The EMBO Journal vol.13 no.9 pp.2075 – 2083, 1994

Reverse transcriptase activity of an intron encoded polypeptide

Stefanie Faßbender, Karl-Heinz Brühl[1], Michael Ciriacy[1] and Ulrich Kück[2]

Lehrstuhl für Allgemeine Botanik, Ruhr-Universität Bochum, D-44780 Bochum, Germany and [1]Institut für Mikrobiologie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany

[2]Corresponding author

Communicated by P.Philippson

A number of group II introns from eukaryotic organelles and prokaryotes contain open reading frames for polypeptides with homology to retroviral reverse transcriptases (RTs). We have used the yeast transposon (Ty) system to express ORFs for RTs from eukaryotic organelles. This includes the mitochondrial *coxI* intron i1 from the fungus *Podospora anserina*, the plastid *petD* intron from the alga *Scenedesmus obliquus* and the mitochondrial *RTL* gene from the alga *Chlamydomonas reinhardtii*. The ORFs were fused with the *TYA* ORF from the yeast retrotransposon Ty to produce virus-like particles in the recipient strains with detectable amounts of the RT-like polypeptides. Analysis of the heterologous gene products revealed biochemical evidence that the *P.anserina* intron encodes an RNA-directed DNA polymerase with properties typically found for RTs of viral or retrotransposable origin. *In vitro* assays showed that the intron encoded RT is sensitive to RT inhibitors such as *N*-ethylmaleimide and dideoxythymidine triphosphate but is insensitive against the DNA polymerase inhibitor aphidicolin. The direct biochemical evidence provided here supports the idea that intron encoded RTs are involved in intron transposition events.

Key words: intron encoded polypeptides/*Podospora anserina*/reverse transcriptase/yeast Ty expression system

Introduction

Since the discovery of reverse transcriptases (RTs) in retroviruses more than 20 years ago (Temin and Mitzutani, 1970), an increasing number of genetic elements has been described with open reading frames (ORFs) for RT-like polypeptides (Doolittle *et al.*, 1989; Xiong and Eickbush, 1990). Mainly structural features led to the identification of RT ORFs in retrotransposons, retrons and even introns from eukaryotic organelles and bacteria (Temin, 1989; Ferat and Michel, 1993; Lambowitz and Belfort, 1993). Reverse transcriptases, or RNA-dependent DNA polymerases, direct essential steps in the life cycle of retroelements and it has been suggested that RTs played an important role in evolution during the transition from an early 'RNA-world' to the present day 'DNA-dominated' world (Gilbert, 1986).

Although many introns have been discovered which carry open reading frames for RT-like enzymes (Lambowitz and Belfort, 1993), it remains an open question whether the intron encoded polypeptides are synthesized and have any function similar to that of retroelements. The discovery of a mitochondrial intron encoding an RT-like protein in the filamentous fungus *Podospora anserina* ~10 years ago has stimulated discussions about the functional role of RT in eukaryotic organelles (Osiewacz and Esser, 1984; Michel and Lang, 1985). It was proposed that circular intron DNAs, which are regularly found in ageing cells of this fungus, may be derived by reverse transcription of processed intron RNA (Kück *et al.*, 1985). The generation of double-stranded DNA molecules may be directed by the intron encoded RT. Since RT-like polypeptides are most probably synthesized at extremely low levels in eukaryotic organelles, no RT polypeptides derived from intron encoded ORFs have so far been isolated from organelle preparations. At least in *P.anserina*, immunochemical analysis has proved that the intron encoded polypeptide is present in detectable amounts in ageing mycelial cells (Sellem *et al.*, 1990). Recently, it has been demonstrated that ribonucleoprotein (RNP) particles from *Neurospora crassa* and yeast mitochondria are associated with a RT activity. In *N.crassa*, mitochondrial plasmids not related to the mitochondrial genome most probably encode an RNA-dependent DNA polymerase (Kuiper and Lambowitz, 1988; Kuiper *et al.*, 1990; Kennell *et al.*, 1993). Further indirect evidence for functional RTs in mitochondria stems from work with yeast mutants. Genetic studies suggest that introns i1 and i2 of the *coxI* gene are involved in intron-excision and intron-insertion after recombination of mRNA derived cDNA with mitochondrial DNA (Gargouri *et al.*, 1983; Levra-Juillet *et al.*, 1989; Meunier *et al.*, 1990). More recently, Lambowitz and coworkers (Kennell *et al.*, 1993) successfully defined and characterized RT activity in yeast mitochondria, which is likely encoded by and highly specific for introns i1 and i2 of the *coxI* gene. However, so far no intron encoded polypeptide has been shown biochemically to be a RT.

Using the yeast Ty system (Müller *et al.*1987; Kingsman *et al.*, 1991), we were able to demonstrate the successful overexpression of RT-like genes from *Podospora* mitochondria and from algal organelles. The enriched polypeptide derived from the *P.anserina* intron is shown to have an RNA-directed polymerase activity, with properties shared with RTs from different retroelements. The biochemical evidence provided here supports the idea that introns can behave as retroelements which need a RT for transposition into new sites and for transmission between different organisms (Dujon, 1989; Cavalier-Smith, 1991; Lambowitz and Belfort, 1993; Roger and Doolittle, 1993).

Results

Construction of an universal code equivalent of the coxI intron ORF

Chloroplasts and mitochondria in algae use the universal genetic code. However, mitochondria in animal and fungi use a deviation of the standard one. The most significant difference can be found in the case of the 'UGA' codon which specifies tryptophan in fungal mitochondria, but is usually used as a termination codon in pro- and eukaryotes (Grivell, 1983). Therefore, the mitochondrial *coxI* intron ORF from *P.anserina* was mutagenized in order to use it in heterologous expression systems. In addition, an *Nco*I restriction site was created at the site of the methionine initiation codon within the intron ORF. As outlined in the Materials and methods, a total of eight different mutations were generated by successive *in vitro* manipulations. Using the methodology developed by Eckstein and coworkers (Taylor *et al.*, 1985a,b; Nakamaye and Eckstein, 1986), we constructed a universal code equivalent to the *coxI* intron ORF, which codes for 788 amino acid residues. Recombinant plasmid pKGE1 (Figure 1) contains a 2.5 kb fragment carrying the mutagenized ORF integrated into the unique *Nco*I restriction site of vector M13mp18NcoI. This plasmid was used to construct recombinant expression plasmids.

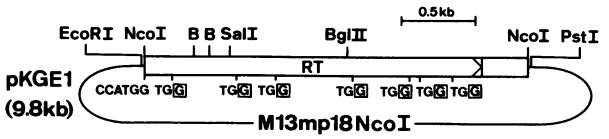

Fig. 1. Restriction map of recombinant vector pKGE1. The universal code equivalent of the RT-like ORF derived from the *Podospora coxI* intron was integrated into sequencing vector M13mp18NcoI. Codons and *Nco*I restriction sites generated by *in vitro* mutagenesis are given. Substituted nucleotides are boxed.

