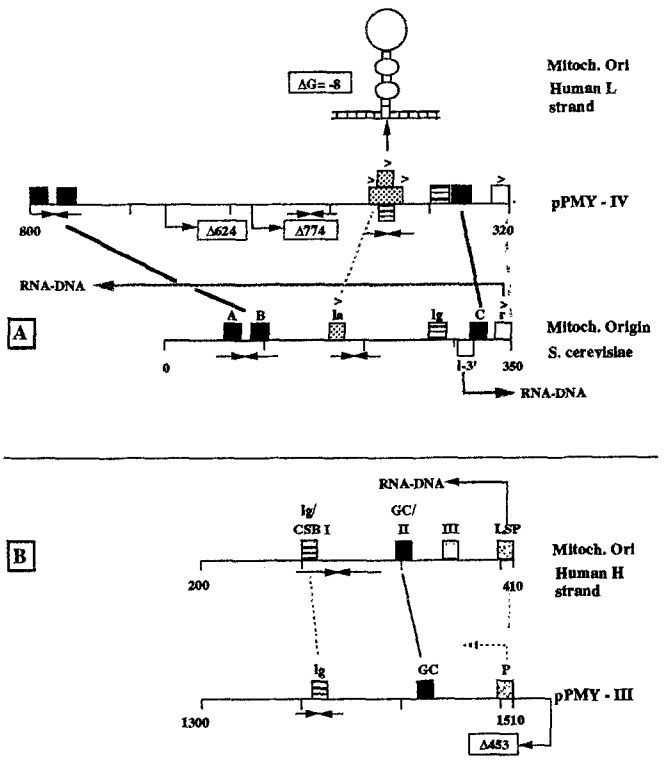


Fig. 2. A schematic overview of the structural features present in the yeast *S. cerevisiae* mtDNA replication origin (bottom line) and analogous structures present in *P. hybrida* pPMY IV (top line). Numbers refer to the nucleotide numbering for pPMY IV according to the bottom strand in Fig. 1 *C* and for *S. cerevisiae* according to de Zamaroczy et al. (1984). "*A*", "*B*"and "*C*" represent GC-rich clusters of nucleotides, "*Ia*" refers to an ars core-consensus sequence. "*Ig*" represents a sequence homologous to the yeast/*E. coli* gyrase-like recognition-site, "*r*" and "*1–3'*" indicate positions of a yeast-transcription-initiation-site (see also text for explanation). The *boxed numbers* in pPMY IV in panel **A** refer to numbers of nucleotides which were deleted in the corresponding deletion clones (see Fig. 1). The blow out of the stem-and-loop structure in pPMY IV is analogous to the T-rich secondary structure present in the mammalian mtDNA replication origin of the light strand. **B** schematic presentation of the structural features present in the human mtDNA heavy (H)-strand replication origin (top line) and analogous structures in pPMY III (bottom line). *Numbers* refer to nucleotide numbering for pPMY III according to the bottom strand in Fig. 1 B and for human mtDNA according to Chang and Clayton (1987). "*LSP*" indicates the position of the light strand promoter, "*P*" the position of the *P. hybrida* promoter. "*CSB*" *I, II and III* represent a putative yeast/*E. coli* gyrase-like sequence ("*Ig*", which is part of a stem-and-loop), a GC cluster ("*GC*") and, in mammals, a conserved A-stretch, respectively

Sequence homology studies with the mammalian mtDNA heavy (H)-strand replication origin revealed structural homology with the bottom strand (Fig. 1 B) of pPMY III. Also in pPMY III the gyrase-like sequence (Ig/CSBI in Fig. 2 B) is part of a stem-and-loop, and the relative spacing of these replication origin structures in pPMY III is approximately the same as in the mammalian H-strand origin. These observed structural homologies of the mtDNA inserts of pPMY III and IV with the yeast and mammalian mtDNA replication origins make these *P. hybrida* regions candidates for being genuine mtDNA origins of replication.

Table 1. Requirements for DNA synthesis in the *P. hybrida* in vitro DNA synthesizing mitochondrial lysate system

Reaction conditions	Activity (%)
Complete system	100
minus $MgCl_2$	1
minus GTP, but with ATP	99
minus GTP	73
minus dATP, dCTP, dGTP	23
minus CTP, GTP, UTP	91
minus mitochondrial lysate	1
with ethidium bromide (25 μM)	7

DNA synthesis was performed and measured as described under Materials and methods in the presence or absence of the indicated reaction components or inhibitor. The complete system refers to the reaction mixture with endogenous mtDNA as template primer. The values are the mean of at least three experiments; 100% corresponds to 2.34 pmol of [α-^{32}P]dTTP or [α-^{32}P]dATP incorporated in 60 min

Organelle-free in vitro DNA synthesis system

In the absence of an effective *in vivo* mitochondrial transformation method, *in vitro* DNA replication systems have proven suitable alternatives to test the functions of potential replication origin DNA templates and to study replication mechanisms (Stillman 1983; Wong and Clayton 1985; Zyskind and Smith 1986). Some characteristics of the developed *P. hybrida* mitochondrial DNA synthesizing system are shown in Table 1. The incorporation of [α-^{32}P]dTTPs into TCA-insoluble material is ATP-dependent. The system is very sensitive to ethidium bromide inhibition which indicates that the DNA polymerase present in this system is similar to the γ-DNA polymerase of mammalian mitochondria. This system, also containing RNA-polymerase-, gyrase- and primase-activity, can use both co-isolated endogenous mtDNA and externally added (plasmid)DNAs as a template.

