Université de Montréal

Identification of a Protein Required to Target the DNA Repair Enzyme Apn1 into Yeast Mitochondria

par

Ratsavarinh Vongsamphanh

Département de microbiologie et immunologie

Faculté de médecine

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de
Maître ès sciences (M.Sc.)
en microbiologie et immunologie

Août 2000

Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé:

Identification of a Protein Required to Target The DNA Repair Enzyme Apn1 into Yeast Mitochondria

présenté par:

RATSAVARINH VONGSAMPHANH

a été évalué par un jury composé des personnes suivantes:

Mémoire accepté le:.....

Abstract

Mitochondria generate constantly reactive oxygen species (ROS) which are known to cause a variety of DNA lesions, namely apurinic/apyrimidinic (AP) sites. In the yeast *Saccharomyces cerevisiae*, the enzyme Apn1 (AP endonuclease) plays a crucial role in the repair of nuclear AP sites. Previous studies have shown that cells lacking Apn1 are hypersensitive to DNA damaging agents that produce AP sites and display increased spontaneous mutation rates. Additional studies have demonstrated that the Apn1 C-terminus (Ct) contains two basic amino acids clusters (I and II) that function as bipartite nuclear localization signals. Deletion of either cluster prevents entry of Apn1 into the nucleus. We postulated that the C-terminal end of Apn1 interacts with at least one protein which is important to translocate Apn1 into the nucleus. We have now demonstrated with the yeast two-hybrid system that Pir1, a protein of previously unknown function, interacts with the Apn1-Ct *in vivo* and *in vitro*. Surprisingly, deletion of pir1 apparently caused accumulation of Apn1 in the nucleus, and a corresponding depletion from the cytoplasm. To explain the biological significance of the Pir1/Apn1 interaction, we postulated that Pir1 may delay the nuclear entry of Apn1 to allow some of the AP endonuclease to be transported into the mitochondria to repair damaged mitochondrial DNA. Enzymatic assays revealed that in the absence of Pir1, Apn1 endonuclease activity is drastically reduced in the mitochondria. Furthermore, the rate of mitochondrial mutations is increased in the *pir1Δ* mutant relative to the parent. Thus, the data clearly indicate that Pir1 is not implicated exclusively in the nuclear import of Apn1, but it is also required to ensure proper cellular distribution of this endonuclease to the mitochondria.

Résumé

Les dérivés réactifs de l'oxygène générés principalement lors de la respiration mitochondriale produisent des lésions à l'ADN, notamment les sites abasiques (AP). Chez la levure *Saccharomyces cerevisiae*, l'enzyme Apn1 (AP endonucléase) joue un rôle crucial dans la réparation des sites AP nucléaires. Des études ont démontré que sans la protéine Apn1 les cellules sont hypersensibles aux agents endommageant l'ADN causant des sites AP et possèdent un haut taux de mutations spontanées. D'autres études ont démontré que l'extrémité C-terminale (Ct) d'Apn1 possédant deux régions basiques (I et II) constitue un signal de localization nucléaire bipartite. Une délétion de la région I ou II empêche l'entrée d'Apn1 dans le noyau. Ainsi, nous postulons que la partie C-terminale d'Apn1 doit interagir avec au moins une protéine qui sera importante pour localiser Apn1 dans le noyau. Nous avons identifié par le système à deux hybrides la protéine Pir1, dont le rôle n'ayant pas encore été établi précédemment, comme interagissant avec Apn1-Ct *in vivo* et *in vitro*. De façon surprenante, en absence de Pir1, Apn1 s'accumule dans le noyau et paralèllement diminue dans le cytoplasme. Pour déterminer l'importance biologique de l'interaction entre Pir1 et Apn1, nous proposons que Pir1 retarde l'entrée nucléaire d'Apn1 pour permettre un certain transport de l'AP endonucléase vers les mitochondries dans le but de réparer l'ADN mitochondrial endommagé. Des essais enzymatiques ont démontré qu'en absence de Pir1, l'activité AP endonucléase d'Apn1 est significativement réduite dans les mitochondries. De plus, la fréquence de mutations mitochondriales est élevée dans un mutant de Pir1 comparativement à la souche parentale. Ces résultats démontrent clairement que Pir1 n'est pas exclusivement impliqué dans le transport nucléaire d'Apn1 mais est aussi nécessaire pour la distribution cellulaire adéquate de l'endonucléase dans les mitochondries.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Ade	adenine
AP	apurinic/apyrimidinic
Apn1	AP endonuclease
Apn1p	AP endonuclease protein
ARM	armadillo
BER	base excision repair
bp	base pair
cDNA	complimentary deoxyribonucleic acid
C. elegans	*Caenorhabditis elegans*
Ct	C-terminus
dRpase	deoxyribose-phosphodiesterase
E. coli	*Escherichia coli*
Endo IV	endonuclease IV
ery^R	erythromycin resistance
Exo III	exonuclease III
FEN1	flap-endonuclease 1
GAD	galactose activation domain
GAL	galactose
GBD	galactose DNA binding domain
GFP	green fluorescent protein
Gst	glutathione-S-transferase
H2A	histone 2A
H2B	histone 2B
his	histidine
H_2O_2	hydrogen peroxide
Kar α	karyopherin alpha
Kar β	karyopherin beta
Kb	kilobase

kDa	kiloDalton
leu	leucine
Mg	magnesium
MMS	methyl methane sulfonate
NER	nucleotide excision repair
NLS	nuclear localization signal
NPC	nuclear pore complex
$^1O_2$	singlet oxygen
O_2^-	superoxide anion
• OH	hydroxyl radical
8-OHG	8-hydroxyguanine
O/N	overnigth
8-OxoG	8-oxo-guanine
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
3'-PGA	3'-phosphoglycolaldehyde
PIR	Protein with Internal Repeats
Pir1p	Protein with Internal Repeats 1 protein
Pol β, δ, ε	polymerase beta, gamma, epsilon
RFC	replication factor-C
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SV40	simian virus 40
URA	uracile
YPD	yeast peptone dextrose
YPEG	yeast peptone ethanol glycerol

Acknowledgments

For giving me the opportunity to do this work and for his guidance and support, I am grateful to Dr. Dindial Ramotar, my research supervisor.

I give thanks to Dr. Jean-Christophe Hoflack for helping me with the two-hybrid system and for his support.

I am thankful to Xiaoming Yang for his technical assistance throughout this project.

I would like to acknowledge Pierre-Karl Fortier for contributing to my work.

I also send thanks to past and present members of the laboratory and the Guy-Bernier Research Centre for making my studies such an enjoyable experience.

A special thank you to Luisa Izzi for your friendship and support.

Finally, I am grateful to my parents, brother and boyfriend for their love and support.

Contributions of Authors

The following describes the contribution of each co-author for the manuscript: Vongsamphanh, R., Fortier, P-K., Ramotar, D. (2000) Pir1p Mediates Translocation of Yeast Apn1p Endonuclease into the Mitochondria to Repair Damaged DNA. Submitted for publication in *Molecular and Cellular Biology* (Chapter 2).

Ratsavarinh Vongsamphanh made the following constructs which were all used in this study: pGFP-APN-CT, pGBD-APN-CT, pGBD-APN1, pGAD-PIR1, pGFP-PIR1, pGFP-APN, and pAPN355-GFP. Expression of Gfp-Apn-Ct in the yeast strain pJ69 was assessed by Western blot (Figure 2-1A) using monoclonal anti-Gfp antibodies and its cellular location was determined by fluorescent microscopy (Figure 2-1B).

Ratsavarinh Vongsamphanh performed the yeast two-hybrid screening with pGBD-APN-CT as the bait plasmid to screen a yeast genomic library. Once the positive clone identified, she did a DNA sequence analysis which revealed that the clone contained 87% of the *PIR1* gene (Figure 2-2). To exclude the possibility that the interaction was only due to the truncated forms of the two proteins, the two-hybrid result was confirmed with both full-length Apn1 and Pir1.

Pierre-Karl Fortier made the pGST-APN-CT construct. He evaluated the expression of the construct in bacteria by Western blot (Figure 2-3B) using monoclonal anti-Gst antibodies and purified the fusion protein from a glutathione-S-transferase affinity column. Miss Vongsamphanh used the pGST-APN-CT plasmid to verify the Apn1/Pir1 interaction *in vitro* by passing either the purified Gst-Apn-Ct (Figure 2-3D) or native Apn1 (Figure 2-3E) onto a Gfp-Pir1 phenyl-agarose column (Figure 2-3C). Prior to the

coupling of Gfp-Pir1 onto the column, she verified its expression in yeast by Western blot (Figure 2-3A) using monoclonal anti-Gfp antibodies.

Ratsavarinh Vongsamphanh determined the Gfp-Pir1 fusion protein stability in the presence or absence of native Apn1 and various C-terminal shorter form of the AP endonuclease by Western blot using yeast total extracts. The blot was probed using either monoclonal anti-Gfp (Figure 2-4A) or polyclonal anti-Apn1 (Figure 2-4B) antibodies. She also performed the MMS gradient plate assays (Figure 2-5).

Miss Vongsamphanh generated the RVY1 and RVY2 mutants by one-step gene targeting using the *KanXM* gene module. The RYV1 mutant and its parental strain (SEY6210) were transformed with pGFP-APN1 so that the cellular localization of the Gfp fusion protein in both strains can be observed under fluorescent microscopy (Figure 2-6).

Ratsavarinh Vongsamphanh prepared nuclear, total, and cytoplasmic extracts from the parent and *pir1Δ* strains carrying pGFP-APN1. She then assessed the amount of Gfp-Apn1 in the nuclear (Figure 2-7A), total (Figure 2-7B), and cytoplasmic (Figure 2-7C) extracts by Western blot analysis using monoclonal anti-Gfp antibodies.

Miss Vongsamphanh prepared mitochondrial extracts from the parent and the *pir1Δ* mutant strains carrying or not the pDR6 plasmid. She examined the Apn1 level by Western blot analysis (Figure 2-8A) using polyclonal anti-Apn1 antibodies. She confirmed the mitochondrial location of Apn1 by proteinase K treatment of purified mitochondria (Figure 2-8B) and also determined the AP endonuclease activity in the mitochondrial extracts (Figure 2-8C).

Ratsavarinh Vongsamphanh determined the rates of mitochondrial mutations by scoring for colonies resistant to erythromycin in different yeast strains (Table 2-1).

CHAPTER 1

GENERAL INTRODUCTION

OXIDATIVE DNA DAMAGE

Living organisms must maintain their genetic integrity. Failure to do so, in eucaryotic organisms, may lead to mutagenesis, carcinogenesis, and aging (1). One of the most prominent forms of cellular stress is the constant exposure to reactive oxygen species (ROS) that cause DNA damage (2).

Production and Detoxification of Reactive Oxygen Species

Reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radicals (O^-_2), and hydroxyl radicals (•OH) are highly unstable molecules which have one or more unpaired electrons and, as such, are stabilized through the attack and removal of electrons from other sources with the concomitant destabilization of the donor molecule. They are formed in living cells during normal metabolism, via mitochondrial respiration and neutrophil/macrophage activation (3). Oxygen-derived species are also produced exogenously following exposure to physical and chemical mutagens such as ionizing radiation, UVA-radiation (4,5) or, 4-nitroquinoline-1-oxide (6,7). An imbalance between production and scavenging of intracellular ROS gives rise to oxidative stress (8).

Among the free radicals, the hydroxyl radical (•OH) is the most reactive toward biological molecules and generates a multitude of modifications in proteins, lipids and DNA (9,10). Since proteins and lipids are readily degraded and resynthesized, the most significant consequence of oxidative stress is thought to be DNA alterations which include base damage, sugar damage, and DNA-protein crosslinks (11,12). Such oxidative DNA damage, has been implicated in the etiologies of several human disorders, including atherosclerosis (13), neurodegenerative diseases such as Down's syndrome (14), Alzheimer's disease (15), and in the normal aging process (3,16). Therefore, it is an

essential requirement for all organisms to detoxify ROS. Indeed, every cell is equiped with a first line of defense consisting of various antioxidants, e.g., gluthathione peroxidase and vitamins, as well as enzymes, e.g., superoxide dismutase and catalase, that minimize the level of ROS (13,17). Yet depending on the genetic state and age of a cell, and whether it is exposed to oxidants, the load of oxidative stress can overwhelm the first line defense mechanisms, thus resulting in an accumulation of oxidative DNA lesions (3).

Types of Lesions Caused by Reactive Oxygen Species

Base Damage

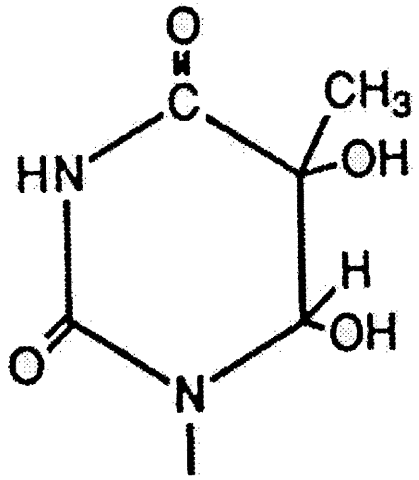
Oxidative base modifications represent the dominant form of DNA lesions whose lethal or mutagenic action has been established *in vitro* and *in vivo*. Thymine glycol (18) and 8-oxo-guanine (8-OxoG) (19,20) are representatives of oxidized pyrimidines and purines, respectively (Figure 1-1). While thymine glycol acts as an inhibitor of DNA synthesis by purified DNA polymerases suggesting a lethal action (2,21), 8-oxoG mispairs with dAMP causing GC-TA tranversions (22,23) making it a highly mutagenic lesion.

Sugar Damage

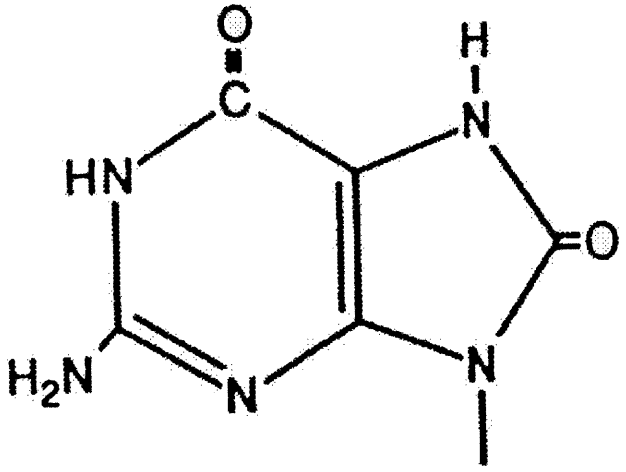
ROS also produce several modifications to the sugar moiety of DNA, namely oxidized apurinic/apyrimidinic (AP) sites and single strand breaks that terminate with 3'-blocked ends (24). 3'-blocked ends prevent the action of DNA polymerases, and, in addition, unrepaired AP sites are targets for mutagenesis because they lack template infomation (2,25-27) (Figure 1-2). Lindahl and Nyberg (28) have estimated that approximatively 10,000 AP sites are formed in each human cell per day. Subsequent studies determined the half life of AP sites at physiological pH to be between 20 and 100 hours (29). As AP sites are both cytotoxic and highly promutagenic due to a lack of

Figure 1-1 Chemical structure of representative oxidative base lesions

Thymine glycol and 8-oxo-guanine (8-oxo-Gua) produced by oxidative agents. Thymine glycol can exist as any of several stereoisomers .



Thymine glycol

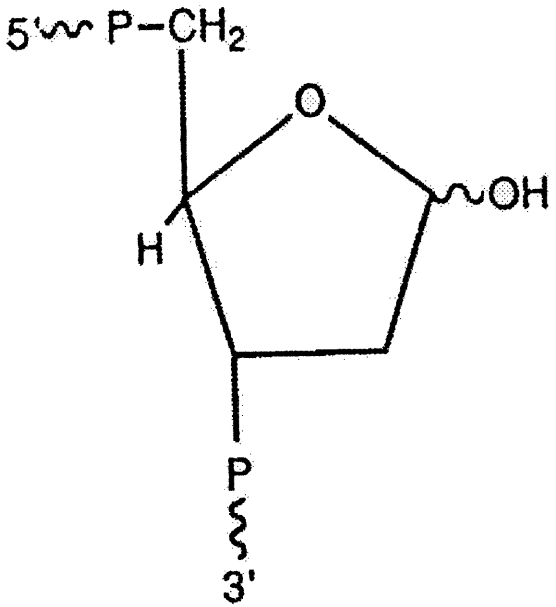


8-oxodG

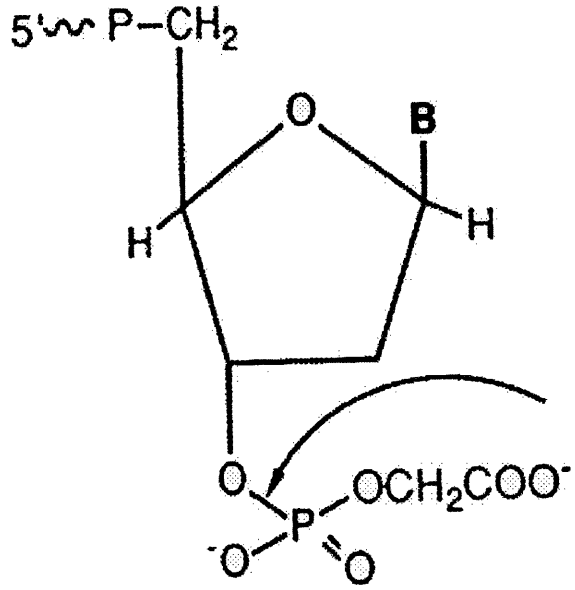
Figure 1-1

Figure 1-2 Sructure of AP site and 3'-phosphoglycolate

Unmodified abasic (AP) site (left) and a DNA strand break with a blocked 3'-terminus (right). The arrow shows the bond that is attacked by 3'-repair diesterases to produce a free 3'-hydroxyl terminus. **B** represents any base and the wavy lines are the continuation of DNA chains (30).



AP site



3'-phosphoglycolate

Figure 1-2

coding information, and given their stability and frequency of formation, the repair of AP sites is essential to cell viability. Thus, to maintain genomic integrity, all organisms have evolved DNA repair mechanisms to ensure proper removal of oxidized DNA lesions.

REPAIR OF OXIDATIVE DNA DAMAGE

Although several discrete repair pathways exist in prokaryotes and eukaryotes, the highly conserved base excision repair (BER) pathway is believed to be the primary defense against oxidative damage (31).

Base Excision Repair

N-Glycosylases and AP Endonucleases

The BER pathway involves several proteins that act to excise a single damaged nucleobase from DNA and replace it with the correct undamaged nucleotide (Figure 1-3). The first step of BER is initiated by any among a variety of DNA glycosylases, whose role is to catalyse hydrolysis of the N-glycosydic bond between a damaged base and the deoxyribose sugar. This results in release of the damaged moiety as a free base, and subsequent generation of an unmodified AP site as secondary lesion (Figure 1-2) (32,33). Although all DNA glycosylases cleave glycosydic bonds, some DNA glycosylases are specific for a particular substrate (e.g. uracil), whereas others are able to recognize several different types of DNA base damage (34). Moreover, a subset of DNA glysosylases possess an additional activity AP lyase (Table 1-1) (35). The resulting abasic site is converted to a single nucleotide gap by the action of either an AP endonuclease or an AP lyase. The AP endonuclease cleaves on the 5' side, while the AP lyase cuts on the 3' side of the AP site (Figure 1-3) (36) .

Figure 1-3 Schematic representation of base excision repair pathways

Enzymes that are reported to be involved in the individual steps are shown. Specific *E. coli* and mammalian proteins are highlighted in *italic* and in **bold**, respectively.

