

**RNA Polymerase II during Transcript Elongation:
dealing with DNA damage and staying phosphorylated**

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ABSTRACT

This thesis covers two topics related to transcript elongation in *Saccharomyces cerevisiae* - the regulation of the phosphatase Fcp1 and transcription-coupled DNA damage repair.

Formation of RNA polymerase II (RNAPII) complexes throughout the transcription cycle is mediated in part by the phosphorylation state of the C-terminal domain (CTD) of the largest RNAPII subunit. Although a multitude of kinases can phosphorylate the CTD, currently only one CTD-specific phosphatase, Fcp1, has been identified. This work studies the possibility that Fcp1 might be associated with the elongating form of RNAPII. The phosphatase co-fractionates with RNAPII in association with the elongation factor Elongator. Furthermore, genetic studies show that a double mutant that carries a deletion of an Elongator gene as well as a temperature sensitive *fcp1* mutation has a synthetic lethal phenotype at the permissive temperature. In vitro assays using crude extracts demonstrate that the CTD of RNAPII becomes dephosphorylated in a Fcp1-dependent manner. In contrast, in a reconstituted DNA-RNA-RNAPII system, the addition of the purified phosphatase does not stimulate such dephosphorylation. These results indicate a close relationship between Fcp1 phosphatase and the elongating form of RNAPII.

Transcription-coupled DNA damage repair is a term applied to the preferential repair of DNA damage on the coding strand within active genes.

The second half of this thesis describes the characterisation of nucleotide excision repair (NER) of the intrastrand 1,3-(pGpTpG)-cisplatin lesion in *Saccharomyces cerevisiae* as well as an attempt to reconstitute a transcription-coupled NER reaction (TC-NER) in vitro. Using modified yeast extracts, the excision products of the above lesion by NER were found to be between 23 and 26 nucleotides long, via incisions around the 15th phosphodiester bond 3' and the 7th phosphodiester bond 5' of the damage. The attempt to reconstitute TC-NER in vitro was hindered by difficulties with the transcription substrate and the functional instability of purified NER proteins.

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ABBREVIATIONS AND NOMENCLATURE

bp.	basepair
BSA	Bovine Serum Albumin
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl Sulfoxide
DTT	dithiothretol
HEPES	N-[Hydroxyethylpiperazine -N '-[2-ethanesulfonic acid]
IP	immunoprecipitation
mins	minutes
NTP	Nucleotide triphosphate
NP-40	Ethylphenyl-polyethylene glycol
o/n	overnight
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PMSF	Phenylmethanesulfonyl fluoride
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate
Tris	Tris[hydroxymethyl aminomethane]
WCE	whole cell extract

Saccharomyces cerevisiae

<i>GENE1</i>	Wild-type or dominant mutant gene
<i>gene1</i>	Recessive mutant gene
Gene1	Protein encoded by <i>GENE1</i>

CHAPTER ONE: INTRODUCTION

1.1. RNA polymerase complexes through the transcription cycle

The action of RNA polymerase II is the crux of gene expression. The core enzyme, made up of 12 polypeptides, has the capability to synthesise RNA on its own *in vitro*. However, regulated and complete transcription of a protein-encoding gene depends on proper recruitment of the polymerase to the promoter, initiation of the RNA chain and promoter escape, transcript elongation, and, finally, transcript termination and release. These steps all require the assistance of specialised accessory factors, such as the general transcription factors, elongation factors, and termination factors.

The first part of this introduction will follow RNA polymerase II through its transcription cycle and discuss the changes in factors with which it associates as well as in its own chemical composition and structure.

1.1.1 RNA polymerase at initiation

RNA polymerase II (RNAPII) and the general transcription factors (GTFs) form a preinitiation complex at the promoter. *In vitro* studies have shown that RNA polymerase and the GTFs, TFIID, TFIIB, TFII E, TFII H, and TFII F, can assemble in a defined order on the promoter [13, 72, 128]. In addition, these factors constitute the minimal system for transcription initiation at a promoter [14]. This preinitiation assembly alone, however, does not allow a response to transcriptional activators or repressors and requires an additional complex, Mediator, to do so.

Mediator was purified as a protein complex of approximately 20 proteins by Kornberg and colleagues [58]. Seven of Mediator's subunits were novel; yet the remaining had been identified in previous suppressor and mutation screens (Reviewed in [83]). Mutations in *SRB2*, *SRB4*, *SRB5*, *SRB6*, and *SRB7* abate the cold sensitivity of cells carrying a C-terminal domain truncation of RNAPII's largest subunit Rpb1 (CTD) [120]. Some of the other Mediator subunits identified had also previously been isolated in genetic screens: Pgd1 was

isolated as a suppressor of hyperrecombination . Mutations in Rox3 cause aerobic expression of the hypoxic response gene CYC7. Nut1 and Rgr1 participate in gene repression, while Gal11, Sin 4, and Nut2 play roles in both gene activation and repression. And lastly, disruptions in the CSE2 gene cause mitotic missegregation of chromosomes screens (Reviewed in [83]).

Mediator has three biochemical functions. In addition to allowing activation or repression, it stimulates basal transcription 8-fold and CTD phosphorylation by the general transcription factor TFIID 12-fold [58]. The exact mechanisms for each of these activities remain vague, although biochemical data provide grounds for interesting speculation. Protein interaction studies indicate that Mediator consists of modules; one that contains Srb2, Srb4, Srb5, Srb6 and Rox3 and another that has Rgr1, Sin4, Gal11, Pgd1, and Med2 as components [65]. In light of the genetic data, one may suggest that these two modules may act as CTD and activator/repressor interaction sites, respectively. In support of this idea, Mediator-RNAPII holoenzyme purified from *med2*, *pgd1*, or *sin4* deletion mutants could not stimulate activated transcription in a pure, *in vitro* system, yet the Mediator-dependent effects on basal transcription and CTD phosphorylation remained similar to the wild type [84]. Recent results from Sakurai and Fukasawa indicate that Gal11 can associate with both TFIID and TFIIE [100]. Potentially partly through this interaction, Mediator stimulates the last steps of transcript initiation, CTD phosphorylation, and the formation of an open template complex. Nut1 can act as a histone acetyltransferase, which suggests a connection between chromatin structure and transcription activation and initiation; however connections between its biochemical activity and its function within Mediator have not been made [68].

Structural data show that Mediator indeed interacts with the CTD as well as with a secondary site on the polymerase. X-ray crystallography studies by the Kornberg lab reveal that core RNA polymerase II resembles a clamp into which DNA binds [28]. Mediator by itself appears as a compact, triangular structure [6]. Yet upon equimolar addition of RNAPII, Mediator unfolds into an extended state and cradles the back of RNAPII, perhaps to contact the CTD as well as other sites. The stability of the holoenzyme might depend on both types

of RNAPII contacts since co-complexes do not form in the absence of the CTD [6].

All together, the RNA polymerase, the general transcriptions factors, and Mediator associate with the promoter, and transcription starts after the TFIIH-dependent melting of about 14 basepairs surrounding the transcription start site [34]. However, this process typically cannot reach productive RNA synthesis until the addition of the tenth nucleotide. Therefore, RNAPII repeatedly forms and releases short RNA fragments until it forms a transcript which is long enough to confer overall stability to the ternary complex [34]. As inferred from its crystal structure, the polymerase can accommodate a DNA-RNA hybrid corresponding to up to 9 nucleotides of RNA transcript. After this point, the DNA-RNA hybrid separates with the RNA reaching down another groove on the enzyme's surface [94]. Protein crosslinking have shown that this RNA exit channel and the CTD juxtapose each other [32]. This suggests a simple structural basis for CTD dependent regulation of transcription and processing of RNA, which will be discussed in more detail next and in section 1.2.

1.1.2 The C-terminal domain of Rpb1 (Part 1): involvement in transcription initiation regulation and promoter escape

The C-terminal domain of the RNAPII largest subunit Rpb1 contains tandem repeats of the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. The sequence has been conserved through evolution, although the number of repeats varies between species. The mammalian CTD consists of 52 quasi-perfect copies of the repeat, yeast 26-27 copies, and other eukaryotes intermediate between the two (Reviewed in [29]). Truncations leaving fewer than 8 heptapeptide repeats cause lethality in yeast [139]. The CTD is the target for phosphorylation, although the repetitive nature of the sequence has hindered the mapping of specific target residues. Nevertheless, predominant phosphorylation exists on serines, with secondary levels on threonines and tyrosines. Mutation of serines in the CTD also causes lethality in yeast [139].

Phosphorylation of the CTD plays a key role in the regulation of at least two steps of the transcription cycle – the formation of the preinitiation complex and elongation. Normal assembly of the preinitiation complex depends on the

presence of the CTD and preferentially incorporates RNAPII with a hypo-phosphorylated CTD, RNAPIIA [69]. The elongating transcription complex, on the other hand, contains a hyperphosphorylated RNA polymerase, designated RNAPIIO [15, 92]. Data from the Young laboratory suggests that CTD phosphorylation by Srb10/11 prior to preinitiation complex formation inhibits transcription [50]. While in this case CTD phosphorylation acts as a negative regulator, it has a positive role in the recruitment of factors which stimulate elongation [88] and mRNA processing such as 5' capping, splicing, and 3' end formation [75] as will be discussed later.

Although there are many CTD kinases in the cell [11, 124], it is most likely TFIIH (which phosphorylates serine-5 of the RNAPII CTD) that acts at the initiation-elongation transition [38, 39, 50, 112, 124]. Data to support this idea come from strains that carry a temperature-sensitive allele for the TFIIH kinase subunit, *KIN28*. At the restrictive temperature, a decrease in levels of hyper-phosphorylated RNAPII, and therefore likely elongating RNAPII, occurs [21].

The list of kinases able to phosphorylate the CTD and our knowledge of their roles in transcriptional regulation continues to grow. For example, CTD kinase I (CTDK-1) can phosphorylate or regulate the phosphorylation of serine-2 in response to the diauxic shift and cause the stimulation of expression of various genes. Addition of CTDK-I has been found to stimulate transcript elongation in *in vitro* transcription reactions [64]. Ctdk-1 also seems to negatively regulate phosphorylation of serine-5, although whether this activity opposes the action of Srb10/11, TFIIH, or another kinase is unclear [89]. Our knowledge of CTD phosphorylation during promoter escape and elongation, therefore, remains incomplete; regulation of these steps may require other kinases, co-factors, and/or biochemical modifications to the RNA polymerase.

1.1.3 Complexes of the elongating RNAPII

The stability of the elongating RNAPII ternary complex with DNA and the nascent transcript is formidable. The complex remains intact even after treatments in very high salt, purification by gel filtration, precipitation with antibodies, and days-long storage at 4°C without nucleotides (Reviewed in [126]). Since elongation is processive and premature disassociation from the

DNA irreversible, the tenacity of the ternary complex likely helps to ensure the complete synthesis of gene transcripts.

Unlike promoter-specific initiation of a transcript, RNA polymerase II can elongate the transcript without any accessory factors at a rate of 20 nucleotides per second *in vitro* [35]. This process, however, does not occur in a continuous fashion -- the polymerase can either temporarily pause or irreversibly arrest. Pausing during elongation may result from intrinsic characteristics in the DNA sequence and may even exist as a point of regulation, as exemplified by the *Drosophila* heat shock genes *hsp70*, *hsp26*, and *hsp27* [67]. Pausing and arrest may also occur as a consequence of other obstacles. These obstacles include DNA-binding proteins, particularly nucleosomal proteins, which form higher orders of DNA packing, as well as DNA adducts (damage sites). In order to transcribe efficiently, RNAPII can sequester co-factors that either suppress pausing or prevent arrest.

1.1.4 Elongation factors

In yeast, TFIIF (factor g), the only GTF known to stimulate transcription elongation, consists of three proteins of 105, 54, and 30 kDa. The genes of the two largest subunits, TFG1 and TFG2, bear 50% and 51% similarity with the human TFIIF subunits RAP74 and RAP30. Structure function studies have shown that Rap30 binds DNA through its carboxyl-terminal end, while its central moiety interacts with RNAPII to affect elongation. Rap74 also stimulates elongation, yet through its amino-terminus [116]. Dahmus and colleagues have also shown that the amino-terminal end of Rap74 can stimulate dephosphorylation of the CTD by the phosphatase Fcp1 (see section 1.2). The biochemical mechanism by which TFIIF enhances elongation efficiency awaits future clarification.

TFIIS (SII) enables RNAPII to traverse through intrinsic arrest sites as well as nucleoprotein complexes [82]. To accomplish this end, TFIIS binds to arrested RNAPII and activates an endoribonuclease activity in the polymerase that degrades the 3' end of the nascent transcript. It has been proposed that this action not only allows repeated attempts at productive elongation but also the realignment of the 3'-OH of the RNA with the catalytic site of RNAPII [140].

RNAPII can be extracted from its native ternary complexes with DNA and RNA found in chromatin extracts. This polymerase is hyperphosphorylated and is free of the initiation-specific Mediator complex. These characteristics and the stability of the RNAPII-DNA complex in high salt indicate that the polymerase is in its elongating form. RNAPII from these native ternary complexes comes associated with a three-subunit complex, Elongator [88]. Yeast strains lacking the genes encoding the constituents of Elongator, *ELP1*, *ELP2*, and *ELP3*, all show slow-adaptation phenotypes as well as temperature and salt sensitivity [40, 88, 142]. These mutants also have delays in transcriptional activation. For example, upon the galactose induction of *GAL1-10* gene, *elp* knockout cells accumulate less than an eighth of the wildtype mRNA levels after half an hour. However, this reduction in transcription is not persistent because similar mRNA levels accumulate in both mutant and wildtype cells after 2-4 hours. This delay is also observed with *INO1*, *PHO5*, and *ENA5*, which are all inducible genes, yet not with constitutive genes such as *RPB2*, *TUB2*, and *ACT1* [88].

The elongator subunits have a genetic interaction with TFIIS. These double mutants are sensitive to the drug 6-azauracil, which blocks nucleotide metabolism and thereby depletes intracellular GTP and UTP pools and hinders transcript elongation[88]. Elongator indeed affects transcript elongation biochemically since the protein complex has been shown to increase the processivity of RNAPII on chromatin templates [J. Svejstrup (ICRF), unpublished data].

The precise function of the Elongator subunits Elp1 and Elp2 are unknown. Elp3, on the other hand, is a histone acetyltransferase [142]. The HAT activity of Elongator functionally overlaps with that of the SAGA complex, which remodels chromatin at promoters [127, 141]. Hence, the role of Elongator might be to condition the chromatin to allow RNAPII access to its DNA template [141].

Yeast Spt16/Cdc68 and its human counterpart FACT also aid elongation by interacting with nucleosomes [73]. Mutation in Spt16 and Cdc68 actually suppress the sensitivity of TFIIS mutants to 6-azauracil [87]. This phenotype may result from a reduced processivity of RNAPII that would allow extra time for transcription through potential barriers. Purified FACT stimulates *in vitro* transcription on remodelled chromatin templates. Orphanides et al. attribute

this activity to FACT's interactions with the H2A/H2B tetramer which could promote nucleosome disassembly during transcription [86, 87].