Initiation of DNA synthesis in the P. hybrida potential mtDNA replication origins inserted in plasmids pPMY III and IV

To determine the possible site of initiation of replication on the potential replication origin-containing plasmids pPMY III and IV it was necessary to enrich the incubation mixtures for partially replicated DNA molecules. This was accomplished by the addition of the chain terminator cytosine-β-D-arabinofuranoside-5′-triphosphate (araCTP) to the reaction mixtures. In the absence of araCTP, synthesis, once initiated, will proceed rapidly to completion and the origin of synthesis can not be determined. In these experiments we used 6 mM araCTP, which inhibits overall DNA synthesis in the lysate to 37%.

When double-stranded pPMY I, II, III or IV is added as a DNA template to the organelle-free *in vitro* DNA synthesizing lysate system, (followed by incubation, re-isolation, vector/insert-digestion, electrophoresis and autoradiography) only the 1 994 bp pPMY III- and the 1 359 bp pPMY IV-inserts were more strongly labelled than the vector. These results suggest that initiation of DNA synthesis takes place in the inserts of both pPMY III and pPMY IV (data not shown). This supports the observed structural homologies with the yeast and mammalian mtDNA replication origins.

The position where the initiation of DNA synthesis started within these mtDNA inserts of pPMY III and IV was further investigated using a set of *Bal*31-deleted single-stranded pPMY IV and III constructs (see Figs. 1 C/3 C and Figs. 1 B, 4 B respectively).

The position where the initiation of DNA synthesis started within these mtDNA inserts of pPMY III and IV was further investigated using a set of *Bal*31-deleted single-stranded pPMY IV and III constructs (see Figs. 1 C, 3 C and Figs. 1 B, 4 B respectively).

After incubating single-stranded (ss) pPMY IV constructs (cloned in M13 in both orientations) as template DNA in the lysate system, these newly labeled template DNAs were isolated and used as probes on a blot containing an *Eco*RI/*Bam*HI/*Hinc*II/*Pvu*II multiple digest (Fig. 3 A1) or a *Eco*RI/*Bam*HI/*Hinc*II/*Pvu*I multiple digest of plasmid pPMY IV (Fig. 3 B1). These hybridizations resulted in an intensive labeling of the left 420 and 208 bp (Fig. 3 A2) or the left 821 and 208 bp fragments (Fig. 3 B2). The same results were also obtained when three Bal31-deleted ss pPMY IV constructs (325, 624 or 774 bp deleted) were used (Fig. 3 A3, 4, 5 and B3, 4, 5 respectively). These experiments suggest that the observed DNA synthesis in pPMY IV is initiated within the left non-deleted 585 bp of pPMY IV-774 (marked by the dotted area in Fig. 3 C). This region comprises only part of the yeast replication origin homology, but does contain the stable (T-rich) stem-and-loop also present in the mammalian mtDNA L-strand replication origin (Fig. 2 A).

When the same experiments were performed with ss-pPMY III (Fig. 4 A2) or ss-pPMY III-453 (Fig. 4 A5; 453 bp deleted) all three pPMY III *Eco*RI/*Hinc*II/*Xho*I insert fragments are labeled. However, when pPMY III-834- or pPMY III-1143-deleted constructs are used as single-stranded DNA template (Fig. 4 A3 and 4) no hybridization with these insert fragments can be observed. The most probable explanation for this phenomenon is that the observed DNA synthesis in pPMY III starts within a 371 bp region between pPMY III-453 and -834 (marked by the dotted area in Fig. 4 B). This region includes the structural homology region with the mammalian mtDNA H-strand replication origin (Fig. 2 B). None of the tested mtDNA recombinant plasmids, which were not scored positively in the yeast transformation screening assay, showed specific initiation of DNA synthesis, as did the pPMY III and IV constructs. These hybridizations are comparable to each other because the applied conditions were standardized; when nick-translated pPMY plasmids were applied as probes in re-hybridization experiments using the same (stripped) pPMY-blots and the same hybridization conditions, stoichiometric labeling of all bands was observed (data not shown).