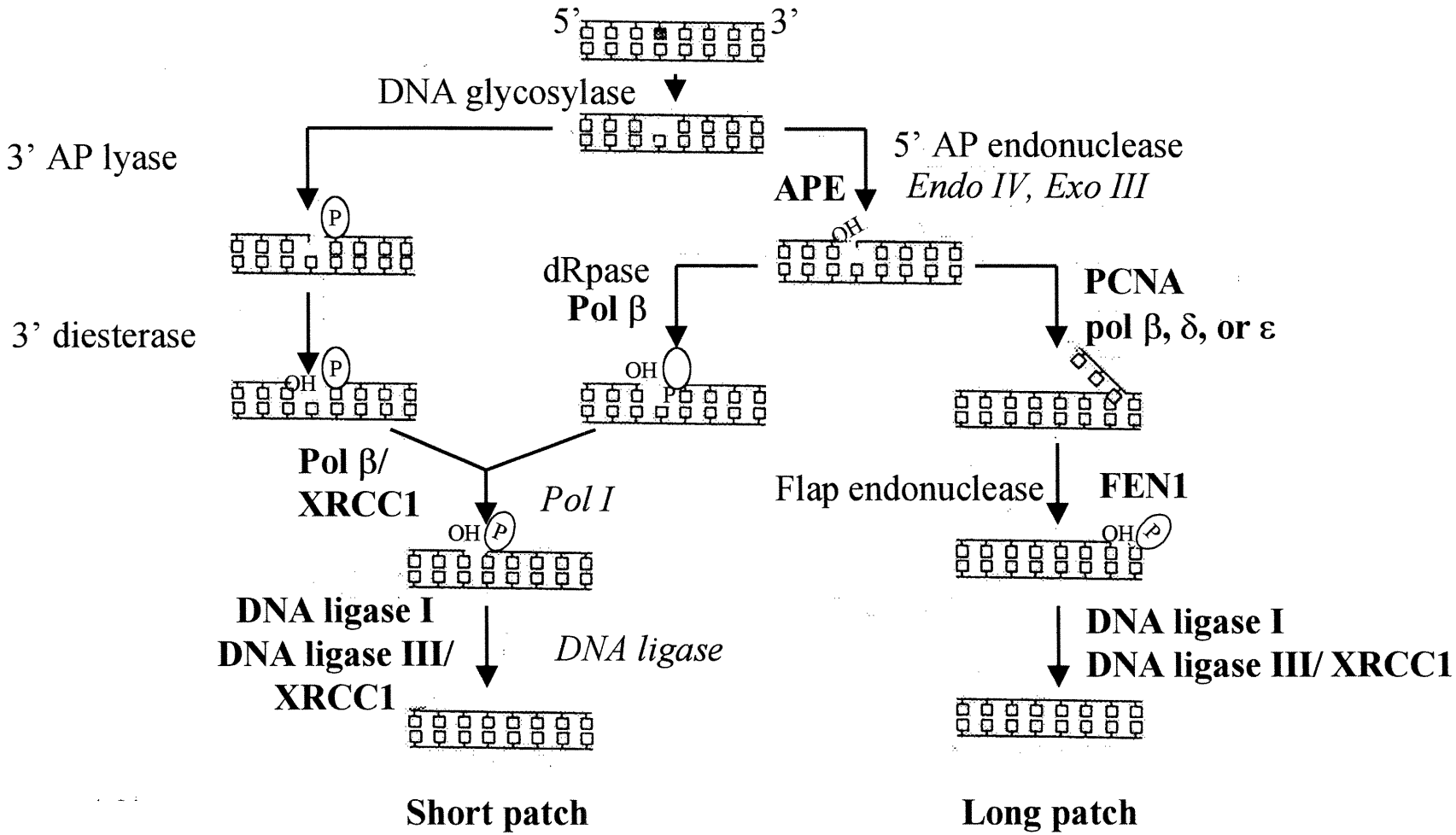

5'
3'
DNA glycosylase
3' AP lyase
5' AP endonuclease
APE
Endo IV, Exo III
P
OH
dRpase
Pol β
PCNA
pol β, δ, or ε
3' diesterase
OH
P
Pol β/
XRCC1
Pol I
Flap endonuclease
FEN1
DNA ligase I
DNA ligase III/
XRCC1
DNA ligase
DNA ligase I
DNA ligase III/ XRCC1
Short patch
Long patch

Figure 1-3

Table 1-1 Summary of the principal BER enzymes

Enzyme	*E.coli*	*S.cerevisiae*	*S.pombe*	Human
DNA glycosylase				
Uracil DNA glycosylase	*ung*	*UNG1*		*UDG1*
3MeA DNA glycosylase	*alkA*	*MAG*	*mag1*	*AAG*[a]
	tag		*mag2*[b]	
8-oxoguanine DNA glycosylase/AP lyase	*fpg*	*OGG1*[a]		*OGG1*[a]
MutY G:A mismatch glycosylase/AP lyase	*mutY*			*MYH*
Thymine glycol DNA glycosylase/AP lyase	*nth*	*NTG1/NTG2*	*ntg1*	*NTG1*
TDG T:G mismatch DNA glycosylase				*TDG*
AP endonuclease				
Exonuclease III	*xth*	*ETH1/APN2*	*eth1*[b]	*APE/REF1/HAP1*
Endonuclease IV	*nfo*	*APN1*	*apn1*[b]	
Other factors				
Flap endonuclease		*RTH1/RAD27*	*rad2*	*FEN1*
Proliferating cell nuclear antigen		*POL 30*	*pcn1*	*PCNA*
Replication Factor C		*RFC*	*rfc*	*RFC*
XRCC1				*XRCC1*
DNA polymerase		*POL IV*		*Pol β*
		POL 3	*pol 3*	*Pol δ*
		POL 2	cdc20	*Pol ε*
DNA ligase	*ligA*	*CDC9*	cdc17	*LIG1, LIG3*

[a] Protein orthologous to the bacterial enzyme.
[b] No enzymatic activity has been assigned yet.

Alternative Pathways in BER

In mammalian cells, completion of BER following cleavage of AP sites can occur by either short patch or long patch BER (37). In the former pathway, where only one nucleotide is replaced, 5'-deoxyribose-phosphate produced by AP endonuclease and 3'-blocking groups left by AP lyase can be removed by mammalian polymerase β (Pol β) and 3'-diesterase activity associated with AP endonuclease, respectively (37). In both cases, the undamaged nucleotide is inserted by Pol β and the DNA backbone is sealed by either DNA ligase I or DNA ligase III (38). The short patch BER can be enhanced by non-essential accessory factors such as the XRCC1 and XPG proteins (Figure 1-3) (38). In the long patch BER, 2-13 nucleotides are excised and replaced by normal nucleotides (39). In mammalians, this nucleotide patch is displaced by either Pol δ or Pol ε, and the resulting "flap" structure is removed by the flap-endonuclease FEN1 (40). This process requires at least two accessory proteins, namely proliferating cell nuclear antigen (PCNA) and replication factor-C (RFC) (Figure 1-3) (40). Although long patch repair has not yet been demonstrated in yeast, both *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*) have homologues to mammalian FEN1, PCNA, and RFC (34). The mechanism by which a cell decides whether to repair oxidative DNA damage via the short or long patch BER is still unknown, although this may be dictated by chromatin structure. However, the ultimate purpose of BER in general is to restore the original DNA sequence with the concerted action of the BER enzymes (Table 1-1) (41,42).

AP Endonuclease Families

In addition to being central damage-general intermediates in BER, AP sites are produced spontaneously and are induced by certain DNA damaging agents, such as methyl methane sulphonate (MMS) (28,43). The efficient repair of AP sites is critical because they are unstable and mutagenic (27). AP sites are repaired primarily by AP endonucleases which comprise two distinct families, exemplified by *Escherichia coli* (*E. coli*) endonuclease IV (Endo IV) and exonuclease III (Exo III) (2,25,44,45).

Exonuclease III Family

Members of the Exo III family include *E. coli* exonuclease III, drosophila Rrp1, and human Ape/Hap1/Ref1 and are magnesium (Mg^{2+})-dependent (2,25). *E. coli* exonuclease III , encoded by the *xth* gene, has been studied extensively. Exonuclease III comprises approximatively 90% of the total AP endonuclease and 3'-repair diesterase activity in *E. coli* cell-free extracts. It has relatively broad substrate specificity, and initiates the repair of both DNA strand breaks with 3'-blocking groups, and AP sites. Strains of *E. coli* bearing mutations in the *xth* gene are hypersensitive to H_2O_2 (2,25).

Endonuclease IV Family

Functionally defined members of the Endo IV family, e.g., *E. coli* endonuclease IV and *S. cerevisiae* Apn1, are Mg^{2+}-independent enzymes. The AP endonucleases belonging to the Endo IV family cleave the sugar phosphate bond 5' to the AP site to generate a 5'-deoxyribose phosphate and a 3'-hydroxyl group (Figure 1-4) (2,25). They also possess a second enzymatic activity, i.e. 3'-phosphodiesterase. This activity removes 3' DNA-blocking groups such as 3'-phosphates, 3'-phosphoglycolates, and 3'-α, β-unsaturated aldehydes that arise from oxidative base damage and the activity of combined

Figure 1-4 Processing of the AP site by either AP endonuclease or AP lyase

Products produced by AP endonuclease and AP lyase. AP endonucleases produce a free 3'-hydroxyl terminus and a 5'-terminal deoxyribose 5-phosphate that can be removed by dRPase. AP lyase produce a 3'-terminus containing α,β-unsaturated aldehyde (4*R*-4-hydroxy-*trans*-2-pentenal) and a 5'-phosphate (30).

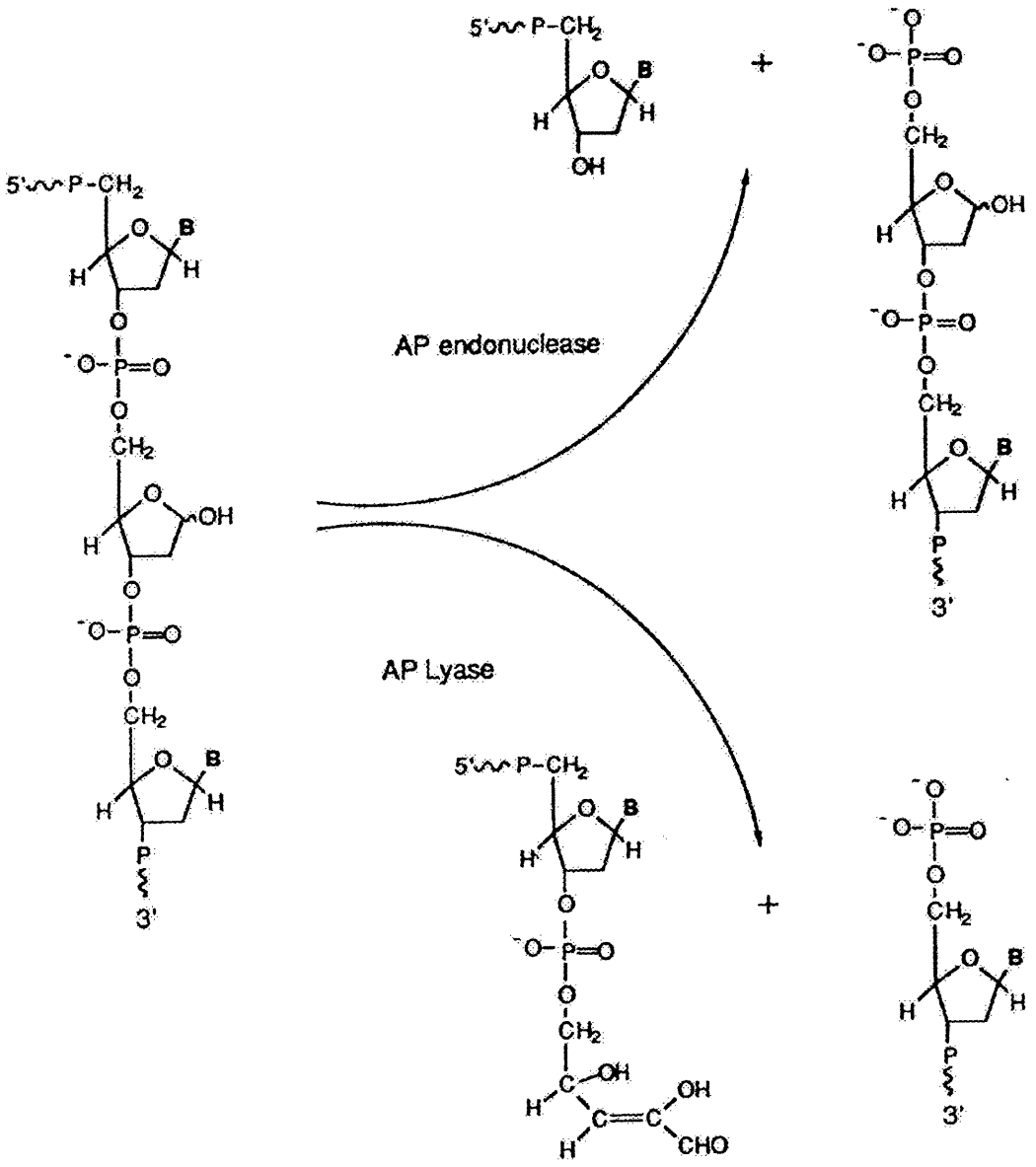


Figure 1-4

glycosylase/lyase enzymes (43). Unlike AP endonucleases, AP lyases cleave on the 3' side of the AP site through a β-elimination reaction to produce the α-β-unsaturated aldehyde product, (4*R*)-4-hydroxy-*trans*-2-pentenal (Figure 1-4) (35,46). This product blocks DNA repair synthesis by preventing DNA replication, and must therefore be removed by 3'-diesterase (2). Thus, AP endonucleases are considered to be far more efficient in processing AP sites than AP lyases.

SACCHAROMYCES CEREVISIAE APN1 GENE

Cloning of *APN1*

In the budding yeast *Saccharomyces cerevisiae*, Apn1, a member of the Endo IV family, is the predominant constitutive AP endonuclease. Johnson and Demple (47) initially characterized Apn1 as a 3'-diesterase in crude extracts using a synthetic DNA substrate containing 3'-phosphoglycolaldehyde (3'-PGA) esters. Although the substrate used in the study is unlikely to be a naturally occurring product in DNA, 3'-PGA is a structural analogue of 3'-phosphoglycolate, the predominant species found at DNA strand breaks after *in vitro* treatment with oxidative damaging agents such as gamma (γ)-rays (26). Further analysis of the purified protein clearly revealed that it also contained AP endonuclease activity (48). Both 3'-repair diesterase and AP endonuclease activities showed no metal requirement. The *in vitro* activites of the yeast enzyme are quite similar to those of the *E. coli* endonuclease IV, suggesting that the enzymes are structurally related (47-49). Since AP-endonuclease/3'-diesterase-deficient yeast mutants were not available, the precise nature of this relationship could not be established. Screening a yeast

λgt11 expression cDNA library in *E. coli* using a polyclonal antibody produced from the purified Apn1 resulted in the successful identification of the gene, designated *APN1* (50).

Importance of the Apn1 Protein in budding yeast

Yeast Apn1 protein (40.5-kDa) is found to be clearly homologous to *E. coli* endonuclease IV (31.6-kDa), with 41% amino acid identity through a 280-residue segment (Figure 1-5) (50). Since then, two other proteins related to bacterial endonuclease IV, from *Caenorhabditis elegans* (*C. elegans*) (51) and *Schizosaccharomyces pombe* (52), have been identified (Figure 1-5).

In crude extracts of the yeast *S. cerevisiae*, Apn1 is the major endonuclease activity that functions in the repair of both oxygen radical damage and AP sites (48,50). Indeed, Ramotar *et al.* (43) demonstrated that yeast mutant cells lacking Apn1 protein are defective in repairing AP sites, as well as DNA strand breaks with blocked 3'-termini. Consequently, Apn1-deficient strains display hypersensitivity to the oxidative agent H_2O_2 and to the alkylating agent MMS which produces DNA strand breaks and apurinic sites, respectively (43). Furthermore, *apn1Δ* deletion mutants have a 10-to-15- fold increase in the spontaneous mutation rate (43). These mutations correspond mainly to AT to CG transversions (53). The high frequency of spontaneous mutations in the *apn1Δ* null mutant is due to unrepaired AP sites (54). The study showed that the high mutation rate can be reduced to the parental level with the expression of endonuclease III, an enzyme that lacks a 3'-diesterase but possesses a β-lyase activity capable of cleaving only AP sites (54). Hence, Apn1 performs an important role in genetic stability in yeast.

Figure 1-5 Comparison of the predicted amino acid sequences of the four members of the Endo IV family

The five highly conserved regions (I, II, III, IV, and V) are boxed. Region I of *S. pombe* Spapn1 is interrupted by an intron (51).

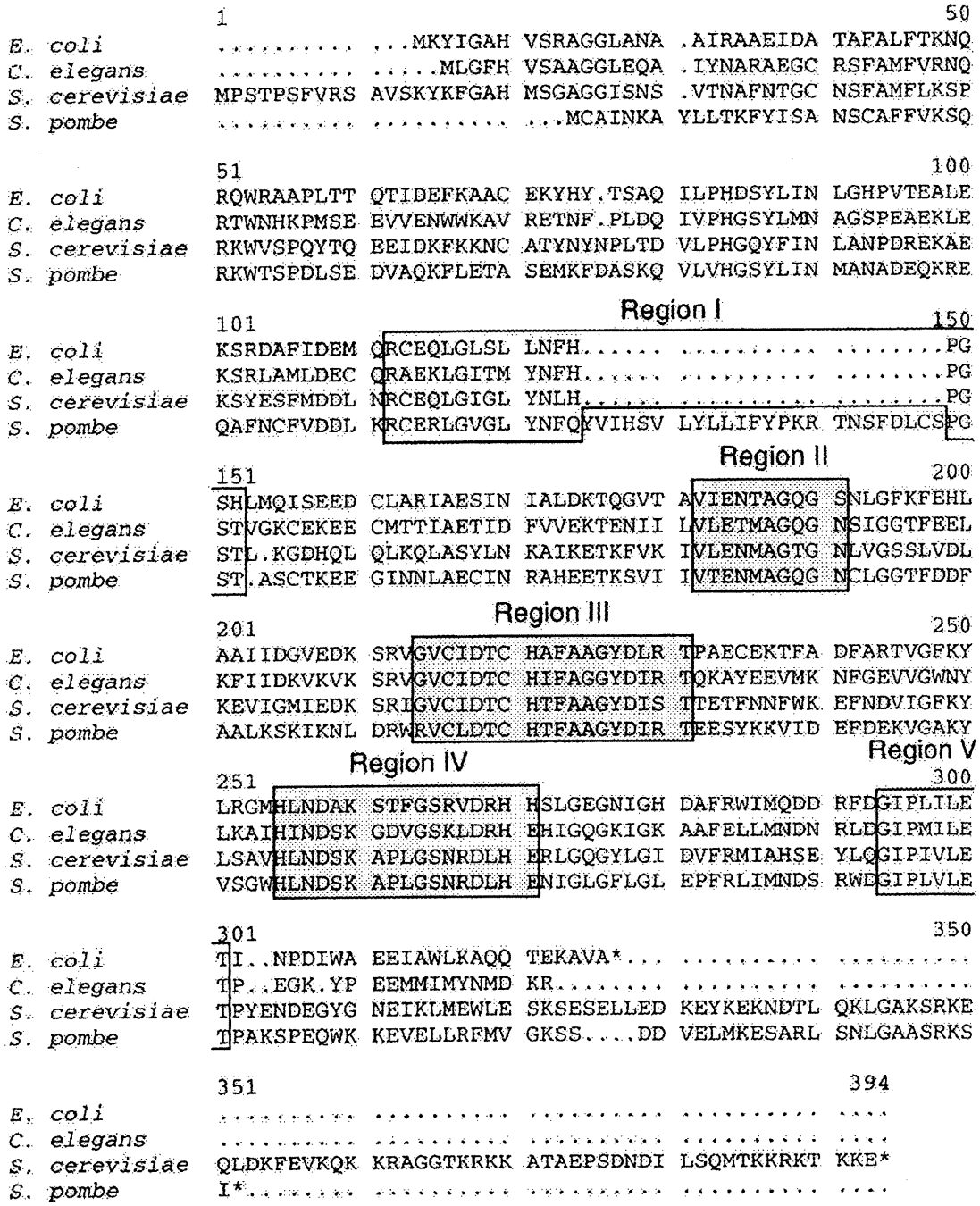


Figure 1-5

Apn1 C-terminus, a Bipartite Nuclear Localization Signal

Comparison of the primary structure of Apn1 with that of its *E. coli* homologue endonuclease IV revealed that the yeast protein bears an extra 82 amino acid residues at the C-terminus (50,55). The Apn1 C-terminal end is especially rich in basic amino acids and three lysine/arginine clusters (1, 2 and 3) can be identified (Figure 1-6) (55).