The identification of several elongation factors stems from studies using the transcription inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). Although originally proposed to inhibit transcription initiation, DRB in all likelihood inhibits transcription by enhancing transcriptional pausing and premature termination of transcription both *in vivo* and *in vitro* [20]. DRB inhibition appears to result from the inhibition of one or more protein kinases required for transcription, such as the TFIIF kinase and pTEFb [145, 146]. This compound therefore affects the phosphorylation of the CTD and subsequently blocks the transition between initiation and elongation. DRB, however, cannot inhibit transcription in a partially purified system but requires the DSIF (DRB sensitivity-inducing factor) to do so [134].

DSIF, when purified from HeLa cells, consists of two polypeptides, p14 and p160. Sequence analysis show that the former is a human homologue of the yeast Spt4 protein and the latter of yeast Spt5 [134]. Mutations in *SPT4* and *SPT5* can be suppressed by mutations *rpb1* and *rpb2*, the latter of which has been characterised to have defects in transcript elongation *in vitro*. In addition, many mutations in *spt4* and *spt5* confer 6-AU sensitivity [48]. The addition of DSIF and its cofactor NELF into *in vitro* transcription reaction without DRB induces transcriptional arrest [134]. This negative regulation affects transcripts longer than 40 nucleotides and occurs via interactions with RNAPII and regulation by the phosphorylation state of the CTD. Recent work by Wada *et al.* proposes a novel transcriptional regulation network in which the FACT complex, in conjunction with the CTD-kinase P-TEFb, counterbalances the DSIF/NELF-dependent transcription inhibition. This group also were able to reconstitute *in vitro* transcription from the adenovirus major late promoter with the general transcription factors TBP, TFIIB, TFIIE, and TFIIF along with DSIF, NELF, P-TEFb and FACT – the latter two complexes replacing the requirement for TFIIF kinase [133]. In this case, FACT has a role in transcript elongation independent of its interactions with nucleosomes.

ELL and ELL2 are two related proteins that stimulate transcript elongation by suppressing transient pausing by RNAPII at various sites on the

DNA [109-111]. This activity is independent of any other factors and requires direct interactions with RNAPII, its template, nascent transcript, or some combination of the three. ELL can bind tightly with RNA polymerase II in solution, and mutants that are not capable of this association are also not able to stimulate elongation [109]. ELL has been found *in vitro* to inhibit RNAPII from assembling with the TATA-binding protein and TFIIB and entering into the pre-initiation complex. However, this activity depends on ELL functional domains that are distinct from those required for elongation. ELL may have a potential role in cancer, and specifically acute myeloid leukemia [109]. In this disease, it has been found that the human ELL gene undergoes a translocation and forms a chimera with another gene called MLL. Although the exact biochemical nature of the chimera protein is not known, introduction of the chimeric protein into mouse myelomonocytic precursor cells led to immortalisation and leukemic transformation (Reviewed in [26]).

Elongin A can stimulate transcript elongation, yet its activity is increased significantly when the protein is complexed with elongin B and elongin C [5, 27]. Elongin B and C can form a complex on their own which binds to the von Hippel-Lindau tumour suppressor gene product, VHL. Upon association with VHL, elongin B and C cannot bind to elongin A and stimulate elongation[90]. Those patients with von Hippel syndrome suffer from a variety of cancers and produce a mutant form of VHL, which cannot bind elongin B/C. The identification of those genes affected by shift in elongin b/c binding steady state will provide insight into the mechanism of tumourigenesis associate with this syndrome (Reviewed in [26]).

DNA damage causes RNAPII to stall and may sequester a separate group of protein complexes to co-ordinate transcription and DNA damage repair. This topic will be discussed in more detail in Section 1.3.

1.1.5 RNA processing and transcript termination

During the synthesis of the nascent transcript, two processing events occur – mRNA capping and splicing. Capping occurs only on RNAPII

transcripts and enhances splicing, transport, translation, and stability of RNA [95]. Caps are made by the conversion of 5'-triphosphate to 5'-diphosphate ends, the addition of GMP, and methylation. In *Saccharomyces cerevisiae*, these modifications require the activities of the RNA triphosphatase Cet1, the RNA guanylyltransferase Ceg1, and the methyltransferase Abd1 [149]. Ceg1 and Abd1 have been observed to associate with the phosphorylated RNAPII CTD both *in vitro* and *in vivo* [18, 75, 102]. Interestingly, *in vitro* this interaction between phosphorylated CTD and Ceg1 inhibits the guanylylation activity of Ceg1, and this block is relieved by the formation of the Ceg1/Cet1 heterodimer [75]. *In vivo*, the Ceg1/Cet1 complex is associated with early elongating polymerase [102]. Abd1, on the other hand, also associates with the phosphorylated CTD independently of the Ceg1/Cet1 heterodimer and travels as far as the 3' end of the gene [102]. The association of both Ceg1/Cet1 and Abd1 with the CTD depend on the TFIIH kinase Kin28, and their disassociation dependent on the CTD phosphatase Fcp1 [18, 102].

The removal of introns occurs within the spliceosome, which consists of small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins. The latter group includes members of the serine/arginine-rich (SR) protein family, which also embraces a set of nuclear matrix factors that interact with the CTD. *In vitro* splicing reactions are inhibited by the addition of CTD peptide or anti-CTD antibodies [150]. Furthermore, inhibition of splicing by the overexpression of phosphorylated CTD and the precipitation of the hyperphosphorylated RNAPII by anti-SR antibodies implicate that elongating polymerase, and in particular the phosphorylated CTD, provides a platform for the binding of RNA processing factors [33].

Before transcript termination, the nascent transcript becomes cleaved and polyadenylated at the 3' end. In yeast, this requires at least three sequence elements (Reviewed in [151]). The UA-rich element, typically of the sequence UAUAUA and defined as the efficiency element, sits at a variable distance from the cleavage site and increases the efficiency of 3' end formation. The A-rich element follows and positions cleavage approximately 20 nucleotides downstream. These A-rich positioning elements, though, function only with an efficiency element present. Polyadenylation then occurs at the PyA_n element, or Poly(A) site. Many yeast genes utilise several PyA_n elements,

while in higher eukaryotes a single AAUAAA site is sufficient. If the A-rich element is mutated in yeast, polyadenylation sites become more scattered. Although the protein complexes required for cleavage and polyadenylation are well characterised in higher eukaryotes, only recently have the details concerning the yeast machinery been described. The factors CFIA, Hrp1, and CFII form a minimal system for accurate RNA cleavage. By analogy to the mammalian system, CFII binds to the positioning element and cleaves at the PyA_n element. Hrp1(CFIB) stabilises the positioning of the cleavage complex and also interacts with the UA-rich motif where CFIA binds. PFI and Pab1 then associate to form a polyadenylation holoenzyme which adds the poly(A) tail (Reviewed in [151]). There are indications that factors in this complex may interact with RNAPII. In the mammalian system, the CTD may stabilise the interaction between complexes which recognise the efficiency and positioning elements [74]. Moreover, addition of phosphorylated CTD conferred more cleavage activity than the addition of hypophosphorylated form in an *in vitro* RNA cleavage system [52]. In yeast, truncations of the CTD effect the 3' processing of some genes and not others; therefore the role of the CTD in this activity in yeast is still not fully defined [76].

3' end processing and RNAPII processivity determine the site of transcript termination. Efficiency of termination depends on the strength of the polyadenylation signal and the presence of a termination site, which may, in some cases, cause transcriptional pausing. Transcription run-on experiments allow the measurement of the number of polymerases along a specific template. Under normal conditions, the polymerase density decreases downstream of the termination site. In temperature-sensitive CFIA mutants, an increased number of RNAPII reads through the termination site under non-permissive conditions [8]. In similar run-on experiments in *Schizosaccharomyces pombe*, deletion of known pause sequences also caused increased density of RNAPII past the termination site [7]. Recent experiments in a mammalian *in vitro* system by Yonaha and Proudfoot suggest that RNAPII needs to reach a pause site to allow time for the termination mechanism to occur. Pre-mRNA cleavage occurs in the presence of a ternary complex (nascent RNA, DNA template, RNAPII). The 3' RNA cleavage product remains associated in the ternary complex but

quickly becomes degraded. Subsequent to this degradation, the elongation complex destabilises and RNAPII is released from the template [147].

1.2 C-terminal domain of Rpb1 (part 2) – phosphorylation cycles, the phosphatase, and other regulatory pathways

As discussed in the Section 1.1, RNAPII interacts with many factors during the transcription cycle via its CTD. Phosphorylation of this domain plays a pivotal role in regulation, from the formation of the pre-initiation complex to transcription termination. Yet for this regulation to occur, phosphorylation must be counterbalanced by dephosphorylation. In addition, since phosphorylation persists through elongation until pre-mRNA cleavage, a phosphatase must act upon RNAPII to return it to the hypophosphorylated form so that it may assemble at the promoter. The negative regulation imposed by Srb10/11 must also be reversed by a CTD phosphatase [29].

1.2.1 Fcp1 – The CTD-phosphatase

Fcp1 is the only CTD-specific phosphatase identified to date. First purified from HeLa cells and then from yeast, Fcp1 can interact with RNAPII via docking sites on the polymerase that are distinct from the CTD. The active site of Fcp1 contains the phosphatase/phosphotransferase motif DXDX(T/V), which occurs in enzymes such as human phosphomannomutase and L-3-phosphoserine phosphatase [24]. In yeast, deletion of *FCP1* or mutation of either aspartic acid residue in the above motif are lethal. In biochemical studies with purified proteins, mutation of the first aspartic acid in the motif confers total inactivity of the enzyme; phosphatase carrying a mutation of the motif's second aspartate still has 40% of wildtype activity. These data suggest that the first aspartic acid acts as the catalytic residue as it does in the other proteins studied with this DXDX(T/V) motif [60].

TFIIF, and more specifically the Rap74 subunit, stimulates the activity of the phosphatase. TFIIB can inhibit this TFIIF-modulated CTD stimulation although it has no effect on Fcp1 activity on its own [4, 17]. Both these factors

bind to the same two independent sites on Fcp1, although with different affinities. While the stronger binding site for TFIIB lies within Fcp1 residues 457 to 666, that for TFIIF lies between amino acids 667 and 732. In addition, regions of TFIIB and Rap74 that interact with Fcp1 also share the motif sequence – KEFGK. Within this sequence, the lysines prove important for Fcp1 binding, and a double substitution mutant is not viable. Interestingly, the residues 499 to 593 of Fcp1 encode a predicted BRCT domain. Some mutations in this BRCT domain confer temperature-sensitivity. At the permissive temperature, these ts-strains had a greater amount of hyperphosphorylated RNAPII than the wildtype. Under non-permissive conditions, the levels of hypophosphorylated Rpb1 quickly dropped below detection [59].

During the purification of Fcp1 from yeast, Chambers and Kane found that the addition of a side fraction could increase the phosphatase activity by over 300 fold. Although this fraction contained TFIIF, addition of purified TFIIF could provide only a 3-5 fold stimulation [16]. Although the initial study regarded the missing factor essential for Fcp1 activity, Kobor et al. found that recombinant Fcp1 purified from baculovirus did not require any additional factors for activity. In these experiments, Fcp1 could dephosphorylate 90% of its substrate when the phosphatase and RNAPII were in equimolar concentrations. Additions of TFIIF and the stimulatory factor did cause Fcp1 to become more catalytic [60]. This missing factor, termed FcpX, has persisted as a focus of purification efforts in the Kane laboratory.

Studies by Lehman and Dahmus show that human RNAPIIO elongating from the adenovirus-2 major late promoter demonstrate a differential resistance to dephosphorylation by Fcp1. As RNAPIIO progresses through the transcript, it becomes more resistant to the phosphatase. Treatment with Sarkosyl causes the elongating polymerase to be susceptible to Fcp1, and the addition of nuclear extract restores the resistance. If RNAPIIO transcribes off of a dC-tailed template, which does not require initiation at a promoter and the addition of general transcription factors, the CTD is not protected from Fcp1 and becomes dephosphorylated to similar levels as free RNAPIIO. These results suggest that RNAPIIO changes conformation as it elongates and that another nuclear factor modulates CTD phosphorylation/dephosphorylation [66].

Although these *in vitro* experiments indicate that RNAPII elongating from a promoter is protected from Fcp1-mediated dephosphorylation, chromatin immunoprecipitations show that serine-5 phosphorylation of the CTD decreases in a Fcp1 dependent fashion as RNAPII moves from 5' to 3' of a gene. In addition, serine-2 phosphorylation of the CTD remains unchanged [61, 102]. The contradiction between *in vivo* and *in vitro* results calls for further investigation on the regulation of dephosphorylation during elongation.

1.2.2 Other modifications to the CTD

Although much of the current research focuses on CTD serine phosphorylation, several other modifications occur on the domain and may bear significance in transcriptional regulation. Two kinases, c-Abl and Arg, can phosphorylate the tyrosine of the CTD. The product of the *c-abl* proto-oncogene stimulates transactivation by GAL4-VP16, and this activity depends on both the DNA-binding and the tyrosine kinase domains. Furthermore, the addition of the retinoblastoma protein blocks the kinase and the supertransactivation by c-Abl, although it does not effect transactivation of GAL4-VP16 alone [138]. Both TFIIH and Ctdk1 can phosphorylate threonines. In studies of the human TFIIH and CTDK1, these enzymes phosphorylated serine and threonine in ratios of 9:1 and 30:1, respectively [91].

The C-terminal domain can also be glycosylated. The addition of the carbohydrate N-acetylglucosamine can occur on the hydroxyl group of serines and threonines. The modification in mammalian RNAPII occurs on fourth residue of the heptad repeat, which is either a serine or threonine, and cannot be detected in RNAPIIO. Hence, in this regard, the CTD can exist in three states: unmodified, glycosylated, or phosphorylated. The role for CTD glycosylation remains unknown. However, considering the mutual exclusiveness of the glycosylated and phosphorylated states, O-GlcNac might aid in the interactions with transcription factors during pre-initiation and is removed before elongation [25, 57].

Prolines in the CTD provide substrate for peptidyl-prolyl *cis/trans* isomerases (PPIases), which catalyse the rotation about the peptide bond proceeding a proline. Such isomerisation can cause significant changes in

protein structure and surface contour and subsequently affect protein-protein interactions. Hence, PPlases can regulate the activity of proteins by affecting active complex assembly (Reviewed in [53]). The PPlase Ess1 specifically targets the CTD and interacts with the form of RNAPII phosphorylated on serine 2 [80, 144]. The isolation of this enzyme evolved from a genetic screen for *trans*-activating factors involved in 3'-end formation of pre-mRNA [46]. Five of the six suppressors of the yeast *ess1* temperature-sensitive mutants are or may be involved in transcription. The most common positive in the suppressor screen has high homology with *Drosophila* TFIIIS. Another suppressor encodes a homologue of a subunit belonging to the human Sin3A-Rpd3 histone deacetylase complex. Overexpression of the gene encoding the Rpb7 subunit also suppresses the temperature-sensitive phenotype. But most interestingly in the context of the CTD, high-copy expression of the CTD phosphate gene *FCP1* suppresses the *ess1* mutants. Furthermore, *ESS1* overexpression could not suppress *fcp1* mutants, indicating that *FCP1* acts genetically downstream from *ESS1*. Wu *et al.* hypothesise that Ess1 may regulate phosphorylation/dephosphorylation through CTD isomer-specificities of kinases and phosphatases [144].