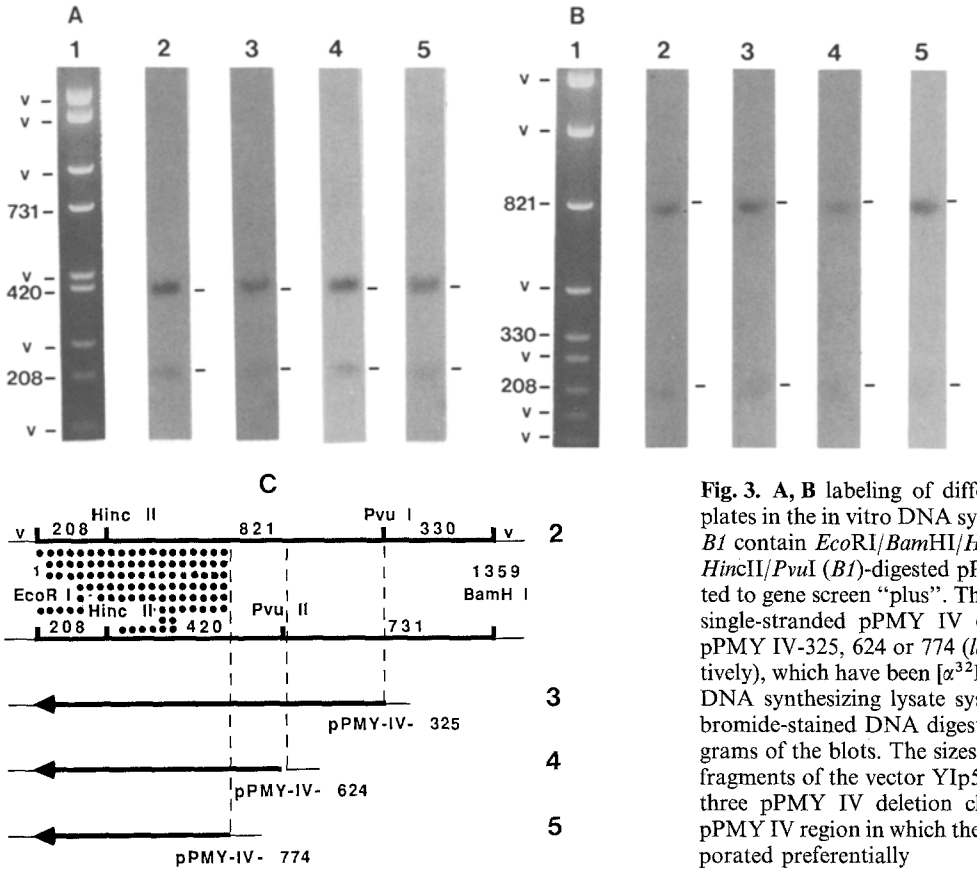


Fig. 3. A, B labeling of different single-stranded pPMY IV templates in the in vitro DNA synthesizing lysate system. *Lanes A1 and B1* contain *Eco*RI/*Bam*HI/*Hin*cII/*Pvu*II (*A1*)- and *Eco*RI/*Bam*HI/*Hin*cII/*Pvu*I (*B1*)-digested pPMY IV respectively, which were blotted to gene screen "plus". The Southern blots were hybridized with single-stranded pPMY IV (*lanes A2/B2*) or with *Bal*31-deleted pPMY IV-325, 624 or 774 (*lanes A3/B3, A4/B4* and *A5/B5* respectively), which have been [α^{32}P]-labeled by incubation in the in vitro DNA synthesizing lysate system. *Lanes 1* represent the ethidium bromide-stained DNA digests, *lanes 2–5* represent the autoradiograms of the blots. The sizes are given in basepairs. "*V*" represent fragments of the vector YIp5. **C** physical map of pPMY IV and of three pPMY IV deletion clones. The *dotted area* indicates the pPMY IV region in which the [α^{32}P]-labeled nucleotides were incorporated preferentially

Direction of DNA synthesis in the mtDNA replication origins inserted in pPMY III and IV and the mutual effect of both origins

In yeast mitochondria, DNA synthesis at each replication origin elongates bi-directionally, whereas in mammalian mitochondria replication elongates at both origins unidirectionally (see Fig. 2). Moreover, in animal mitochondria the H-strand replication origin and the L-strand replication origin are localized in two different strands of the same genomic circle. In the animal mtDNA genome the L-strand replication is not initiated until the H-strand replication has elongated into the direction of the L-origin (Clayton 1982). The question arises whether such phenomena also occur in the mitochondrial DNA of higher plants. This can be investigated in the *in vitro* DNA synthesizing lysate system by studying DNA synthesis on plasmid templates in which both the *P. hybrida* pPMY III and IV origin are present. In Fig. 5 the physical map of two recombinant plasmids carrying both the III and IV origins are presented. The constructs consist of both the pPMY III- and IV-inserts cloned in the YIp5 vector in two different orientations: according to the sequence in the 5′-3′ "opposite" orientation (pPMY III-IV-6600, Fig. 5A) and in the "same" orientation (pPMY III-IV-5241; Fig. 5B).

After *in vitro* DNA synthesis incubations, the reactions were stopped at the indicated times (Fig. 6: 5–45 min). The [α^{32}P]-labeled newly synthesized DNAs were digested with *Eco*RI-*Xba*I-BstEII-*Bam*HI-*Sac*I, electrophoresed and exposed to X-ray films. The data of the pPMY III-IV-6600-construct incubations indicate that DNA synthesis in the pPMY III-IV-6600 plasmids initiates in the 261 bp pPMY III-derived origin fragment (which overlaps the H-strand-like origin in the 371 bp fragment; most specific labeling after 5 min; Fig. 6 A2), followed by elongation in the counterclockwise direction into the 682 and 734 bp pPMY III fragments (Fig. 6 B-C2). After elongation into the 2 294 bp fragment a second DNA synthesis initiation site becomes active resulting in a more than stoichiometric labeling of the 903 bp pPMY IV-derived origin fragment (which includes the 585 bp L-strand-like origin region; Fig. 6 D2). This second DNA initiation site elongates clockwise in the direction of the 2 294 bp, 734 bp and 682 bp fragments (doubling of the band intensities; Fig. 6 E2), whereas the counter-clockwise pPMY III-strand DNA synthesis elongates into the 456 bp and 2 869 bp fragments.