Cloning and expression of reverse transcriptase-like open reading frames using the Ty system in yeast

For assaying the biochemical activity of various RT-like open reading frames, our strategy was to clone the putative RT into an appropriate expression vector based on the retrotransposon Ty1 of *Saccharomyces cerevisiae*. In this expression system the TYA protein encoded by the retrotransposon Ty1 is used as a specific carrier for recombinant proteins. The putative RTs are packaged as TYA fusion proteins into Ty-VLPs. The key feature of this expression system is the ease with which hybrid Ty-VLPs can be purified by simple centrifugation steps (Kingsman *et al.*, 1991). The basic vector for overproduction of the RT ORFs, pFM2IIBglII, consists of the structural *TYA* gene from the Ty element, which encodes a *gag*-like protein. The TYA protein alone is able to assemble into VLPs in the absence of any other Ty encoded protein (Adams *et al.*, 1987; Müller *et al.*, 1987). In order to achieve high level transcription of the *TYA* ORF, the vector contains the *ADH1* promoter region upstream of the *TYA* gene. As shown by Garfinkel *et al.* (1985) and Müller *et al.* (1987) overexpression of the *TYA* gene leads to the intracellular accumulation of large numbers of VLPs. Intron encoded and *RTL* gene encoded putative RTs were fused, in-frame, just downstream of the *TYA* ORF to obtain plasmids pPA405, pSOB148 and pCRB11, respectively (Figure 2). As a control, the *RTL* gene was placed in an inverse orientation in plasmid pCRB9. Cloning was achieved by a series of manipulations described in detail in Materials and methods. Plasmid pCD1, which contains a functional Ty1 element (Müller *et al.*, 1991), was used as a positive control. In this

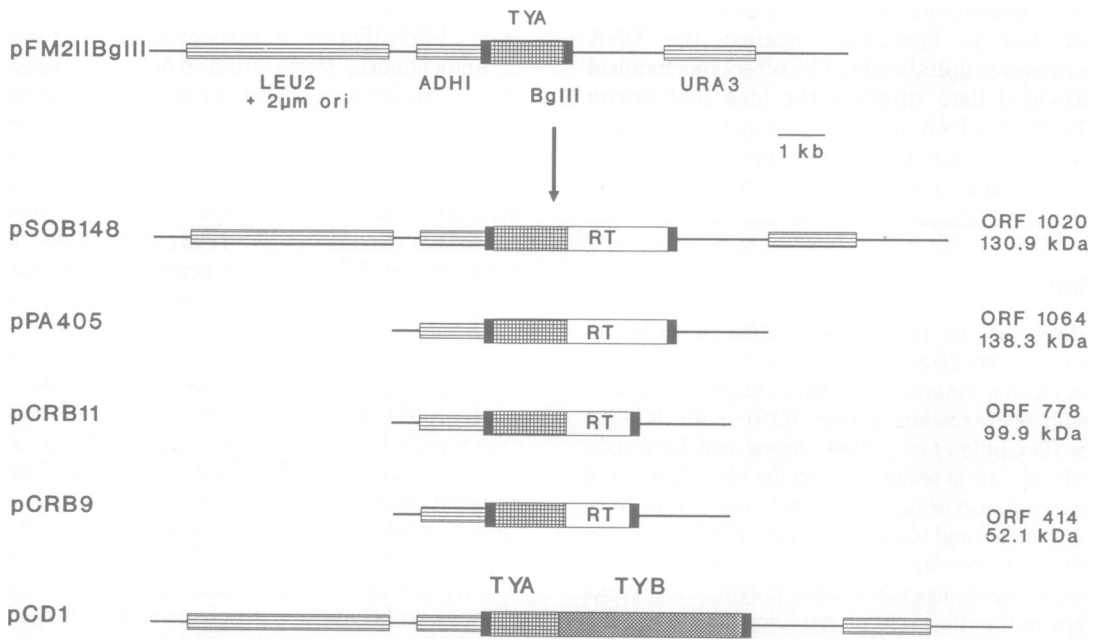

Fig. 2. Vectors for expression of RT-like ORFs in yeast. Except for plasmids pFM2IIBglII, pCRB9 and pCD1, all vectors encode TYA–RT fusion proteins. The number of amino acid residues and the calculated molecular weight are given for polypeptides encoded by the chimeric open reading frames. Thin lines represent sequences derived from vector pBR322, boxes indicate those derived from yeast DNA. Abbreviations: *LEU2*, yeast *LEU2*^{d} gene; *URA3*, yeast *URA3* gene; *ADH1*, *ADH1* promoter; 2μm *ori*, the origin of replication of the 2μm yeast plasmid; *TYA* and *TYB*, open reading frames *TYA* and *TYB* derived from the yeast Ty1 element. RT indicates ORFs for RT-like polypeptides from *S.obliquus* (pSOB148), *P.anserina* (pPA405) or *C.reinhardtii* (pCRB11, pCRB9).

plasmid the second Ty1 gene *TYB*, encoding a multifunctional protein with protease, integrase and RT activity, is expressed as a TYA−TYB fusion protein via a specific frameshifting event occurring within the overlapping region between the two ORFs during translation (Xu and Boeke, 1990). The TYA−TYB fusion protein is subsequently processed to the TYB protein by a protease encoded by the *TYB* ORF.

Synthesis and detection of TYA−RT fusion products

Yeast cells PS1-11 were transformed with constructs shown in Figure 2. After partial purification of Ty-VLPs, accumulating in yeast transformants, the isolated proteins of pellet fractions were separated electrophoretically. Figure 3A demonstrates that TYA fusion proteins are clearly visible in the Coomassie blue stained gel. The TYA protein of ~52 kDa is overexpressed in yeast cells transformed with plasmids pFM2IIBglII and pCD1. The larger 180 kDa unprocessed TYA−TYB fusion protein does not accumulate in cells transformed with pCD1 because the translation frameshift is a relatively rare event and the fusion protein is subsequently processed by the protease to individual TYB protein (Xu and Boeke, 1990). In extracts from yeast transformants overexpressing TYA fusion proteins (pSOB148, pPA405, pCRB11) additional bands which correspond in size to TYA fusion proteins could easily be identified among yeast proteins. The pellet fraction of yeast cells containing the plasmid pCRB9 shows a protein that is insignificantly larger than the TYA protein. Further identification of TYA and TYA fusion proteins was performed by Western blot analysis using a polyclonal antiserum raised against TYA protein (Figure 3B). In lanes pFM2IIBglII and pCD1 bands with an apparent size of 52 kDa can be seen; this is consistent with previous findings

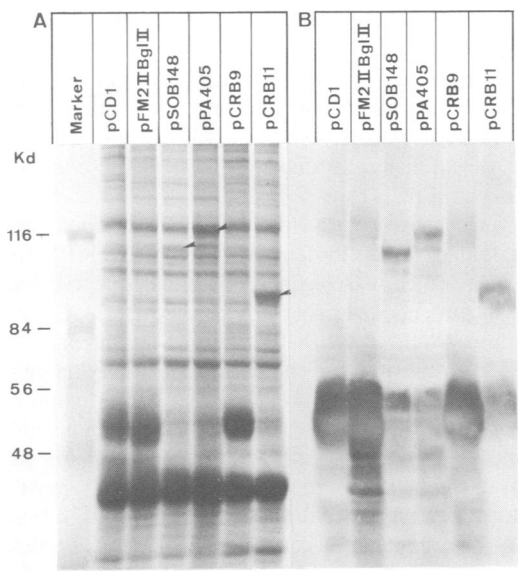

Fig. 3. Expression of RT-like ORFs in *S.cerevisiae*. Partially purified Ty-VLPs and hybrid Ty-VLPs from pellet fractions were electrophoretically separated through a SDS−polyacrylamide gel. (**A**) Coomassie blue stained gel. (**B**) Immunoblot of the protein gel probed with anti-TYA antiserum. Samples were taken from ultracentrifuge pellets derived from cells containing pCD1, pFM2IIBglII, pPA405, pSOB148, pCRB9 and pCRB11. Arrowheads mark proteins corresponding in size to expected TYA fusion proteins. The TYA protein is present as a 52 kDa (pCD1, pFM2IIBglII) or 53 kDa (pCRB9) protein respectively.

that TYA electrophoretically shows a larger size than predicted (44 kDa; see Boeke and Sandmeyer, 1991). Depending on the fragment cloned in the vector pFM2IIBglII, additional bands (pSOB148, pPA405, pCRB11) already observed in the Coomassie stained gel reacted specifically with the TYA antiserum. Proteins overexpressed in yeast transformants pSOB148 (114 kDa), pPA405 (130 kDa), pCRB11 (100 kDa) are therefore undoubtedly TYA fusion proteins. The predicted molecular sizes of the TYA−RT fusion proteins are indicated in Figure 2. In the case of the fusion protein derived from pSOB148, the size of the polypeptide seems to be smaller than predicted. However, as mentioned before, the TYA protein itself has unusual migration properties during electrophoresis. Beside that, we cannot exclude proteolytic activity in the yeast host, leading to shortened fusion proteins. The weak signal comigrating with TYA (pPA405, pSOB148, pCRB11) may originate from the low level of expression of genomic Ty elements, as previously shown by Müller *et al.* (1987).