Given the structural and functional similarities between Apn1 and endonuclease IV, the C-terminal extension would be expected to fulfill some specific physiological role in eucaryotic cells. Indeed, through indirect immunofluorescence and deletion analysis, it was demontrated that the extreme C-terminal of Apn1 encompassing cluster 1 is essential for nuclear targeting (55). Elimination of just the last 12 amino acids (SQMTKKRKTKKE) from Apn1 (Apn_{355}) does not alter the stability or enzymatic activity of the protein, but leaves over 90% of the protein in the cytoplasm (55). In addition, this delocalized truncated Apn1 failed to restore wild-type resistance to oxidative or alkylating agents in the *apn1Δ* null mutant (55).

Although cluster 2 (KQKKRAGGTKRKK) also ressembles a nuclear localization signal (NLS), it cannot by itself target Apn1 to the yeast nucleus (56). In fact, clusters 1 and 2 function as a bipartite nuclear localization signal necessary for efficient translocation of Apn1 to the nucleus (56). While cluster 1 alone cannot target the *E. coli* endonuclease IV to the yeast nucleus, both basic clusters 1 and 2 are efficient for nuclear localization of the bacterial enzyme (56). The role of basic cluster 3 remains to be investigated. However Apn_{334} which contains only cluster 3 is very unstable relative to Apn_{355} which possesses clusters 2 and 3 (55). Thus cluster 3 may contain a proteolysis site which is exposed when cluster 2 is deleted. Surprisingly, attachment of the nuclear targeting signal of SV40 T-

Figure 1-6 Schematic comparison of Apn1, endonuclease IV, and Apn derivatives

The *nfo* and the *APN1* genes are represented by *bars* and *vertical* and *hatched lines*, respectively. The *number* above each *bar* indicates the length of the polypeptide chain. The *black squares* (1, 2 and 3) represent basic amino acid clusters. The basic cluster 1 and 2 at the bottom of the figure, with basic residues *underlined*, are the sequences that resemble nuclear localization signals (NLS) (55). The defined NLS of *S. cerevisiae* histone H2B (57) is also indicated.

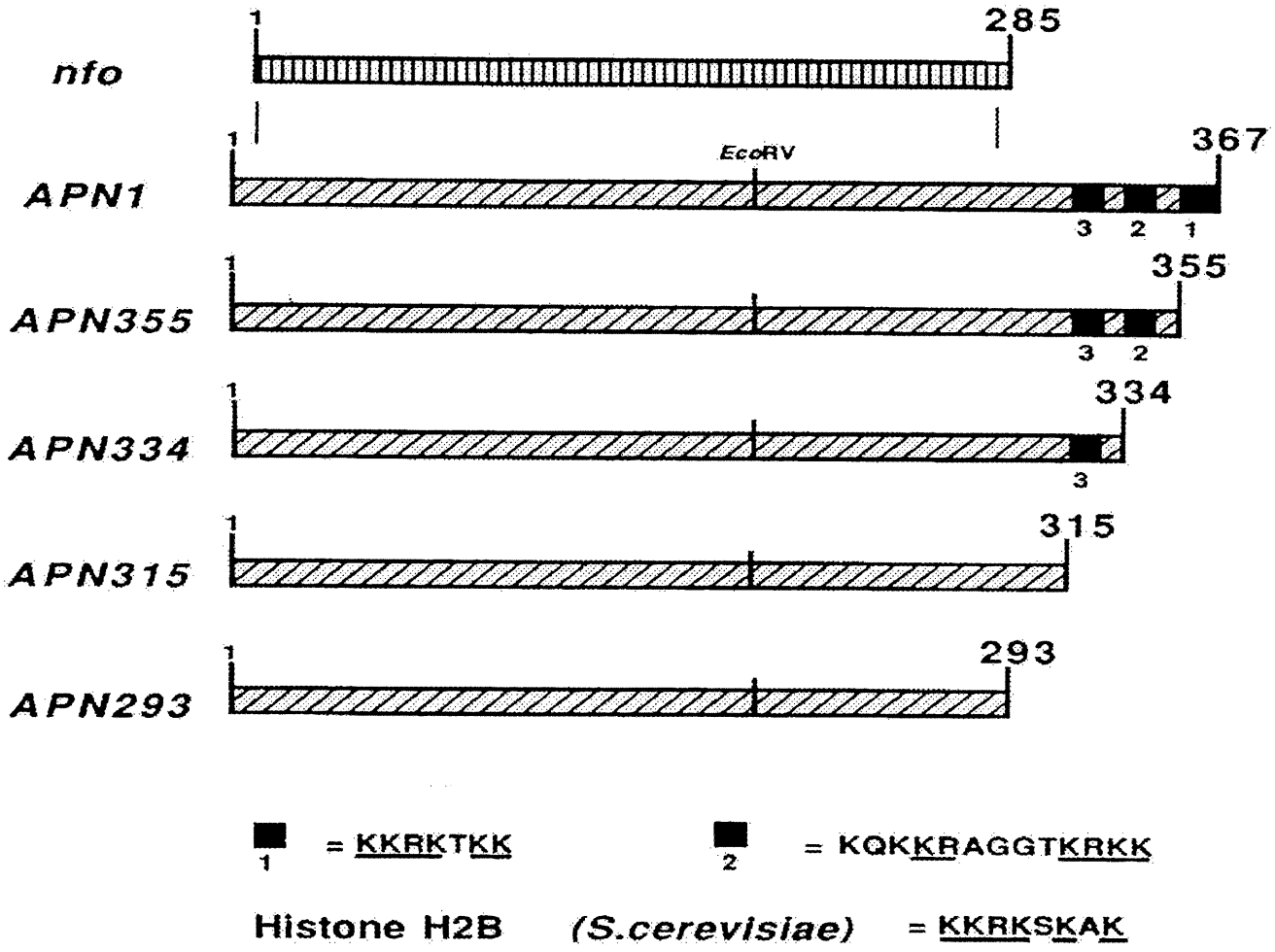

nfo
1
285
APN1
1
EcoRV
367
3 2 1
APN355
1
355
3 2
APN334
1
334
3
APN315
1
315
APN293
1
293
1 = KKRKTKK
2 = KQKKRAGGTKRKK
Histone H2B (S.cerevisiae) = KKRKSKAK

Figure 1-6

antigen to the truncated Apn1 did not restore nuclear localization of the yeast protein (55). This suggests that translocation of Apn1 in yeast may be operating through a very specific nuclear transport mechanism.

NUCLEAR TRANSPORT

Nuclear Protein Import

In the past few years, major progress has been accomplished in our understanding of the cellular factors that mediate signal-dependent nuclear transport. Multiple pathways have been discovered where different types of proteins are transported either into or out of the nucleus through the action of cytosolic receptors molecules called karyopherins/importins which recognize distinct targeting signals (58). In eukaryotic cells, the nuclear transport process occurs in two sequential steps (59). First, karyopherin (kar) α recognizes targeting signals and kar β1 docks the karyopherin/transport substrate complex to the nuclear pore complex (NPC) in an energy-independent fashion (60). For classical nuclear import pathways, the kar α/β1 heterodimer is involved, whereas most other newly discovered pathways require an kar β homolog alone (61). Second, the monomeric guanine nucleotide-binding protein Ran together with several other key Ran-interacting and -regulating proteins mediate the active translocation of the transport substrate through the NPC and the release into the nucleus (62).

Target sequences and importins

Different types of NLSs exist, and can be seperated into three broad classes (60). The first group contains NLSs resembling the NLS of the SV40 large T antigen which consists of a short stretch of basic amino acids (63). The second class of NLSs is also

highly basic in nature. It encompasses proteins with bipartite NLS which contains two interdependent positively charged clusters seperated by a spacer of 10-12 amino acids, as found in nucleoplasmin NLS (64). The third group of NLS includes those resembling those of the yeast homeodomain containing protein Mat α2 where charged/polar residues are interspersed with non-polar residues, or the protooncogene c-myc where as few as three positively charged amino acids flanked by specific nonbasic residues are important for nuclear targeting (60). All classes of NLSs are believed to be recognized specifically by the α/β1-karyopherin heterodimer (61). In yeast, only a single essential Kar α subunit (SRP1/KAP60) exists, whereas at least 5 isoforms of Kar α have evolved in humans and mouse (60). Through a conserved amino-terminal karyopherin β-binding domain, all Kar αs interact specifically with Kar β1 to effect high affinity NLS binding and docking at the NPC (60). The presence of more than one form of Kar α in higher mammals suggests that there is specialisation in their cellular role, and that distinct Kar α isoforms could bind particular target proteins (65). In fact, no complementation is observed with any single human Kar α in a *S. cerevisiae* SRP1 mutant (66). As mentioned above, signal-dependent nuclear import is not carried out exclusively by the Kar α/β1 heterodimer (61). There are at least 12 different Kar β isoforms in eukaryotic cells in addition to Kar β1, each of which has a specific role in the transport of particular classes of proteins (61). For example, Kar β4 mediates the import of ribosomal proteins into the nucleus (67).

HYPOTHESIS AND OBJECTIVES

Ramotar *et al.* (55) have clearly demonstrated that the bipartite nuclear localization signal of Apn1 is essential for nuclear transport. However, before the nucleocytoplasmic transport pathway of Apn1 can be determined, the karyopherin that recognizes and binds to the Apn1 bipartite NLS must first be identified. We, thus propose that there is at least one karyopherin which will bind to the bipartite nuclear localization signal of Apn1 and translocate the enzyme through the nuclear pore. The initial goal of this thesis was to identify the karyopherin using the Apn1p C-terminal end as a bait in the yeast two-hybrid screen. However, the focus of this project changed when, we found instead, unexpectedly, a cell wall protein, Pir1p, which interacts with the Apn1 bipartite NLS. In this thesis, we show that Pir1 interacts with Apn1 C-terminal end and mediates Apn1 translocation into the mitochondria. We further show that the biological significance of Apn1/Pir1 interaction is to maintain mitochondria genome stability.

CHAPTER 2

Pir1p Mediates Translocation of the Yeast Apn1p Endonuclease into the Mitochondria to Repair Damaged DNA

Submitted for publication in *Molecular and Cellular Biology*

Pir1p Mediates Translocation of the Yeast Apn1p Endonuclease into the Mitochondria to Repair Damaged DNA

RATSAVARINH VONGSAMPHANH, PIERRE-KARL FORTIER,

AND DINDIAL RAMOTAR ‡

Guy-Bernier Research Centre, University of Montreal,

5415 Boul. de l'Assomption, Montreal, Quebec, Canada HIT 2M4.

‡ Corresponding author:

University of Montreal

Guy-Bernier Research Centre

5415 Boul. de l'Assomption

Montreal, Quebec, Canada, H1T 2M4.

ABSTRACT

The mitochondrial genome is continuously subjected to attack by reactive oxygen species generated through aerobic metabolism. This leads to the formation of a variety of highly genotoxic lesions, including abasic sites. In the case of nuclear DNA, yeast Apn1 functions to cleave abasic sites, and *apn1Δ* mutants are hypersensitive to agents such as methyl methane sulfonate (MMS) that induce abasic sites. Here we demonstrate for the first time that yeast Apn1 is also localized to the mitochondria. We found that Pir1p, initially isolated as a cell wall constituent of unknown function, interacts with the C-terminal end of Apn1 bearing a bipartite nuclear localization signal. Further analysis revealed that Pir1p is required to cause Apn1 mitochondrial localization, presumably by competing with the nuclear transport machinery. *pir1Δ* mutants displayed a striking accumulation (~3-fold) of Apn1 in the nucleus, which coincided with drastically reduced levels in the mitochondria. To explore the functional consequences of the Apn1/Pir1 interaction, we measured the rate of mitochondrial mutations in the wild-type, *pir1Δ*, and *apn1Δ* mutants. Both *pir1Δ* and *apn1Δ* mutants exposed to MMS exhibited 3.6- and 5.8-fold increases, respectively, in the rate of mitochondrial mutations, underscoring the importance of Apn1 in the repair of the mitochondrial genome. We conclude that Pir1 interacts with Apn1, either at the level of the cytoplasm or nucleus, and facilitates Apn1 transport into the mitochondria to repair damaged DNA.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* possesses a 40.5-kDa DNA repair enzyme, Apn1, that is localized to the nucleus (43,55). Apn1 is a key enzyme in the base excision repair pathway and it functions by hydrolyzing apurinic/apyrimidinic (AP) sites produce either spontaneously or upon removal of damaged bases (43,48). This enzyme also possesses a 3'-diesterase activity that removes blocked 3'-ends from single strand DNA breaks. Cells lacking Apn1 are hypersensitive to the alkylating agent methyl methane sulfonate (MMS) due to defective repair of MMS-induced AP sites (43). *apn1Δ* mutants also display a 10- to 15-fold increase in the rate of spontaneous single base-pair mutations arising strictly as a result of unrepaired AP sites (53,54). Recent studies have shown that Apn1 homologues also exist in the fission yeast *Schizosaccharomyces pombe*, as well as in the nematode *Caenorhabditis elegans*, underscoring the importance of Apn1 family members in the repair of damaged DNA (52). To date, no homologue of Apn1 has been found in human cells (52).

Deletion analysis and immunofluorescence studies established that the extreme C-terminal end of Apn1 possesses a bipartite nuclear localization signal (NLS), which is characterized by two interdependent positively charge clusters separated by a spacer of 10 to 12 amino acid residues (55,56). Removal of a portion of the NLS, i.e., the last 12 amino acid residues (SQMTKKRKTKKE) from the C-terminus of Apn1p (Apn_{355}) had no effect on the enzymatic activities of the protein, but abolished the nuclear localization of Apn1p leading to extranuclear accumulation (55). Consequently, the Apn_{355} variant is unable to restore MMS resistance to an *apn1Δ* mutant. Addition of the SV40 nuclear localization signal to Apn_{355} failed to target this protein to the nucleus, suggesting that the

Apn1 nuclear transport mechanism is highly specific (55). Proteins with classical monopartite NLS, such as SV40, and bipartite NLS, such as nucleoplasmin are imported in the nucleus by a heterodimer consisting of karyopherin α (Kar α, or importin α) and karyopherin β (Kar β, or importin β). Kar α recognizes the classical NLS and Kar β binds to Kar α thus docking the heterotrimeric complex to the nuclear pore complex for subsequent nuclear transport (61,68,69). Whether Apn1 is imported into the nucleus through the Kar α/β complex, or directly by one of the several Kar β-like proteins that bind to NLS (67,70,71), is not known.

The N-terminal region of Apn1p possesses a putative mitochondrial transit sequence as revealed by the PSORT[1] program. This raises the possibility that Apn1p could also be targeted to the mitochondria and involved in the repair of mitochondrial DNA. In support of this notion, many DNA repair enzymes are targeted to both the nucleus and the mitochondria. These include:(i) the *S. cerevisiae* Ntg1, which repairs oxidative DNA lesions such as thymine glycol, (72), (ii) the *S. pombe* Uvde protein that incises a variety of DNA lesions including pyrimidine dimers and AP sites (73), and several human DNA glycosylases e.g., uracil DNA glycosylase, OGG1, hNth1, and MUTY (74-77). Additional DNA repair enzymes belonging to the base excision repair pathway such as DNA polymerase and ligase also exist in the mitochondria. Recent studies show that the entire base excision repair pathway can be reconstituted from purified enzymes derived from *Xenopus laevis* mitochondria, and complete repair of uracil opposite guanine has been demonstrated with rat liver mitochondrial extract (78,79). Since the mitochondrial DNA is in close proximity with the electron transport chain, which produces as by-products reactive oxygen species that are known to generate a variety of DNA lesions

including AP sites (80,81), it is therefore imperative that DNA repair enzymes exist in the mitochondria to maintain stability of the genome. In yeast, mutants lacking the mitochondrial mismatch repair protein, Msh1p, exhibit a high rate of mitochondrial mutations (82). Thus, defects in mitochondrial DNA repair could contribute to human disorders (83,84). In fact, a multitude of mitochondrial mutations have been identified and some have been associated with a variety of human diseases including Parkinson's, Alzheimer's, and some forms of diabetes mellitus (85-87).

We initially set out to identify the karyopherin that recognizes the bipartite NLS of Apn1p for subsequent translocation into the nucleus by using the Apn1p C-terminal end as a bait in the yeast two-hybrid screen. We report the unexpected identification of Pir1p, previously isolated as a cell wall protein, which interacts with Apn1p. Deletion of the *PIR1* gene from two different parental backgrounds did not hamper Apn1p translocation into the nucleus, but instead decreased cytoplasmic and mitochondrial Apn1p levels. Our findings support a model where Pir1p binds to Apn1p, either in the cytoplasm or nucleus, and facilitates its entry into the mitochondria to prevent genetic instability.

MATERIALS AND METHODS

Strains, media, genetic analysis, and transformation

The *S. cerevisiae* strains used in this study were PJ69-4A (*MAT***a** *trp-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ)*, SEY6210 (*APN1*$^{+}$, laboratory stock), DRY377 (*apn1Δ::HIS3*), YAT1530 (*PIR1*$^{+}$), and YAT1529 (*pir1Δpir2Δ*) (kindly provided by Dr. Toh-E, Japan). The *pir1Δ::KanMX* mutant strains RVY1 and RVY2 were derived from SEY6210 and YAT1530, respectively, by one-step gene targeting using the *KanMX* gene module (88). Yeast cells were grown in either complete yeast peptone dextrose (YPD) or minimal synthetic (SD) medium, to which nutritional supplements were added at 20 μg/ml (89). Standard genetic analysis and transformation were carried out as described previously (90,91). The *E. coli* strain used for plasmid maintenance was DH5α.

Construction of the bait plasmid pGBD-APN-CT

The plasmid YEpAPN1, which contains the entire *S. cerevisiae APN1* gene with its transcriptional termination sequence (55), was used as the template to amplify by polymerase chain reaction (PCR) (92) the 3' end of the *APN1* gene (base pairs +780 to +1435) using the primers APN1-1 (5'-$^{+780}$GCGCACTCTGAATTCCTGCAGGG^{+803}-3') and DR2 (5'-$^{+1435}$CCAGCGGTCG-ACATTACAAGTA^{+1413}-3') bearing the restriction sites (underlined) for *EcoRI* and *SalI*, respectively. This procedure yielded a 600-bp fragment containing basic clusters 1 and 2 of the *APN1* gene, which was digested with *EcoRI* and *SalI* and subcloned next to the *GAL4* DNA binding domain in the *S. cerevisiae* expression vector pGBDUC2 to produce pGBD-APN-CT (93).