1.3 DNA damage, transcription, and repair

Many DNA adducts obstruct RNAPII transcription. Current hypotheses suggest that the stalled polymerase acts as a sequestering signal to repair mechanisms in the cell since, for many damages, injury to the transcribed strand of active genes is repaired faster than the same type of lesion in the global genome. So far, two pathways, base excision and nucleotide excision repair, have been shown to have distinct transcription-coupled sub-pathways. In addition, in human cells, these transcription-coupled systems share several protein complexes, which may provide insight into the mechanisms [62]. However, before discussing the DNA damage repair in the context of gene expression, the basic pathways must be introduced.

1.3.1 DNA damage repair – an overview

Natural products of every-day intracellular life as well as exogenous chemical assailants attack the DNA on a regular basis. Ubiquitous water causes the spontaneous hydrolysis of nucleotide bases while reactive oxygen species from aerobic metabolism lead to potentially mutagenic oxidation of DNA. Similar reactive oxygen species may evolve from an electron transfer off of a foreign redox-cycling compound such as paraquat, which was once a common herbicide. Other common environmental hazards such as benzo[a]pyrene, found in cigarette smoke and charcoal-broiled beef, and aflatoxins, a mold product found on edible nuts (peanuts, pistachios, almonds), oil seeds, and grains, form covalent adducts on DNA. The high-energy UV rays such as those from the sun cause chemical modifications such as pyrimidine-pyrimidine dimers. If these damages are not repaired, their accumulation can lead to defects in cellular regulation and eventually to cancer. The well-established links between sun exposure and skin cancer as well as tobacco use and lung cancer prove this case (Review in [2]). To tolerate the constant formation of lesions, DNA relies on the cell's repair system to maintain its genetic integrity.

Repair of chemical DNA damage may follow either of two general mechanisms – the reversal of the chemical modification or the excision of the DNA damage. As an example of the first category, photolyase in lower organisms such as *E. coli*, *H. halobium*, and *S. cerevisiae* reverses pyrimidine-pyrimidine dimers by absorbing a photon by one of its chromophores, and the subsequent excited state transfers to the catalytic co-factor FADH⁻, which initiates the monomerisation (Reviewed in [41]). Methylation damage repair also occurs through a reversal mechanism. For example, O⁶-methylguanine transferase accepts methyl groups from the O⁶ position of guanines onto one of its cysteine residues; although this allows the DNA to return to its native chemical state, it also causes the irreversible inactivation of the transferase. Depending on the type of damage, the excision pathways may result in the removal of a single base or of a stretch of nucleotides. DNA polymerase and ligase resolve the resultant gap. The base excision repair pathway (BER) removes the single damaged base and repairs many oxidative base damages and alkylation damage such as 3-methyladenine. The details of

the versatile nucleotide excision repair pathway and protein complexes involved therein will be described in the next section.

1.3.2 Nucleotide excision repair

Base excision and damage-reversal repair pathways use enzymes that are specific and selective for the damages they recognise and remove, as the vast number of DNA glycosylases required for BER clearly demonstrates. The clearance of bulkier and more varied DNA damage relies on the nucleotide excision repair pathway. In *E. coli*, the main players include the gene products of *UvrA*, *UvrB*, and *UvrC* (Figure 1.3.2). Mutants of these genes show sensitivity not only to UV irradiation, but also to a myriad of other compounds such as mitomycin, nitrogen mustard, psoralen, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The proteins UvrA and UvrB associate into a complex UvrA₂UvrB₁ and then bind to DNA; both steps require the hydrolysis of ATP. The current model envisions that this complex binds at a DNA site removed from the damage. UvrA₂UvrB₁ then patrols around the DNA for damage in a unidirectional manner courtesy of its 5'-3' helicase activity, which can be inhibited by bulky base damage. This inhibition leads to the docking of the UvrA₂UvrB₁ complex on the damage, the ATP-hydrolysis dependent release of the UvrA dimer, and the sequestering of UvrC. UvrB/UvrC then invoke incisions to each side of the damage. The spacing between the 5' and 3' cuts varies depending on the types of damages and the DNA sequence, yet the gap size averages around 12 nucleotides in length. UvrD, or Helicase II, directs the displacement of the subsequent damaged oligonucleotide and release of UvrC. The removal of UvrB from the gapped DNA occurs upon repair synthesis by PolI, and the 3' nick is sealed by DNA ligase.

NER in eukaryotes require more than 20 proteins. In the *S. cerevisiae* model, Rad14 and Rad4 initiate repair by recognising DNA damage. Rad23 can stimulate this activity *in vitro* [45]. The helicase activity of core TFIIH catalyses the formation of a DNA bubble, and RPA aids in stabilising this open complex [36, 37]. Rad1/Rad10 and Rad2 respectively incise 5' and 3' of the damage [44].

Gentle purification of a tagged TFIIH yields a multimeric complex that includes at least Rad14, Rad4, Rad1, Rad10, and Rad2. Addition of this complex can reconstitute NER activity of whole cell extracts made from mutants deleted of any of these constituents [115]. These data suggests that, unlike in the *E. coli* system, the proteins required for NER in eukaryotes can assemble into a so-called repairosome independent of DNA damage. A more recent affinity purification of a histidine-tagged Rad14 from nuclear extracts produces an even larger complex that includes the aforementioned proteins as well as Rad7, Rad16, Rad23, Rpb1 and RPA. Furthermore, the fraction containing these proteins has functional activity in that it can carry out the incision step of NER. However, the results from the tagged Rad14 bear a caveat since the activity was assayed only after a single purification step and several independent complexes containing NER proteins could have co-purified in this process [98]. Controversy surrounds the repairosome model as not all groups can purify TFIIH in association with any of the NER proteins. In addition, Guzder *et al.* show with purified proteins that there exists no physical association between TFIIH and a complex designated nucleotide excision repair factor 1 (NEF-1) containing Rad1, Rad10, and Rad14. As an alternative to the repairosome model, Guzder and colleagues propose that the protein complexes NEF-1, NEF-2 containing the Rad4-Rad23 complex, NEF-3 consisting of Rad2 and TFIIH, and NEF-4 including Rad7-Rad16, assemble sequentially at the site of damage [43].

The identification of NER proteins in man provides an interesting historical prospective. Hebra and Kaposi first described the disease xeroderma pigmentosum (XP) in 1870. The disorder is characterised by skin photosensitivity and high incidence of skin cancer among other clinical characteristics. In 1968, Cleaver UV-irradiated skin fibroblast cells from XP patients prior to a three hour incubation with tritiated thymidine, fixation, and exposure to film. The subsequent autoradiographs allowed the visualisation of de novo DNA repair synthesis. These experiments led to the conclusion that XP cells could not repair DNA damage as efficiently as normal cells [23]. Later experiments found XP mutations can be divided into seven complementation groups, XP-A to XP-F and XP-V.

Nucleotide excision repair in humans follows a similar mechanism to that in yeast. Either XPC-hHR23B (human homologues of *S. cerevisiae* Rad4-Rad23) or RNA polymerase initially sense the damage. Which particular sensor participates depends on whether the damage occurs on the transcribed strand of an active gene or elsewhere on the genome and will be discussed in more detail later. XPA (Rad14) binds to the damage, while the TFIIH helicases, XPB and XPD, asymmetrically unwind the DNA. Structure dependent endonucleases, XPG (Rad2) and XPF-ERCC1 (Rad1-Rad10), respectively, incise 3' and 5' of the damage near the junction of single- and double-stranded DNA. The resultant oligonucleotide product ranges between 24 and 34 nucleotides long. The DNA gap is filled in by either a polymerase δ or ϵ holoenzyme in co-operation with replication proteins PCNA, RPA, and RFC. Lastly, a DNA ligase, most probably DNA Ligase I, seals the nick (Reviewed in [143]). The existence of a mammalian repairosome is also controversial. Most experiments base their conclusions on co-immunoprecipitations and pull-down experiments, which may only indicate physical affinity between proteins rather than required pre-assembly (Reviewed in [3]). More studies are required to clarify these issues.

1.3.3 Transcription-coupled nucleotide excision repair in *E. coli* – a paradigm?

Although preferential repair of transcriptionally active genes was discovered first in mammalian cells, the mechanism in eukaryotes remains a conundrum. *E. coli* TC-NER presents a better defined mechanism and acts as a possible paradigm for the pathway.

Selby and Sancar initially tested the hypothesis that RNA polymerase stalled on a DNA damage would act as a signal for repair. However, this polymerase actually inhibited the activities of purified *UvrA*, *UvrB*, and *UvrC*. The search for a factor that could alleviate this inhibition and direct strand specific repair led to the identification of the “transcription-repair coupling factor,” or TRCF. The gene *mdf⁺*, which encodes TRCF, had been identified in the 1970s - twelve years before Selby and Sancar discovered TRCF. At that

time, Bockrath and colleagues had already concluded that MDF was involved in the excision repair of premutational lesions located only in the transcribed strand of DNA [9].

TRCF can bind to both the RNA polymerase and the UvrA₂UvrB₁ complex. The binding of TRCF to the RNAP-RNA-DNA ternary complex causes its disruptions and the release of the polymerase. By its interactions with UvrA, TRCF recruits UvrA₂UvrB₁ to damage and then disassociates with the UvrA dimer so NER may occur as described earlier.

1.3.4 Transcription-coupled nucleotide excision repair in eukaryotes

The suggestion that cells could preferentially repair damage in active genes emerged after observations of the “rodent repair paradox.” Twenty-four hours after UV irradiation, rodent cells in culture remove only 10-20% of their pyrimidine dimers while human cells have cleared the majority of these lesions. However, rodent cells prove resistant to killing by UV. Bohr and Hanawalt hypothesised that these cells selectively repair DNA damage in regions essential for survival, which are active genes [10]. Furthermore, this efficient repair is specific for the transcribed strand of active genes [77, 78].

Cells from patients with Cockayne’s syndrome (CS) lack transcription-coupled nucleotide excision repair (TC-NER), while their repair of damage in the global genome is similar to that in normal human cells [131]. CS breaks down into at least five complementation groups. CS-A and CS-B are individual to the syndrome, while the other three overlap with XP (XP-B, XP-D, and XP-G). The helicases XPB and XPD belong in the TFIIH complex, and extracts made from XP-B/CS cells show reduced RNAPII transcription. A case comparison between an XP-G/CS patient and two XP siblings suggest links between CS and protein interactions with the XPG protein [85]. A recent study by La Page *et al.* indicates that transcription-coupled base excision repair also require CS-B, XPB, XPD, and XPG [62].

Drawing parallels from the *E. coli* paradigm, the CS-A and CS-B could potentially have the same function as TRCF. CSA belongs to the WD-repeat protein family, the members of which regulate a variety of cellular functions such as cell division, signal transduction, and mRNA modification; it can interact

with the p44 subunit of TFIIH and CSB [51]. CSB contains putative ATPase/helicase motifs resembling the SWI/SNF protein family, which include the chromatin remodeling complex SWI2/SNF2 [125]. CSB can bind to the damage recognition protein XPA, TFIIH, TFIIIE, and XPG [54] [108]. Furthermore, after the hydrolysis of ATP, CSB associate with the RNAPII-RNA-DNA ternary complex [118]. Unlike the *E. coli* TCRF, CSB does not enable in the disruption of the polymerase ternary complex but rather sequesters TFIIH to the stalled polymerase [108] [117].

Although the above interaction experiments support an eukaryotic TC-NER model similar to that in the *E. coli*, they cannot provide a mechanism to resolve the *in vivo* results found with the carcinogen N-acetoxy-2-acetylaminofluorene (NA-AAF). When XP-D and XP-D/CS cells are exposed to this compound, prolonged RNA synthesis repression is observed in the XP-D/CS but not in the XP-D cells. Albeit both cell lines are repair deficient, the XP-D/CS cells manifest additional difficulties in transcription in response to DNA damage. Hence, the defect in TC-NER found in CS cells might reflect deficiencies in RNA synthesis rather than the lack of repair-specific functions. Consistent with the existence of a link between CS and transcription, CSB can stimulate the rate of RNA polymerase II elongation on a promoter-independent, dC-tailed template in the absence of additional transcription factors [107]. Furthermore, recent data presented by Rockx *et al.* showed that CS cells contain a reduced level of hypophosphorylated RNA polymerase II after UV irradiation. Since the pre-initiation complex recruits only RNAPIIA, its decrease would potentially lead to a reduction in transcript initiation and the observed RNA synthesis defect [97].

The mechanism of transcription-coupled repair may include yet another element, that of ubiquitination. After UV-irradiation, RNA polymerase II, and specifically the hyperphosphorylated form, becomes ubiquitinated in normal cells. This modification does not occur in CS cells unless transformed with the appropriate complementing CS gene [12]. Furthermore, proteasome inhibitors block an UV-induced decline in RNA polymerase II levels [96], perhaps indicating that CS-dependent RNAPII-ubiquitination leads to degradation of the polymerase. If RNAPII ubiquitination is an obligatory step in TC-NER then that

presumably also means loss of both polymerase and its nascent transcript from an active gene.

Hanawalt and co-workers have suggested a mechanism for TC-NER that allows RNAPII to remain on its template. In this model, the nascent transcript of RNAPII stalled at a damage may become a substrate of TFIIIS-induced cleavage, which allows the polymerase to reverse on the DNA away from the lesion. This action provides space for the NER machinery, or photolyase as in the case of the experiments presented by Tornaletti *et al* [123]. In this scenario, the polymerase remains transcription competent and may continue to elongate after repair. However, data from Brouwer and co-workers has shown that TFIIIS is not essential for TC-NER in yeast {Verhage, Heyn, et al. 1997 #3167}.

In *S. cerevisiae*, Rad26 is the homologue to human CSB, and deletion of *RAD26* does indeed affect transcription-coupled NER [129]. As in the case of CSB, the mechanism by which Rad26 affects TC-NER is unclear. Questions also remain regarding to where along the transcript the requirement for Rad26 begins. While investigations of repair in the *URA3* locus showed that TC-NER requires Rad26 only during transcript elongation, studies in the *MFA2* gene demonstrate that Rad26 is necessary for TC-NER even during initiation [119, 122]. These differences may reflect relative transcript levels and possibly the chromatin structure in the promoter region – both of these factors can affect the accessibility of DNA to protein complexes. In support of this hypothesis, recent data by Citterio *et al.* demonstrate that CSB can bind DNA and alter double helix conformation [22]. In addition, it can remodel chromatin, an activity which is severely reduced in the absence of histone tails. Possibly CSB has two roles: one which is the rearrangement of chromatin to facilitate access to DNA damage by the repair proteins and the second which is to weaken the RNAPII-DNA interaction and stimulate the displacement or removal of the stalled polymerase [22].

In addition to the CS group of proteins, other factors may play roles in TC-NER. In the absence of Rad26, a residual level of preferential repair remains [132] yet the responsible factors have not been uncovered in *S. cerevisiae*. Transcription factor 2 (N-TEF), an elongation factor originally identified in *Drosophila*, can release RNAPII stalled at a thymine cyclobutane

dimer, and Hara *et al.* suggest that it might be the functional counterpart of *E. coli* Mfd [47].