Detection of RT activity associated with TYA−RT VLPs

After partial purification of VLPs by glycerol step-gradient centrifugation, RT activity was assayed by incorporation of

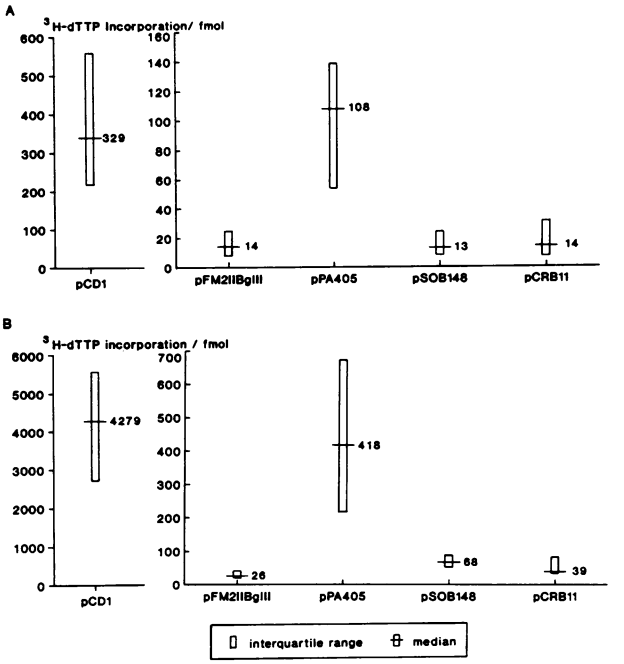

Fig. 4. Exogenous RT activity of different hybrid TyVLPs. In the standard assay, incorporation of [^{3}H]dTTP was followed using poly(rA)−d(pT)$_{15}$ as a template−primer. The incorporation of radiolabelled [^{3}H]dTTP into the homopolymeric template is given in fmol [^{3}H]dTTP. Buffer 1 (**A**) and buffer 2 (**B**) were used for the RT assays. Calculation of the median value and interquartile range combine different numbers of measurements; the number of measurements is given and the numbers of independent VLP preparations are indicated in parentheses. pFM2IIBglII: A, 34 (15), B, 14 (8); pCD1: A, 22 (8), B, 10 (7); pPA405: A, 34 (17), B, 14 (7); pSOB148: A, 6 (6), B, 4 (4); pCRB11: A, 10 (4), B, 4 (4). There are three values which separate the total frequency of a distribution into four equal parts; the central value is called the median and the other two the upper and lower quartiles respectively. The median of a set of values is that value which divides the frequency exactly into two halves. If the set was even in number the median value was taken between the two central values. The interquartile range is a measure of variability that is used when the distribution of values is asymmetrical and is calculated as the distance from the upper to the lower quartile (Kendall and Buckland, 1960).

[^{3}H]dTTP into an artificial homopolymer poly(rA)·oligo(dT)$_{12-15}$ template–primer complex. Resuspended pellet fraction proteins (5 μg) were used under different conditions. The diagram in Figure 4 documents the data of the RT assay. The distribution of absolute incorporation rates was asymmetrical in independently conducted experiments and shows quite a high variation. Therefore, instead of the mean values and standard deviations, the median values and the interquartile ranges are given in the diagram. Yeast strains normally harbour 20–50 endogenous copies of Ty1 and Ty2 elements (Boeke, 1989). A background level of RT activity is therefore assayed with extracts from cells transformed with plasmid pFM2IIBglII, which overexpress the *TYA* gene only. Pellet fractions which were obtained from yeast cells overexpressing an intact Ty1 element (pCD1) served as positive controls. The overexpression of an intact Ty1 element leads to a significant increase in the exogenous RT activity, as was already shown by other investigators (Garfinkel *et al.*, 1985; Mellor *et al.*, 1985; Müller *et al.*, 1987). VLP preparations of yeast cells containing plasmid pCD1 gave a significant incorporation of [^{3}H]dTTP into the homopolymer primer–template complex. The RT assay shows clearly that pellet fractions of yeast transformants overexpressing the intron encoded polypeptide of *P.anserina* (pPA405) possess a significant RT activity. Incorporation of the intron encoded RT is increased 10- or 20-fold, depending on the incubation buffer, when compared with the negative control (pFM2IIBglII). However, activity of the *TYB* encoded RT (pCD1) is 3- or 10-fold higher than the activity of the intron encoded polypeptide of *P.anserina*. In pellet fractions of yeast transformants overexpressing the RT ORFs of *Chlamydomonas reinhardtii* (pCRB11) and *Scenedesmus obliquus* (pSOB148), RT activity is not significantly increased; they showed no incorporation significantly above the background.

Reverse transcriptases require divalent cations Mn^{2+} and/or Mg^{2+} for optimal activity (Varmus and Brown, 1989). The intron encoded RT of *P.anserina* prefers Mg^{2+} (6 mM) to Mn^{2+} (0.6 mM) cations like the Ty RT and is even slightly inhibited by Mn^{2+} (data not shown). No [^{3}H]dTTP was incorporated in the absence of either divalent cations, or the primer, or the template molecule. Similarly, no incorporation of dNTP was observed when proteins were denatured by boiling before assaying the enzymatic activity (data not shown).

In addition to the scintillation measurements, an RT activity test was developed to make the exogenous RT products directly visible (Figure 5). With this approach RT activity was demonstrated by visualization of extension products of the oligo(dT)$_{12-15}$ primer. The radiolabelled cDNA products of this reaction were separated electrophoretically and visualized after autoradiography. With this gel assay the results of the scintillation measurements could be confirmed.

(i) As a control the *TYB*-encoded RT of an intact Ty1 element was overexpressed. In lane pCD1 of Figure 5 a ladder of bands is visible which represents the extension products of the oligo(dT)$_{12-15}$ primer. The occurrence of a pattern of extension products demonstrates that radioactive [α-^{32}P]dTTP was incorporated into the primer–template complex.

(ii) Pellet fractions of yeast cells transformed with the plasmid pF2IIBglII and therefore not overexpressing RT were used to measure background activity. Only very faint bands were visible due to the very low RT activity of endogenous Ty elements.

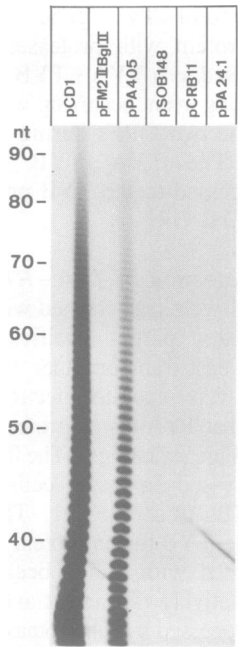

Fig. 5. Polyacrylamide gel electrophoresis of RT extension products. Ty-VLPs and hybrid Ty-VLPs of different yeast transformants were incubated for 30 min with oligo(dT)–poly(rA) primer–template mixture in the presence of [α-^{32}P]dTTP and the extension products were then separated on an 8% polyacrylamide DNA sequencing gel. Labelled products were visualized by autoradiography.