The same time-course experiments performed with pPMY III-IV-5241 (both origin elongation directions in the same orientation; Fig. 5B) resulted in a similar [α-^{32}P]dTTP incorporation pattern until 20 min. However, after 30 min no second DNA synthesis initiation in the 903 bp fragment can be observed (Fig. 6 I2; the 903 bp fragment labels stoichiometric compared to the 682 bp and 734 bp fragments). This might be explained by the

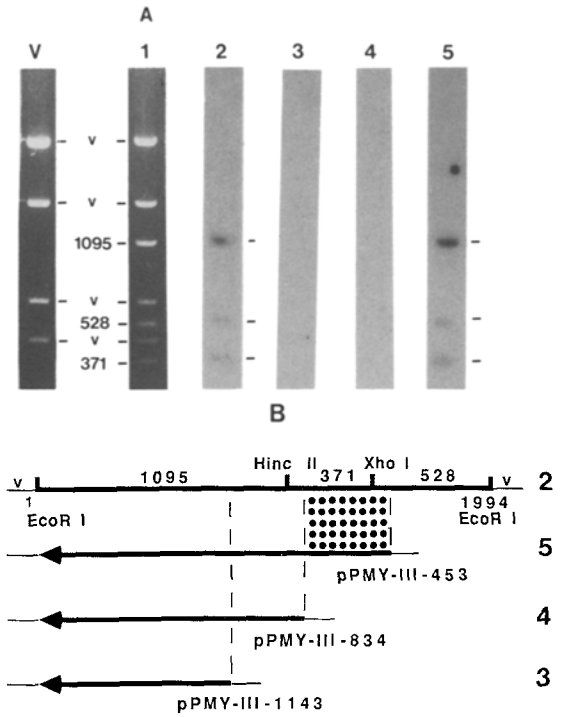
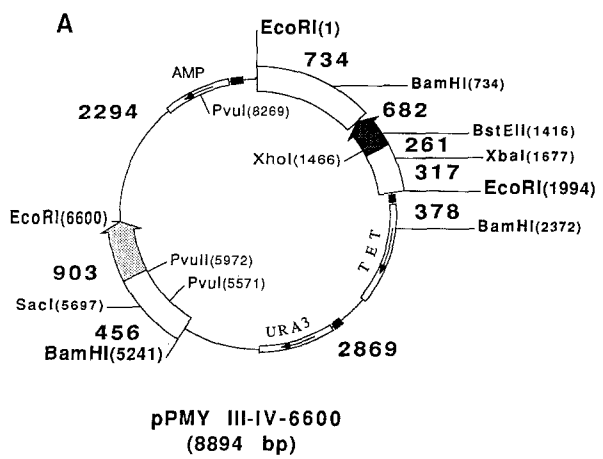


Fig. 4. **A** labeling of different single-stranded pPMY III templates in the in vitro DNA synthesizing lysate system. *Lane V* represents the ethidium bromide-stained *Eco*RI/*Hin*cII/*Xho*I digest of vector YIp5. *Lane A1* contains *Eco*RI/*Hin*cII/*Xho*I-digested pPMY III, which was blotted to gene screen "plus". The Southern blots were hybridized with single-stranded pPMY III (*lane A2*) or with *Bal*31-deleted pPMY III-1143, 834 or 453 (*lanes A3, A4* and *A5* respectively), which have been [α^{32}P]-labeled by incubation in the in vitro DNA synthesizing lysate system. *Lane 1* represents the ethidium bromide-stained DNA digest, *lanes* 2–5 represent the autoradiograms of the blots. The sizes are given in kilobasepairs. *V* represents fragments of the vector YIp5. **B** physical map of pPMY III and of three pPMY III deletion clones. The *dotted area* indicates the pPMY III region in which the [α^{32}P]-labeled nucleotides were incorporated preferentially

fact that this second initiation site in the 903 bp pPMY IV-derived fragments is located on the same strand in this plasmid which is already being used as template for the III-1 994 strand daughter DNA synthesis. All lanes show (just as in the gels presented in Figs. 3 and 4) labeled DNA at the origins of the lanes (data not shown) which could not enter the gel; most probably this represents partial (branched) restriction fragments which fail to enter the gel (Fuller et al. 1981).

Electron microscopic analysis of the pPMY III and IV plasmids incubated in the in vitro mitochondrial lysate system

The initiation process in the mammalian H-strand origin displaces the 'mother'-H-strand resulting in a D-loop which can be observed by EM analysis. Such D-loop structures cannot be observed in the L-strand origin because of the lack of strand displacement (Wong and Clayton 1985). To determine whether the *P. hybrida* H-strand-like origin (inserted in pPMY III) shows D-loop initiation of DNA synthesis, like the mammalian H-strand replication origin counterpart, pPMY III and IV double-stranded plasmids were incubated in the lysate system for 10 min under the same conditions as reported for the experiments with the pPMY III-IV constructs in Fig. 6 (including araCTP and non-labeled dTTP instead of [α-^{32}P]dTTP). After isolation, the pPMY III and IV plasmids were digested with *Pvu*II. This enzyme has only one recognition site present in these plasmids; consequently, in pPMY III *Pvu*II creates linear molecules showing the possible replication loop in the middle. Finally these DNA molecules were analyzed by electon microscopy to determine the presence of D-loops in these plasmids. The pPMY IV plasmids did not show any loops; however, pPMY III constructs did show the presence of one D-loop in the middle of the molecules (Fig. 7 B).



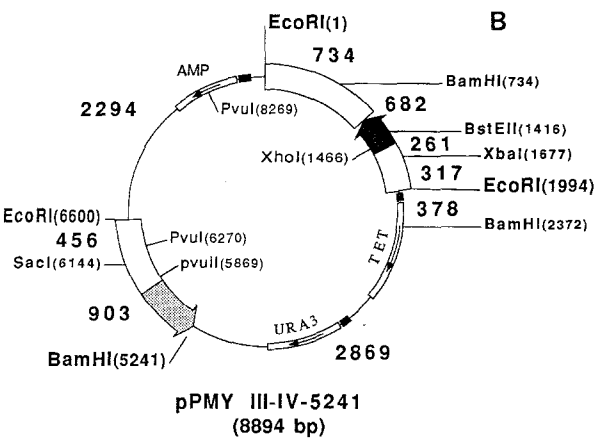


Fig. 5A, B. Physical maps of pPMY III-IV double constructs with the III and IV replication initiation regions in the same strands (**B**; pPMY III and pPMY IV DNA synthesis elongations in the same orientation) or in complementary strands (**A**; opposite elongation directions) *TET, AMP* and *URA3* refer to the coding regions of the ampicilin-and tetracyclin resistance genes and the yeast uracil3 gene respectively. *Numbers in bold* refer to the restriction fragments after digestion with the restriction enzymes presented outside the circle which were used in time-course experiments (Fig. 6). The *shadowed regions* indicate the minimal in vitro determined functional replication origin region (Figs. 3 and 4); the *large open arrows* indicate the DNA synthesis elongation directions as determined in vitro (Fig. 6)