Yeast two-hybrid screening

The yeast two-hybrid screening was performed according to the technique described by James *et al.* (93). The bait plasmid pGBD-APN-CT was introduced in the two hybrid strain PJ69 (*ura3*$^-$, *leu2*$^-$, *ade*$^-$, *his*$^-$), which bears three reporter genes, i.e., *ADE*, *HIS*, and *lacZ*. This strain cannot grow in the absence of adenine and histidine, unless the reporter genes are activated upon association of the Gal4 DNA binding and activation domains (93). A genomic library consisting of yeast DNA fragments fused to the *GAL4* activation domain (GAD) bearing the selective marker LEU2 was subsequently introduced into strain PJ69 already carrying plasmid pGBD-APN-CT. The transformed cells were allowed to grow in 100 ml of selective SD medium (lacking leucine and uracil) O/N at 30°C to allow expression of both plasmids. The cells were resuspended in 3 ml of H_2O and 100 µl were plated on SD medium lacking leucine, adenine, and uracil (LAU). The resulting transformants (60,000 colonies *Ura3*$^+$, *Leu2*$^+$) were screened for colonies growing in the absence of adenine. At least 20 Ade$^+$ colonies were obtained and only one (PJ69/pGBD-APN-CT/pGAD-RV2) strongly expressed the two additional reporter genes *HIS3* and *lacZ* (data not shown). In these experiments, the *IMP2* gene encoding a transcriptional co-activator that interacts with the product of the YLR368w gene was used as control (94). The plasmid pGAD-RV2 was extracted from the positive colony and subjected to DNA sequence analysis.

DNA sequence analysis

The primers 2H-GADUP (5'-TTCGATGATGAAGATACC-3') and 2H-GADDOWN (5'-TGAAGTGAACTTGCGGGG-3') were used to sequence the 5'- and

3'-ends, respectively, of the insert. The insert was subsequently sequenced completely by the dideoxy chain termination method (95).

Construction of the full-length *PIR1*

Yeast genomic DNA was used as template to amplify the full-length *PIR1* gene (base pairs -23 to +1208) using the primers PIR1-UP(B) (5'-$^{-23}$CCCCTATAGTGAATTCAAGAAAATGC^{+4}-3') and PIR1-DO (5'-$^{+1208}$GTGAAGAACTGTCGACCTCATTTCATAGG^{+1180}-3') bearing the restriction sites (underlined) for *EcoRI* and *SalI*, respectively. The PCR-amplified 1.2 kb fragment contains the entire coding region of the *PIR1* gene. After digestion with *EcoRI* and *SalI*, the fragment was subcloned next to the *GAL4* activation domain in the yeast plasmid pGADC3 (93).

Protein extracts

Total extracts were prepared as previously described in yeast extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5% glycerol, 1 mM each of EDTA, PMSF, and DTT, and 0.5 μg/ml of pepstatin, aprotinin, and leupeptin (54).

Attachment of Apn1-Ct, Apn1 full-length, Pir1 87%, Pir1 full-length, and Imp2 to *GFP*

The plasmids pGBD-APN-CT, pGAD-pRV2, and pGAD-PIR1, pNlexA-IMP2 (96) were digested with *EcoRI* and *SalI* to release the DNA fragments encoding the Apn1 C-terminal basic clusters, 87% of *PIR1*, and the full-length *PIR1* and *IMP2* genes, respectively. Each fragment was subcloned next to the *GFP* gene which was under the *GAL1* promoter in the yeast expression vector pYES2.0 to produce pGFP-APN-CT, pGFP-pRV2, pGFP-PIR1, and pGFP-IMP2. To produce pGFP-APN1, the plasmid

pGBDU-APN1 was digested with *EcoR1* and the *APN1* fragment was inserted next to *GFP*.

Phenyl-agarose column

For the preparation of the phenyl-agarose column, 1 ml of the phenyl-agarose slurry (Pharmacia) was added to an eppendorf tube and centrifuged to pellet the beads which were then washed twice with 2 M $(NH_4)SO_4$ in yeast extraction buffer. The equilibrated matrix was transferred to a 2-ml column (Bio-Rad). Crude extracts (500 μg) derived from yeast strains expressing the indicated Gfp-fusion protein was allowed to bind to the column in a final concentration of 1.7 M $(NH_4)SO_4$ for 20 min at room temperature. The column was washed with extraction buffer until the OD280 was baseline. The Gst-Apn-CT fusion protein (200 ng) or total extract (500 μg) derived from strain SEY6210 carrying the Apn1 overproducing plasmid (YEpAPN1) was slowly applied onto the column at 4°C over a period of 8 h. The column was washed with 1X PBS to remove non-specific interactions and directly analyzed by Western blot.

Preparation of subcellular fractions from yeast

Cells were grown in 1 litre of -URA 2% raffinose at 30°C O/N. The next day, the cultures were induced where indicated with 0.5% galactose for 0, 2 and 4h. Sample cultures of 300 ml were taken at each time course for nuclear and cytoplasmic extractions. The cell pellets are weighed, washed with H_20, resuspended (0.3 g/ml) in 100 mM TRIS-SO_4 pH 9.3 buffer, containing 10 mM DTT, incubated with gentle shaking at 30°C for 10 min, and centrifuged 3,000 x g/5 min at room temperature (RT). The pellets were washed once with buffer B (1.2 M D-sorbitol, 20 mM KPB pH 7.4), following centrifugation, and addition of zymolyase at 2.5 mg/g of cell pellet in 0.1 g cell pellet/ml of buffer B. The

pellets were incubated for 60 min at 30°C with gentle shaking until the cell wall was completely digested. The spheroplasts were collected at 3,000 x g/5 min at RT and washed 3 times (1g/10 ml buffer B). From this stage, all manipulations were done at 4°C. The spheroplasts were suspended at 1 g/2 ml of MIB (0.6 M D-mannitol, 20 mM HEPES-KOH, pH 7.4, 0.5 mM PMSF) and broken in Dounce Homogenizer with 15 strokes using the pestle (glass and teflon). The homogenate was then diluted in 2-fold in MIB and centrifuged at 3,000 x g/5 min. The pellet contained crude nuclei and the supernatant represents the crude cytoplasm and mitochondria. The supernatant was spun at 9,500 X g/10 min and the resulting pellet contained the crude mitochondria and the supernatant was named the cytoplasmic fraction.

To obtain purified mitochondria, the crude mitochondria were diluted in 200 µl of MIB buffer and layered on a two-step Nycodenz (Sigma) gradient made in a 14 x 89 mm Ultra-Clear centrifuge tube. The bottom layer of the gradient contained 5 ml of 18% and the top layer contained 5 ml of 14% Nycodenz in MIB buffer. The tubes were spun at 40,000 rpm in an SW41 rotor for 30 min and the purified mitochondria were recovered between the two layers as a light brown band. The purified mitochondria were diluted 5-fold in MIB and centrifuged at 9,500 x g for 10 min. The purified mitochondria were lyzed by addition of 200 µl of yeast extraction buffer to produce the mitochondrial fraction. Proteinase K treatment was carried out before and after lysis of the mitochondria at 37°C for 30 min using 1 µg/ml of proteinase K.

To obtain purified nuclei, the crude nuclei were washed three times with the MIB buffer and each time spun at 3,000 x g for 5 min. The final washed pellet was resuspended in MIB buffer and loaded onto a 30 to 50% Ficoll 400 (Pharmacia) step gradient in

Ultraclear tubes (97) . The gradient was spun in a Beckman SW28 rotor at 18,000 rpm for 60 min at 2°C. The nuclei band in the 40% layer and were collected using a 20-ml syringe with a 16-gauge needle.

Immunoblotting

The antibodies used in this study were monoclonal anti-Gfp and anti-Gst, and polyclonal anti-Apn1, which was raised against purified Apn1 (55). For Western analysis, the antibodies were used at a dilution of 1:5000, 1:5000 and 1:2500, respectively, in 10 mM Tris-HCl (pH 7.5)-150 mM NaCl-5% powdered milk (98); 10 ml of this mixture was used to probe nitrocellulose blots (8 by 10 cm) overnight at 4°C. The secondary antibodies were anti-mouse for the Gfp monoclonal antibodies and anti-rabbit for the Gst and Apn1 antibodies. Anti-mouse and anti-rabbit were used at a dilution of 1:2500 and 1:5000, respectively, and detected by the enhanced chemiluminescence system (Dupont, NEN).

Gradient plate assays

This assay was performed as previously described (99).

Enzyme assays

β-Galactosidase activity was determined from crude extracts as previously described (96). AP endonuclease activity was assayed using an AP site substrate prepared by incorporation of [γ-^{32}P]dUTP, derived by deamination of [γ-^{32}P]dCTP, into poly [d(A-T)] using DNA polymerase (Klenow fragment) (100). **The AP sites were produced by removal of** the uracil by uracil DNA glycosylase. A typical assay contains 1 pmol of substrate in 25 μl reaction mix containing 50 mM Hepes. KOH (pH 7.6), 50 mM KCl, 1 mM EDTA, 100 μg of BSA, and the indicated concentration of protein extracts or

purified protein. One unit of enzyme cleaves 1 pmol of the substrate/min under the standard reaction condition (100).

Mutation rate assay

The frequency of *ery*^{R} colonies was determined from 15-20 independent cultures and used to compute the rates according to Lea and Coulson's method. Cells were cultured into log phase (OD_{600}= 0.8 to 1.0), treated with and without MMS for 1 h, washed, and plated onto YPEG plates containing erythromycin (2 mg/ml). Erythromycin resistant colonies were scored after 10 days of incubation at 30°C.

RESULTS

Apn-Ct interacts with Pir1

The *APN1* C-terminal DNA segment (APN-CT), encoding the last 103 amino acid residues which encompasses the entire bipartite nuclear localization signal of Apn1, was selected as bait for a yeast two-hybrid screen. To verify that the bait retained nuclear localization, the *APN-CT* fragment was fused to the C-terminal end of the *GFP* gene and placed under the control of the galactose inducible *GAL1* promoter. The resulting plasmid pGFP-APN-CT was introduced into the strain PJ69 and examined for the production of the Gfp-Apn-Ct fusion protein using monoclonal anti-Gfp antibody. Western blot analysis revealed that the plasmid pGFP-APN-CT directed the expression of a polypeptide that corresponded to the expected size (38-kDa) of the Gfp-Apn-Ct fusion protein, as compared to the pGFP vector that produced the native (~26-kDa) Gfp (Figure 2-1A, lane 3 vs. 2). Immunofluorescent analysis revealed that the Gfp-Apn-Ct was localized to the nucleus as expected, whereas the Gfp alone was distributed throughout the cell (Figure 2-1B, and data not shown). These data clearly indicate that Apn-Ct harbors a functional NLS.

The *APN-CT* DNA fragment was used in the yeast two-hybrid screen and one positive clone was identified (see materials and methods), which upon direct DNA sequence analysis revealed that it contained 87% of the *PIR1* gene encoding a Protein with Internal Repeats (Figure 2-2) (101,102). This partial *PIR1* gene, designated *PIR1Δ-44*, lacked the sequence that corresponded to the N-terminal 44 amino acid residues (Figure 2-2). The native *PIR1* gene was predicted to encode a polypeptide with 341 amino acid residues and a calculated molecular weight of 34.6-kDa. The *PIR1* gene product was

Figure 2-1 Expression and cellular location of Gfp-Apn-Ct in yeast cell.

Panel A, total extracts were derived from the two-hybrid strain PJ69 bearing the pYES2.0 vector (lane 1), and the plasmids pGFP (lane 2), and pGFP-APN-CT (lane 3) after 2 h induction (see below). Each lane contained 50 μg of total extract and the blot was probed with monoclonal anti-Gfp antibody. Panel B, immunofluorescent analysis of strain PJ69 expressing Gfp-Apn-Ct. Cells were grown in selective medium with 2% raffinose as carbon source followed by induction with 0.5% galactose for 2 h. Molecular weight standards are shown on the right.

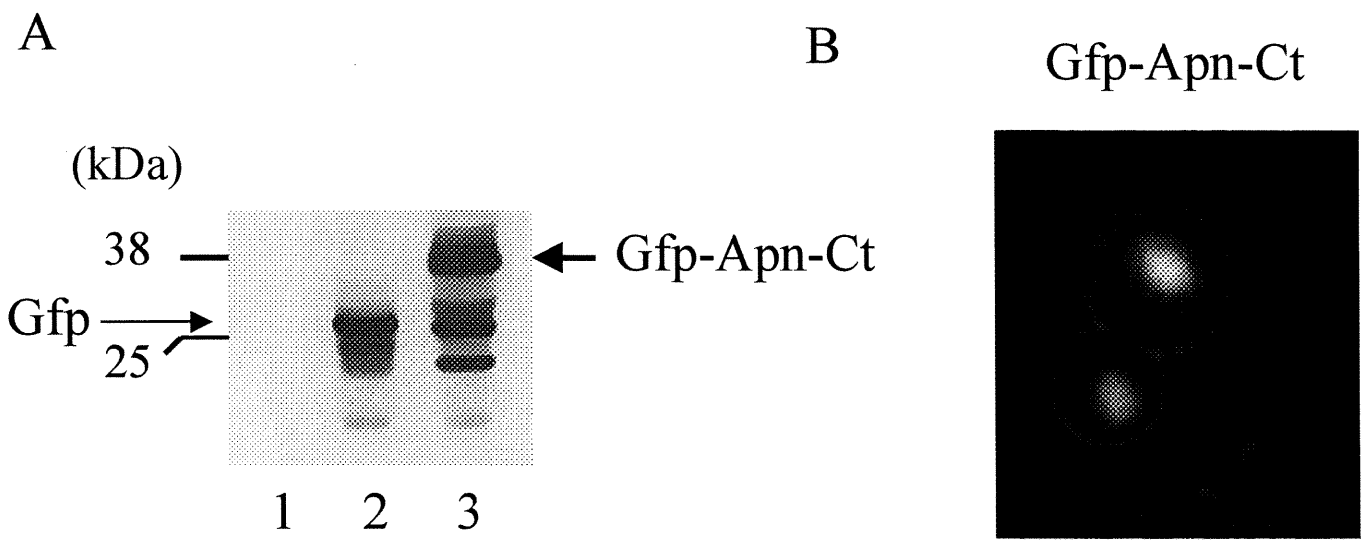


Figure 2-1. Vongsamphanh et al., 2000

Figure 2-2 Deduced amino acid sequence of Pir1.

The seven tandemly repeated sequences are shown in bold. The N-terminal 44 amino acid residues (underlined) are missing in plasmid pGAD-RV2 and the remaining portion is designated *PIR1Δ-44*.

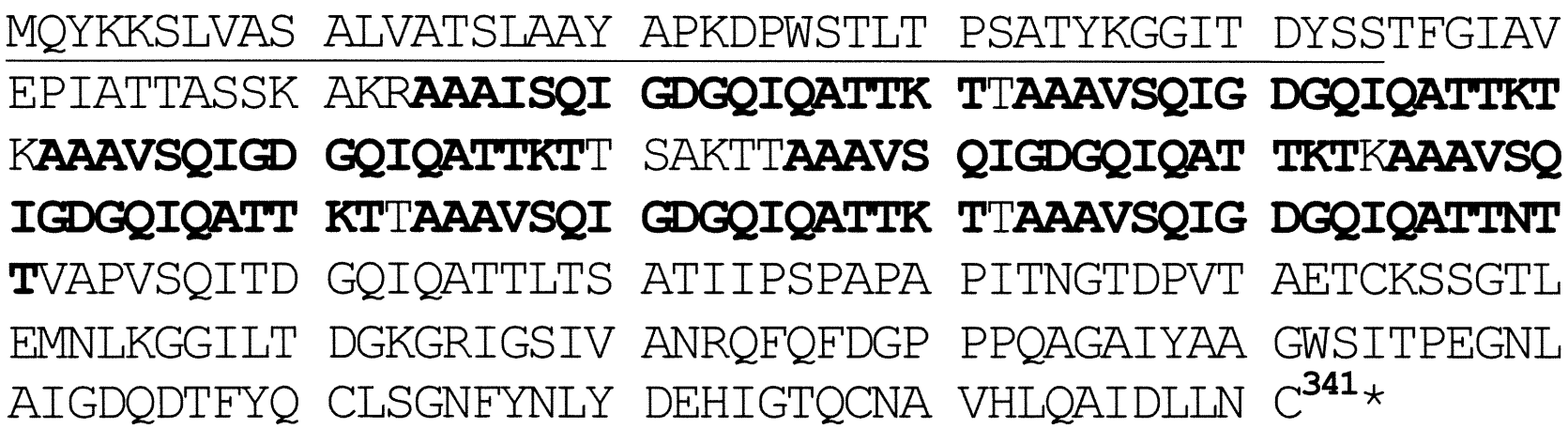


Figure 2-2. Vongsamphanh et al., 2000

previously identified as a cell wall protein, but without any known specific biological function (101,102). The observed interaction between Apn-Ct and Pir1 was not restricted to truncated forms of the two proteins, as the full-length genes *APN1* and *PIR1* also produced a strong interaction in the two-hybrid assay (see materials and methods, and below). The control gene used throughout these experiments was *IMP2*, which encodes a transcriptional co-activator that interacts with the product of the *YLR368w* gene (94,99). The above data indicate that the C-terminal end of Apn1 may directly or indirectly interact with Pir1 *in vivo* and that the interaction is specific for the combination of Apn1 and Pir1.

Pir1 interacts with Apn1 *in vitro*

To test if Pir1 is associated with Apn1 *in vitro*, two independent expression plasmids, pGFP-PIR1 and pGST-APN-CT, were designed to produce different tagged forms of the proteins, Gfp-Pir1 (~62-kDa) and Gst-Apn-Ct (~38-kDa) in wild-type yeast and in *E. coli*, respectively (Figure 2-3A, lane 3 and Figure 2-3B, lane 1). The strongly hydrophobic nature of Gfp was exploited to couple the Gfp-Pir1 fusion protein onto phenyl-agarose columns to serve as an affinity step to bind Gst-Apn1-Ct (see materials and methods). Binding of Gfp-Pir1 onto the column, or the control Gfp-Imp2 fusion protein (~63-kDa), was detected by directly analyzing the column matrix by Western analysis using monoclonal anti-Gfp antibody (Figure 2-3C, lanes 2 and 1, respectively). To assess whether Gst-Apn-Ct can bind to the Gfp-Pir1 column, a fixed amount of Gst-Apn-Ct (200 ng) was loaded onto both columns, extensively washed, and the matrices were directly subjected to Western analysis using monoclonal anti-Gst antibodies (Figure 2-3D). While Gst-Apn-Ct was bound to the Gfp-Pir1 column (Figure 2-3D, lane 2), it

Figure 2-3 Expression of Gfp-Pir1 and Gst-Apn-Ct and interaction detected by affinity column.

Panel A, total extracts were derived from strain SEY6210 harboring either the vector pYES2.0 (lane 1), or the plasmids pGFP (lane 2), or pGFP-PIR1 (lane 3) and probe with anti-Gfp monoclonal antibody. Panel B, total extracts derived from bacteria carrying either plasmid pGST-APN-CT (lane 1) or pGST (lane 2) were subjected to purification on glutathione-S-transferase affinity column and stained with red Ponceau. Panel C, binding of either the control protein Gfp-Imp2 (lane 1) or Gfp-Pir1 (lane 2) to the phenyl agarose column. The matrices were probe by Western analysis using anti-Gfp antibody. Panel D, equal amount of Gst-Apn-Ct (200 ng) was separately loaded onto Gfp-Imp2- (lane 1) and Gfp-Pir1- (lane 2) phenyl-agarose columns, followed by extensive washing, and direct analysis of the column matrix by Western blot probed with anti-Gst antibody. Panel E, same as panel D, except total extract (500 μg) derived from strain SEY6210 overproducing Apn1 was used and the column matrix was probed by Western blot using anti-Apn1 polyclonal antibodies.