(iii) Extracts from yeast cells containing the plasmid pSOB148 or pCRB11 show no significant RT activity. The signal intensities seen in lanes pSOB148 and pCRB11 of Figure 5 were similar to that of the background control (pFM2IIBglII).

(iv) Enriched hybrid Ty-VLPs derived from yeast transformants which overexpress the intron encoded polypeptide of *P.anserina* (pPA405) produced a distinct ladder of extension products, similar to the control assay with plasmid pCD1.

(v) We constructed plasmid pPA24.1, a deletion derivative of plasmid pPA405, to demonstrate that the RT activity of cells transformed with plasmid pPA405 is due to the activity of the intron encoded protein. Plasmid pPA24.1 contains a deletion of 26 bp in the N-terminus of the intron ORF (for details see Materials and methods). This deletion generates a −1 frameshift and the TYA–intron ORF fusion protein of this construct was not expressed with this plasmid (data not shown). As seen in lane pPA24.1 (Figure 5) VLP preparations which were derived from yeast cells transformed with plasmid pPA24.1 lacked a significant RT activity. The pattern of extension products was almost as weak as in the background control (lane pFM2IIBglII). This result demonstrates that the expression of the intron encoded ORF from *P.anserina* can be directly linked to the observed RT activity.

Characterization of the intron encoded RT activity of P.anserina

To test whether the intron encoded RT of *P.anserina* has inhibition profiles like other typical RTs, assays were done

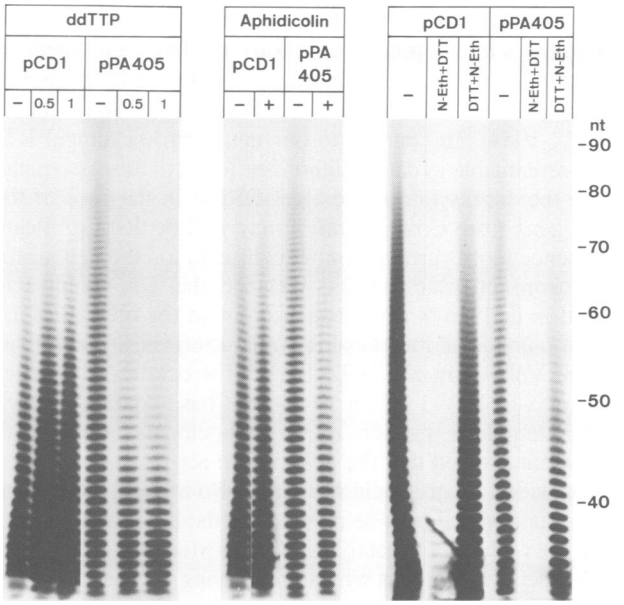

Fig. 6. Effect of different inhibitors of RTs and DNA polymerases on exogenous RT activity. Inhibition of Ty RT and the intron encoded RT of *P.anserina* by *N*-ethylmaleimide (N-Eth), ddTTP and aphidicolin. After incubation of VLP fractions in the presence of [^{32}P]dTTP RT extension products were separated on an 8% polyacrylamide DNA sequencing gel. RT assays were done as described. *N*-ethylmaleimide (10 mM) or DTT (50 mM) were incubated with samples for 10 min on ice before assaying, or increasing ddTTP concentrations (expressed in μM) or aphidicolin (100 μg/ml) were added to the standard RT reaction.

in the presence of different RT inhibitors. Activity of the *Podospora* RT and the yeast Ty1 RT were measured in parallel in the presence of RT inhibitors. Sulfhydryl blocking agents which oxidize the sulfhydryl group of cysteine residues can abolish enzyme activity when a cysteine residue is involved at the active site. In this regard, *N*-ethylmaleimide is a useful tool for identifying the activity of RTs and DNA polymerases, which usually do have cysteine residues at their active sites. Activity of many RTs is completely inactivated by incubation with 10 mM *N*-ethylmaleimide for 10 min on ice (Hizi *et al.*, 1991b). Most DNA polymerases from eukaryotes are *N*-ethylmaleimide-sensitive (Wang, 1991). Preincubation at 50 mM DTT prevents the oxidation of cysteine and therefore abolishes the inhibitory effect of *N*-ethylmaleimide. As shown in Figure 6 and Table I, the RT activity of the intron encoded RT was nearly completely inhibited by incubation with 10 mM *N*-ethylmaleimide for 10 min on ice and the activity of Ty-RT was similarly sensitive to this compound. Preincubation of the VLP proteins with 50 mM DTT neutralized the negative effect of *N*-ethylmaleimide on the RT activity.

A remarkable feature of RTs is their sensitivity to inhibition by dideoxythymidine triphosphate (ddTTP). ddTTP is a strong inhibitor of retroviral RTs even at low concentrations when using a poly(rA)−oligo(dT) template−primer complex. By contrast, both pro- and eukaryotic DNA polymerases are not affected by low amounts of ddTTP (Smoler *et al.*, 1971). VLP fractions isolated from yeast cells transformed with plasmid pCD1 or pPA405 were incubated in the standard RT assay with increasing amounts of ddTTP (Figure 6 and Table I). In the presence of 20 nM ddTTP the incorporation of [^{3}H]dTTP into the homopolymeric template−primer complex was 50% inhibited (Table I). As shown in Figure 6, cDNA fragments of decreasing size can be observed when the amount of ddTTP was increased and Ty-RT (pCD1) appeared to be less sensitive to ddTTP than the intron encoded RT of *P.anserina* (pPA405).

Table I. [^{3}H]dTTP incorporated in the standard RT assay containing buffer 2 in the presence of different inhibitors

Inhibitor	[^{3}H]dTTP incorporated (fmol)/(%)	
	pPA405	pCD1
***N*-ethylmaleimide**		
−N-Mal; −DTT	735 (100)	2395 (100)
+N-Mal; −DTT	36 (4.9)	184 (7.7)
+DTT; +N-Mal	995 (135)	3700 (154.5)
ddTTP (nM)		
10	786 (73.9)	2837 (62.9)
20	598 (56.2)	2543 (56.4)
100	631 (59.3)	2295 (50.9)
500	388 (36.4)	1936 (42.9)
Aphidicolin		
−	1136 (100)	4046 (100)
+	530 (46.8)	2610 (64.52)

Inhibitors used were: 10 mM *N*-ethylmaleimide (N-Mal), 50 mM dithiothreitol (DTT); dideoxythymidine triphosphate (ddTTP, various concentrations) and aphidicolin (100 μg/ml). Mean values of duplicated determinations are given. Numbers in parentheses are percentages of activity.

In subsequent experiments an inhibitor for eukaryotic DNA polymerases was used to distinguish whether the intron encoded polypeptide from *Podospora* is a DNA-dependent or a RNA-dependent DNA polymerase. Eukaryotic polymerases (e.g. α-polymerase of animals, α-like polymerase of plants, viral polymerases and yeast DNA polymerase) are completely inhibited at an aphidicolin concentration of 10 μg/ml, whereas RNA-dependent DNA polymerases are resistant to aphidicolin (Ikegami *et al.*, 1978; Spadari *et al.*, 1982). As shown in Figure 6 and Table I, the activity of the intron encoded and Ty encoded RTs were only 50% inhibited at an aphidicolin concentration of 100 μg/ml and therefore, like RTs from other sources, are insensitive to the inhibitory effect of aphidicolin.