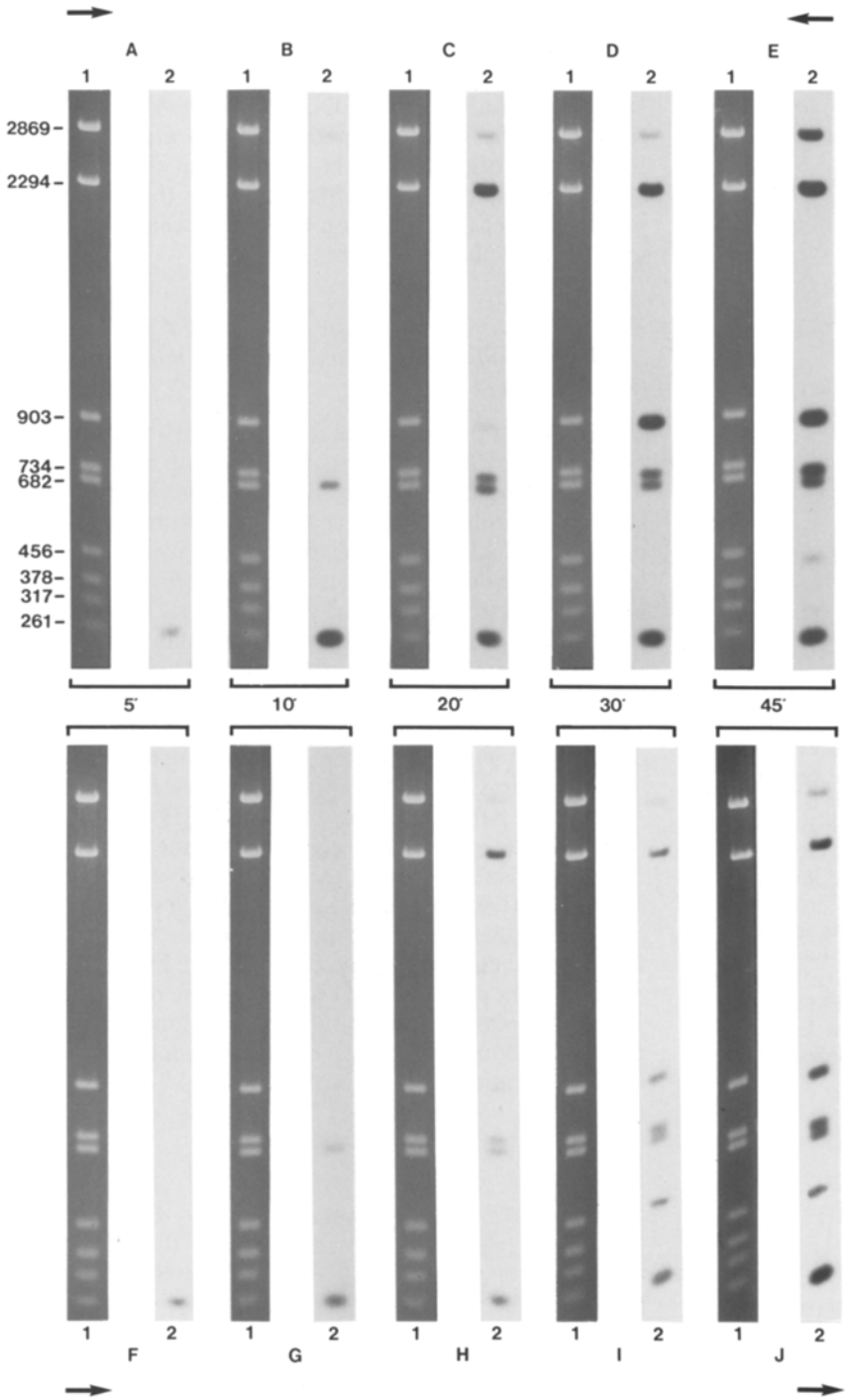


Fig. 6. Determination of the direction of DNA synthesis, and the mutual effect in the *P. hybrida* pPMY III and IV mtDNA replication origins on the process of initiation of DNA synthesis, using time-course experiments in the *P. hybrida* mtDNA synthesizing lysate system with pPMY III-IV constructs as templates. Plasmid pPMY III-IV-6600 (III and IV origins in opposite orientation in different strands; *lanes A–E*) and pPMY III-IV-5241 (III and IV origins in the same orientation in the same strands; lanes *F–J*) were incubated in the presence of 6 mM araCTP and 6% PVA, re-isolated, digested with *Eco*RI-*Xba*I-*Bst*EII-*Bam*HI-*Sac*I, electrophorezed and autoradiographed. *Lanes 1* represent the ethidium bromide-stained DNA digests, *lanes 2* represent the autoradiographs of the agarose gels. The incubation times were 5′, 10′, 20′, 30′, 45′ for *lanes A2–E2* and *F2–J2* respectively. The sizes are given in kbp (see for physical maps Fig. 5A and B). The *arrows* pointing to each other (around lanes *A1–E2*) indicate that in the template constructs used the III and IV origins are orientated in the opposite directions. The arrows around lanes *F1–J2* indicate that the III and IV origins are orientated in the same direction

Localization of P. hybrida mtDNA sequences containing potential replication origins

Recently a physical map of the *P. hybrida* mtDNA has been constructed (Folkerts and Hanson 1989). The master circle is 442 kbp in length and contains three large repeats (Fig. 8) which act as recombination sites resulting in four different master circles, each with a different arrangement of the unique regions, and in three subgenomic circles.