1.4 Open questions and the scope of this thesis

The phosphorylation state of the CTD is integral in the regulation of not only transcription but also in activities intimately linked to transcription. The first half of this thesis focuses on CTD dephosphorylation by the *S. cerevisiae* Fcp1 phosphatase. The enzyme associates with the elongating, hyperphosphorylated polymerase as shown through co-purification. Ternary complexes isolated from chromatin fractions also carry Fcp1 and remain insensitive to Fcp1 unless RNA polymerase is encouraged to run off its template. *In vitro* transcription-phosphatase assays demonstrate that yeast Fcp1 is unable to dephosphorylate the polymerase while it is in a ternary complex with DNA and RNA. Under the conditions here, this resistance is independent of any other factors, and dephosphorylation cannot be stimulated by TFIIS or Elongator.

The second half of this work presents studies on nucleotide excision repair with the goal of establishing a biochemical TC-NER assay. A method for making whole cell extracts competent for transcription and repair as well as other biochemical functions is described. Using these extracts, yeast NER of a specific cis-platin damage is characterised and compared human NER. Furthermore, methods are established that allow the fast assessment of extracts and protein fractions for DNA for NER activity. These methods are combined during the purification of a large and active NER complex. Lastly, constructs of TC-NER DNA substrates are described.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

Materials were obtained from Sigma or BDH, unless stated otherwise, at the highest grade available. Most molecular biology techniques were performed according to [101] while yeast manipulations were according to either [1] or [99]. Modified or less standard techniques are described below.

2.2 Frequently used buffers

3x SDS sample buffer

Glycerol	20% (v/v)
2-mercaptoethanol	15% (v/v)
SDS	9% (w/v)
Tris-HCl pH 6.8	187.5mM
Bromophenol Blue	0.1% (w/v)

100x Protease Inhibitor Cocktail

Leupeptin	28.4 µg/ml
Pepstatin A	137 µg/ml
PMSF	17 mg/ml
Benzamidine	33 mg/ml

Formamide loading buffer

Formamide	90% (v/v)
Tris-borate	90 mM
EDTA	1 mM
Bromophenol Blue	0.05% (w/v)
Xylene Cyanol	0.05% (w/v)

1x Phosphatase Inhibitor Cocktail

EDTA	5 mM
EGTA	2 mM
Sodium Fluoride	5 mM
Potassium	
Phosphate, pH 7.5	5mM
Sodium	
Pyrophosphate pH 7.5	5mM
Okadaic Acid	25 nM

2.3 Biological strains

Table 2.3.1 Yeast Strains

W303-1a	ade2-1, trip1-1, can1-100, leu2-3, leu2-112, his3-11, his3-15, ura3,
DC14	mat a

DC17	MAT a
YMK110	MAT α W303a, fcp1::LEU2, fcp1 W575A:pRS314 [TRP]
BJ2168	MAT a, leu 2, trp1, ura3-52, prb1-1122, pep 4-3, prc1-407, gal2
W303 ELP3 Y540A	W303 MAT α elp3::ELP3 Y540A::LEU2

Table 2.3.2 *E. coli* Strains

TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F[traD36 proAB⁺ lacI^q lacZΔM15]</i>
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2.4 DNA Manipulation

Mini preps

E. coli cultures were grown overnight in 5 mls LB containing 100 μ g/ml ampicillin and then serially pelleted in an eppendorf. Cells were resuspended in 150 μ l Solution I, vortexed, and allowed to sit at room temperature for 5 minutes. Cells were then lysed by the addition of 300 μ l Solution II, gently inverted several times, and incubated on ice for 5 minutes. 200 μ l Solution III was then added, and the mixture vortexed vigorously and placed on ice for an addition 5 minutes. The slurry was centrifuged at 14,000 rpm for 3 minutes., and the clear supernatant transferred to a fresh eppendorf tube containing 4 μ l 10 mg/ml Ribonuclease A (RNase A), approximately 20 Kuntz units, and incubated for 15 minutes at 37°C. The resultant DNA solution was then extracted with 500 μ l 1:1::equilibrated phenol (pH 8.0):chloroform, and the aqueous layer precipitated by the addition of 350 μ l isopropanol, incubation at –20°C, and centrifugation at 14,000 rpm for 10 minutes. The pellet was washed with 70% ethanol and then resuspended in 30 μ l TE.

Solution I

Tris-HCl pH 7.5	25 mM
EDTA	10 mM
Sucrose	15% (w/v)

Solution II

NaOH	200 mM
SDS	1% (w/v)

Solution III

Potassium Acetate, pH 5 3M

PCR

PCR reactions were performed in a DNA Engine (GRI) thermal cycler in 50 µl reaction volumes. Templates were either plasmids or yeast chromosomal DNA, and oligonucleotides were as listed in Table 2.4. The template was denatured by 3 minutes at 95°C and was typically amplified by 25 cycles of

95°C	1 min.
55°C	1 min.
72°C	3 min.

followed by 10 minutes at 72°C.

Reaction conditions

1x PCR buffer (supplied with enzyme)	5 µl
Template	100 ng
Oligonucleotides	10 pmoles each
dATP, dCTP, dGTP, dTTP (Pharmacia)	1.25 mM each
PFU polymerase (Roche)	1 unit

Large Scale Purification of Single and Double Strand M13

F' carrying *E. coli*, TG1, was streaked onto a M9 plate from frozen culture and allowed to grow for 24–48 hours at 37°C. 50 ml LB was then inoculated with a single colony and grown for 6–8 hours at 37°C with aeration (1:5::media:flask volume shaken at 300 rpm). 2.5 ml of culture was infected by the addition 50 µl M13 stock and incubation at room temperature for 5 minutes. Cultures were then diluted into 250 ml prewarmed LB and grown for 6–8 hours with aeration. Cells were harvested by centrifugation at 4,000 rpm for 15 minutes at 4°C (Sorvall, J4). Double stranded M13 was extracted from the cell pellet by large scale DNA purification and caesium chloride gradient as described by [101].

Single strand M13 was purified from the cell harvest supernatant which had been centrifuged 4 times at 8,000 rpm (Sorvall, GSA) and filtered through a 0.22µ filter (Pall/Gelman). The phage DNA was precipitated by the slow,

sequential addition of PEG 8000 and NaCl at 40 g and 30 g per litre supernatant respectively. The solution was then stirred at room temperature for 30–60 minutes and then centrifuged at 8,000 rpm for 20 minutes at 4°C (Sorvall, GSA). After removal of the supernatant, the dry pellet was resuspended in 40 ml/litre supernatant 10 mM Tris-HCl, pH 8.0. This solution was then extracted three times in a 1:1::equilibrated phenol (pH 8.0):chloroform, and the aqueous phase precipitated by the addition of 1/10 volume 3 M sodium acetate, pH 5.2 and 2.5 volumes ethanol followed by incubation at –20°C for 1 hour. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes at 4°C (Sorvall, SS-34) and washed with 70% ethanol. After the resultant pellet was air-dried for 10 minutes, it was dissolved in 1–2 ml TE, pH 8.0 and stored at –20°C.

Purification of Plasmid DNA on Sucrose Gradients

Using a peristaltic pump (Verder 2000), 40 ml of fresh buffered 20% sucrose solution was slowly added and mixed into 40 ml of fresh buffered 5% sucrose solution. Simultaneously and at the same rate, the solution that had started at 5% sucrose solution was pumped into the bottom of two gradient centrifuge tubes. The gradients were then left for 1 hour at 4°C before 1.0 ml of DNA solution (≤ 500 ug) was carefully layered on top of the sucrose. Gradients were then centrifuged at 25,000 rpm in a SW28 (Beckman) for 19 hours at 4°C. 1.5 ml fractions were collected from the bottom of each gradient via a capillary tube connected to a peristaltic pump. DNA was analysed by agarose gel electrophoresis.

Buffered 5% sucrose

NaCl	1.0 M
Tris-HCl , pH 7.5	25 mM
EDTA, pH 8.0	5 mM
Sucrose	5% (w/v)

Buffered 20% sucrose

NaCl	1.0 M
Tris-HCl , pH 7.5	25 mM
EDTA, pH 8.0	5 mM
Sucrose	20% (w/v)

Preparative Purification of Oligonucleotides from Acrylamide Gels

Denaturing acrylamide (29:1::acrylamide:bis-acrylamide) urea 2 mm thick gels were prepared at percentages that were appropriate for separation of full length

oligonucleotides from their n-1 by-product, or for separation of drug-adducted oligonucleotides from those not carrying the lesion. Oligonucleotides were loaded onto gels as a 1:1 mix with formamide loading. The resulting bands, corresponding to the different oligonucleotide forms, were removed from the gel with a clean scalpel after visualisation by UV shadowing. The acrylamide slices were homogenised in 500 µl TE in an eppendorf tube and the DNA allowed to diffuse out overnight whilst being shaken at 37°C. To remove the acrylamide pieces, the bottom of the eppendorf tube was punctured and the contents centrifuged through a 5 ml syringe containing 3.0 ml TE-equilibrated Sephadex G25 (Pharmacia). The purified oligonucleotides were precipitated by the addition of 1/10 volume sodium acetate, pH 5.0 and 2.5 volumes ethanol and centrifugation at 14,000 rpm for 20 minutes at 4°C (Eppendorf). After washing the pellet with 70% ethanol, the DNA was resuspended in 20-100 µl TE and stored at –20°C.

³²P Labelling of Oligonucleotides

For applications requiring radioactively labelled oligonucleotides other than Southern blotting, between 50ng and 1.0 µg of oligonucleotide was incubated in 1xT4 PNK buffer, 10 µCi γ ³²P ATP (3000Ci/mmol), and 1-5 units T4 polynucleotide kinase (New England Biolabs) for 1 hour at 37°C in a 10 µl reaction volume. The labelled DNA can either be run directly on a denaturing urea polyacrylamide gel or purified as follows for applications such as sequencing and bandshifts. After the reaction's completion, the volume was brought up to 100 µl, and the enzyme was inactivated by addition of EDTA to a final concentration of 50 mM and heating to 70°C for 20 minutes. The free γ ³²P ATP was removed and buffers exchanged to TE via a 1.0 ml TE-equilibrated Sephadex G25 column.

For Southern blot probes, 1.0 µg of oligonucleotide was incubated with in 1xT4 PNK buffer, 70 µCi γ ³²P ATP (3000Ci/mmol), and 1-5 units T4 polynucleotide kinase (New England Biolabs) for 1 hour at 37°C in a 10 µl reaction volume. The labelled probe was then purified as described above.

Preparation of Cisplatin Oligonucleotides

Cisplatin was dissolved in 2x Platination buffer to a concentration of 5.7 mM, or 1.8 mg/ml. One volume of cisplatin solution was mixed with one volume of HPLC and gel purified oligonucleotide so that the final concentrations of drug and DNA were 3 mM and 2 mM respectively. After an overnight incubation at 37°C in the dark, the reaction was stopped by the addition of sodium chloride to a final concentration of 0.5 M.

Free cisplatin was removed from solution by purifying the oligonucleotide solution on a TE-equilibrated Sephadex G25 column. Addition of lesions was confirmed by end-labelling treated oligonucleotides with T4 polynucleotide kinase (see above) and running products on a denaturing urea-polyacrylamide gel. If the level of platination was appropriate, the oligonucleotide was purified via a preparative urea-polyacrylamide gel (see above).

2x Platination buffer

NaCl	6 mM
Na ₂ HPO ₄	1 mM
NaH ₂ PO ₄	1 mM

Synthesis of Double-Stranded M13 Cisplatin Substrate

Single-stranded M13-GTGx and a five-molar excess of platinated oligonucleotide (see Table 2.4) were heated to 65°C for 5 minutes and allowed to anneal by gradual cooling to 30°C over 1 hour, then to room temperature over 20 minutes, and finally in ice during synthesis reaction set-up. The annealed oligonucleotide/single-stranded M13 was mixed into a reaction cocktail containing 1x NEB buffer 1 (New England Biolabs), 0.6 mM of each dNTP, 2 mM ATP, 33 µg/ml BSA, 0.3 units/µl T4 DNA polymerase (New England Biolabs), and 16 units/µl T4 DNA ligase (NEB). The closed-circle substrate was synthesised by incubation for 4 hours at 37°C. To check for a complete reaction, an aliquot was taken and subjected to separation on a 0.8% 1xTBE agarose gel containing 0.25 µg/ml ethidium bromide run at 50 V overnight. The substrate was purified on a 0.75 g/ml caesium chloride gradient containing 740 µg/ml ethidium bromide, followed by butanol extraction and

buffer exchange to TE via a Centricon 100 (Amicon) and two TE-equilibrated Sephadex G25 columns. The presence of a lesion was confirmed through the substrate's resistance to ApaLI digestion. Stocks were stored at -80°C .

Preparation of UV DNA Substrate

Plasmids from freshly transformed cells were prepared using the Qiagen method following the manufacturer's instructions and diluted to a final concentration of 50 $\mu\text{g/ml}$ TE. Depending on volume, either 20-50 μl drops or 15 millilitre aliquots in a shaking petri dish were UV irradiated at a dose rate of 0.5J/m^2 . After the addition of 1/9 volume of 10x Nth protein buffer, DNA was incubated at 37°C for 30 minutes with Nth protein. Closed circle DNA was then purified by sucrose gradient.

Preparation of Tailed Transcription Templates

DNA, normally pB100-Uless was digested to completion with SmaI, extracted with 1:1::phenol:chloroform, and precipitated with 0.3 M sodium acetate and 2.5 volumes ethanol. After washing with 70% ethanol, the pellet was resuspended to 3.3 mg/ml in 10 mM Tris-HCl, pH 8, and the DNA stored at -20°C . Tailing reactions contained the linear DNA diluted to 1.9 mg/ml in 1x TdT buffer as supplied with the terminal transferase enzyme (Roche) and 0.75 mM CoCl_2 . Small aliquots were used for small-scale terminal transferase reactions using a range of dCTP concentrations and incubation times to find optimum conditions for a single strand tail-length of approximately 20 nucleotides. Large-scale tailing reactions were then performed with the optimum dCTP concentration. The products were then digested with a restriction enzyme that would remove the extraneous 3' tail on the non-coding strand, and the appropriate fragment isolated by either agarose gel electrophoresis or gel filtration chromatography.

Coupling Biotin Oligonucleotides to Streptavidin Magnetic Beads

Streptavidin magnetic beads (Dynabeads, Dynal) were washed with Buffer BW. They were then incubated with gel purified biotin-labelled oligonucleotides at a ratio of 2 pmoles DNA / μl magnetic beads for 15 minutes at room temperature in a 4x bead volume of 0.5x Buffer BW. The beads were then washed three times in buffer BW and resuspended in one bead volume of H_2O .

Buffer BW

Tris-HCl, pH 7.5	10 mM
EDTA	1 mM
NaCl	2 M

Sequencing near a Primer

Sequencing primer was labelled with ^{32}P and purified (see above). 1.0 pmole of template in 6.0 μl H_2O was denatured by the addition of 4.0 μl denaturing buffer and incubation for 5 minutes at room temperature. 0.5 pmoles of labelled primer was annealed to the template by the addition of 3.0 μl 3M sodium acetate, pH 5.0 and 100 μl ethanol and incubation for 15 minutes on dry ice. After a 15 minute centrifugation at 14,000 rpm in a microcentrifuge at 4°C, the pellet was washed with 70% ethanol and resuspended in 15 μl Reaction cocktail. The primer was extended by the addition of 3.25 U of Sequenase v 2.0 (Amersham) and incubation either on ice or at room temperature for 5 minutes. Reactions were terminated by mixing 3.5 μl aliquots of the sequencing mix with 2.5 μl of each ddNTP (8 μM), incubating for 5 minutes at 37°C, and adding of 4.0 μl formamide loading buffer. Samples were heated to 95°C for 3 minutes before loading onto the appropriate denaturing urea acrylamide gel.