Discussion

We provide direct biochemical evidence that an intron encoded fungal polypeptide possesses an RNA-dependent DNA polymerase activity. The *in vitro* activity assay became feasible when a sufficient amount of the enzyme was synthesized in the heterologous Ty expression system from yeast. Although synthesis of retroviral RTs has been demonstrated in *Escherichia coli* (Tanese *et al.*, 1985; Hizi *et al.*, 1988; Soltis and Skalka, 1988), all our attempts failed to detect RT activities in prokaryotic systems after expression of RT ORFs from eukaryotic organelles (S.Faßbender, H.D.Osiewacz and U.Kück, unpublished data). In the Ty system, fusion proteins can be overexpressed and isolated as VLPs, even when the C-terminus is derived from heterologous polypeptides (Adams *et al.*, 1987). Protection against degradation by cellular proteases most probably

makes possible the efficient expression of RTs, which are otherwise difficult to isolate from prokaryotic expression systems (Sellem *et al.*, 1990; Osiewacz, 1992). The yeast Ty system has already been used to demonstrate RT activities when ORFs from LINE elements of human or trypanosomatid origin were expressed (Gabriel and Boeke, 1991; Mathias *et al.*, 1991). Other yeast systems have also been used for the successful expression of RTs from the HIV-1 retrovirus or from the CaMV pararetrovirus (Takatsuji *et al.*, 1986; Hizi *et al.*, 1988).

Direct demonstration of RT activity has been shown for retroviral and retrotransposon particles and recently even for fungal mitochondria (Goff *et al.*, 1981; Shiba and Saigo, 1983; Kennell *et al.*, 1993). Lambowitz and coworkers (Kuiper and Lambowitz, 1988; Kennell *et al.*, 1993) have isolated RNP particles from *N.crassa* and yeast mitochondria and succeeded in the partial purification of a chromatographic fraction with RT activity.

As an alternative to this experimental approach we have followed the activity of an RNA-directed DNA polymerase by direct expression of a mitochondrial intron ORF. This RT activity was demonstrated by several lines of evidence. First, a frameshift mutation in the intron ORF abolished any RT activity in gel assays used to visualize extension products due to RT activities. Second, the *P.anserina* RT enzyme is highly dependent on Mg^{2+} ions as a cofactor. Optimal activity was observed in the presence of Mg^{2+}, but in the absence of Mn^{2+} ions, distinguishing this RT from DNA-dependent DNA polymerases, which need a high Mn^{2+} concentration for *in vitro* transcription of synthetic homopolymeric RNA molecules (Lazcano *et al.*, 1992). In addition, the *P.anserina* enzyme is strictly dependent on the presence of a primer and template molecule for incorporation of nucleotides. Therefore it can be concluded that the intron ORF does not encode a template-independent terminal deoxynucleotidyltransferase (Nakamura *et al.*, 1981). Third, inhibitors like *N*-ethylmaleimide and ddTTP abolish the activity of the intron encoded polypeptide. It is known that RTs require reduced sulhydryl groups for their activity (Hizi *et al.*, 1991b). *N*-ethylmaleimide oxidizes the SH-groups of cysteine residues and thus inhibits the RT enzyme almost completely. For instance, the RTs from the yeast Ty element or from retroviruses such as MoMLV and AMV are inhibited at a concentration of 10 mM *N*-ethylmaleimide (Roth *et al.*, 1985; Soltis and Skalka, 1988). The only exception observed so far is the RT from the HIV retrovirus. In contrast to other RTs, it has a rather low content (0.3%) of cysteine residues, compared with 1.3% in the *P.anserina* enzyme which is very similar to many other RTs (Hizi *et al.*, 1991a).

While the DNA polymerases from *E.coli* and from eukaryotes are resistant to low ddTTP concentrations (Smoler *et al.*, 1971; Wang, 1991), retroviral RTs are highly sensitive even when low ddTTP concentrations of 5–50 nM are present in the activity assays. The activity of the Ty RT and intron encoded RT is 50% inhibited in the presence of 20 nM ddTTP. Interestingly, the *P.anserina* RT is more strongly inhibited at an increasing concentration (500–1000 nM ddTTP) than the *TYB* encoded RT. The same was observed by Gabriel and Boeke (1991) comparing RT activities from the LINE element of the trypanosomatid *Crithidia fasciculata* with that from the Ty enzyme. Final evidence that the intron encoded polypeptide represents a RT came from assays in which aphidicolin was tested. This inhibitor acts specifically on eukaryotic DNA polymerases α and related enzymes, but has no effect on RTs of diverse origin (Hsu and Taylor, 1982; Spardari *et al.*, 1982; Pfeiffer *et al.*, 1984). In contrast to our data with the fungal RT, we were unable to detect significant RT activities associated with the expression of the algal ORFs. In the case of the *RTL* gene from *C.reinhardtii* it may well be that significant sequence motifs are missing which seem to be essential for RTs from retroelements. For instance, the 'DN' amino acid motif in domain V is not consistent with the obligate 'DD' motif found in all functional RTs characterized so far (Argos, 1988; Velasco *et al.*, 1992). Crystal structure of a ternary complex of HIV-1 RT, a 19 base/18 base double-stranded DNA template–primer, and a monoclonal antibody Fab fragment showed that the polymerase active site is defined by a triad of aspartic acid residues. Two of these are located in domain V and may be involved in the binding of cations that are required for catalysis (Jacobo-Molina *et al.*, 1993). Other sequence blocks which are missing are the X domain and a Zn^{2+} finger-like motif, which are present in the *P.anserina* and *S.obliquus* ORFs (Lambowitz and Belfort, 1993). These two intron ORFs can be distinguished by their localization within the mosaic gene. While the ORF from the *P.anserina* intron is in-frame with the upstream exon and most probably expressed as a fusion protein in fungal mitochondria, the chloroplast ORF is free-standing. However, the latter is expressed as a fusion protein in the heterologous yeast cell. It is possible that the N-terminal part of the TYA–petD fusion polypeptide leads to conformational changes which abolish any RT activity. An experimental test of this may be feasible by proteolytic cleavage of the TYA domain from the chloroplast RT-like polypeptide (Kingsman *et al.*, 1991). Functional differences between the two RTs can be seen when nucleic acid preparations from both organisms are compared. While no intron derived plasmid-like DNA can be detected in *S.obliquus* (U.Kück, unpublished observation), amplified circular intron DNA was isolated from mitochondria of senescent *P.anserina* mycelia (Kück, 1989a). It has been suggested that a reverse transcriptase may be involved in the generation of this plasmid-like molecule (Kück *et al.*, 1985). Processed intron RNA may be transcribed into a circular cDNA copy by a similar mechanism to that proposed for the generation of circular CaMV DNA molecules from a linear RNA genome (Hohn *et al.*, 1985).

Another unsolved question to be answered concerns the function of a reverse transcriptase in senescent mitochondria of *P.anserina*. The intron encoded polypeptide which was shown to exist in ageing mycelia (Sellem *et al.*, 1990) may contribute to the transposition and amplification of the circular intron DNA. In this context it should be mentioned that long-living mutants from *P.anserina* which lack any amplified circular intron DNA contain mtDNA deletions that remove all or part of the homologous *coxI* intron (Jamet-Vierny *et al.*, 1984; Kück *et al.*, 1985). From recent PCR experiments it was suggested that group II intron mobility takes place at the RNA level by reverse splicing, followed by reverse transcription of the chimeric RNA molecules (Mueller *et al.*, 1993). In the case of *P.anserina*, an erroneous reverse splicing mechanism (Sellem *et al.*, 1993) may contribute to mtDNA rearrangements observed in mitochondria from senescent mycelia (Belcour *et al.*, 1981;