Southern blot hybridizations were performed with the inserts of pPMY I, II, III and IV as probes for blots containing digested cosmid clones covering the complete *P. hybrida* mitochondrial genome. The results of these localization studies are schematically presented in Fig. 8. The inserts of pPMY I and II are localized on the "bottom" and "middle" subgenomic circles, whereas the origin of replication-containing pPMY III and IV inserts are localized in the "upper" subcircle of the master genome. The pPMY IV replication origin [B1] is localized in the 1.6 kbp *Bam*HI fragment 3′ downstream from the atpA-1 gene approximately 60 kbp away from the pPMY III origin, which is mapped on the 3.7 kbp *Bam*HI fragment (pPMY III[A]).

The 1 359 bp *Bam*HI/*Eco*RI pPMY IV insert probe also resulted in seven other (weak) hybridization signals. After using a minimal-origin probe of pPMY IV five additional weak hybridization signals could still be detected (pPMY IV B2-B6 in Fig. 8).

Discussion

P. hybrida mtDNA sequences, containing general origin of replication characteristics, have been selected, isolated and characterized (pPMY I, II, III and IV) in order to study the plant mtDNA replication mechanism(s). Structural homology with the yeast and mammalian mitochondrial replication origins could only be found in pPMY III and IV (Fig. 2). Based on these structural replication characteristics the pPMY III and IV regions are the best candidates for representing genuine *P. hybrida* mtDNA replication origins. In order to verify these structural replication characteristics the pPMY I, II, III and IV constructs were analyzed for their ability to initiate DNA synthesis in an *in vitro* DNA synthesizing lysate system isolated from purified *P. hybrida* mitochondria. Such plant organellar systems are capable of catalyzing specific initiation of replication reactions on externally added plasmid DNA templates possessing a potential organelle replication origin (Wu et al. 1986; de Haas et al. 1987; Carrillo and Bogorad 1988), whereas purified DNA polymerases are incapable of catalyzing replication (Gold et al. 1987).

When supercoiled pPMY I, II, III or IV constructs served as DNA templates in this system incorporation of [α-^{32}P]dTTP occurs preferentially only in the pPMY III and IV insert fragments, whereas the pPMY I and II restriction fragments or the vector DNA were relatively less labeled (data not shown). The observed preferential labeling patterns are most probably not due to repair or nick translation at labile template sites because such labile sites are generally located in regions containing secondary structures (Kornberg 1980, 1982). As in pPMY I, the preferred DNA synthesis regions (segments within pPMY III and IV) contain many other secondary structures apart from the preferred regions theirselves. Therefore, these results suggest that, in addition to a certain level of repair DNA synthesis, a significant amount of *de novo* DNA synthesis was initiated in these specific mtDNA regions of plasmids pPMY III and IV.

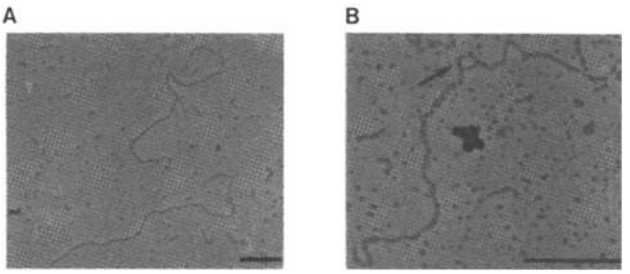


Fig. 7 A, B. Electron microscopic analysis of pPMY III plasmids which have been incubated in the *P. hybrida* in vitro mtDNA synthesizing system under the same conditions as described in Fig. 6. After isolation the molecules were linearized, resulting in molecules where the replication origin region is located in the middle. A control (incubation without DNA synthesizing system) is presented in panel **A**. The *horizontal bar* represents 1 kbp. Magnifications: 20 000 × (**A**) and 50 000 × (**B**). The *arrow* indicates a replication loop