Denaturing buffer

NaOH	1 M
EDTA	1 mM

Reaction cocktail

Tris-HCl, pH 7.5	40 mM
MgCl_2	20 mM
NaCl	50 mM
DTT	10 mM
dCTP, dGTP, dTTP	0.5 μM each
MnCl_2	10 mM
Sodium isocitrate	15 mM
$\alpha^{32}\text{P}$ dATP (3000Ci/mmol)	5 μCi

Primer Extension

Primer extension of repair products was carried out in a similar manner to sequencing near a primer yet with the following modifications. First, the template was first linearised with PvuI (New England Biolabs). Second, the reaction cocktail was changed to that described below. The extension continued for 10 minutes at 37°C. Lastly, the reaction was terminated only by formamide loading buffer and without ddNTPs.

Reaction cocktail

Tris-HCl, pH 7.5	40 mM
MgCl ₂	20 mM
NaCl	50 mM
DTT	10 mM
dCTP, dGTP, dTTP	80 µM each

Table 2.4.1 Oligonucleotides

Txp1 - Transcription Template – non-transcribed strand

AAGGAAGAGGAAAGAGGAGGAAGAGGGAAGGACACGTGCCCCGACGTA
CACAGGAGAAGGAGAAGG

Txp2 - Transcription Template – transcribed strand

CCTTCTCCTTCTCCTGTGTACGTGCGGGCACGTGTCCTTCCCTCTTCCTC
CTCTTTCCTCTTCCTTTCTTCCCTCTTTCCTC

Cisplatin GTG Oligonucleotide

TCTTCTTCTGTGCACTCTTCTTCT

NER Southern Probe

GAAGAGTGCACAGAAGAAGAGGCCTGG

NER 3' Incision – Primer Extension Primer

CAGGAAACAGCTATGAC

NER Dual Incision – HeLa End Labelling Primer

GGGAAGAGTGCACAGAAGAAGAGGCCTGGTCG

NER Dual Incision – Yeast End Labelling Primer

AAATGGGGAGAAGAAGAGTGCACAGAAGAAGAGGCCTGGTCG

TCR Substrate – TS 5'

GCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCACTAG
TAGGCC

TCR Substrate – TS 3'

CCCCAGGAATTCFGGTCATAGCTGTTTCTTGCTCGAGGAGCTCGAATT
CACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGT

TCR Substrate – NCS

AGTTGTAAAACGACGGCCAGTGAATTCGAGCTCCTCGAGCAGGAAACA
GCTATGACCGAATTCCTGGGGAGAAGAAGAGTGCACAGAAGAAGAGG
CCTACTAGTGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGC
AAGC

2.5 Protein manipulation and chromatography

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels containing 8-10% 37.5:1::acrylamide:bis-acrylamide were cast as according to Laemmli [63] in Mini-Protean II gel system (Bio Rad). Samples to be loaded were mixed with 3x SDS loading buffer and denatured by incubation at 100°C in a heat block for 3 minutes. Gels were subjected to 200 V in 1x Laemmli buffer until size markers (Broad Range Protein Markers, New England Biolabs) were appropriately separated.

Western Blotting Analysis

After proteins were separated by SDS-PAGE, they were transferred onto either nitrocellulose (Hybond-C, Amersham) or PVDF membrane (Hybond-P, Amersham) using the Mini-Protean II Western system according to manufacturers instruction. The membrane was then blocked for one hour in 4% milk powder (Marvel, Tesco) in 1x PBS, 0.02% Tween before incubation with primary antibodies (Table 2.4) for either overnight at 4°C or two hours at room temperature. The blot was then washed three times, 10 minutes in 1x PBS, 0.02% Tween and then probed with secondary antibody (Table 2.4) for 45 minutes at room temperature. After washing again as above, the signal was visualised either with NBT/BCIP (BioRad) or chemiluminescence (ECL or ECL-plus, Amersham) according to manufacturer's instructions.

Silver Staining

After proteins were separated on a SDS-PAGE gel, the gel was fixed in Solution 1 for at least 1 hour and then in Solution 2 for 20 minutes. It was then rinsed for 1 minute in Solution 3 and washed in H₂O three times over 1 minute. The gel was then incubated in Solution 4 for 20 minutes before another wash in H₂O as above. The signal was developed during incubation in Solution 5 until the desired intensity, and the reaction was stopped by incubation in 1% acetic acid for at least 1 hour.

Solution 1		Solution 2	
Methanol	50% v/v	Ethanol	50%
Acetic Acid	12% v/v		
Formaldehyde	0.05% v/v		
Solution 3		Solution 4	
Na ₂ S ₂ O ₃	0.02% w/v	AgNO ₂	0.2% (w/v)
		Formaldehyde	0.075% (v/v)
Solution 5			
Na ₂ CO ₃	6% (w/v)		
Na ₂ S ₂ O ₃	0.0004% w/v		
Formaldehyde	0.05% v/v		

Whole Cell Extractions (Ingles [49])

An overnight yeast inoculum was used to seed cultures so that cells would reach mid-late log phase after 12-16 hours. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in a Beckman J-4 or equivalent rotor at 4°C. The cells were washed in 1/10 volume ice cold H₂O and then in 1/10 volume extraction buffer. The pellet was drained, scraped into either a syringe or plastic icing bag, and extruded into liquid nitrogen. The resulting spaghetti-like strands were crushed under liquid nitrogen until a fine powder using a mortar and pestle. When checked under a microscope, at least 75% of all cells were lysed. After the addition of 1 pellet volume of cold extraction buffer, the extract was allowed to thaw before being centrifuged at 8,000 rpm in a Sorvall GSA for 20 minutes at 4°C. The supernatant was centrifuged further at 40,000

rpm in a Beckman Ti45 for 2 hours at 4°C. Solid ammonium sulphate was added to the clarified extract to a final concentration of 2.94 M at 4°C, and the pellet retrieved after ultracentrifugation at 40,000 rpm in a Beckman Ti70.1 for 15 minutes. This was then resuspended in minimal amount of buffer, approximately 50 µl/g cell pellet, and dialysed for 12-16 hours at 4°C against dialysis buffer. The product was clarified by a 10 minute, 14,000 rpm centrifugation at 4°C in a microcentrifuge, quick-frozen in liquid nitrogen, and stored at –80°C.

Extraction buffer		Dialysis Buffer	
Tris-HCl, pH 7.5	0.2 M	HEPES, pH 7.5	20 mM
Ammonium sulphate	0.39 M	Glycerol	20% (v/v)
MgSO ₄	10 mM	MgSO ₄	10 mM
Glycerol	20% (v/v)	EGTA	10 mM
EDTA	1 mM	DTT	5 mM
DTT	1 mM	1x Protease Inhibitors	
1x Protease Inhibitors			

Whole Cell Extracts by Spheroplasts

An overnight yeast inoculum was used to seed cultures so that cells would reach mid-late log phase after 12-16 hours. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in a Beckman J-4 or equivalent rotor at 4°C. The cells were washed in 1/10 volume ice cold H₂O, resuspended in 1/10 pellet volume 0.1 M EDTA, 10 mM DTT, and incubated for 10 minutes at 30°C with gentle shaking (100 rpm). The cell suspension was centrifuged at 6,000 rpm for 10 minutes in a Beckman JA-14, resuspended to 1g cell pellet/ml YPS+E, and incubated for 1-2 h at 30°C with gentle shaking. Digestion was stopped by a 10-fold dilution of the cells with ice cold YPS and by three washes with YPS, the last containing 1x protease inhibitors. The washed cell pellet was then resuspended into 4ml Spheroplast Buffer 1/g cell pellet. The same volume of Spheroplast Buffer 2 was added drop-wise while the suspension was stirred gently. The extract was then allowed to stir for an additional 20 minutes on ice before neutralised 4 M ammonium sulphate was added to a final concentration of 0.9 M. The precipitate was cleared by ultracentrifugation in a Beckman Ti45

rotor at 32,000 rpm for 1 hour at 4°C. The supernatant was removed and 0.35 g ammonium sulphate was added for each millilitre of supernatant. This precipitate was recovered by centrifugation at 7,000 rpm in a Beckman Ti45 for 15 minutes, resuspended in the minimum amount of Dialysis Buffer, and dialysed overnight against the same buffer. Extract was then quick-frozen in liquid nitrogen and stored at -80°C.

YPS+E

Yeast extract	1% (w/v)
Bactopeptone	2% (w/v)
Sorbitol	1 M
Yeast lytic enzyme (ICN)	70,000u

YPS

Yeast extract	1% (w/v)
Bactopeptone	2% (w/v)
Sorbitol	1 M

Spheroplast Buffer 1

Tris-HCl pH 8.0	10 mM
EDTA	1 mM
DTT	5 mM
Protease Inhibitors	1x

Spheroplast Buffer 2

Tris-HCl pH 8.0	50 mM
MgCl ₂	10 mM
DTT	2 mM
Sucrose	25% (w/v)
Glycerol	50% (v/v)

Dialysis Buffer

HEPES, pH 7.5	20 mM
Glycerol	20% (v/v)
MgSO ₄	10 mM
EGTA	10 mM
DTT	5 mM
1x Protease Inhibitors	

Bead-beaten Whole Cell Extracts

An overnight yeast inoculum was used to seed cultures so that cells would reach mid-late log phase after 12-16 hours. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in a Beckman J-4 or equivalent rotor at 4°C. The cells were washed in 1/10 volume ice cold H₂O and resuspended in 1/3 volume 3x Lysis Buffer along with DTT and EDTA, both to a final

concentration of 5 mM. This cell suspension was then bead beaten with either a KDL-A Dyno-Mill (Willy A. Bachofen) or a bead-beater (Stratech), and the extract clarified by centrifugation at 8,000 rpm in a Sorvall GSA rotor for 30 minutes at 4°C. Potassium acetate concentrations were adjusted to approximately 500 mM by the addition of 1/9 volume 5 M potassium acetate, pH 7.8, and DNA precipitated out by the slow addition of polyethyleneimine (PEI), pH 7.8, to a final concentration of 0.3%. The supernatant was removed from the chromatin pellet after 1 hour of PEI treatment by ultracentrifugation at 42,000 rpm in a Beckman Ti 45 for 2 hours at 4°C, and both were frozen in liquid nitrogen and stored at –80°C.

3x Lysis Buffer

Glycerol	60% (v/v)
Tris-Acetate, pH 7.8	450 mM
Potassium Acetate	150 mM

Chromatin Extracts

Chromatin extracts for phosphatase assays were prepared in a similar manner to bead-beaten whole cell extracts. However, the addition of PEI was omitted. Furthermore, after the last ultracentrifugation, the supernatant was discarded and the loose, cloudy chromatin pellet was washed with 10 pellet volumes of Buffer A300. This chromatin extract was quick-frozen in liquid nitrogen and stored at –80°C.

Buffer A300

Glycerol	20% (v/v)
HEPES, pH 7.6	40 mM
DTT	1 mM
EDTA	1 mM
Potassium Acetate, pH 7.8	300 mM
Protease Inhibitors	1x

HeLa Whole Cell Extracts (HeLa WCE)

HeLa cells from 2 litres of actively growing suspension cultures ($6\text{-}8 \times 10^5$ cells/ml) were washed four times in ice cold PBS and the packed cell volume

(PCV) measured. Cells were then resuspended in 4 PCVs cold TE, 1x Protease Inhibitors and allowed to swell for 20 minutes on ice before homogenisation with 30 strokes of a homogeniser. Four PCVs of Sucrose-Glycerol Buffer were added dropwise while stirring the extract slowly in an ice bath at 4°C; to this was added 1 PCV of cold saturated, neutralised ammonium sulphate solution. After allowing the slurry to stir for an additional 30-50 minutes, centrifuged at 42,000 rpm in a Beckman SW50 rotor for 3 hours at 4°C. The resultant supernatant was removed and precipitated with 0.33g ammonium sulphate/ml supernatant and neutralised by the addition of 10 µl 1M NaOH/g ammonium sulphate added. The precipitate was recovered by centrifugation at 11,000 rpm in a Beckman SW55 rotor for 1 hour, scraped into dialysis tubing, and dialysed over 12 hours against Dialysis Buffer. The extract was then clarified by centrifugation at 14,000 rpm in a microcentrifuge for 10 minutes, quick-frozen in 20µl aliquots in liquid nitrogen and stored at –80°C.

Sucrose-Glycerol Buffer

Tris-HCl	50 mM
MgCl ₂	10 mM
Sucrose	25% (w/v)
Glycerol	50% (v/v)

Dialysis Buffer

Hepes-KOH, pH 7.9	25 mM
KCl	0.1 M
MgCl ₂	12 mM
Glycerol	17% (v/v)
DTT	2 mM
Protease Inhibitors	1x

Baculovirus Extracts

SF9 insect cells were transformed and processed according to the Bac-N-Blue baculovirus protocol (Invitrogen).

Bio-Rex 70 Chromatography

For every 90 mg of protein to be loaded onto the column, 1 g of BioRex 70 (Bio Rad) was prepared by swelling and washing in Buffer A1200 three times. The resin was then redissolved in A0 and equilibrated with 3-5 column volumes of either Buffer A150 or Buffer A200. Protein was loaded onto the column in a buffer of comparable salt concentrations and eluted stepwise, both at a rate of 2 column volumes per hour. Fractions were collected in 1/10 column volume

volumes and either applied to the next column or quick-frozen in liquid nitrogen and stored at -80°C . BioRex 70 resin could be regenerated by a 3 column volume wash of 0.5 M KOH and intensive rinsing with H_2O until the pH dropped to pH 7.5. It was then stored at 4°C in 40 mM HEPES, pH 7.6, 1 mM EDTA, 0.02% sodium azide.

Buffer A

Glycerol	20% (v/v)	For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer A1200 is Buffer A containing 1200 mM potassium acetate.
HEPES, pH 7.6	40 mM	
DTT	1 mM	
EDTA	1 mM	
Protease Inhibitors	1x	

Designated salt: potassium acetate, pH 7.8

Phosphocellulose (P11) Chromatography

For every 20 mg of protein to be loaded onto the column, 0.22 g of P11 (Whatman) was dissolved in 20 volumes of 0.5 M KOH and immediately filtered dry using a Buchner funnel. The resin was then washed with copious volumes of H_2O until the filtrate pH dropped to below 11. This process was repeated, yet with 0.5 M HCl and washed until the filtrate pH was above 3. The resin was then resuspended in P11 Suspension Buffer and titrated to pH 7.6 with KOH and then poured into a column support. After equilibration with A150, protein was loaded at 2 column volumes per hour and eluted stepwise into 1/10 column volume fractions. Fractions were then applied to the next column or quick-frozen in liquid nitrogen and stored at -80°C .

NB: If a Nickel-Agarose column were to follow the P11, Buffer A did not contain EDTA and contained 5 mM 2-mercaptoethanol instead of 1 mM DTT.