Table II. Nucleotide sequences of oligonucleotides used in this investigation

No.	Sequence	Position
3	5'GTTTCAGATGAACCAGCAACGCCAATAAGCCAATCATCAGCATATCTTAC	1456–1407[a]
5	5'AATAAACCATTTAATACC	941–923[a]
12	5'TAACCGTTCCAGATCATCT	1841–1821[a]
14	5'ACTGAGTATCCAGTAGATAA	1903–1883[a]
20	5'TTTCAAACCATTCCTTAGA	681–662[a]
21	5'CCTTAATGACCATTCTACTAC	2118–2097[a]
25	5'GAGATCTTAATCAATACCCAATATAGGGCGATAGCGATC	272–233[a]
40	5'CCTTTCGCCATGGGATAAGTGCGATC	98–78[a]
143	5'TTCTTAGATATCCATGCTATTTT	1310–1333[b]
203	5'GGGGATCCCCTGCA	
204	5'CTAACACCGTATATGAT	5660–5643[b]
212	5'GGCCTCGAGATCTTTAATAGCTACTTTGTGAGGT	5313–5292[c]
213	5'GGCCTCGAGATCTATGAAAAGAATTAAATTAAAA	3145–3166[c]
226	5'GGCCTCGAGATCTATGTTTACTGTAAACTACATTTGGAT	8753–8779[d]
227	5'GGCCTCGAGATCTCTAGAAACGGCGCCAATCTGCATTCAA	9845–9818[d]

[a]Nucleotide position in the *coxI* intron *P.anserina* (Osiewacz and Esser, 1984)
[b]Nucleotide position in the Ty1-9C element of *S.cerevisiae* (Brühl, 1991)
[c]Nucleotide position in the *petD* intron of *S.obliquus* (Kück, 1989b)
[d]Nucleotide position in the *RTL* gene from *C.reinhardtii* (Boer and Gray, 1988)
Oligonucleotides Nos 3, 5, 12, 14, 20, 21, 25 and 40 were used for the *in vitro* mutagenesis, Nos 143 and 204 as sequencing primers, and Nos 212, 213, 226 and 227 as PCR primers to generate expression constructs. Oligonucleotide No. 203 was used as a *PstI*–*Bam*HI–*PstI* linker to construct plasmid pKGEBamHI.

Kück *et al.*, 1981). This evidence together with a number of other data support the assumption that functional RTs are expressed in mitochondria from *P.anserina* and other eukaryotes. For instance, multiple ORFs in mitochondrial and plastid genomes code for polypeptides with significant similarity to RTs from various retroelements (Lambowitz and Belfort, 1993), and mitochondrial introns have been shown to have transposition properties similar to retrotransposons (Levra-Juillet *et al.*, 1989; Meunier *et al.*, 1990; Mueller *et al.*, 1993; Sellem *et al.*, 1993). These examples indicate that functional RTs from eukaryotic organelles are involved not only in evolutionary but also in developmental processes.

Materials and methods

Strains and growth conditions

Escherichia coli strain XL1 Blue (Stratagene) was used for cloning and DNA propagation. *S.obliquus*, strain KS3/2 (Kück, 1989b) and *C.reinhardtii*, strain CC406 (*cw15*, *mt*$^-$; Davis and Plaskitt, 1971) grown for 3 days under continuous light in TAP medium (Gorman and Levine, 1965) were used for preparation of total DNA according to Rochaix *et al.* (1989). Expression experiments were done in *S.cerevisiae*, strain PS1-11 (*Mata ura3-52 his4 leu2-3,112 pep4-3*) (Müller *et al.*, 1991). Transformation was performed according to the method of Klebe *et al.* (1983) with slight modifications (Dohmen *et al.*, 1989). Transformants were selected by growth on synthetic complete medium lacking uracil (Sherman *et al.*, 1986). Cells were grown by shaking at 30°C.

Oligonucleotides

Oligonucleotides (Table II) were synthesized by the β-cyanoethyl phosphoramidite method (Sinha *et al.*, 1984) using an Applied Biosystems Model 381A DNA synthesizer. Several individual oligonucleotides were used for *in vitro* mutagenesis (Nos 3, 5, 12, 14, 20, 21, 25 and 40), DNA sequencing (Nos 143 and 204) and PCR experiments (Nos 212, 213, 226 and 227).

In vitro mutagenesis

The intron RT ORF from *P.anserina* contains seven 'TGA' codons, specifying tryptophan in fungal mitochondria. Using individual oligonucleotides as outlined in Table II, eight *in vitro* mutagenesis steps were performed to generate a universal equivalent of the intron ORF. For all *in vitro* mutagenesis manipulations, the phosphorothioate method developed by Eckstein and coworkers was used (Taylor *et al.*, 1985a,b; Nakamaye and Eckstein, 1986). In our initial experiments, two copies of the *coxI* intron inserted in head-to-tail orientation in M13 sequencing vectors were used for *in vitro* mutagenesis (Stahl *et al.*, 1980). Using oligonucleotide 40, an *NcoI* site was generated at the first methionine codon of the intron ORF at position 87 (Osiewacz and Esser, 1984). The *in vitro* mutagenesis created a 2.5 kb *NcoI* fragment which was subcloned into vector M13mp18NcoI. This M13 vector contains a unique *NcoI* restriction site for DNA cloning (I.Godehardt and U.Kück, unpublished). In successive *in vitro* manipulations, seven 'TGA' codons were mutagenized. The corresponding A to G transversions at positions 254, 654, 935, 1427, 1832, 1896 and 2108 (Osiewacz and Esser, 1984) created the desired TGG codons. All *in vitro* manipulations were confirmed by DNA sequencing of the complete mutagenized intron ORF. The resulting plasmid pKGE1 (Figure 1) was used for further cloning and expression experiments.

Nucleotide sequence analysis

The dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) was used for DNA sequencing. Cloning of fragments was verified by sequencing with a T7 DNA polymerase sequencing kit (Pharmacia), using oligonucleotides Nos 143 and 204 as primers.

DNA amplification by the PCR

The PCR technique was modified according to Mullis and Faloona (1987); 10–100 ng of total DNA in a volume of 50 μl containing 67 mM Tris–HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.17 mg/ml BSA and 33 μM dNTPs was amplified with 0.3 μg of each primer and two units of replitherm DNA polymerase (Biozym, Hamelin, Germany). Prior to amplification the samples were denatured for 5 min at 95°C. The amplification reaction involved denaturation at 92°C for 2 min, annealing at 50–60°C for 2 min and polymerization at 72°C for 1–5 min, for 30 cycles.

Plasmids

Plasmid pFM2ΔBglII is a derivative of plasmid pFM2 previously described (Müller *et al.*, 1987). In order to remove the overlap of *TYA* and *TYB* reading frames, a 312 bp *Ban*II–*Bgl*II fragment from pFM2 was removed, the ends were made blunt by T4 polymerase and subsequently ligated in the presence of a *Bgl*II linker (5'-CAGATCTC). This resulted in an in-frame fusion of *TYA* and *TYB* and consequently, in the production of Ty particles consisting only in TYA–TYB fusion proteins in pFM2ΔBglII yeast transformants (Brühl, 1991). Construction of plasmid pCD1 has been described elsewhere (Müller *et al.*, 1991). Digesting pFM2ΔBglII with *Bgl*II followed by intramolecular re-ligation of the largest 11.9 kb *Bgl*II fragment results in

recombinant plasmid pFM2IIBglII (Figure 2). Chimeric genes were constructed by digesting pFM2IIBglII with *BglII* followed by insertion of RT ORFs from different sources. These RT ORFs were produced as follows. (i) pKGE*Bam*HI is a derivative of plasmid pKGE1 with a *PstI*–*Bam*HI–*PstI* linker (oligonucleotide 203) in the unique *PstI* site. (ii) A 1.7 kb fragment from pKGE*Bam*HI, containing the *coxI* intron ORF was used to construct the yeast expression plasmid pPA405. p24.1 is a derivative of plasmid pPA405 which contains an internal deletion of 26 bp resulting in a frame-shift mutation inside the coding sequence. The deletion was generated by *Bal*31 nuclease treatment of plasmid pPA405 restricted with *SalI*. The *SalI* site is located within the RT ORF at position 636. (iii) pSOB148 was constructed as follows: a 1.8 kb fragment containing the *pet*D intron ORF with *BglII* overhangs was amplified by PCR using total DNA from *S.obliquus* and oligonucleotides Nos 212 and 213. The resulting fragment was inserted into the *BglII* site of plasmid pFM2IIBglII. (iv) For the construction of pCRB11 and pCRB9 a 1.1 kb fragment containing the mitochondrial RT-like ORF (RTL) from *C.reinhardtii* was amplified by PCR with oligonucleotides Nos 226 and 227 using total DNA from *C.reinhardtii*. The resulting PCR product was cloned into the *BglII* site of pFM2IIBglII. Using pCRB11, the *RTL* gene is overexpressed as a TYA fusion protein. The *RTL* gene has an inverse orientation in plasmid pCRB9. The recombinant constructions were verified by restriction mapping and DNA sequencing across the TYA–RT junctions.