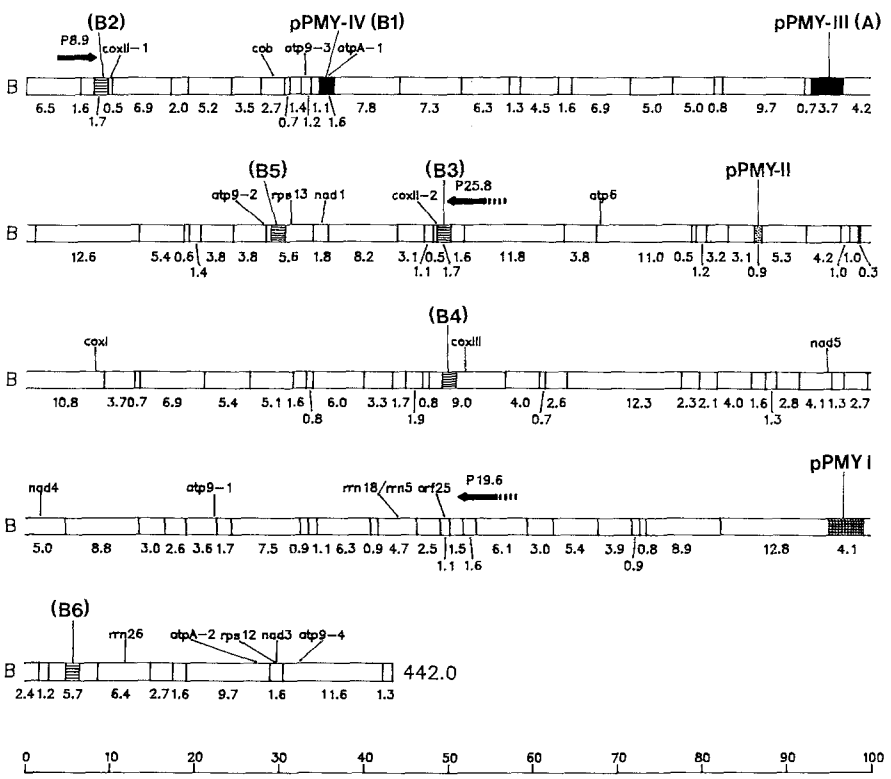


Fig. 8. Localization of *P. hybrida* mtDNA fragments inserted in pPMY I and II (ars fragments), pPMYIII (*P. hybrida* H-strand-like replication origin located in strand A, indicated as pPMY III-A) and pPMY IV (*P. hybrida* L-strand-like origin located in the B-strand, indicated as pPMY IV-B1) on the physical map of the *P. hybrida* (line 3704) mitochondrial genome (Folkerts and Hanson 1989). Restriction maps for *Bam*HI (*B*) of overlapping cosmids were used to construct a physical map of the mitochondrial genome. The location and orientation of the recombination repeat copies is indicated by *arrows*. The homology extending upstream of the repeat in two of the three copies is indicated by the *dashed line*. The numbers of the repeat copies (P8.9, P25.8 and P19.6) correspond to the size of *Pst*I fragments covering the repeat copies indicated. "*pPMY IV-B1–B6*" indicate weaker hybridization signals with the insert of pPMY IV as a probe; these regions might include additional pPMY IV-like origins of replication

Programming this system with single-stranded pPMY III or IV-DNAs we were able to narrow down the putative replication origins of pPMY III and IV (Figs. 3 and 4). DNA synthesis initiates in pPMY IV within the first 585 bp, which comprises the T-rich stem-and-loop which is also present in the mammalian mtDNA light-strand replication origin (Fig. 2A) but which only partly overlaps the structural characteristics described for the yeast mtDNA replication origins (for example, the essential GC-stem-and-loop "A-B" is missing; see Fig. 1C and 2A). In pPMY III the region involved in the initiation of DNA synthesis was limited to a 371 bp region between nucleotides 1 160 and 1 540 (see Fig. 1B) comprising the structural origins of replication characteristics which are also present in the mammalian mtDNA heavy-strand replication origin (Fig. 2 B). Both the presence of these structural mammalian mtDNA replication origin characteristics and the *in vitro* DNA synthesis data brought us to the most likely hypothesis that the 371 bp region present in pPMY III functions as a mammalian *heavy (H)strand-like* replication origin and that the 585 bp region in pPMY IV might represent a mammalian *light (L)strand-like* replication origin. An alternative is that both pPMY III and IV act as a yeast-like replication origin.

To prove the correctness of this hypothesis, EM experiments, DNA synthesis time-course experiments, with both potential origins present in the same construct, and replication origin mapping experiments, have been performed. In the *in vitro* system EM analysis of, the incubated pPMY III template confirmed the mammalian mtDNA-like replication model: D-loop structures were observed but, as expected according to the supposed animal analogy, only in the pPMY III constructs in the region overlapping the 371 bp minimal origin region (Fig. 7).

In order to investigate whether the pPMY III and IV origins did influence each other analogous to the mtDNA H- and L-strand origins, time-course experiments were performed in the *in vitro* DNA synthesizing system. Double-stranded constructs consisting of both the pPMY III and IV origin regions in the opposite orientation (that means in the two complementary DNA strands; see Fig. 5A, this is the natural situation in the mtDNA H- and L-strand origins), or in the same orientation (in the same strand; see Fig. 5B), were applied as templates in this system. After using the "same orientation" template it turned out that initiation of DNA synthesis occurred specifically only in the pPMY III fragment (in the 371 bp H-strand-like origin region) followed by unidirectional elongation (Fig. 6 lanes F-J). However, in the complementary construct ("opposite orientation"), DNA synthesis first specifically inititates in the pPMY III origin region and elongates counterclockwise into the direction of the pPMY IV origin; then the IV origin (located in the 585 bp L-strand-like origin region) also shows DNA synthesis initiation and clockwise elongation into the direction of the III origin (see Fig. 6 lanes A-E). Both, the uni-directional elongation directions and the following order of the III and IV initiation events fit with the animal mtDNA H- and L-strand replication mechanism (Clayton 1982).