Buffer A

Glycerol	20% (v/v)
HEPES, pH 7.6	40 mM
DTT	1 mM
EDTA	1 mM
Protease Inhibitors	1x

For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer A1200 is Buffer A containing 1200 mM potassium acetate.

Designated salt: potassium acetate, pH 7.8

P11 Suspension buffer

Glycerol	20% (v/v)
HEPES, pH 7.6	200 mM
DTT	1 mM
EDTA	1 mM
Potassium Acetate	600 mM

Nickel Agarose Chromatography

Typically, 0.5 ml Nickel Agarose (Qiagen) was first washed with Buffer I-0 before the protein was loaded. The resin was then washed at a rate of 2 column volumes/hour with 10 column volumes of Buffer I-10, 10 mM imidazole, pH 7.5 followed by 10 column volumes of Buffer I-100, 10 mM imidazole, pH 7.5. The affinity column was then eluted by 10 column volumes of Buffer I-100, 100 mM imidazole, pH 7.5 into 1/5 column volume fractions which were then quick-frozen under liquid nitrogen and stored at -80°C .

Buffer I

Glycerol	20% (v/v)
HEPES, pH 7.6	40 mM
2-mercaptoethanol	5 mM
Protease Inhibitors	1x

Designated salt: potassium acetate, pH 7.8

Heparin-Sepharose Chromatography

For every 240mg of protein to be loaded, 1 g of Heparin-Sepharose (Pharmacia) resin was swollen in Buffer K100. After the column was equilibrated with 3-5 column volumes of K100, the protein was loaded and eluted stepwise at a rate of 2 column volumes an hour in 1/10 column volume fractions. Fractions were then applied directly onto the next column or quick-frozen in liquid nitrogen and stored at -80°C . The resin was regenerated by two washes, the first being 15-20 column volumes of 0.1M Tris-HCl, pH 8.5, 0.5 M KCl and the second being 15-20 column volumes of 0.1 M sodium acetate buffer, pH 5, 0.5 M KCl. It was then re-equilibrated with at least 5 column volumes of K100, 0.02% sodium azide and stored at 4°C .

Buffer K

Tris-HCl, pH 7.9	50 mM	For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer K100 is Buffer K containing 100 mM potassium chloride.
Glycerol	20% (v/v)	
DTT	1 mM	
EDTA	1 mM	
Protease Inhibitors	1x	

Designated salt: potassium chloride

Single-strand DNA Cellulose Chromatography

Single-strand DNA cellulose was equilibrated in buffer D500. Protein in 500 mM NaCl was loaded and washed with 2 column volumes of D500 and D750 at a rate of 2 column volumes an hour. Proteins with strong affinities for single strand DNA were eluted with 5 column volumes of D1500 with 50% ethylene glycol. Peak protein fractions were pooled and dialysed against Buffer D200 with 20% sucrose. After clarification, these fractions were quick-frozen in liquid nitrogen and stored at -80°C .

Buffer D

Glycerol	10% (v/v)
Tris-HCl, pH 7.5	25 mM
DTT	1 mM
EDTA	1 mM
Protease Inhibitors	1x

For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer D100 is Buffer D containing 100 mM sodium chloride.

Designated salt: sodium chloride

MonoQ Chromatography

MonoQ PC 1.6/5 or Mono QHR 5/5 (Pharmacia) were run at 4°C on respectively SMART or ÅKTA chromatography systems. These columns were first washed with 5-10 column volumes of Buffer Q1200 and then equilibrated with start buffer, typically Buffer Q100. Protein was loaded and eluted by salt gradient at generally 1 column volume/minute into 1/10 column volume fractions.

Buffer Q

Glycerol	20% (v/v)
Tris-HCl (or Acetate), pH7.5	50 mM
DTT	0.5 mM
EDTA	0.1 mM
Protease Inhibitors	1x

For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer Q1200 is Buffer Q containing 1200 mM sodium chloride or potassium acetate, depending on the protein purified.

Designated salt: sodium chloride or potassium acetate

DEAE-Sepharose Chromatography

Progel TSK DEAE-5PW (Supelco) was run at 4°C on an ÅKTA chromatography system. The column was first washed with 5-10 column volumes of Buffer TEZ1000 and then equilibrated with start buffer, typically Buffer TEZ60. Protein was loaded and eluted by a 10 column volume salt gradient ranging from 60

mM to 1000 mM ammonium sulphate at a rate of 0.5 ml/minute into 0.8 ml fractions.

Buffer TEZ

Glycerol	10% (v/v)
Tris-HCl (or Acetate), pH7.5	50 mM
DTT	1 mM
MgSO ₄	6 mM
ZnSO ₄	10µM
Protease Inhibitors	1x

For chromatography buffer, the number following buffer name denotes concentration of designated salt in millimolar units. For example, Buffer Q1200 is Buffer Q containing 1200 mM sodium chloride or potassium acetate, depending on the protein purified.

Designated salt: ammonium sulphate

Superose 6 Chromatography

Superose 6 (Pharmacia) columns were run at 4°C on a SMART chromatography system generally using Buffer S6 at a rate of 10-30 µl/minute. Size standards were Gel Filtration standards from BioRad.

Buffer S6

Glycerol	10% (v/v)
Tris-HCl, pH 7.5	50 mM
NaCl	200 mM
NP-40	0.01% (v/v)
Protease Inhibitors	1x

Crosslinking of Antibodies onto Protein A Resin

Protein A beads (Pharmacia) were washed 3 times in PBS and incubated at 4°C with 0.25 ml ascites or monoclonal antibodies/ml resin overnight. The beads were gently centrifuged at 3500 rpm in a Beckman J-1 for 5 minutes and washed twice with PBS. The beads were then washed twice with 10 volumes 200 mM sodium borate, pH 9.0 and resuspended in 10 volumes 200 mM sodium borate, 20mM dimethylpimedilate. The crosslinking took place while being rocked at room temperature over an hour. The reaction was stopped by one wash of 5 volumes 200 mM ethanolamine, pH 8.0 and rocking incubation

with the same volume and solution for 3 hours at room temperature. The beads were then washed twice briefly with 10 volumes PBS, twice for 10 minutes with 10 volumes 50 mM glycine pH 2.3, 150 mM NaCl. During the last wash, two column volumes of the glycine buffer were left on top of the beads and titrated to pH 7.5 by the addition of 1/20 volume 1 M Tris-Acetate, pH 8.5. The beads were washed again with 10 volumes PBS and stored in 2 volumes PBS, 0.02% sodium azide.

2.6 Yeast techniques

Yeast Transformations

An overnight culture was diluted to 5×10^6 cells/ml in 100 ml YPD and allowed to grow at 30°C with proper aeration. When a cell density of 2×10^7 was reached, the culture was centrifuged at 3000 rpm for 5 minutes. The pellet was then washed in 50 ml sterile H₂O and resuspended into 2 ml 100 mM lithium acetate. The cells were split into two eppendorf tubes and pelleted again by a quick spin at top speed in a microcentrifuge. The supernatant was removed and cells brought up to a cell density of 2×10^9 cells/ml, or approximately 0.5 ml per tube, with 100 mM lithium acetate. For each transformation, 1×10^8 prepared cells were pelleted and the following solutions were layered over the cells in the order listed:

- 240 µl 50% (w/v) PEG
- 36 µl 1.0 M lithium acetate
- 25 µl 2.0 mg/ml boiled and sheared single-stranded DNA
- 50 µl plasmid DNA (0.1-10 µg) in H₂O.

The mix was then vortexed vigorously until the cells had become fully resuspended and incubated at 30°C with shaking for 30 minutes. The cells were heat shocked in a 42°C water bath for 20-25 minutes, centrifuged for 15 seconds at 8000 rpm in a microcentrifuge, and resuspended gently in 1 ml H₂O. Between 2 and 200 µl of transformed cells were plated on selective media and grown for up to 4 days at 30°C.

Tetrad dissection

Diploid yeast were streaked onto sporulation medium for 5 days or until sufficient numbers of asci were obtained. Asci were digested with a 5% glucuronidase solution (DuPont) until suitable for micromanipulation, approximately 30 minutes. The spores were separated onto YPD agar with a Singer MSM system, and at least 16 asci were analysed. Genotype and phenotype were assessed by replica plating on selective plates; mating type was tested by abilities to cross with DC17 (mat α) or DC14 (mat a) tester strains.

Sporulation Medium

Potassium Acetate	1% (w/v)
Bacto-agar (Difco)	2%

Generation of Knock-in Yeast Mutants

For small-scale mutations such as amino acid switches and short deletions or insertions, plasmids were constructed to contain a selective marker and the mutation of interest centered between at least 300 bp of the flanking 5' and 3' sequences of the chosen gene. The plasmid was then linearised within the 5' homology and transformed into diploid yeast as described above. Colonies were then checked by PCR for the correct construct integration, and positives were tetrad dissected. Mutant haploids were identified by growth on selective media and manifestation of mutant phenotypes and were tested for mating type.

2.7 Activity assays

Transcription Elongation Assay

Transcription activity of whole cell extracts and purified proteins was assayed in a 25 μ l reaction volume with conditions as described below for 30 minutes at room temperature. The reaction was stopped by addition 200 μ l of Stop Buffer, 0.2% SDS, 0.4 mg/ml Protease K and incubation at 40°C for 30 minutes. RNA was then precipitated with 500 μ l ethanol and recovered by centrifugation at 4°C at 14,000 rpm in a microcentrifuge. Transcripts were resolved by a

denaturing urea-acrylamide gel after the pellet was resuspended in 10-20 μ l Formamide loading buffer.

Transcription of a Guanine-less cassette was assayed in a similar manner to that described above except that the Stop buffer contained 40U/ml RNase T1 (5'-3'Prime) and was incubated for 5 minutes at room temperature before the addition of SDS and Protease K.

Typical Assay Conditions		Stop Buffer	
Hepes, pH 7.5	40 mM	Tris-HCl, pH 7.5	10 mM
MgCl ₂	10 mM	NaCl	0.3 M
2-mercaptoethanol	5 mM	EDTA	5 mM
ATP, CTP, UTP	1 mM each	Glycogen	0.1 mg/ml
GTP	20 μ M		
RNase Inhibitor (5' 3')	1u/25 μ l		
Glycerol	5% (v/v)		
α^{32} P-GTP (3000 Ci/mmol)	5 μ Ci/25 μ l		
Template DNA	1 pmole		
(Polymerase	100 ng/25 μ l		
or whole cell extract	25 μ g/25 μ l)		

TFIIH Kinase Activity Assay

The kinase activity of TFIIH was assayed by incubating it with RNA polymerase II for 30 minutes at 30°C in the below buffer conditions. After the addition of SDS-loading buffer to 1x and incubation at 95°C for 3 minutes, the proteins were separated on a 7.5% SDS PAGE gel. The gel was then dried and exposed to either a phosphorimager screen or BioMax film with an intensifying screen.

Typical Assay Conditions

Hepes, pH 7.5	40 mM
MgCl ₂	10 mM
2-mercaptoethanol	5 mM
Glycerol	5% (v/v)
$\gamma^{32}\text{P}$ -ATP (3000 Ci/mmol)	10 $\mu\text{Ci}/20\ \mu\text{l}$
ATP (Roche)	125 μM
Polymerase	200 ng/20 μl

Fcp1 Phosphatase Activity Assay

Fcp1 phosphatase activity was assayed with RNA polymerase II as a substrate. RNA polymerase II had previously been phosphorylated with TFIIH-kinase (see above). Fcp1 was incubated under the conditions described below for 30 minutes at 30°C. Reactions were stopped by the addition of SDS loading buffer to 1x. Samples were incubated at 95°C for 3 minutes before loading onto a 7.5% SDS PAGE gel.

Typical Assay Conditions

Tris-Acetate, pH 7.8	40 mM
MgCl ₂	10 mM
DTT	5 mM
Glycerol	20% (v/v)
EDTA	0.1 mM
Tween-20	0.025%
Potassium Acetate	10 mM
2-glycerophosphate	10 mM
Okadaic Acid	10 mM
Polymerase	200 ng/20 μl

Chromatin assembly assay

Plasmids purified from caesium chloride gradients were relaxed by Topoisomerase I treatment. Typically, 10 μg of plasmid was incubated for 1 hour at 37°C in a 50 μl reaction volume containing 1x Topo I buffer and 20 units of Topoisomerase (Promega). SDS was added to the reaction to 0.4% and

proteinase K to 250 µg/ml and incubated for at least 30 minutes at 42°C. The DNA was extracted twice with 1:1::phenol:chloroform, and the aqueous layer ethanol precipitated with 0.3 M sodium acetate. After the precipitate was washed with 70% ethanol, it was resuspended in TE to a concentration of 0.3 mg/ml.

Each chromatin assembly reaction consisted of 1x Assembly buffer, 2 mM ATP, 30 mM KCl, 0.5mg/ml creatine phosphokinase (rabbit muscle) and 150 - 300 ng of relaxed plasmid. Typically 100 µg of whole cell extract was added to each 50 µl reaction and incubated for up to 3 hours at room temperature. Reactions were stopped by the addition of EDTA to 20 mM, NaCl to 0.2 M, and 10 u RNase and incubated for 15 minutes at 37°C. SDS was then added to 0.4% and protease K to 250µg/ml and incubated for at least 30 minutes at 37°C. Reactions were then extracted with 1:1::phenol:chloroform and precipitated with ethanol. The DNA was then washed with 70% ethanol and resuspended in 10 µl TE. The products were then visualised after being separated in a 1.5% TBE gel that had been stained for 1 hour in 3 µg/ml ethidium bromide and rinsed for 10 minutes in H₂O.

1x Topo I buffer

Tris-HCl, pH 7.5	50 mM
MgCl ₂	10 mM
DTT	0.5 mM
NaCl	50 mM

5x Assembly Buffer

HEPES-KOH, pH 7.5	200 mM
MgCl ₂	25 mM
DTT	2.5 mM
Phosphocreatine	110 mM
BSA	9.0 µg/ml

Nucleotide Excision Repair – Repair Synthesis Assay

Nucleotide excision repair synthesis activity of whole cell extracts was assayed using the 3.0 kb pBluescript KS(+) and the 3.7 kb pHM14. Both plasmids were treated with E. coli Nth protein, and closed circular plasmids isolated by caesium chloride and sucrose gradients. Each reaction of 50 µl contained 250 ng of either irradiated pBluescript KS(+) or platinated and 250 ng of undamaged pHM14, 1x Reaction buffer, 2 mM ATP, 30 mM KCl, 0.5mg/ml creatine phosphokinase (rabbit muscle), 20 µM of each dGTP, dCTP, and TTP, 8 µM

dATP, 74 kBq of α -³²P-dATP (110 TBq/mmol) and repair proteins or whole cell extracts as indicated. Reactions were incubated for 3 hours at either room temperature or 30°C for respectively yeast or HeLa extracts. Reactions were stopped by adding EDTA to concentration of 20 mM, SDS to 0.6% and proteinase K to 250 µg/ml and by incubating at 37°C for at least 30 minutes. After the addition of 39 µl TE to each reaction, the NER products were extracted with 1:1::phenol:chloroform. The DNA was precipitated from the aqueous layer with the addition of 1/50 volume 5 M NaCl, 5 µg glycogen (Roche), and 2.5 volumes ethanol. After a 20 minute incubation on dry ice and centrifugation at 14,000 rpm for 30 minutes at 4°C, the pellet was washed with 500 µl 70% ethanol and dried briefly in a vacuum centrifuge. The DNA was then linearised before separation by electrophoresis overnight on a 1% agarose gel containing ~0.3 µg/ml ethidium bromide. Data were analysed by autoradiography with intensifying screens and densitometry.