Protein extraction from yeast cells and purification of virus-like particles (VLPs)

Ty-VLPs were prepared as described previously (Müller *et al.*, 1987). Cells were disrupted with glass beads. After differential centrifugation to remove cell debris and glass beads, the recovered supernatant was loaded on top of a step gradient consisting of 30%/50% (w/w) glycerol in RTB buffer (50 mM Tris–HCl, pH 7.9, 1 mM EDTA, 10 mM β-mercaptoethanol) and centrifuged at 4°C for 15 h at 40 000 r.p.m. in a Ti50 rotor to enrich for VLPs. The resulting pellet was resuspended in 500 μl RTB buffer. This preparation was used to measure the RT activity of Ty-VLPs and hybrid Ty-VLPs.

SDS–PAGE and immunoblotting

Fractionation of yeast proteins on denaturating SDS–polyacrylamide gels (7.5% acrylamide) was performed according to Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) with a protein assay from Bio-Rad (München, Germany). After electrophoresis, proteins were visualized by Coomassie blue staining. For immunoblots, proteins were transferred directly to Immobilon P transfer membrane (Millipore, Eschborn, Germany) by electroblotting using a Fastblot System (Biometra, Göttingen, Germany). To detect TYA fusion proteins the membranes were probed with rabbit antisera directed against TYA protein using a phosphatase-coupled colour reaction described by Towbin *et al.* (1979). TYA antigen was purified from Ty-VLP preparations by SDS (1%) denaturation and subsequent FPLC chromatography in RTB buffer. Purified TYA (100–500 μg) was used to immunize rabbits. Alkaline phosphatase conjugated to anti-rabbit IgG was obtained from Dianova (Hamburg, Germany) and applied as recommended. 5-Bromo-4-chloro-3-indolyl phosphate (100 μl/ml) (Boehringer, Mannheim, Germany) was used as a substrate for the phosphatase.

Assay of reverse transcriptase activity

Enzymatic activity was assayed by measuring the incorporation of labelled nucleotide substrate using a synthetic template–primer complex. A 50 μl standard mixture contained 5 μg of pellet fraction protein, 10 μl of buffer 1 (250 mM Tris–HCl, pH 8.3, 250 mM KCl, 30 mM $MgCl_2$ 3 mM $MnCl_2$, 30 mM dithiothreitol) or 10 μl of buffer 2 (250 mM Tris–HCl, pH 8.3, 30 mM $MgCl_2$, 30 mM dithiothreitol), 10 μl of 50% glycerol, 0.4 μM [^{3}H]dTTP (1.7 Bq/mmol, Amersham, Braunschweig, Germany) and 0.02 A_{260} units poly(rA)–$d(pT)_{15}$ (from Pharmacia, Freiburg, Germany). After incubation at 23°C for 30 min the assay was stopped by adding 1 ml of cold 10% (w/v) trichloracetic acid (TCA), 1% (w/v) sodium pyrophosphate. The product was allowed to precipitate at 0°C for 30 min, and subsequently vortexed and filtered through 2.4 mm GF/C glassfibre filters (Schleicher and Schüll, Dassel, Germany) and rinsed with 10×2 ml of chilled 5% (w/v) TCA, 1% (w/v) sodium pyrophosphate and rinsed once with 2 ml of cold 95% ethanol. After drying filter-bound radioactivity was counted in scintillation fluid.

Polyacrylamide gel electrophoresis of RT extension products

Ty-VLPs were incubated with oligo(dT)–poly(rA) primer–template in a 10 μl mixture containing 50 mM Tris–HCl, pH 8.3, 6 mM $MgCl_2$, 10% glycerol and 0.02 A_{260} units poly(rA)–$d(dT)_{15}$ in the presence of [α-^{32}P]dTTP at 110 MBq/mmol (Amersham, Braunschweig, Germany) for 30 min at 23°C. The reaction was stopped by adding 5 μl of sequencing stopper (Pharmacia, Freiburg, Germany) and separation of RT extension products was done on an 8% polyacrylamide DNA sequencing gel. Labelled products were visualized by autoradiography.

Acknowledgements

We express our thanks to Ms B.Ebeling and I.Godehardt for their help in the *in vitro* mutagenesis, Ms S.Bär, M.Luber for technical assistance, Professor Eckstein (Göttingen) for advice in the *in vitro* mutagenesis and his gift of bacterial strains. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg, Germany). This paper is dedicated to Professor Esser on the occasion of his 70th birthday.

References

Adams,S.E., Dawson,K.M., Gull,K., Kingsman,S.M. and Kingsman,A.J. (1987) *Nature*, **329**, 68–70.
Argos,P. (1988) *Nucleic Acids Res.*, **16**, 9909–9916.
Belcour,L., Begel,O., Mossé,M.O. and Vierny,C. (1981) *Curr. Genet.*, **3**, 13–21.
Boeke,J.D. (1989) In Berg,D.E. and Howe,M.M. (eds), *Mobile DNA*. American Society for Microbiology, Washington DC, pp. 335–375.
Boeke,J.D. and Sandmeyer,S.B. (1991) In Broach,J.R., Jones,E.W. and Pringle,J. (eds), *The Molecular and Cellular Biology of the Yeast Saccaromyces*. pp. 193–261.
Boer,H.P. and Gray,M.W. (1988) *EMBO J.*, **7**, 3501–3508.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Brühl,K.H. (1991) Ph.D. Thesis. Heinrich-Heine-Universität, Düsseldorf.
Cavalier-Smith,T. (1991) *Trends Genet.*, **7**, 145–148.
Davis,D.R. and Plaskitt,A. (1971) *Genet. Res.*, **17**, 33–43.
Dohmen,R.J., Strasser,A.W.M., Zitomer,R.S. and Hollenberg,C.P. (1989) *Curr. Genet.*, **15**, 319–325.
Doolittle,R.F., Feng,D.F., Johnson,M.S. and McClure,M.A. (1989) *Q. Rev. Biol.*, **64**, 1–30.
Dujon,B. (1989) *Gene*, **82**, 91–114.
Ferat,J.L. and Michel,F. (1993) *Nature*, **364**, 358–361.
Gabriel,A. and Boeke,J.D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9794–9798.
Garfinkel,D.J., Boeke,J.D. and Fink,G.R. (1985) *Cell*, **42**, 507–517.
Gargouri,A., Lazowska,J. and Slonimski,P.P. (1983) In Schweyen,R., Wolf,K. and Kaudewitz,F. (eds), *Mitochondria 1983*. Walter de Gruyter, Berlin, pp. 259–268.
Gilbert,W. (1986) *Nature*, **271**, 501.
Goff,S., Traktman,P. and Baltimore,D. (1981) *J. Virol.*, **38**, 239–248.
Gorman,D.S. and Levine,R.P. (1965) *Proc. Natl Acad. Sci. USA*, **54**, 1665–1669.
Grivell,L.A. (1983) In Schweyen,R.J., Wolf,K. and Kaudewitz,F. (eds), *Mitochondria 1983*. Walter de Gruyter, Berlin.
Hizi,A., McGill,C. and Hughes,S.H. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 1218–1222.
Hizi,A., Tal,R., Shaharabany,M. and Loya,S. (1991a) *J. Biol. Chem.*, **266**, 6230–6239.
Hizi,A., Shaharabany,M., Tal,R. and Hughes,S.H. (1991b) *J. Biol. Chem.*, **267**, 1293–1297.
Hohn,T., Hohn,B. and Pfeiffer,P. (1985) *Trends Biochem. Sci.*, **10**, 205–209.
Hsu,T.W. and Taylor,J.M. (1982) *J. Virol.*, **44**, 493–498.
Ikegami,S., Taguchi,T., Ohashi,M., Oguro,M., Nagano,H. and Mano,Y. (1978) *Nature*, **257**, 458–460.
Jacobo-Molina,A. *et al.* (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6320–6324.
Jamet-Vierny,C., Begel,O. and Belcour,L. (1984) *Eur. J. Biochem.*, **143**, 389–394.
Kendall,M.G. and Buckland,W.R. (1960) *A Dictionary of Statistical Terms*. Oliver and Boyd Ltd, Edinburgh.
Kennell,J.C., Moran,V.J., Perlman,P.S., Butow,R.A. and Lambowitz,A.M. (1993) *Cell*, **73**, 133–146.
Kück,U. (1989a) *Exp. Mycol.*, **13**, 111–120.
Kück,U. (1989b) *Mol. Gen. Genet.*, **218**, 257–266.
Kück,U., Stahl,U. and Esser,K. (1981) *Curr. Genet.*, **3**, 151–156.
Kück,U., Osiewacz,H.D., Schmidt,U., Kappelhoff,B., Schulte,E., Stahl,U. and Esser,K. (1985) *Curr. Genet.*, **9**, 373–382.
Kingsman,A.J., Adams,S.E., Burns,N.R. and Kingsman,S.M. (1991) *Trends Biotechnol.*, **9**, 303–309.