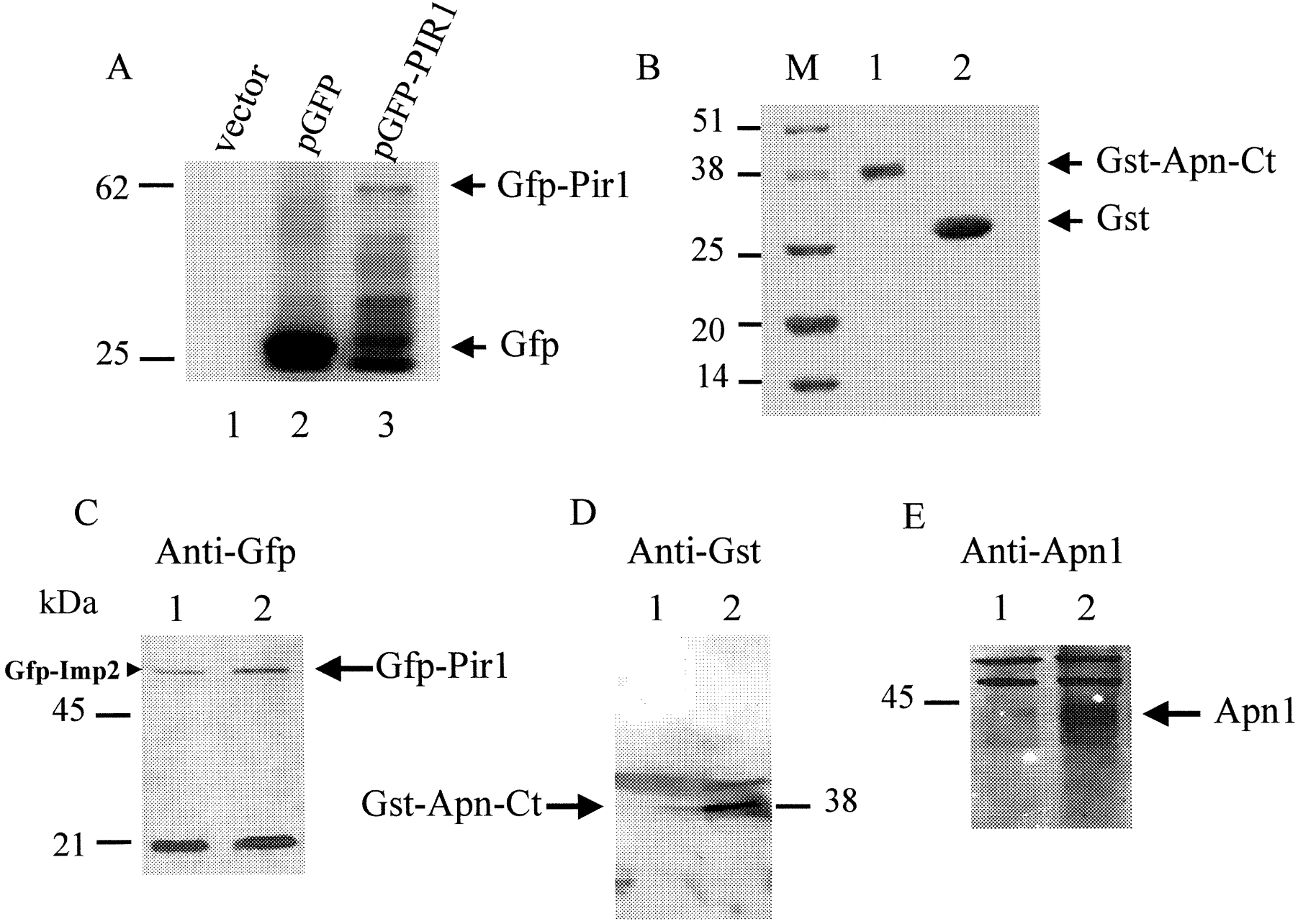


Figure 2-3. Vongsamphanh et al., 2000

showed no detectable binding to the control column Gfp-Imp2 (Figure 2-3D, lane 1). In the case of the control Gfp-Imp2 column, the Gst-Apn1-Ct was recovered in the flow-through washed fractions (data not shown). The Gfp-Pir1 column, but not the Gfp-Imp2 column, also specifically retained the native Apn1 when total yeast extract derived from an Apn1 overproducing strain SEY6210/YEpAPN1 was applied to either columns (Figure 2-3E, lane 2 vs 1). In this latter experiment, the two additional polypeptides detected by the anti-Apn1 antibodies were non-specific and retained by both columns (Figure 2-3E). Collectively, the data suggest that Pir1 interacts with Apn1, and that the interaction involves the C-terminal end of Apn1. Consistent with this observation, Apn1 and Gfp-Pir1 were found to copurify on two separate columns, DEAE sepharose and single-stranded DNA agarose (data not shown).

Pir1 stability depends on the presence of Apn1 *in vivo*

We tested if the steady state level of the Gfp-Pir1 fusion protein is dependent on the presence or absence of Apn1. As shown above, the parent strain SEY6210 harboring pGFP-PIR1 expressed the full length Gfp-Pir1 protein, as well as several fragmented forms of the fusion protein (Figure 2-3A, lane 3). In contrast, an *apn1Δ* mutant expressed predominantly truncated forms of Gfp-Pir1 (Figure 2-4A, lane 2). However, the full-length Gfp-Pir1 was detected in the *apn1Δ* mutant only upon reintroduction of the native *APN1* gene (Figure 2-4A and B, lane 3) or the bait plasmid pGBD-APN-CT (data not shown). The full-length Gfp-Pir1 was not seen in the *apn1Δ* mutant carrying either of two plasmids, pAPN355 or pAPN334, expressing the Apn1 protein with progressively shorter C-terminal ends (Figure 2-4A and B, lanes 4 and 5). While the data clearly indicate that

Figure 2-4 Gfp-Pir1 stability is dependent upon Apn1 with an intact C-terminal end.

Panel A, total extracts were derived from the *apn1Δ* strain harboring the vector Yep352 (lane1); and the plasmid pGFP-PIR1 together with either the vector Yep352 (lanes 2); or the plasmid YEpAPN1 (lane 3), pAPN355 (lane 4), pAPN334 (lane 5), and pAPN315 (lane 6), which expressed an unstable variant of Apn1. The blot was probed using anti-Gfp antibody. Panel B, total extracts were the same as panel A, except probed with anti-Apn1 polyclonal antibodies. Each lane contained 50 μg of total extracts.

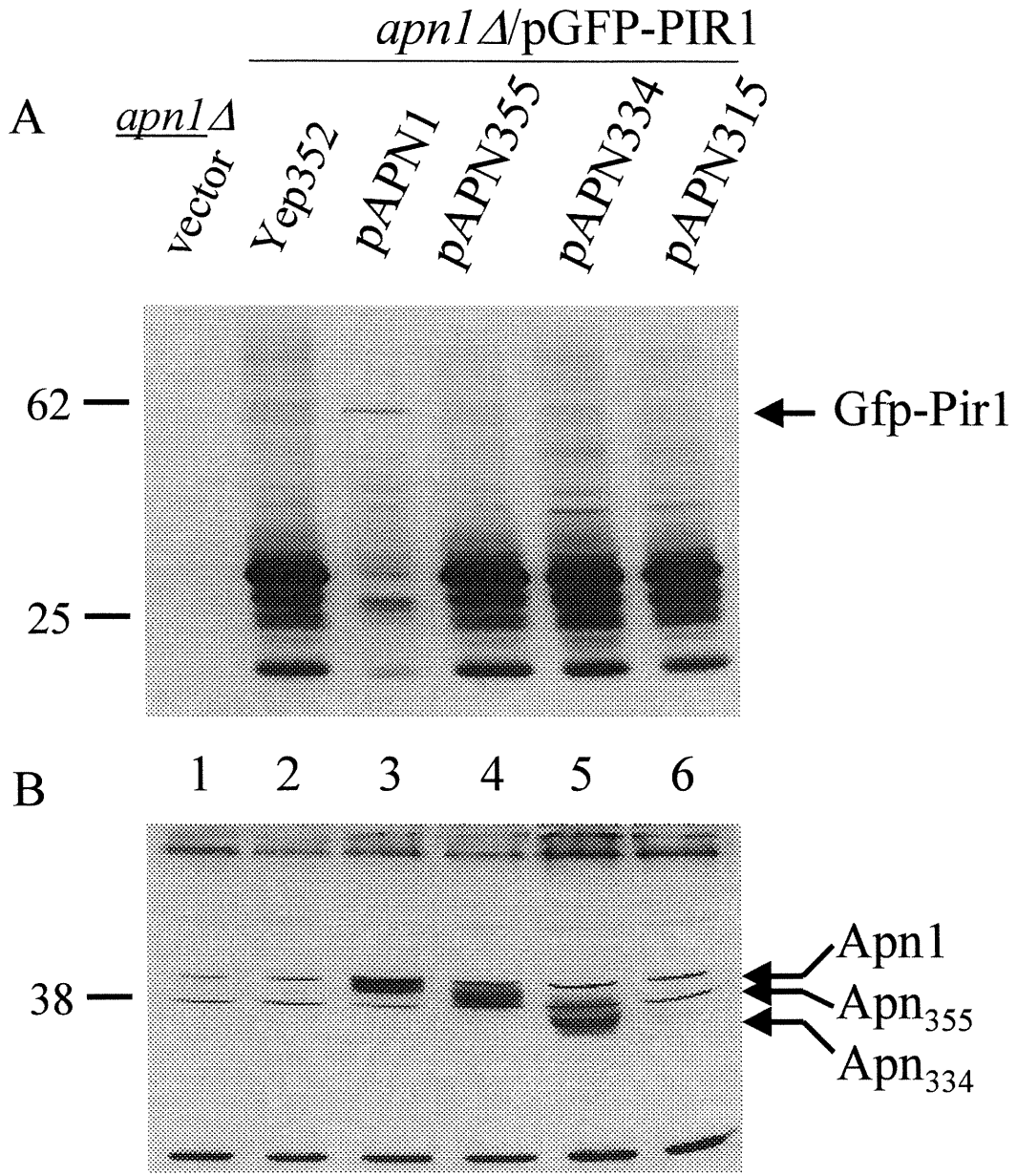


Figure 2-4. Vongsamphanh et al., 2000

stability of the Gfp-Pir1 absolutely requires the intact C-terminal end of Apn1, it also supports the notion that Pir1 and Apn1 exist in the form of a complex *in vivo*. It should be noted that in reciprocal experiments, Apn1 stability was unaffected by the presence or absence of Pir1 (see below).

pir1Δ mutants are not sensitive to MMS

To explore the possible biological role of Apn1/Pir1 interaction, we first tested if *pir1Δ* mutants were similarly hypersensitive as the *apn1Δ* mutants to MMS. The *PIR1* gene was deleted from two different parental strains SEY6210 and YAT1530, to produce the *pir1Δ* strains RVY1 and RVY2, respectively. Interestingly, the *pir1Δ* mutants showed parental resistance to MMS, as compared to the *apn1Δ* mutant (Figure 2-5; (43)). Moreover, an *apn1Δ pir1Δ* double mutant was no more sensitive to the single *apn1Δ* mutant. It would thus appear that Pir1 is not absolutely required for Apn1 function in the repair of MMS-induced DNA lesions.

Pir1 deficiency causes Apn1 to accumulate in the nucleus

Since Pir1 does not directly influence Apn1-modulated DNA repair, we examined if it affects the distribution of Apn1 in the cell. In this experiment, a plasmid pGFP-APN1 was designed to express a N-terminal Gfp-Apn1 functional fusion protein under the control of the *GAL1* promoter. Introduction of the pGFP-APN1 plasmid into either the parent strain SEY6210 or the *pir1Δ* mutant, following induction, revealed that the Gfp-Apn1 was present in the nucleus (Figure 2-6A and B). Strikingly however, the Gfp-Apn1 protein was at least 3 times more intense in the nucleus of the *pir1Δ* mutant as compared

Figure 2-5 Comparison of the MMS resistance of the wild-type and *pir1Δ* mutant carrying various plasmids.

The strains are referred by the gene deletion. The results were obtained from gradient plate assays where the bottom layer contained 0.13 mmol of MMS. Growth all along the gradient is considered to be 100 %.

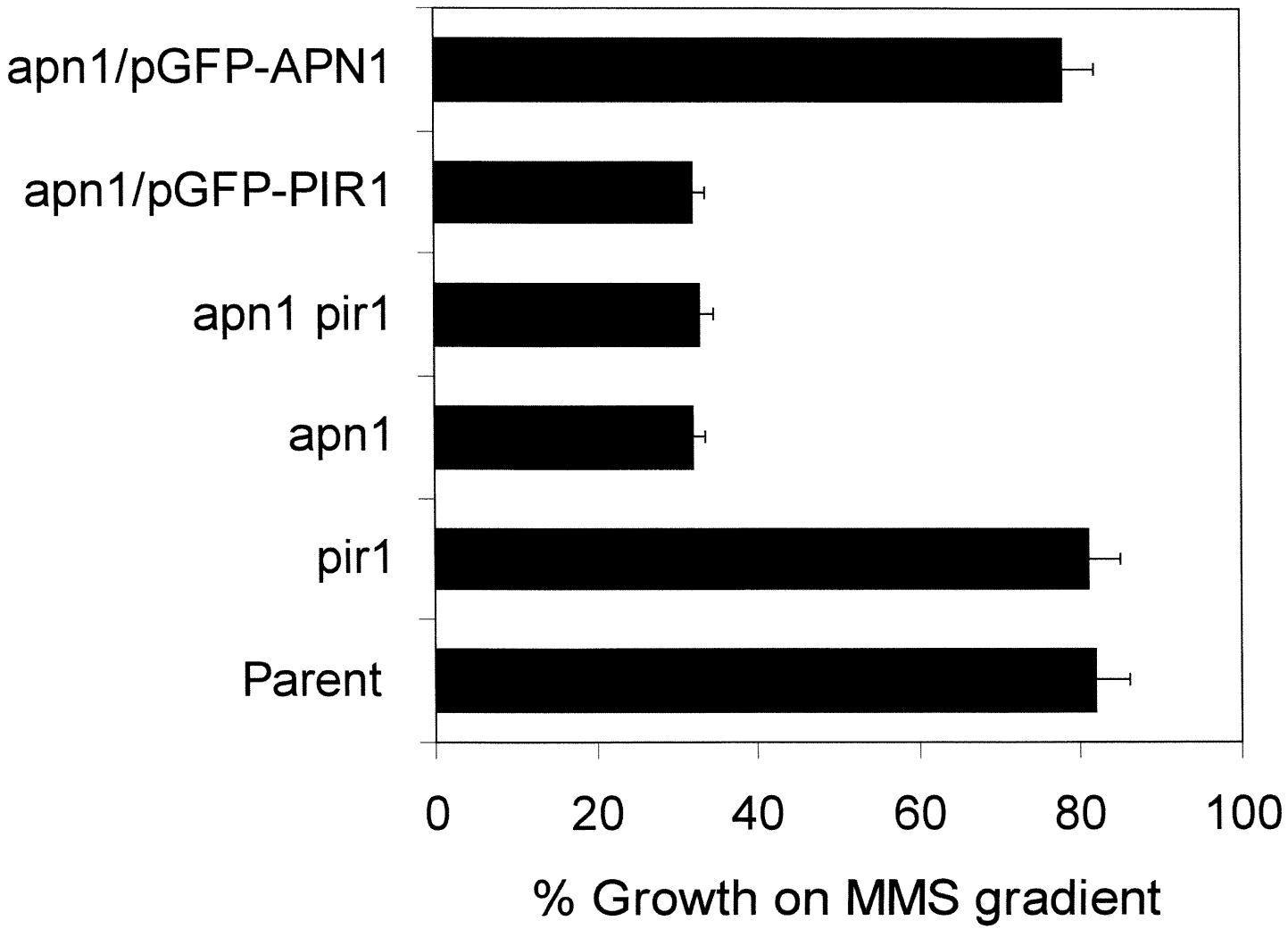


Figure 2-5. Vongsamphanh et al., 2000

Figure 2-6 Increased staining intensity of Gfp-Apn1 in the nucleus of *pir1Δ* mutant. Panels A-D, strain SEY6210(parent)/pGFP-APN1, RVY1(*pir1Δ*)/pGFP-APN1, SEY6210/pGFP-IMP2, and RVY1/pGFP-IMP2, respectively. Cells were grown in selective medium with 2% raffinose and induced with 0.5% galactose for 2h before photographed at 100 times magnification by CoolSnap camera attached to a Leitz immunofluorescent microscrope.

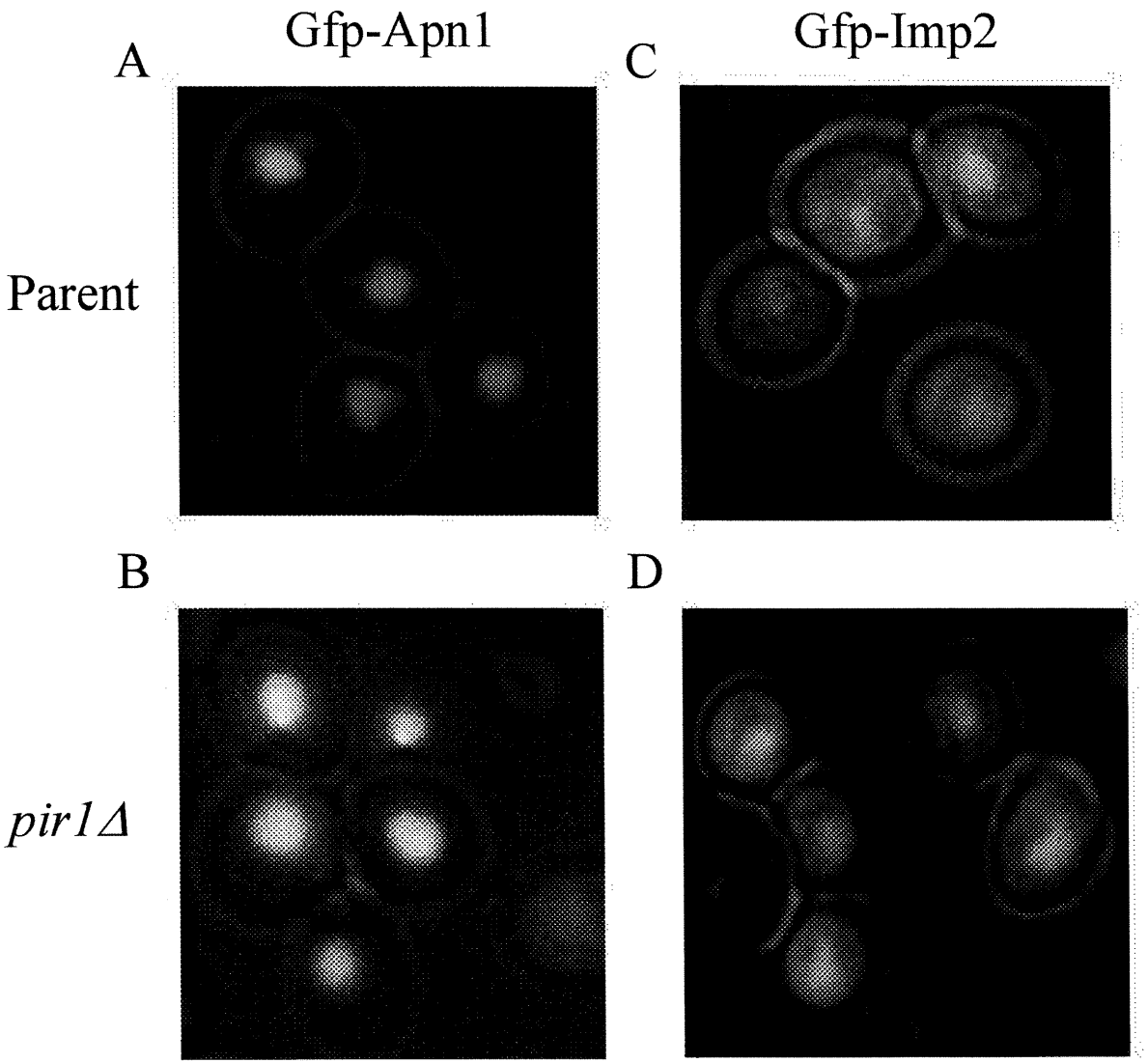


Figure 2-6. Vongsamphanh et al., 2000

to the parent strain (Figure 2-6B vs. 2-6A). In contrast, the control protein Gfp-Imp2 showed both cytoplasmic and nuclear localization, and most importantly, the pattern of staining was not different between the parent (Figure 2-6C) and the *pir1Δ* mutant (Figure 2-6D). It appears that Gfp-Apn1 accumulated in the nucleus of the *pir1Δ* mutant.