5x Reaction Buffer

HEPES-KOH, pH 7.5	200 mM
MgCl ₂	25 mM
DTT	2.5 mM
Phosphocreatine	110 mM
BSA	9.0 µg/ml

Dilution Buffer

HEPES-KOH, pH 7.5	200 mM
MgSO ₄	10 mM
EGTA	10 mM
DTT	5 mM
Glycerol	20% (v/v)
Protease Inhibitor	1x

Nucleotide Excision Repair – Dual Incision Southern-based Assay

Nucleotide excision repair activity of whole cell extracts was assayed in 50 µl reaction volumes. Each reaction consisted of 1x Reaction buffer, 2 mM ATP, 30 mM KCl, 0.5mg/ml creatine phosphokinase (rabbit muscle) and 250 ng platinated M13-GTGx substrate and was incubated for 30 minutes at either room temperature or 30°C for respectively yeast or HeLa extracts. Reactions were stopped by adding EDTA to concentration of 20 mM, SDS to 0.6% and proteinase K to 250 µg/ml and by incubating at 37°C for at least 20 minutes. After the addition of 39 µl TE to each reaction, the NER products were extracted with 1:1::phenol:chloroform. The DNA was precipitated from the aqueous layer

with the addition of 1/50 volume 5 M NaCl, 5 µg glycogen (Roche), and 2.5 volumes ethanol. After a 20 minute incubation on dry ice and centrifugation at 14,000 rpm for 30 minutes at 4°C, the pellet was washed with 500 µl 70% ethanol and dried briefly in a vacuum centrifuge. The DNA was then digested in a 10 µl reaction volume with HindIII and XhoI (New England Biolabs) for 2 hours at 37°C. Before loading onto a 12% denaturing urea acrylamide gel, reactions were mixed with 8 µl formamide buffer and heated to 95°C for 3 minutes.

After electrophoresis, the DNA was transferred by capillary action for 90-120 minutes from the gel to a nylon membrane (Hybond-N⁺, Amersham) that had been soaked in 0.9 M Tris borate, 20 mM EDTA. The DNA was then fixed for 20 minutes to the membrane with 0.4 M NaOH and then washed for 2 minutes with 5x SSC. The blots were then hybridised with a labelled complementary probe (see Table 2.4) in hybridisation buffer for 16 hours, washed for 10 minutes in 2x SSC, 0.1% SDS before exposure to either a phosphoimager screen or BioMax MS film (Kodak) with intensifying screen.

5x Reaction Buffer

HEPES-KOH, pH 7.5	200 mM
MgCl ₂	25 mM
DTT	2.5 mM
Phosphocreatine	110 mM
BSA	9.0 µg/ml

Dilution Buffer

HEPES-KOH, pH 7.5	200 mM
MgSO ₄	10 mM
EGTA	10 mM
DTT	5 mM
Glycerol	20% (v/v)
Protease Inhibitor	1x

Hybridisation Buffer

SDS	7% (w/v)
PEG-8000	10% (w/v)
NaCl	250 mM
Potassium Phosphate Buffer, pH 7.0	130 mM

Nucleotide Excision Repair – Dual Incision End-labelling Based Assay

Repair reactions were carried out in a similar manner to that described for the Southern-Based assay, but the reaction volumes were reduced to 10 µl. After the completion of the repair reaction, template oligonucleotides (see Table 2.4)

were added to a final concentration of 0.5 pM and the reaction was heated to 95°C for 3 minutes. The oligonucleotides and repair products were allowed to anneal while cooling to room temperature over 30 minutes and on ice for 5 minutes. The following was then added to each repair reaction and incubated for 5 minutes at 37°C:

32P-TTP/32PdCTP(3000 Ci/mmol)*	0.2 µl	*depending on template sequence
Sequenase Dilution Buffer (supplied		
with enzyme by Amersham)	1.2 µl	
Sequenase v. 2 (Amersham)	0.2 µl	

The labelling was then chased with the addition of 1 µl 25 mM dNTPs (Roche) and incubation at 37°C for 12 minutes. The reaction was stopped upon the addition of 10 µl formamide buffer. The labelled repair products were then resolved on a 15% denaturing urea polyacrylamide gel and visualised by autoradiography onto BioMax MS film with an intensifying screen.

CHAPTER THREE: FCP1 FUNCTION

3.1 Background

The phosphorylation and dephosphorylation of the C-terminal domain (CTD) of Rpb1, the largest subunit of RNA polymerase II, regulates transcription as well as other transcription-linked cellular functions. Although many CTD kinases have been identified, only one phosphatase, Fcp1, is known. The regulatory role of the phosphatase in the context of the transcription cycle remains unclear. For example, it could counteract Srb10-Srb11 repression and thereby increase the levels of hypophosphorylated polymerase able to enter into pre-initiation complexes. On the other hand, it might act as a repressor by counteracting phosphorylation by TFIIH kinase and preventing the transition into transcript elongation. Lastly, Fcp1 might work during elongation or near transcript termination to recycle the phosphorylated elongating polymerase to a form which can return to the promoter for another round of transcription.

Experiments prior to the commencement of this thesis studied Fcp1-mediated dephosphorylation of free RNAPIIO and did not address phosphatase activity in the context of transcription [16, 30]. Recent work by Cho *et al.* and Lehman and Dahmus demonstrate that within a defined *in vitro* transcription system, human Fcp1 can dephosphorylate the elongating form of polymerase as a substrate [19, 66]. This section focuses on the CTD-phosphatase of *S. cerevisiae*, its ability to co-purify with the elongating polymerase complex, and its activity in the context of native RNAPII elongation complexes and *in vitro* generated RNAPIIO ternary complexes.

3.2 Identification of Fcp1 within the elongating RNAPIIO complex

Elongating polymerase when purified from chromatin exists as a multiprotein complex. The most outstanding components are subunits of RNA polymerase II itself as well as the Elongator complex consisting of three novel proteins designated Elp1, Elp2, and Elp3 with respective apparent molecular weights of 150, 90, and 60 kDa. This elongating holo-polymerase complex had

proved difficult to purify. Its integrity depended on the maintenance of a hyperphosphorylated CTD and the polymerase became increasingly dephosphorylated during the first steps of purification even in the presence of the phosphatase inhibitor okadaic acid. These results prompted us to test the possibility that Fcp1, a CTD-specific phosphatase which is not inhibited by okadaic acid, might be associated with the elongating RNAPII holo-enzyme. Therefore, fractions from the fifth purification step of Elongator, chromatography through a MonoQ column, were separated on a 7.5% SDS polyacrylamide gel and immunoblotted using antibodies specific for Fcp1 (a kind gift of M. Kobor, U. Toronto). The peak of Fcp1 was found to overlap with that of Rpb1 and Elp1 (Figure 3.2.1). The phosphatase profile, however, trails, which suggests that not all of Fcp1 could be associated with polymerase and/or Elongator.

3.3 Genetic interactions between Fcp1 and Elongator

The observation that Fcp1 co-fractionates with Elongator and the elongating RNAPII complexes raised the possibility that these entities functionally interact. To investigate possible genetic interactions between Fcp1 and Elongator, the phenotypes of double mutants were studied in a collaborative effort with M. Kobor and J. Greenblatt at the Banting and Best Institute, University of Toronto. While knockouts of any of the Elongator genes are viable [40, 88, 142], deletion of *FCP1* is lethal [60]. Individual Elongator mutants, *elp1*, *elp2*, and *elp3*, were therefore crossed with a *FCP1* knockout carrying one of three temperature sensitive alleles on a CEN/ARS/TRP1 plasmid [60] and the wildtype *FCP1* on a URA3 plasmid. These strains failed to grow on the drug 5-FOA (which forces cells to lose the wildtype URA allele), even at the permissive temperature (Figure 3.3.1). This indicates that partial loss of Fcp1 function is lethal in the absence of Elongator. To investigate whether this synthetic lethality was due to the elimination of a physical interaction or a deletion of redundant functions, a functional mutant of Elongator was made and then crossed with the same temperature sensitive strains. Elp3 is a histone acetyltransferase (HAT). Mutation of either one of two tyrosines, Y540 or Y541, to alanine confers the same phenotypes as an Elongator gene deletion, which include slow adaptation to growth conditions and salt- and

temperature-sensitivity [142]. Interestingly, preliminary evidence indicates that the Elp3 Y540A/Fcp1^{ts} double mutant is viable and shows no additional phenotypes from the parental strains [M. Kobor, personal communication]. This is consistent with the possibility that the association between Fcp1 and polymerase depends on a physical interaction with Elongator, which can still occur in the Elp3 HAT mutant but not in the absence Elp3.

3.4 *In vitro* interactions between Fcp1, RNA polymerase II, and Elongator

Work by Chambers et al. indicated that FCP1 can dephosphorylate the CTD in the context of RNAPII but cannot use a hyperphosphorylated peptide as a substrate [17]. The addition of a CTD-less RNA polymerase inhibits the CTD phosphatase activity, which suggests that Fcp1 utilises docking sites on RNAPII that are distinct from the CTD [17]. However, these studies did not demonstrate a physical interaction between Fcp1 and RNAPII. The current study investigated whether Fcp1 interacts strongly with RNAPII and whether yeast Elongator proteins could affect this putative interaction. Purified proteins were incubated together and resolved on a Superose-6 gel filtration column. Stable protein associations would be expected to cause a shift in the elution profile.

Core polymerase was purified as described by Thompson and Burgess [121]. Histidine-tagged Elp1 was purified by a nickel-affinity column from baculovirus cells transfected with an *ELP1-6His* bearing plasmid. Elp2 and Fcp1 were kind gifts of, respectively, J. Walker (ICRF) and M. Kobor (U. Toronto). Approximately 10 pmoles of each protein were incubated in different combinations before being subjected to gel filtration chromatography.

Resolved alone on the sizing column, core polymerase, Fcp1, and Elp2 eluted as approximately 650 kDa, 100 kDa, and 90 kDa proteins as expected. Elp1, interestingly, behaved as a protein much larger than its calculated molecular weight, 150 kDa. This suggests that Elp1 either multimerises or has an elongated structure (Figure 3.4.1a). The elution profile of these proteins did not change when any combination of the four proteins resolved by gel filtration as shown in Figures 3.4.1b and 3.4.1c. These data indicate that none of these

proteins form tight pairwise or multi-component complexes and that Elp1 and Elp2 do not enable a strong interaction of Fcp1 with RNAPII.

3.5 Stimulation of Fcp1 activity within elongating RNAPIIO complexes in chromatin fractions

The elongating polymerase is hyperphosphorylated [55, 88, 92], yet both the biochemical and genetic data presented above suggest that Fcp1 can associate with the elongating polymerase *in vivo*. To avoid dephosphorylation of elongating polymerase, the activity of Fcp1 must, therefore, be held in check at least in early elongation complexes by either an inhibiting factor or an opposing kinase. To explore these possibilities, the phosphatase activity in native elongation complexes derived from chromatin extracts was examined.

Chromatin fractions were extracted from yeast cells as described by Otero et al. [88] and incubated in a variety of conditions. The phosphorylation state of RNAPII was monitored with antibodies directed against phosphorylated serine-5 of the CTD heptapeptide repeat. Since the C-terminal domain is prone to proteolysis, relative phosphorylation in these experiments was normalised against the signal generated by 8WG16, an anti-CTD antibody which preferentially recognises hypo-phosphorylated RNA polymerase II. The level of the Rpb3 RNAPII subunit was used as a loading control.

CTD dephosphorylation occurred upon incubation with $MgCl_2$ (Figure 3.5.1, compare lanes 1 and 2). This activity became more pronounced upon the addition of DNase (Figure 3.5.1, lane 3). A similar effect was seen upon addition of both DNase and nucleotides (Figure 3.5.1, lane 4). Addition of a large excess of exogenous Fcp1 did not significantly stimulate CTD dephosphorylation (Figure 3.5.1, lane 7). These results might suggest that a small percentage of RNAPII studied in these experiments are in native complexes that do not contain Fcp1 and that the CTDs of these polymerases are protected, possibly through contacts with other proteins, from dephosphorylation by exogenous Fcp1.

To confirm that Fcp1 caused the observed dephosphorylation, the phosphatase activities of chromatin fractions from wildtype W303 and temperature-sensitive Fcp1 YMK110 strains were compared. While CTD-

phosphorylation levels remained unaltered in the temperature-sensitive strain even at temperatures that are permissive for growth of this strain, levels of RNAPIIO decreased in wildtype (Figure 3.5.2).

To demonstrate that inability of YMK110 extracts to dephosphorylate the CTD was not due to a general lack of biochemical activity, non-specific transcription activity of the chromatin fraction was assayed at both 25°C and 30°C. Incorporation of $\alpha^{32}\text{P}$ -UTP indicated that the polymerases in the YMK110 chromatin fraction were transcription competent at both temperatures (Figure 3.5.3). Hence the absence of phosphatase activity in the YMK110 chromatin fractions was not due to general lack of biochemical activity but specifically due to the absence of Fcp1 phosphatase activity. Therefore, the decrease in CTD phosphorylation observed in the wildtype chromatin extracts can be attributed either to the direct action of Fcp1 or to a CTD phosphatase activity that is mediated by Fcp1.

3.6 *In vitro* Fcp1 activity within elongating RNAPIIO complexes

The stimulation of dephosphorylation in chromatin as described above suggests that the CTD is normally somehow protected from the activity of the phosphatase during initiation and transcript elongation. To determine what holds Fcp1 activity in check, Fcp1 phosphatase activity was studied in a defined *in vitro* system, and the phosphatase sensitivities of RNA polymerase that was free in solution or in a DNA-RNA ternary complex were compared.

Histidine-tagged holo-TFIIF was purified as described by Svejstrup *et al.* [113] and used to phosphorylate and ^{32}P -label the CTD of RNA polymerase II. This kinase reaction also labelled other proteins, which were removed together with TFIIF by nickel-agarose chromatography (Figure 3.6.1). Free γ - ^{32}P -ATP was then removed from the labelled polymerase via two Sephadex G25 columns, which also allowed an exchange into phosphatase activity buffer.

Ternary RNAPIIO-RNA-DNA complexes were formed by allowing the polymerase to transcribe a biotin-tagged transcription template made from annealed oligonucleotides, Txp1 and Txp2, in the absence of UTP and CTP (Figure 3.6.2). The DNA template had a 16-nucleotide 3' overhang, which

allowed polymerase to start transcription without the requirement of any general transcription initiation factors [31]. The omission of the two nucleotides caused RNAPIIO to stall after transcribing 36 nucleotides. A gel-mobility shift assay confirmed that ternary complexes were formed, and quantification of shifted product indicated approximately 65% of the polymerase were incorporated onto the DNA (Figure 3.6.3). Biotin on the transcription template allowed these ternary complexes to be separated from free polymerase by the use of streptavidin beads (Figure 3.6.4).

Free RNAPIIO and elongating RNAPIIO were then compared in their sensitivity to Fcp1. The polymerase in the ternary complex was much more resistant to dephosphorylation than free polymerase (Figure 3.6.5).