Klebe,R.J., Harriss,J.V., Sharp,Z.D. and Douglas,M.O. (1983) *Gene*, **25**, 333–341.
Kuiper,M.T.R. and Lambowitz,A.M. (1988) *Cell*, **55**, 693–704.
Kuiper,M.T.R., Sabourin,J.R. and Lambowitz,A.M. (1990) *J. Biol. Chem.*, **265**, 6936–6943.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lambowitz,A.M. and Belfort,M. (1993) *Annu. Rev. Biochem.*, **62**, 587–622.
Lazcano,A., Valverde,V., Hernández,P., Gariglio,P., Fox,G.E. and Or,J. (1992) *J. Mol. Evol.*, **35**, 524–536.
Levra-Juillet,E., Boulet,A., Seraphin,B., Simon,M. and Faye,G. (1989) *Mol. Gen. Genet.*, **217**, 168–171.
Mathias,S.L., Scott,A.F., Kazazian,H.H., Boeke,J.D. and Gabriel,A. (1991) *Science*, **254**, 1808–1810.
Mellor,J., Malim,M.H., Gull,K., Tuite,M.F., McCready,S., Dibbayawan,T., Kingsman,S.M. and Kingsman,A.J. (1985) *Nature*, **318**, 583–586.
Meunier,B., Tian,G.L., Macadre,C., Slonimski,P.P. and Lazowska,J. (1990) In Qualiariello,E., Papa,S., Palmieri,F. and Saccone,C. (eds), *Structure, Function and Biogenesis of Energy Transfer Systems*. Elsevier Science Publishers, Amsterdam, pp. 169–173.
Michel,F. and Lang,F.B. (1985) *Nature*, **316**, 641–642.
Mueller,M.W., Allmaier,M., Eskes,R. and Schweyen,R.J. (1993) *Nature*, **366**, 174–176.
Müller,F., Brühl,K.H., Freidel,K., Kowallik,K.V. and Ciriacy,M. (1987) *Mol. Gen. Genet.*, **207**, 421–429.
Müller,F., Laufer,W., Pott,U. and Ciriacy,M. (1991) *Mol. Gen. Genet.*, **226**, 145–153.
Mullis,K.B. and Faloona,F.A. (1987) *Methods Enzymol.*, **155**, 335–350.
Nakamaye,K.L. and Eckstein,F. (1986) *Nucleic Acids Res.*, **14**, 9679–9698.
Nakamura,H., Tanabe,K., Yoshida,S. and Morita,T. (1981) *J. Biol. Chem.*, **256**, 8745–8751.
Osiewacz,H.D. (1992) *Genom Analysen bei Hyphenpilzen*. Habilitationsschrift, Ruhr University, Bochum.
Osiewacz,H.D. and Esser,K. (1984) *Curr. Genet.*, **8**, 299–305.
Pfeiffer,P., Laquel,P. and Hohn,T. (1984) *Plant Mol. Biol.*, **3**, 261–271.
Rochaix,J.D., Mayfield,S., Goldschmidt-Clermont,M. and Erickson,J. (1989) In Shaw,C.H. (ed.), *Plant Molecular Biology*. IRL Press, Oxford, pp. 263–275.
Roger,A.J. and Doolittle,W.F. (1993) *Nature*, **364**, 289–290.
Roth,M., Tanese,N. and Goff,S.P. (1985) *J. Biol. Chem.*, **260**, 9326–9335.
Sanger,F., Nicklen,S. and Coulsen,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Sellem,C.H., Sainsard-Chanet,A. and Belcour,L. (1990) *Curr. Genet.*, **224**, 232–240.
Sellem,C.H., Lecellier,G. and Belcour,L. (1993) *Nature*, **366**, 176–178.
Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Laboratory Course Manual for Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shiba,T. and Saigo,K. (1983) *Nature*, **302**, 119–124.
Sinha,N.D., Bierat,J., McManus,J. and Köster,H. (1984) *Nucleic Acids Res.*, **12**, 4539–4557.
Smoler,D., Molineux,I. and Baltimore,D. (1971) *J. Biol. Chem.*, **246**, 7697–7700.
Soltis,D.A. and Skalka,M. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3372–3376.
Spadari,S., Sala,S. and Pedrali-Noy,G. (1982) *Trends Biochem. Sci.*, **7**, 29–32.
Stahl,U., Kück,U., Tudzynski,P. and Esser,K. (1980) *Mol. Gen. Genet.*, **178**, 639–646.
Takatsuji,H., Hirochika,H., Fukushi,T. and Ikeda,J. (1986) *Nature*, **319**, 240–243.
Tanese,N., Roth,M. and Goff,S.P. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 4944–4948.
Taylor,J.W., Schmidt,W., Cosstick,R., Okruszek,A. and Eckstein,F. (1985a) *Nucleic Acids Res.*, **13**, 8749–8764.
Taylor,J.W., Ott,J. and Eckstein,F. (1985b) *Nucleic Acids Res.*, **13**, 8765–8785.
Temin,H.M. (1989) *Nature*, **339**, 254–255.
Temin,H.M. and Mitzutani,S. (1970) *Nature*, **226**, 1211–1213.
Towbin,H., Staelin,T. and Gorden,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354
Varmus,H. and Brown,P. (1989) In Berg,D.E. and Howe,M.M. (eds), *Mobile DNA*. American Society for Microbiology, Washington DC, pp. 53–108.
Velasco,A.M., Medrano,L., Lazcano,A. and Or,J. (1992) *J. Mol. Evol.*, **35**, 551–556.
Wang,T.S. (1991) *Annu. Rev. Biochem.*, **60**, 513–552.
Xiong,Y. and Eickbush,T.H. (1990) *EMBO J.*, **9**, 3353–3362.
Xu,H. and Boeke,J.D. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8360–8364.

Received on December 28, 1993; revised on February 4, 1994