Using cosmid clones covering the complete *P. hybrida* mitochondrial genome (Folkerts and Hanson, 1989) in hybridization experiments with pPMY III and IV as probes it appeared that both pPMY III and IV are localized in the same subgenomic circle of the master circle, separated by 60 kbp from each other. Moreover, extensive restriction enzyme mapping showed that the inserts of pPMY III and IV are located in opposite directions on the *P. hybrida* mitochondrial genome. The same localizations are also determined on the physical map of cytoplasmic male-sterile (CMS) *P. hybrida* plants suggesting conservation for functional use (Folkerts and Hanson, 1989 and personal communication). From now on we shall refer to the replication origins inserted in pPMY III and IV as the A-origin (oriA) and B-origin (oriB) respectively (see Figs. 8 and 9). In the *P. hybrida* mitochondrial

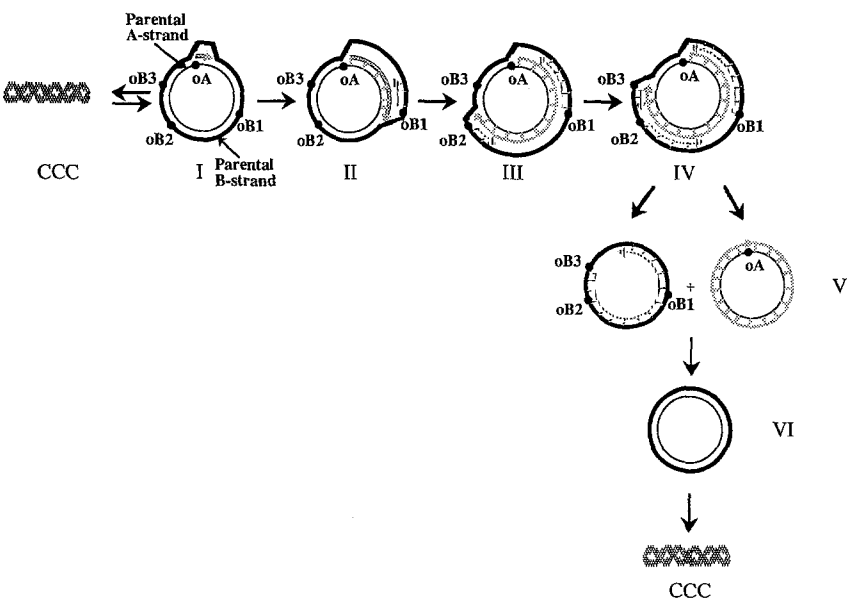


Fig. 9. Hypothetical replication model for *P. hybrida* mitochondrial DNA. "*oA*" and "*oB*" represent the A- and B-strand origin of replication (present respectively in pPMY III and IV). "*ccc*" represents the covalently closed circular mtDNA configuration. Replication starts with transcription initiation in ori A (*I*) followed by processing and elongation (*II*). When the newly synthesized A-daughter strand reaches respectively oris *B1*, *B2* and *B3* in the opposite strand, primase-mediated RNA synthesis starts (*III/IV*) and elongates in the direction of ori A (*IV*). After strand separation (*V*) the B-strand DNA synthesis will be completed and two new daughter strands are created (*VI*) which will be converted to the "*ccc*"-form [In this model only two additional "B"-origins (*B2* and *B3*) are shown in order to keep the model simple; however, hybridization experiments – see Fig. 8 – indicate that, in addition to the B1-origin, there are five additional B-origins present in the *P. hybrida* mitochondrial genome]

genome oriA is unique whereas oriB hybridizes strongly within a region of the same subgenomic circle with which oriA hybridizes (Fig. 8 B1) and hybridizes weakly within several other locations (B2–B6 in Fig. 8); oriB might be present in multicopies on the mitochondrial genome. This is to be expected when considering that the *P. hybrida* genome (442 kbp) is much larger than the animal mtDNA genome (16 kbp) and that oriB (L-strand-like origin) in the B-strand (Fig. 9) initiates (and terminates) always later than the oriA (H-strand-like origin) in the A-strand. In order to compensate for the time difference needed for a complete A- and B-strand replication round, B-strand DNA synthesis should initiate several times in the respective B-origins.

Envisaging a mammalian-like model, *P. hybrida* mtDNA replication will start with transcription initiation at the promoter in the 371 bp-oriA region (in the bottom III-1 994 strand in Fig. 1 B, designated as the A-strand in Fig. 9), followed by processing of the synthesized RNA molecule into a RNA primer in the GC cluster (around nucleotides 1 430; Fig. 1 B). When DNA synthesis on the A-strand template elongates uni-directionally into the T-rich stem-and-loop structure in the 585 bp-oriB1 region (located in the bottom IV-1 359 strand in Fig. 1 C, designated as the B-strand in Fig. 9) this region will be converted to the single-stranded conformation. This is a trigger for primase-mediated RNA or DNA priming in this stem-and-loop, followed by initiation of DNA synthesis on the B-strand template and by unidirectional elongation of this daughter B-strand towards oriA. When the newly synthesized A-daughter strands reaches the additional B-strand origins (B2-B6), (re-)initiation in these sites most likely will take place followed by unidirectional elongation towards oriB1.

The localization data of the "A"- and "B1"-origins imply that in higher plant mitochondria only the master genome (containing both origins) replicates. In each replication round excision should be necessary for the production and maintenance of the subgenomic circles (which contain no initiating oriA). The presence in higher plant mitochondria of an active recombination system (Pring and Lonsdale 1985), including most likely topoisomerase I and gyrase activity (Echeverria et al. 1986), favours such a dynamic replication mechanism.

Using this crude system as reference, reconstitution of a mtDNA replication system consisting of purified replication enzymes and accessory enzyme fractions (like purified systems of *E. coli* and SV40; Baker et al. 1986; Wobbe et al. 1987) will provide a strong tool for studying the proposed replication steps in detail in order to delineate the molecular mechanism of the mtDNA replication process and to verify the hypothetical dynamic genome organization.

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