To further support the above finding, nuclear extracts were prepared from purified nuclei derived from the four strains as in Figure 2-6 after various time of induction, and the levels of Gfp-Apn1 and Gfp-Imp2 were quantified by Western blot analysis using anti-Gfp antibody. In the absence of induction, i.e., time 0, a basal level of Gfp-Apn1 expression was detected in both the parent and *pir1Δ* mutant due to leaky expression from the *GAL1* promoter (Figure 2-7A; (55)). After 2 h of induction, a substantial amount of Gfp-Apn1 accumulated in the nucleus of the *pir1Δ* mutant (at least 4-fold), as compared to the parent (Figure 2-7A). The accumulated Gfp-Apn1 in the *pir1Δ* nucleus is likely higher than 4-fold as a considerable amount of the protein existed as truncated forms, presumably due to nuclear degradation (Figure 2-7A). The difference in the nuclear amount of Gfp-Apn1 between the two strains cannot be due to the protein being more readily lost from the parent nuclei during preparation of the nuclei, as the same phenomenon was observed in the living cells (Figure 2-6A and B). Moreover, parallel experiments conducted with the parent and *pir1Δ* mutant showed no difference in the nuclear amount of the control protein Gfp-Imp2 (Figure 2-7A, bottom panel).

In a separate experiment, the level of AP endonuclease activity was measured in the nuclear extracts using a synthetic substrate containing AP sites (100). After 2 h of induction, AP endonuclease levels in the extracts derived from purified nuclei isolated from the four strains SEY6210/pGFP-APN1, RVY1/pGFP-APN1, SEY6210/pGFP-

Figure 2-7 Altered distribution of Gfp-Apn1 in the nucleus and cytoplasm of *pir1Δ* mutant.

Panel A, nuclear extract (NE) was derived from strains SEY6210 and RVY1 bearing either plasmid pGFP-APN1 (top) or plasmid pGFP-IMP2 (bottom). Panel B, total extract (TE) prepared from strain SEY6210 and RVY1 carrying plasmid pGFP-APN1. Panel C, cytoplasmic extract (CE) derived from strains SEY6210 and RVY1 bearing either plasmid pGFP-APN1 (top) or plasmid pGFP-IMP2 (bottom). The extracts were prepared from cells after the indicated time of induction with galactose, as in Figure 2-6, and all panels were probed with anti-Gfp monoclonal antibodies. The amount of NE, TE, and CE assessed by Western blot analysis was 30, 50, and 150 μg, respectively.

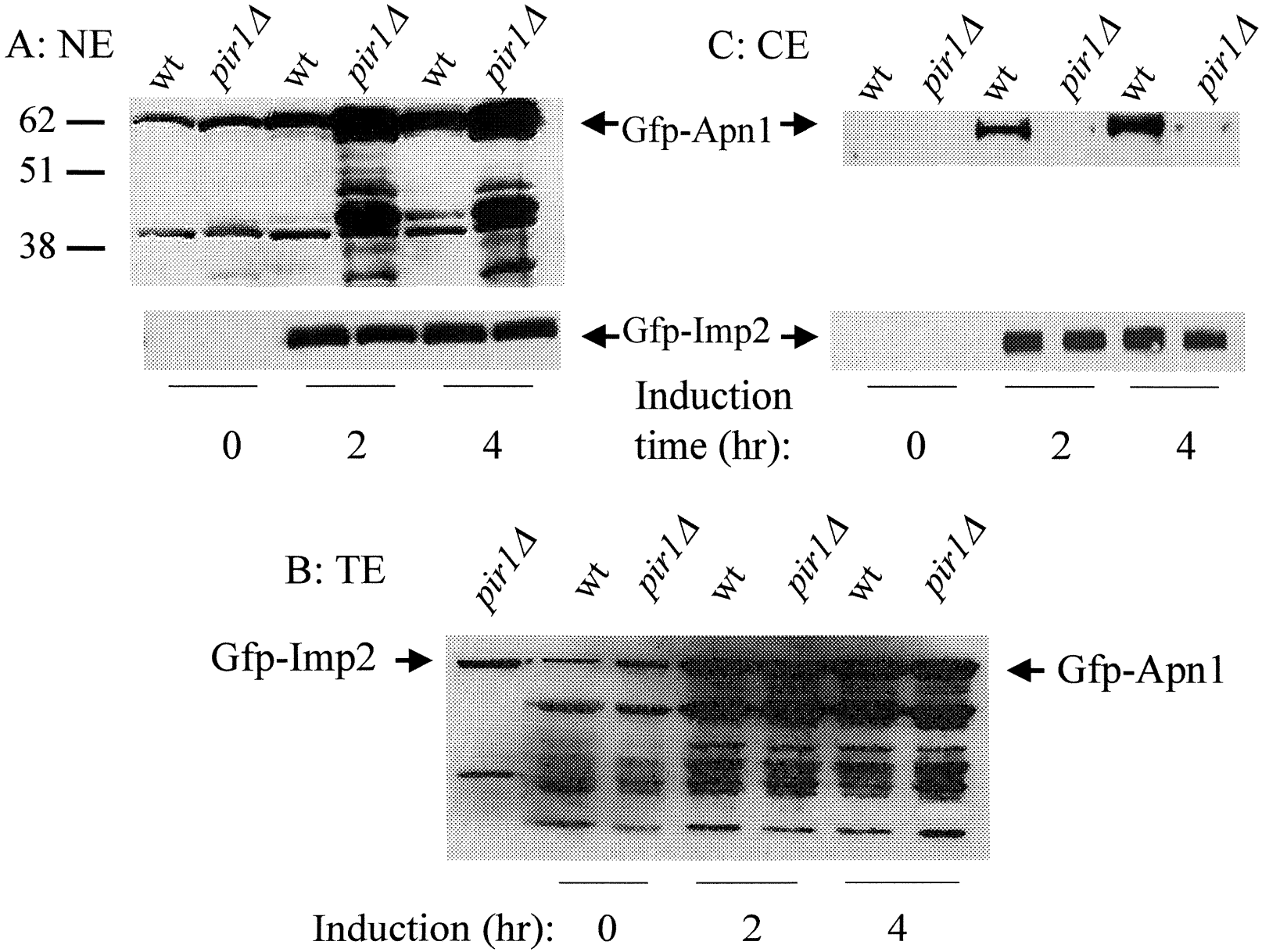


Figure 2-7. Vongsamphanh et al., 2000

IMP2, and RVY1/pGFP-IMP2 were 169.1, 1015.6, 42.3, and 189.2 units per mg of protein, respectively. Induction had no effect on the levels of AP endonuclease in the two latter strains bearing plasmid pGFP-IMP2, and in nuclear extracts from an *apn1Δ* mutant, which expressed extremely low level of AP endonuclease, < 1.0 units per mg protein. The data is consistent with the increased level of Gfp-Apn1 in the *pir1Δ* nucleus. Moreover, it indicates that the endogenous expression of Apn1, i.e., in the absence of the overproducing plasmid pGFP-APN1, is also accumulating in the *pir1Δ* nucleus. Since the accumulated Apn1 in the *pir1Δ* nucleus is also fully active, Pir1 cannot play a role in modulating Apn1 activity. In spite of the higher level of Apn1 in the *pir1Δ* nucleus, this mutant was no more resistant to MMS than the parent. This finding was entirely expected, as Apn1 is not a limiting enzyme in parent strains (43).

To exclude the possibility that the accumulation of Gfp-Apn1 in the nucleus of the *pir1Δ* mutant was not a reflection of increased protein expression or decreased turnover, the level of Gfp-Apn1 was determined in total cell extracts derived from the parent and *pir1Δ* mutant. Western analysis revealed that the amount of Gfp-Apn1 expressed was the same in both strains after 2h induction (Figure 2-7B). In addition, the extent of degradation of Gfp-Apn1 was no different between the two strains (Figure 2-7B). Therefore, the accumulated level of Gfp-Pir1 within the nucleus of *pir1Δ* mutant must reflect an imbalance of Apn1 level from another organelle.

pir1Δ mutant has drastically reduced levels of cytoplasmic and mitochondrial Apn1

Since Apn1 is synthesized in the cytoplasm and translocated to the nucleus, we examined the amount of Gfp-Apn1 in the cytoplasm in both the parent and *pir1Δ* strains

harboring pGFP-APN1 after various time of induction. In the absence of induction, no Gfp-Apn1 was detected in the cytoplasm of either strain (Figure 2-7C). However, after 2h induction, Gfp-Apn1 was detected in the cytoplasm of the parent, but not in the *pir1Δ* mutant (Figure 2-7C). In contrast, the control Gfp-Imp2 protein was detected at the same level in the cytoplasm of the parent and *pir1Δ* mutant bearing plasmid pGFP-IMP2 (Figure 2-7C bottom panel). These data indicate that Pir1 is not required to target Apn1 into the nucleus *per se*, but rather regulate its distribution in the cell.

We therefore considered the possibility that Pir1 may target Apn1 to the mitochondria. Extracts were prepared from purified mitochondria isolated from the parent, *pir1Δ*, and *apn1Δ* strains and look for the Apn1 polypeptide by Western blot analysis using anti-Apn1 polyclonal antibodies (Figure 2-8A). Mitochondria derived from the *pir1Δ* mutant contained substantially lower amounts of the Apn1 cross-reactive polypeptide, at least 5-fold less as compared to the parent (Figure 2-8A, lane 3 vs. 1). No Apn1 reactive polypeptide was detected in the mitochondria derived from the *apn1Δ* mutant (Figure 2-8A, lane 5). A plasmid pDR6, overproducing Apn1, increased the level of the protein in the mitochondria and bypassed the need for Pir1 (Figure 2-8A, lane 4). Thus, in normal cells, Pir1 is required to ensure that Apn1 is efficiently distributed to the mitochondria, but this function can be compensated by overproduction of Apn1. To ascertain that Apn1 is indeed in the mitochondria, and not attached to the outer membranes, intact mitochondria derived from strain SEY6210/pDR6 were either untreated or treated with proteinase K, and extensively washed before extract preparation (Figure 2-8B). The proteinase K pretreatment did not alter the amount of detectable Apn1 polypeptide, strongly indicating that Apn1 is present in the mitochondria (Figure 2-

Figure 2-8 Diminished level of Apn1 in the mitochondria derived from *pir1Δ* mutant.

Panel A and B, Western blot analysis of mitochondrial extracts. For panel A, mitochondrial extracts were prepared from the parent strain SEY6210 and the *pir1Δ* mutant bearing the indicated vector or plasmid. Plasmid pDR6 contained the entire coding region of Apn1 and placed under the control of the *GAL1* promoter in vector pYES2.0 (50). For panel B, intact or disrupted mitochondria were untreated and treated with proteinase K. Each lane (panel A and B) contained 200 μg of mitochondrial extract and the blot was probed with anti-Apn1 antibodies. Panel C, levels of AP endonuclease activity in the mitochondrial extracts derived from the indicated strains.

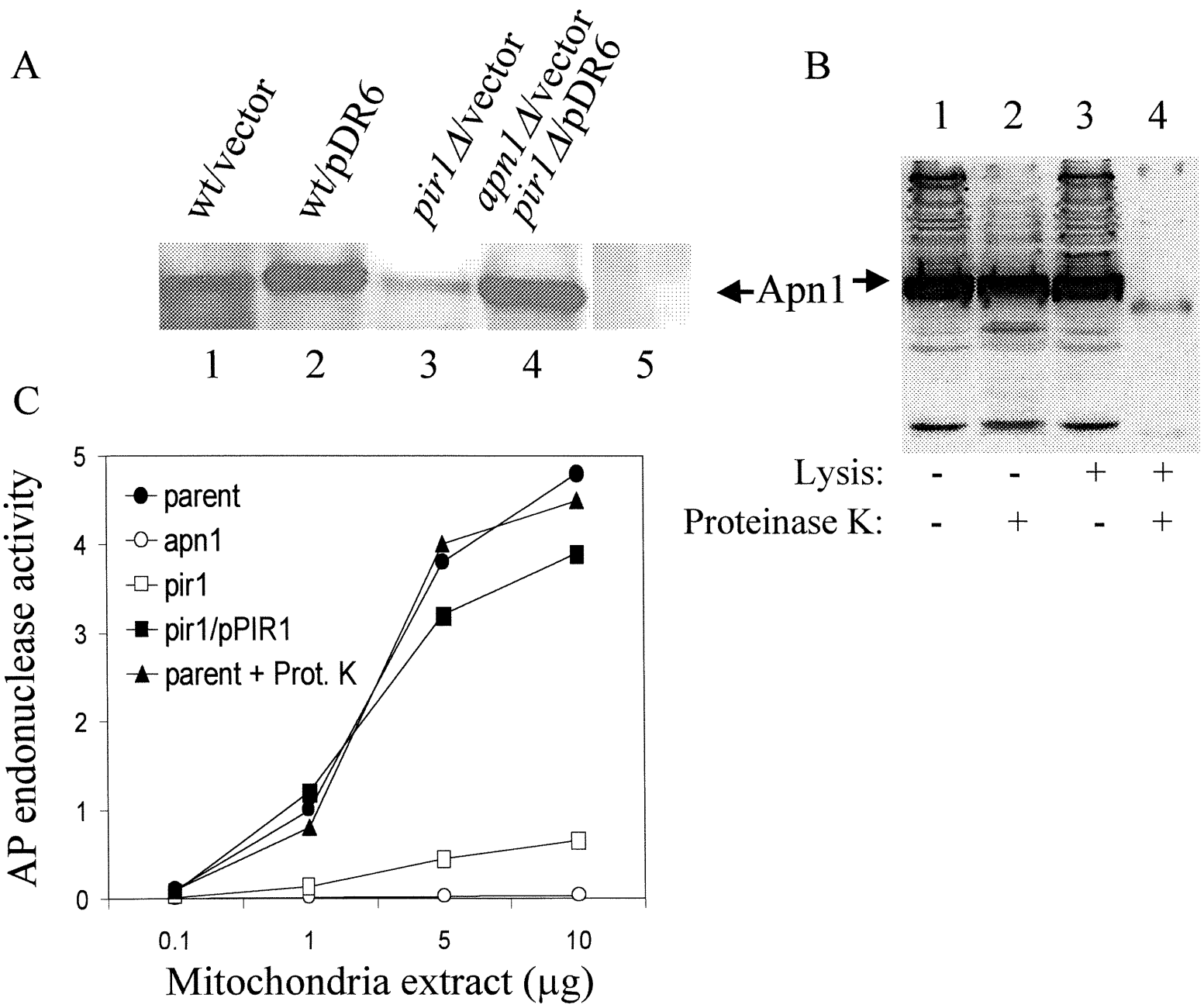


Figure 2-8. Vongsamphanh et al., 2000

8B, lane 2). However, if the mitochondria were first disrupted and pretreated with proteinase K, the amount of Apn1 polypeptide detected was sharply reduced, as compared to no pretreatment (Figure 2-8B, lane 4 vs. 3).

To assess if the level of Apn1 polypeptide detected in the mitochondrial extracts corresponded to the AP endonuclease activity, the extracts were quantified for AP endonuclease using the synthetic AP site substrate. Mitochondrial extracts derived from the *pir1Δ* mutant contained substantially lower levels (~10%) of AP endonuclease activity, as compared to the parent (Figure 2-8C). No AP endonuclease activity was detected in the mitochondrial extract derived from the *apn1Δ* mutant (Figure 2-8C). Introduction of a plasmid pPIR1 carrying the *PIR1* gene into the *pir1Δ* mutant restored the level of AP endonuclease in the mitochondria to nearly parental levels (Figure 2-8C). These data strongly indicate that Apn1 level in the mitochondria is dependent upon Pir1 function.

pir1Δ mutant exhibits increased level of mitochondrial mutations, which can be prevented by Apn1 overproduction

Apn1 is essential to maintain the genetic stability of the nuclear genome. We tested if Apn1 plays a similar role in the mitochondria. The rate of mitochondrial mutations was determined in the parent, *pir1Δ*, and *apn1Δ* mutants by scoring independent colonies for resistance to erythromycin (ery^R). ery^R colonies can occur as a result of mutation in the mitochondrial gene encoding the large ribosomal RNA (21S rRNA) (103). Interestingly, the spontaneous mutation rate of ery^R colonies was similar for the parent, *pir1Δ*, and *apn1Δ*, suggesting that these mutations originate independently of Apn1 function (Table 2-1). However, if the cells were exposed to a low dose of MMS

(0.05 % for 30 min), the level of *ery*^{R} mutations increased by 3.6- and 5.8-fold in the *pir1Δ* and *apn1Δ* mutants, respectively, as compared to the parent (Table 2-1). Overproduction of Apn1 or introduction of the *PIR1* gene into the *pir1Δ* mutant prevented the MMS-induced mutations (Table 2-1). Collectively, the data strongly indicate that Pir1 facilitates Apn1 translocation into the mitochondria, where it can act to process excess AP sites in the mitochondrial genome.

[1] **http://psort.nibb.ac.jp/**

Table 2-1. MMS-induced erythromycin (*Ery*^{R}) resistant colonies (rate 10^8/cell division)

Strains	MMS 0%	MMS 0.05%
SEY6210 (parent) *APN1 PIR1*	6.1 ± 0.5	6.3 ± 0.4
apn1Δ	7.3 ± 0.3	42.3 ± 3.2
apn1 Δ /pPIR1	6.4 ± 0.4	38.8 ± 2.3
pir1 Δ	7.7 ± 0.5	26.6 ± 1.2
pir1 Δ /YEpAPN1	6.3 ± 0.6	7.2 ± 0.2
pir1 Δ /pPIR1	6.8 ± 0.3	8.3 ± 0.4

Rates were calculated based on the frequency obtained from 10 to 15 independent cultures (see materials and methods).

DISCUSSION

We demonstrate that the C-terminal end of the DNA repair enzyme Apn1, comprising a bipartite NLS, interacts either directly or indirectly with the previously uncharacterized Pir1 protein. *In vivo*, an intact C-terminal end of Apn1 was required to stabilize a fusion form of the Pir1 protein. Thus, Pir1 must execute an important intracellular function in the presence of Apn1. We believe that one of the roles of Pir1 is to facilitate Apn1 translocation into the mitochondria. This is supported by our findings that Pir1-deficient cells manifest an imbalance in the subcellular distribution of Apn1, i.e., an accumulation in the nucleus and concomitant reduction in the mitochondria. An apparently dire consequence of Pir1 deficiency is the elevated levels of MMS-induced mitochondrial mutations due to the reduced level of mitochondrial Apn1. We conclude that Apn1 adds to the plethora of DNA repair enzymes that maintain genome stability in the mitochondria. Apn1 may serve in the elimination of excess AP sites, and/or to repair specific oxidative DNA lesions (43), as well as remove 3'-blocking lesions that are created after the action of AP lyases such as Ntg1 (43,104).

A major challenge is to determine the mechanism by which Pir1 facilitates Apn1 distribution into the mitochondria. At least two models can be envisaged. In one model, Pir1 competes with kar α for binding to Apn1 bipartite NLS, or, alternatively, it directly binds to the NLS and sequesters a fraction of the Apn1 protein to be translocated into mitochondria. Pir1 contains seven tandem repeats of a stretch of 18 to 19 amino acid residues (AAAVSQIGDGQIQATTKTT/K) and constituting 38% of the protein. These repeats may engage in the interaction with the basic amino acid residues of Apn1 NLS so as to compete or prevent Kar α from accessing the NLS. Kar α itself consists of ten

tandemly repeated modules termed armadillo (ARM) motifs that form an α helical architecture that creates several contacts with the basic amino acid residues of monopartite and bipartite NLS (61). Although Pir1 repeats are not identical to those of Kar α, three short amino acid stretches VSQ and QIQA, and KTK belonging to the Pir1 repeats are present in ARM 9 and ARM 10 of Kar α, respectively. These short amino acid stretches of Pir1 could compete for the NLS of Apn1.

In a second model, Pir1 can be viewed as a transport receptor or exportin like the Kar β superfamily that shuttles between nucleus and the cytoplasm (67,70,71). Pir1 could mediate export of Apn1 through the nuclear pore complexes and facilitate Apn1 uptake into the mitochondria. In such a model, *pir1Δ* mutant is expected to also favor a nuclear distribution of Apn1, and concomitant depletion in the mitochondria, as observed here. This model is supported by a recent study showing that overproduction of either Pse1/Kar β121 or Kar β123, two members of the Kar β family, facilitates the translocation of hydrophobic proteins into the mitochondria (105). These proteins include the mitochondrial ABC transporter, Atm1p, and a reporter protein fused to the transmembrane domains of apocytochrome B (105). Based on this model, it would be expected that overproduction of Pir1 would deplete the nuclear level of Apn1 and display a DNA repair-deficient phenotype. However, this was not observed, as yeast cells do not appear to sustain higher levels of Pir1 expression from the *GAL1* control promoter. This second model further predicts that Pir1 would be recycled into the nucleus, thereby exhibiting a nucleocytoplasmic distribution. In fact, many exportins, e.g., Crm1 and Msn5 that export Yap1 and Pho4, respectively, are known to cycle between the cytoplasm and nucleus (106,107).

One caveat associated with the second model is that it is energetically less feasible, since it requires that Apn1 cross the nuclear membrane twice before entry into the mitochondria, as opposed to the first model where Apn1 would directly enter from the cytoplasm. However, considering the high rate at which spontaneous mutagenic AP sites are generated in the nuclear genome, the cell may opt to first import Apn1 into the nucleus followed by its distribution to the mitochondria. This seems feasible, as yeast cells retain at least 50 mitochondria under normal growth conditions and may be more tolerant of mitochondrial DNA damage, as opposed to nuclear DNA damage. To distinguish between the above two models, it is imperative to define a single amino acid substitution (perhaps involving the unique proline in the spacer region of the bipartite NLS) that blocks Apn1 entry into the nucleus, but still allowing it to interact with Pir1. According to the first model, the modified Apn1 should be able to enter the mitochondria, unless Apn1 nuclear entry precedes mitochondrial translocation.

Although Apn1 has a putative N-terminal presequence, this is apparently not sufficient to target the protein to the mitochondria. Such a notion is supported by the observation that Apn_{355}, which lacks a portion of the bipartite the NLS, was predominantly localized to the cytoplasm as determined by indirect immunofluorescent microscopy and by Gfp tagged Apn_{355}-Gfp (55)(Vongsamphanh, R. and Ramotar, D., unpublished). In any case, the putative N-terminal presequence is not likely to be a strong signal for targeting Apn1 to the mitochondria. An independent study demonstrated that attachment of the presequence of *S. pombe* SpApn1 to Gfp was sufficient to target the protein to the mitochondria of HeLa cells, but not the presequence of *S. cerevisiae* Apn1 (personal communication, J. Connolly, R. Perez-Jannotti, and D. Bogenhagen). Since *S.*

pombe lacks Pir proteins, this organism may have evolved to effectively use the N-terminal presequence. Thus, any experimental design to demonstrate that the Apn1 N-terminal presequence is capable of targeting a heterologous protein into the mitochondria will only work if the fusion protein also bears the stretch of amino acids residues that promote interaction with Pir1.

Acknowledgments

We sincerely thank Drs. Eric Alani, Dan Bogenhagen, Brian Burke, Michael Weinfeld, and Elliot Drobetsky for their critical comments. This work was supported by a research grant OGP0138503 to D. R. from the Natural Science and Engineering Research Council of Canada. D. R. is a career Scientist of the National Cancer Institute of Canada.

CHAPTER 3

GENERAL DISCUSSION

DISCUSSION

Over the past 20 years, a major focus of carcinogenesis research has been the investigation of oxidative DNA damage (108). In all organisms, the repair of oxidative DNA damage is thought to be primarily mediated by the base excision repair pathway (BER), although there is some evidence for the involvement of other pathways, e.g. the nucleotide excision pathway (NER) (109). In the budding yeast *Saccharomyces cerevisiae*, Apn1 (AP endonuclease) is a key enzyme in the BER pathway (48,50). *apn1Δ* mutants are unable to repair nuclear damaged DNA and show high spontaneous mutation rates (43). In addition, the Apn1 C-terminus functions as a bipartite nuclear localization signal (56). Removal of a portion of the bipartite NLS and fusion of the SV40 T-antigen monopartite NLS to the truncated Apn1 failed to restore nuclear localization of the yeast Apn1. These data suggest that translocation of Apn1 in yeast operates via a nuclear transport mechanism involving the bipartite NLS, similar to the native signal reported for a variety of nuclear proteins, such as nucleoplasmin (64). In addition, Silver (110) reported the presence of "NLS binding proteins" in the yeast cytosol which recognize proteins destined for the nucleus and transport them to the nuclear pore for uptake. The primary objective of this thesis was to identify at least one NLS binding protein that interacts with the C-terminal end of Apn1 to translocate the AP endonuclease into the nucleus. Once the interaction established, the second goal of this work was to determine its biological significance.

The data clearly indicate that Pir1 (Protein with Internal Repeats) interacts, either directly or indirectly, with the C-terminal end of Apn1 both *in vivo* and *in vitro*. The exact interaction site of the two proteins is still unknown but it certainly involves the entire C-

terminal bipartite NLS of Apn1 which spans 103 amino acid residues, as several protein-protein interactions require only a few amino acid residues. For example, a region of 40 amino acids towards the C-terminus of Rad18 is suficient for interaction with Rad6 (111). Moreover, *in vivo*, only native Apn1 can stabilized Pir1 but not truncated forms of Apn1 that lack various portions of the C-termial end. The full length Apn1 possibly maintains Pir1 in a proper conformation such that proteases cannot degrade the Pir1 protein. A Kex2-like protease cleavage site have been found in Pir1 (112). In contrast, Apn1 stability is independent on the presence of Pir1. Site-directed mutagenesis or single amino acid substitution of the Apn1 C-terminal end may allow the identification of the amino acid residues that are crucial for the interaction with Pir1 and also for its stability.

Pir1, together with Pir2, Pir3, and Pir4 belongs to the Pir family of proteins that all contain a sequence consisting of 18-19 amino acid residues (AAAVSQIGDGQIGATTKTT/K) repeated tandemly two to ten times (102). The Pir proteins have been reported to be cell wall proteins of unknown function (101,102). Under normal growth condition, single deletion mutants do not display any striking phenotypes which is indicative of the Pir genes being non-essential. However, the *pir1Δpir2Δpir3Δpir4Δ* quadruple mutant showed a slight growth defect, irregular shape, and susceptibility to cell wall synthesis inhibitors like Calcofluor white and Congo red (102). Furthermore, the Pir2 protein seems important for tolerance to heat shock and resistance to tobacco antifungal protein osmotin (101,113). As for the other Pir proteins, no protective role against environmental or chemical stress have been assigned yet.

The purpose of the intraction between Pir1 and Apn1 is to facilitate the AP endonuclease distribution to the mitochondria and consequently, to maintain the

mitochondrial genome stability. This is supported by the fact that *pir1Δ* null mutants have a reduced level of Apn1 in the mitochondria that correlates with an accumulation in the nucleus and an increased mitochondrial mutation frequency, which can be suppressed by the overproduction of Apn1. Thus, it appears that Apn1 possesses the same function both in the nucleus and in the mitochondria. The present data, therefore, represent the first evidence that Apn1 is present in the mitochondria to maintain stability of the genome.

The mere implication of a cell wall protein, Pir1, in the subcellular distribution of a nuclear DNA repair enzyme, Apn1, is unexpected. A simple and logical explanation that would support this observation is to evoke the possibility that Pir1 has an additional cellular localization besides the cell wall. Using fluorescence microscopy, the Gfp-Pir1 fusion protein was detected everywhere in the cell. However, the data are only preliminary and a detailed experiment is needed to examine additional locations of Pir1.

During the course of this study, we discovered that *pir1Δ* null mutants are uniquely sensitive to arsenite, a toxic metalloid . The arsenite defect of *pir1Δ* mutants can be complemented by the Gfp construct containing the full-length Pir1 but not the Apn1 interacting two-hybrid clone (Pir1 87%) which lacks the first 44 amino acid. The N-terminal region, thus, appears important to target Pir1 to the cell wall and enable it to perform a protective role against arsenite. However, the exact mechanism by which Pir1 protects yeast cells against arsenite remains to be elucidated. Interestingly, although Pir1 stability is dependent on Apn1, its susceptibility to arsenite is independent of the AP endonuclease function as *apn1Δ* null mutant is resistant to arsenite. Perhaps Pir1 is highly expressed so that sufficient amounts of the proteins can reach the cell wall to provide resistance to arsenite. In fact, Toh-E *et al.* (101) suggested that the *PIR* genes are among

those expressed abundantly since codons used preferentially in highly expressed genes are also used in the *PIR* genes. However, it should be noted that this suggestion was made based on an assumption that there is a correlation between bias of codon usage in a gene and its high expression, that was put forward by Bennetzen and Hall (114), and Ikemura (115) in 1982. The *PIR1* gene may be highly transcribed but poorly translated. Among the transcripts that get translated, the majority of the Pir1 proteins may be targeted to the cell wall, with relatively few remaining intracellular. Thus, a possible Pir1 post-transcriptionnal regulation which favors primarily its cell wall function could account for its initial identification as a cell wall protein.

Although it is important to determine the exact physiological role of the Pir proteins in the cell wall, and especially the mechanism by which they provide resistance to arsenite, which in the form of arsenic trioxide has been shown to be an effective treatment for acute promyelocytic leukemia (116), the major challenge now is to determine the precise mechanism for translocation of Apn1 into the mitochondria through the interaction with Pir1. At least two models can be proposed. In the first model, Pir1 has a cytoplasmic location and promotes Apn1 mitochondrial translocation through either competition with karyopherin α for binding to Apn1 bipartite NLS or direct sequestration of a portion of Apn1 through direct binding to the NLS. A recent study has demonstrated, using crystallographic analysis, that the nuclear import factor karyopherin α with its 10 tandem Armadillo (arm) repeats effectively constitute a binding site for the SV40-T antigen NLS motif (61). Similarly, the repeating architectural pattern of Pir1 tandem repeats can be utilized by the protein to generate an array of binding sites for the bipartite NLS motif of Apn1. Three short amino acid sequences (VSQ, QIQA, and KTK) within the Pir1 repeats

are found in two arms of the karyopherin α (ARM 9 and ARM 10), although the two proteins are not homologues.

In the second model, Pir1 has a cytoplasmic and also a nuclear location, and promotes Apn1 uptake into the mitochondria by mediating the transport of Apn1 through the nuclear pore complexes. According to this model, Pir1 would limit the amount of Apn1 into the nucleus to allow some of the AP endonuclease to also enter the mitochondria. Indeed, as observed in this study, *pir1Δ* null mutant has drastically reduced level of Apn1 in the mitochondria that parrallels with the accumulation of the enzyme in the nucleus. Thus, Pir1 can be viewed as a karyopherin β-like protein that shuttles between the nucleus and the cytoplasm. In fact, overproduction of the karyopherin PseI/Kap121 in yeast have been demonstrated to facilitate the mitochondrial translocation import of hydrophobic proteins like the mitochondrial ABC transporter, Atm1p (105).

To distinguish between the two models, an export assay can be performed with purified nuclei, containing the accumulated Apn1, by adding Pir1 and other member of the nuclear transport machinery. If there is a decreased of Apn1 in the nucleus, then this observation would support the second model. On the other hand, the identification of the Apn1 karyopherin α protein and the demonstration that purified Pir1 can compete for binding to the Apn1 bipartite NLS would allow us to eliminate the second model. We favored the second model as it is more important to repair the nuclear genome, as opposed to the mitochondrial genome of which there are at least 50 copies.

It is not uncommon for DNA repair enzymes to be targeted to both the nucleus and the mitochondria. For example, in *S. cerevisiae*, a N-glycosylase that recognizes and excises a broad spectrum of oxidative base damage lesions such as thymine glycol, 8-

hydroxyguanine (8-OHG), and abasic sites (117,118), is targeted to both the mitochondria and the nucleus (72). A 17 amino acid sequence at the N-terminus of Ntg1 has been proposed to be a mitochondrial signal sequence (104). In contrast, multiple subcellular localization of *S. pombe* Uvde enzyme the repairs UV-induced DNA damage including cyclobutane pyrimidine dimers and (6-4) photoproducts is accomplished by translation at the multiple methionine start codons (73). The uvde nucleotide sequence possesses three putative initiation methionine codons at its N-terminus (119). When the second or the third methionine codon was used as the translation initiation site, uvde was observed exclusively in the nucleus. Mutation of the same two codons, however, localized the uvde only to the mitochondria (73). Since Apn1 lacks multiple methionine start codons, its mitochondria translocation must operate via its unique N-terminal mitochondrial signal sequence.

Analysis of the Apn1 N-terminal sequence by the PSORT[1] program revealed that it indeed contains a putative mitochondrial signal sequence. However, the Apn1 mitochondrial signal may not be strong and therefore requires the interaction with the Pir1 protein, as Apn355 lacking the bipartite NLS was localized in the cytoplasm (55). In fact, it has been shown that the N-terminal sequence of Apn1 fused to *GFP* is not sufficient to transport the fusion protein into the mitochondria of Hela cells, whereas the presequence from *S. pombe* Apn1 (SpAPN1) homologue was able to translocate the *GFP* construct to the Hela cells mitochondria (personal communication, J. Connolly, R. Perez-Jannotti, and D. Bogenhagen). Thus, to demonstrate that Apn1 mitochondrial signal sequence has the potential to target an heterologous protein to the mitochondria, the fusion protein must contain the binding site of Pir1.

Although it has never been shown that Apn1 is present in the mitochondria, other DNA repair proteins have been found in this organelle. This is where the oxidative phosphorylation occurs and consequently, a large number of reactive oxygen species are generated (80). Because mitochondria are vital organelles and mitochondrial DNA is subjected to relatively high amount of oxidative damage, it seems that mitochondria would need efficient DNA repair activity to remove oxidative DNA damage (79). Recently, the complete reconstitution of base excision repair of abasic sites in DNA by using highly purified enzymes from mitochondria of *Xenopus laevis* has been reported (78). The results suggest that the pathway employs a mitochondrial class II AP endonuclease, a deoxyribosephosphodiesterase, mt DNA polymerase γ, and a mt DNA ligase. These findings demonstrate that mitochondria are well equiped to perform base excision repair. Since Apn1 is the major AP endonuclease in the budding yeast *S. cerevisiae,* its presence in the mitochondria is more than probable. Further characterization of the interaction between Apn1 and Pir1 will certainly help elucidate the mechanism by which Apn1 is translocated into the mitochondria.

[1] **http://psort.nibb.ac.jp/**

FUTURE DIRECTIONS

The findings that Pir1, a cell wall protein, is involved in the mitochondrial distribution of Apn1, a nuclear DNA repair enzyme, provide a new insight concerning how subcellular translocation of nuclear proteins may be accomplished. Before the exact mechanism of translocation can be elucidated, the Apn1/Pir1 interaction needs to be further characterized. Site-directed mutagenesis or single amino acid substitution in either

the Apn1 bipartite NLS or the Pir1 tandem repeats should allow the identification of the exact site of interaction between these two proteins.

In order to assess which of the two mechanisms speculated upon earlier is more likely to occur under physiological conditions, the cellular distribution of Pir1 needs to be defined more precisely. Based on our findings, Pir1 may be either a cytoplasmic karyopherin α-like protein or a nucleocytolasmic karyopherin β-like receptor, besides its presence in the cell wall.

Future experiments should also be designed to determine if other proteins may be involve in the Apn1/Pir1 interaction. Both Apn1 and Pir1 could be assemble with other proteins into a larger complex, which may then be recognized by other proteins that transport Apn1 to its different cellular compartments. Identification of other interacting cellular partners will undoubtly provide new and exciting information on the mechanism used for Apn1 subcellular distribution in *S.cerevisiae.*

REFERENCES

1. Gilchrest, B.A. and Bohr V.A. (1997) Aging processes, DNA damage, and repair. *Faseb J*, **11**, 322-30.
2. Demple, B. and Harrison L. (1994) Repair of oxidative damage to DNA: enzymology and biology. *Annu Rev Biochem*, **63**, 915-48.
3. Ames, B.N., Shigenaga M.K. and Hagen T.M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A*, **90**, 7915-22.
4. Hutchinson, F. (1985) Chemical changes induced in DNA by ionizing radiation. *Prog Nucleic Acid Res Mol Biol*, **32**, 115-54.
5. von Sonntag, C. (1987) New aspects in the free-radical chemistry of pyrimidine nucleobases. *Free Radic Res Commun*, **2**, 217-24.
6. Ramotar, D., Belanger E., Brodeur I., Masson J.Y. and Drobetsky E.A. (1998) A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide. *J Biol Chem*, **273**, 21489-96.
7. Yamamoto, K., Inoue S. and Kawanishi S. (1993) Site-specific DNA damage and 8-hydroxydeoxyguanosine formation by hydroxylamine and 4-hydroxyaminoquinoline 1-oxide in the presence of Cu(II): role of active oxygen species. *Carcinogenesis*, **14**, 1397-401.
8. Gutteridge, J.M. and Halliwell B. (1989) Iron toxicity and oxygen radicals. *Baillieres Clin Haematol*, **2**, 195-256.
9. Jaruga, P. and Dizdaroglu M. (1996) Repair of products of oxidative DNA base damage in human cells. *Nucleic Acids Res*, **24**, 1389-94.
10. Halliwell, B. and Gutteridge J.M. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol*, **186**, 1-85.
11. Teoule, R. (1987) Radiation-induced DNA damage and its repair. *Int J Radiat Biol Relat Stud Phys Chem Med*, **51**, 573-89.
12. Dizdaroglu, M. (1992) Oxidative damage to DNA in mammalian chromatin. *Mutat Res*, **275**, 331-42.
13. Jackson, R.L. (1993) Anti-oxidants for the treatment and the prevention of atherosclerosis. *Biochem Soc Trans*, **21**, 650-1.
14. Busciglio, J. and Yankner B.A. (1995) Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature*, **378**, 776-9.
15. Halliwell, B. (1992) Reactive oxygen species and the central nervous system. *J Neurochem*, **59**, 1609-23.

16. Sohal, R.S., Agarwal S., Dubey A. and Orr W.C. (1993) Protein oxidative damage is associated with life expectancy of houseflies. *Proc Natl Acad Sci U S A*, **90**, 7255-9.

17. Fridovich, I. (1997) Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem*, **272**, 18515-7.

18. Laspia, M.F. and Wallace S.S. (1988) Excision repair of thymine glycols, urea residues, and apurinic sites in Escherichia coli. *J Bacteriol*, **170**, 3359-66.

19. Dizdaroglu, M. (1985) Formation of an 8-hydroxyguanine moiety in deoxyribonucleic acid on gamma-irradiation in aqueous solution. *Biochemistry*, **24**, 4476-81.

20. Grollman, A.P. and Moriya M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet*, **9**, 246-9.

21. Boiteux, S. (1993) Properties and biological functions of the NTH and FPG proteins of Escherichia coli: two DNA glycosylases that repair oxidative damage in DNA. *J Photochem Photobiol B*, **19**, 87-96.

22. Moriya, M. and Grollman A.P. (1993) Mutations in the mutY gene of Escherichia coli enhance the frequency of targeted G:C-->T:a transversions induced by a single 8-oxoguanine residue in single-stranded DNA. *Mol Gen Genet*, **239**, 72-6.

23. Shibutani, S., Takeshita M. and Grollman A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*, **349**, 431-4.

24. Kozarich, J.W., Worth L., Jr., Frank B.L., Christner D.F., Vanderwall D.E. and Stubbe J. (1989) Sequence-specific isotope effects on the cleavage of DNA by bleomycin. *Science*, **245**, 1396-9.

25. Barzilay, G. and Hickson I.D. (1995) Structure and function of apurinic/apyrimidinic endonucleases. *Bioessays*, **17**, 713-9.

26. Henner, W.D., Grunberg S.M. and Haseltine W.A. (1983) Enzyme action at 3' termini of ionizing radiation-induced DNA strand breaks. *J Biol Chem*, **258**, 15198-205.

27. Loeb, L.A. and Preston B.D. (1986) Mutagenesis by apurinic/apyrimidinic sites. *Annu Rev Genet*, **20**, 201-30.

28. Lindahl, T. and Nyberg B. (1972) Rate of depurination of native deoxyribonucleic acid. *Biochemistry*, **11**, 3610-8.

29. Lindahl, T. (1990) Repair of intrinsic DNA lesions. *Mutat Res*, **238**, 305-11.

30. Ramotar, D. (1997) The apurinic-apyrimidinic endonuclease IV family of DNA repair enzymes. *Biochem Cell Biol*, **75**, 327-36.

31. Lindahl, T. (1993) Instability and decay of the primary structure of DNA [see comments]. *Nature*, **362**, 709-15.

32. Cunningham, R.P. (1997) DNA glycosylases. *Mutat Res*, **383**, 189-96.

33. Sakumi, K. and Sekiguchi M. (1990) Structures and functions of DNA glycosylases. *Mutat Res*, **236**, 161-72.

34. Krokan, H.E., Standal R. and Slupphaug G. (1997) DNA glycosylases in the base excision repair of DNA. *Biochem J*, **325**, 1-16.

35. Kim, J. and Linn S. (1988) The mechanisms of action of E. coli endonuclease III and T4 UV endonuclease (endonuclease V) at AP sites. *Nucleic Acids Res*, **16**, 1135-41.

36. Friedberg, E.C., Walker G.C. and Siede W. (1995) DNA Repair and Mutagenesis. ASM Press, Washington, D.C.

37. Dantzer, F., Nasheuer H.P., Vonesch J.L., de Murcia G. and Menissier-de Murcia J. (1998) Functional association of poly(ADP-ribose) polymerase with DNA polymerase alpha-primase complex: a link between DNA strand break detection and DNA replication. *Nucleic Acids Res*, **26**, 1891-8.

38. Kubota, Y., Nash R.A., Klungland A., Schar P., Barnes D.E. and Lindahl T. (1996) Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. *Embo J*, **15**, 6662-70.

39. Matsumoto, Y., Kim K. and Bogenhagen D.F. (1994) Proliferating cell nuclear antigen-dependent abasic site repair in Xenopus laevis oocytes: an alternative pathway of base excision DNA repair. *Mol Cell Biol*, **14**, 6187-97.

40. Wu, X., Li J., Li X., Hsieh C.L., Burgers P.M. and Lieber M.R. (1996) Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA. *Nucleic Acids Res*, **24**, 2036-43.

41. Parikh, S.S., Mol C.D. and Tainer J.A. (1997) Base excision repair enzyme family portrait: integrating the structure and chemistry of an entire DNA repair pathway. *Structure*, **5**, 1543-50.

42. Matsumoto, Y. and Kim K. (1995) Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. *Science*, **269**, 699-702.

43. Ramotar, D., Popoff S.C., Gralla E.B. and Demple B. (1991) Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation. *Mol Cell Biol*, **11**, 4537-44.

44. Saporito, S.M. and Cunningham R.P. (1988) Nucleotide sequence of the nfo gene of Escherichia coli K-12. *J Bacteriol*, **170**, 5141-5.

45. Saporito, S.M., Smith-White B.J. and Cunningham R.P. (1988) Nucleotide sequence of the xth gene of Escherichia coli K-12. *J Bacteriol*, **170**, 4542-7.

46. Mazumder, A., Gerlt J.A., Absalon M.J., Stubbe J., Cunningham R.P., Withka J. and Bolton P.H. (1991) Stereochemical studies of the beta-elimination reactions at aldehydic abasic sites in DNA: endonuclease III from Escherichia coli, sodium hydroxide, and Lys-Trp-Lys. *Biochemistry*, **30**, 1119-26.

47. Johnson, A.W. and Demple B. (1988) Yeast DNA diesterase for 3'-fragments of deoxyribose: purification and physical properties of a repair enzyme for oxidative DNA damage. *J Biol Chem*, **263**, 18009-16.

48. Johnson, A.W. and Demple B. (1988) Yeast DNA 3'-repair diesterase is the major cellular apurinic/apyrimidinic endonuclease: substrate specificity and kinetics. *J Biol Chem*, **263**, 18017-22.

49. Levin, J.D., Johnson A.W. and Demple B. (1988) Homogeneous Escherichia coli endonuclease IV. Characterization of an enzyme that recognizes oxidative damage in DNA. *J Biol Chem*, **263**, 8066-71.

50. Popoff, S.C., Spira A.I., Johnson A.W. and Demple B. (1990) Yeast structural gene (APN1) for the major apurinic endonuclease: homology to Escherichia coli endonuclease IV. *Proc Natl Acad Sci U S A*, **87**, 4193-7.

51. Masson, J.Y., Tremblay S. and Ramotar D. (1996) The Caenorhabditis elegans gene CeAPN1 encodes a homolog of Escherichia coli and yeast apurinic/apyrimidinic endonuclease. *Gene*, **179**, 291-3.

52. Ramotar, D., Vadnais J., Masson J.Y. and Tremblay S. (1998) Schizosaccharomyces pombe apn1 encodes a homologue of the Escherichia coli endonuclease IV family of DNA repair proteins. *Biochim Biophys Acta*, **1396**, 15-20.

53. Kunz, B.A., Henson E.S., Roche H., Ramotar D., Nunoshiba T. and Demple B. (1994) Specificity of the mutator caused by deletion of the yeast structural gene (APN1) for the major apurinic endonuclease. *Proc Natl Acad Sci U S A*, **91**, 8165-9.

54. Masson, J.Y. and Ramotar D. (1997) Normal processing of AP sites in Apn1-deficient Saccharomyces cerevisiae is restored by Escherichia coli genes expressing either exonuclease III or endonuclease III. *Mol Microbiol*, **24**, 711-21.

55. Ramotar, D., Kim C., Lillis R. and Demple B. (1993) Intracellular localization of the Apn1 DNA repair enzyme of Saccharomyces cerevisiae. Nuclear transport signals and biological role. *J Biol Chem*, **268**, 20533-9.

56. Ramotar, D. and Demple B. (1996) Functional expression of Escherichia coli endonuclease IV in apurinic endonuclease-deficient yeast. *J Biol Chem*, **271**, 7368-74.

57. Moreland, R.B., Langevin G.L., Singer R.H., Garcea R.L. and Hereford L.M. (1987) Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol Cell Biol*, **7**, 4048-57.

58. Wozniak, R.W., Rout M.P. and Aitchison J.D. (1998) Karyopherins and kissing cousins. *Trends Cell Biol*, **8**, 184-8.

59. Fabre, E. and Hurt E. (1997) Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking. *Annu Rev Genet*, **31**, 277-313.

60. Jans, D.A., Xiao C.Y. and Lam M.H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays*, **22**, 532-44.

61. Conti, E., Uy M., Leighton L., Blobel G. and Kuriyan J. (1998) Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. *Cell*, **94**, 193-204.

62. Moore, M.S. and Blobel G. (1992) The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell*, **69**, 939-50.

63. Kalderon, D., Roberts B.L., Richardson W.D. and Smith A.E. (1984) A short amino acid sequence able to specify nuclear location. *Cell*, **39**, 499-509.

64. Robbins, J., Dilworth S.M., Laskey R.A. and Dingwall C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell*, **64**, 615-23.

65. Hu, W. and Jans D.A. (1999) Efficiency of importin alpha/beta-mediated nuclear localization sequence recognition and nuclear import. Differential role of NTF2. *J Biol Chem*, **274**, 15820-7.

66. Nachury, M.V., Ryder U.W., Lamond A.I. and Weis K. (1998) Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor. *Proc Natl Acad Sci U S A*, **95**, 582-7.

67. Jakel, S. and Gorlich D. (1998) Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *Embo J*, **17**, 4491-502.

68. Gorlich, D., Henklein P., Laskey R.A. and Hartmann E. (1996) A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. *Embo J*, **15**, 1810-7.

69. Vetter, I.R., Arndt A., Kutay U., Gorlich D. and Wittinghofer A. (1999) Structural view of the Ran-Importin beta interaction at 2.3 A resolution. *Cell*, **97**, 635-46.

70. Henderson, B.R. and Percipalle P. (1997) Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. *J Mol Biol*, **274**, 693-707.

71. Truant, R. and Cullen B.R. (1999) The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol Cell Biol*, **19**, 1210-7.

72. You, H.J., Swanson R.L., Harrington C., Corbett A.H., Jinks-Robertson S., Senturker S., Wallace S.S., Boiteux S., Dizdaroglu M. and Doetsch P.W. (1999) Saccharomyces cerevisiae Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria. *Biochemistry*, **38**, 11298-306.

73. Yasuhira, S. and Yasui A. (2000) Alternative excision repair pathway of UV-damaged DNA in Schizosaccharomyces pombe operates both in nucleus and in mitochondria. *J Biol Chem*, **275**, 11824-8.

74. Muller-Weeks, S., Mastran B. and Caradonna S. (1998) The nuclear isoform of the highly conserved human uracil-DNA glycosylase is an Mr 36,000 phosphoprotein. *J Biol Chem*, **273**, 21909-17.

75. Croteau, D.L., ap Rhys C.M., Hudson E.K., Dianov G.L., Hansford R.G. and Bohr V.A. (1997) An oxidative damage-specific endonuclease from rat liver mitochondria. *J Biol Chem*, **272**, 27338-44.

76. Rosenquist, T.A., Zharkov D.O. and Grollman A.P. (1997) Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. *Proc Natl Acad Sci U S A*, **94**, 7429-34.

77. Takao, M., Aburatani H., Kobayashi K. and Yasui A. (1998) Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage. *Nucleic Acids Res*, **26**, 2917-22.

78. Pinz, K.G. and Bogenhagen D.F. (1998) Efficient repair of abasic sites in DNA by mitochondrial enzymes. *Mol Cell Biol*, **18**, 1257-65.

79. Stierum, R.H., Dianov G.L. and Bohr V.A. (1999) Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts. *Nucleic Acids Res*, **27**, 3712-9.

80. Richter, C. (1992) Reactive oxygen and DNA damage in mitochondria. *Mutat Res*, **275**, 249-55.

81. Grishko, V.I., Druzhyna N., LeDoux S.P. and Wilson G.L. (1999) Nitric oxide-induced damage to mtDNA and its subsequent repair. *Nucleic Acids Res*, **27**, 4510-6.

82. Sia, E.A., Butler C.A., Dominska M., Greenwell P., Fox T.D. and Petes T.D. (2000) Analysis of microsatellite mutations in the mitochondrial DNA of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **97**, 250-5.

83. Wallace, D.C. (1999) Mitochondrial diseases in man and mouse. *Science*, **283**, 1482-8.

84. Schapira, A.H. (1999) Mitochondrial disorders [editorial; comment]. *Biochim Biophys Acta*, **1410**, 99-102.

85. Bandmann, O., Sweeney M.G., Daniel S.E., Marsden C.D. and Wood N.W. (1997) Mitochondrial DNA polymorphisms in pathologically proven Parkinson's disease. *J Neurol*, **244**, 262-5.

86. Davis, R.E., Miller S., Herrnstadt C., Ghosh S.S., Fahy E., Shinobu L.A., Galasko D., Thal L.J., Beal M.F., Howell N. and Parker W.D., Jr. (1997) Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. *Proc Natl Acad Sci U S A*, **94**, 4526-31.

87. Rotig, A., Bonnefont J.P. and Munnich A. (1996) Mitochondrial diabetes mellitus. *Diabetes Metab*, **22**, 291-8.

88. Wach, A., Brachat A., Pohlmann R. and Philippsen P. (1994) New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. *Yeast*, **10**, 1793-808.

89. Sherman, F., Fink G. and Hicks J. (1983) Methods in yeast genetics, *Cold Spring Harbor Laboratory*. Cold Spring Harbor, New York.

90. Guthrie, C. and Fink G.R. (1991) Guide to yeast genetics and molecular biology. *Meth Enzymol*, **194**, 3-37.

91. Gietz, R.D. and Schiestl R.H. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast*, **7**, 253-63.

92. Sambrook, J., Fritsch E.F. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, *Cold Spring Harbor Laboratory Press*. Cold Spring Harbor, New York.

93. James, P., Halladay J. and Craig E.A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*, **144**, 1425-36.

94. Uetz, P., Giot L., Cagney G., Mansfield T.A., Judson R.S., Knight J.R., Lockshon D., Narayan V., Srinivasan M., Pochart P., Qureshi-Emili A., Li Y., Godwin B., Conover D., Kalbfleisch T., Vijayadamodar G., Yang M., Johnston M., Fields S. and Rothberg J.M. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae [see comments]. *Nature*, **403**, 623-7.

95. Sanger, F., Nicklen S. and Coulson A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*, **74**, 5463-7.

96. Masson, J.Y. and Ramotar D. (1996) The Saccharomyces cerevisiae IMP2 gene encodes a transcriptional activator that mediates protection against DNA damage caused by bleomycin and other oxidants. *Mol Cell Biol*, **16**, 2091-100.

97. Dove, J.E., Brockenbrough J.S. and Aris J.P. (1998) Isolation of nuclei and nucleoli from the yeast Saccharomyces cerevisiae. *Methods Cell Biol*, **53**, 33-46.

98. Gershoni, J.M. and Palade G.E. (1983) Protein blotting: principles and applications. *Anal Biochem*, **131**, 1-15.

99. Masson, J.Y. and Ramotar D. (1998) The transcriptional activator Imp2p maintains ion homeostasis in Saccharomyces cerevisiae. *Genetics*, **149**, 893-901.

100. Yang, X., Tellier P., Masson J.Y., Vu T. and Ramotar D. (1999) Characterization of amino acid substitutions that severely alter the DNA repair functions of Escherichia coli endonuclease IV. *Biochemistry*, **38**, 3615-23.

101. Toh-e, A., Yasunaga S., Nisogi H., Tanaka K., Oguchi T. and Matsui Y. (1993) Three yeast genes, PIR1, PIR2 and PIR3, containing internal tandem repeats, are related to each other, and PIR1 and PIR2 are required for tolerance to heat shock. *Yeast*, **9**, 481-94.

102. Mrsa, V. and Tanner W. (1999) Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (members of the Pir protein family) in stability of the Saccharomyces cerevisiae cell wall. *Yeast*, **15**, 813-20.

103. Cui, Z. and Mason T.L. (1989) A single nucleotide substitution at the rib2 locus of the yeast mitochondrial gene for 21S rRNA confers resistance to erythromycin and cold-sensitive ribosome assembly. *Curr Genet*, **16**, 273-9.

104. You, H.J., Swanson R.L. and Doetsch P.W. (1998) Saccharomyces cerevisiae possesses two functional homologues of Escherichia coli endonuclease III. *Biochemistry*, **37**, 6033-40.

105. Corral-Debrinski, M., Belgareh N., Blugeon C., Claros M.G., Doye V. and Jacq C. (1999) Overexpression of yeast karyopherin Pse1p/Kap121p stimulates the mitochondrial import of hydrophobic proteins in vivo. *Mol Microbiol*, **31**, 1499-511.

106. Yan, C., Lee L.H. and Davis L.I. (1998) Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *Embo J*, **17**, 7416-29.

107. Kaffman, A., Rank N.M., O'Neill E.M., Huang L.S. and O'Shea E.K. (1998) The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature*, **396**, 482-6.

108. Ames, B.N. (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Radic Res Commun*, 7, 121-8.

109. Torres-Ramos, C.A., Johnson R.E., Prakash L. and Prakash S. (2000) Evidence for the involvement of nucleotide excision repair in the removal of abasic sites in yeast. *Mol Cell Biol*, **20**, 3522-8.

110. Silver, P.A. (1991) How proteins enter the nucleus. *Cell*, **64**, 489-97.

111. Bailly, V., Prakash S. and Prakash L. (1997) Domains required for dimerization of yeast Rad6 ubiquitin-conjugating enzyme and Rad18 DNA binding protein. *Mol Cell Biol*, **17**, 4536-43.

112. Mrsa, V., Seidl T., Gentzsch M. and Tanner W. (1997) Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of Saccharomyces cerevisiae. *Yeast*, **13**, 1145-54.

113. Yun, D.J., Zhao Y., Pardo J.M., Narasimhan M.L., Damsz B., Lee H., Abad L.R., D'Urzo M.P., Hasegawa P.M. and Bressan R.A. (1997) Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. *Proc Natl Acad Sci U S A*, **94**, 7082-7.

114. Bennetzen, J.L. and Hall B.D. (1982) Codon selection in yeast. *J Biol Chem*, **257**, 3026-31.

115. Ikemura, T. (1982) Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and Escherichia coli with reference to the abundance of isoaccepting transfer RNAs. *J Mol Biol*, **158**, 573-97.

116. Chen, G.Q., Shi X.G., Tang W., Xiong S.M., Zhu J., Cai X., Han Z.G., Ni J.H., Shi G.Y., Jia P.M., Liu M.M., He K.L., Niu C., Ma J., Zhang P., Zhang T.D., Paul P., Naoe T., Kitamura K., Miller W., Waxman S., Wang Z.Y., de The H., Chen S.J. and Chen Z. (1997) Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. *Blood*, **89**, 3345-53.

117. Senturker, S., Auffret van der Kemp P., You H.J., Doetsch P.W., Dizdaroglu M. and Boiteux S. (1998) Substrate specificities of the ntg1 and ntg2 proteins of Saccharomyces cerevisiae for oxidized DNA bases are not identical. *Nucleic Acids Res*, **26**, 5270-6.

118. Bruner, S.D., Nash H.M., Lane W.S. and Verdine G.L. (1998) Repair of oxidatively damaged guanine in Saccharomyces cerevisiae by an alternative pathway. *Curr Biol*, **8**, 393-403.

119. Takao, M., Yonemasu R., Yamamoto K. and Yasui A. (1996) Characterization of a UV endonuclease gene from the fission yeast Schizosaccharomyces pombe and its bacterial homolog. *Nucleic Acids Res*, **24**, 1267-71.