To address whether RNAPII interactions with the DNA or RNA could physically restrict access of proteins to the CTD in this *in vitro* system, RNAPIIA was used to form elongating polymerase-ternary complexes and was then incubated with TFIIH in the presence of γ -³²P-ATP. As shown in Figure 3.6.6, the kinase was actually more efficient in phosphorylating an elongating polymerase than one free in solution. This indicated that the CTD does not become inaccessible for protein interactions in ternary complexes.

The general transcription factor TFIIIF can stimulate Fcp1 activity on RNA polymerase II that is free in solution as shown by Archambault *et al.* and demonstrated in Figure 3.6.7, lanes 2 and 3 [4]. The addition of TFIIIF, however, did not stimulate the dephosphorylation of RNAPIIO incorporated into a ternary complex (Figure 3.6.7, lane 7). Since Fcp1 is a subunit of elongating polymerase, interactions with other elongating factors may influence dephosphorylation of elongating RNAPIIO. The addition of Elongator (gift of B. Winkler (ICRF)) did not significantly affect Fcp1 activity (Figure 3.6.7, lane 8).

3.7 Discussion

Fcp1 has been identified as a phosphatase belonging to a small family of phosphatases and phosphotransferases containing the motif DXDX(T/V) [60]. After five steps of purification, the phosphatase co-fractionates with Elongator and elongating RNAPII on a gel filtration column. Fcp1 is also shown to have a

genetic interaction with Elongator. Synthetic lethality occurs when temperature-sensitive phosphatase mutants are crossed with Elongator subunit knockouts but not with an otherwise non-functional Elongator HAT mutant. The viability of an histone acetyltransferase defective Elongator/temperature sensitive *fcp1* double mutant suggests that the enzymatically-dead Elongator might provide a structural function. Fcp1 might thus have physical interactions with Elongator and could depend on these contacts for efficient access to the CTD.

The inability to reconstitute a partial elongating polymerase complex from purified RNA polymerase II, Elp1, Elp2, and Fcp1 could have several explanations. First, Elp3 may be required for a stable elongating polymerase complex. This protein was not included in these interactions studies because, when over-expressed on its own, it was insoluble and could not be purified outside of the Elongator complex. The Elongator complex itself was not included in these studies for practical reasons. At the time of these experiments, there was not an ample supply of the complex.

RNA polymerase II used for these interaction studies was not highly phosphorylated. Although the Elongator is capable of forming a complex with hypophosphorylated RNAPII[88], a stable complex of the individual subunits with RNAPII might require a hyperphosphorylated CTD. Lastly, other factors might be needed to load these proteins or for their stable association with RNA polymerase II.

The CTD must maintain a hyperphosphorylated state in order to stimulate activities that work in parallel with transcript elongation such as RNA capping and splicing [74, 81]. The phosphatase activity, however, in ternary complexes isolated from chromatin could be stimulated by the addition of magnesium and/or DNase. The *in vitro* assays demonstrated that RNAPIIO becomes resistant to Fcp1 upon incorporation into a DNA-RNA complex. Furthermore, although TFIIIF can stimulate the Fcp1-mediated dephosphorylation of free RNAPIIO, the transcription factor has no effect on sensitivity of elongating RNAPIIO to Fcp1. The absence of any effect on Fcp1 dephosphorylation of Elongator addition might, unfortunately, be due to limited activity of the complex tested. Until very recently, there has not been a reliable biochemical assay to assess whether the purified Elongator complex has

activity *in vitro*. At this time, a possible role for Elongator in the modulation of Fcp1 activity cannot be ruled out.

The results with yeast Fcp1 presented here differ from those found in the mammalian system by Lehman and Dahmus and Cho *et al.* [19, 66]. In the *in vitro* system of Cho *et al.*, RNAPII elongating from the adenovirus late promoter was washed in Sarkosyl to remove general transcription factors and treated with Fcp1; dephosphorylation of the CTD was observed [19]. In Lehman and Dahmus' system, core RNAPIIO elongating along a dC-tailed template and in the presence of the RAP74 subunit of TFIIF was found to be as sensitive to Fcp1 dephosphorylation as free RNAPIIO. Resistance to dephosphorylation was, however, observed when transcription was initiated in nuclear extracts, which suggests that additional factors modulate the Fcp1 sensitivity of mammalian RNAPIIO.

The observed differences between the yeast and mammalian phosphatase systems might be explained in two ways. During the initial characterisation of yeast Fcp1, Chambers and Kane found that an additional factor, now designated FcpX, significantly stimulated the activity of the CTD phosphatase [16]. This factor may also be required for the efficient activity of Fcp1 when elongating yeast RNAPIIO is its substrate. In the experiments presented here, the formation of a phospho-protein that co-migrates with Fcp1 is observed (Figure 3.6.4 and Figure 3.6.6). It is therefore possible that Fcp1 acts as a phosphotransferase which removes phosphates from the CTD and requires FcpX for efficient enzymatic turnover. Alternatively, the resistance of the elongating polymerase to Fcp1 could be caused by a sequence specific or system-specific RNA-DNA structure which interferes with Fcp1 docking onto the polymerase.

Recent work by the Buratowski and Bentley laboratories have used yeast chromatin immunoprecipitations to examine the *in vivo* phosphorylation states of RNAPII through the transcription cycle [61, 102]. They found that serine-5 of the CTD became increasingly dephosphorylated while serine-2 phosphorylation was seen to increase as the polymerase transcribed along the coding region [61]. Schroeder *et al.* compared the amount of CTD phosphorylation at the 5' and 3' ends of transcripts. They found that in wildtype cells, the 5':3' ratio of phosphorylated serine-5 was 25:1 while in a Fcp1-temperature sensitive strain

at a restrictive temperature, this ratio was close to 4:1 [102]. These data indicate that the serine-5 dephosphorylation observed along a gene is indeed due to Fcp1 activity *in vivo* [102].

The data presented in this chapter can be combined with the results of others to formulate the following model for phosphorylation states during the transcription cycle (Figure 3.7.1). RNA polymerase is recruited to the promoter in its hypophosphorylated state and associated with the Mediator complex [114]. During the transition between initiation and elongation, the CTD becomes phosphorylated at serine-5 by TFIIF in a reaction stimulated by Mediator [58], and RNAPII then associates with Elongator instead [88]. As has been shown in this work, Fcp1 may be a constituent of elongating polymerase and might require specific signals to become activated. Therefore, during elongation, Fcp1 acts to dephosphorylate serine-5 while other CTD-kinases such as CTDK-1 phosphorylates serine-2. This progressive change in CTD phosphorylation might turn off phospho-serine-5 stimulated functions, such as capping [61, 102], and also trigger phospho-serine-2 stimulated activities, such as 3' end formation [80].

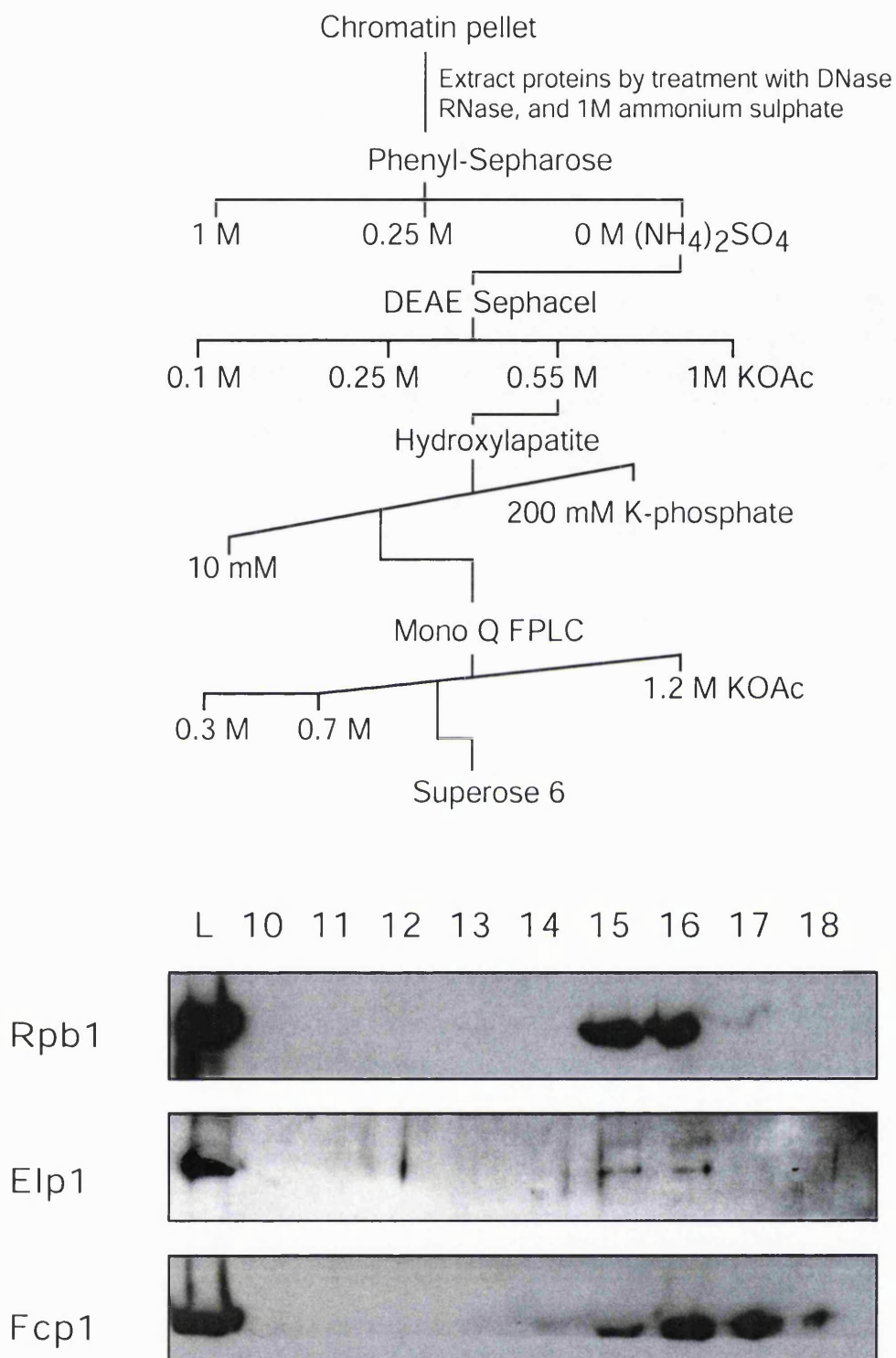


Figure 3.2.1. **Co-purification of Fcp1 and the Elongating polymerase.** Elongating polymerase purified from chromatin extracts by the scheme diagrammed above. Superose 6 fractions were loaded onto a 7.5% SDS-PAGE, blotted, and probed with anti-CTD, anti-Elp1, and anti-Fcp1 antibodies.

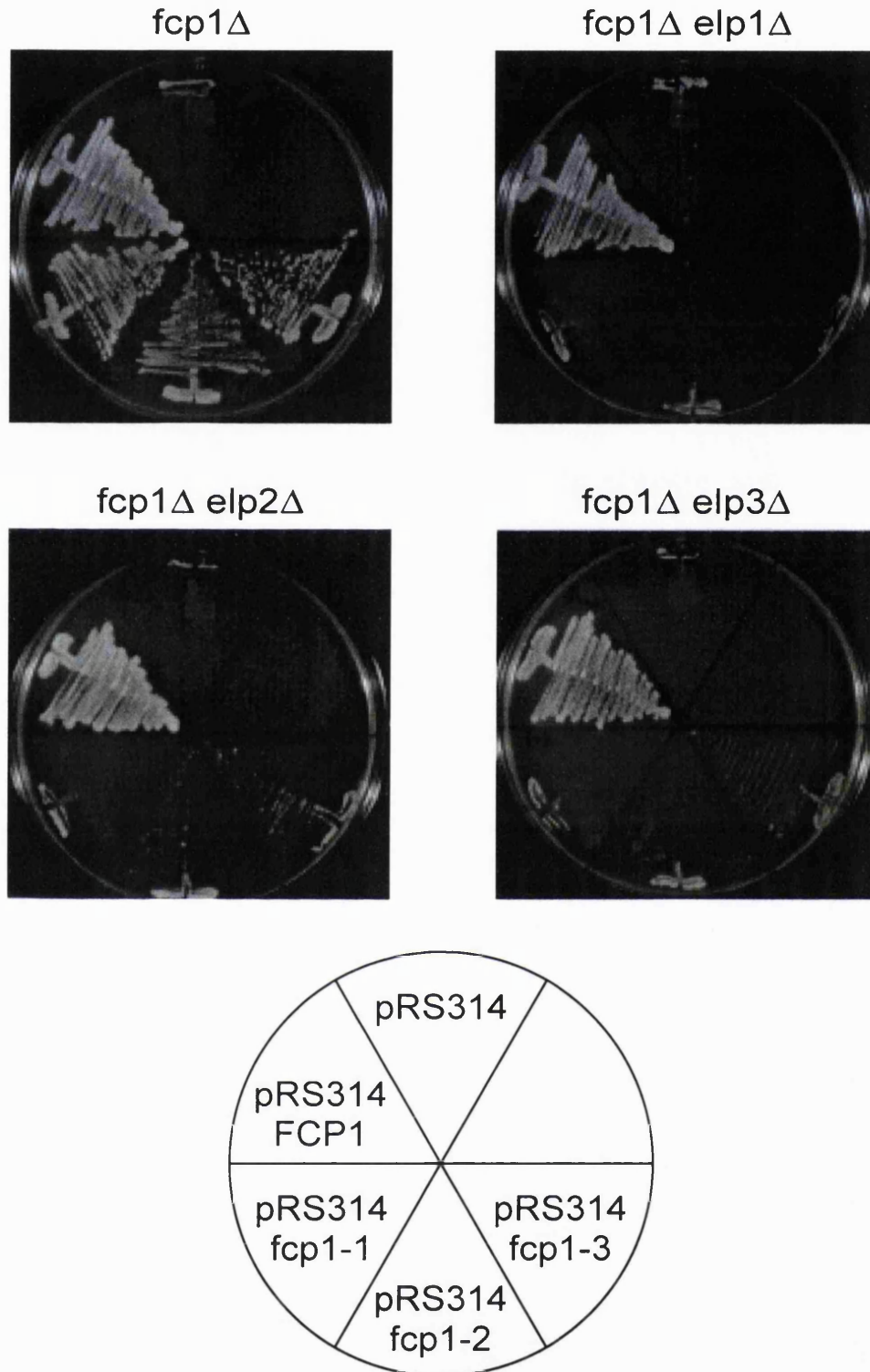


Figure 3.3.1. **Genetic interaction between FCP1 and Elongator.** Knockout mutants of Elongator subunits *elp1*, *elp2*, and *elp3* were crossed with the *fcp1Δ* strain carrying a CEN/ARS/URA plasmid carrying the wildtype *FCP1* and CEN/ARS/TRP1 plasmid with one of three temperature sensitive *fcp1* mutations: *fcp1-1*(R250A, P251A), *fcp1-2* (L177A, L181A, H187A), and *fcp1-3*(Δ2-134), which is truncated at amino acid 134. Strains were streaked on synthetic complete media lacking tryptophan and containing 5-FOA and grown at 30°C.

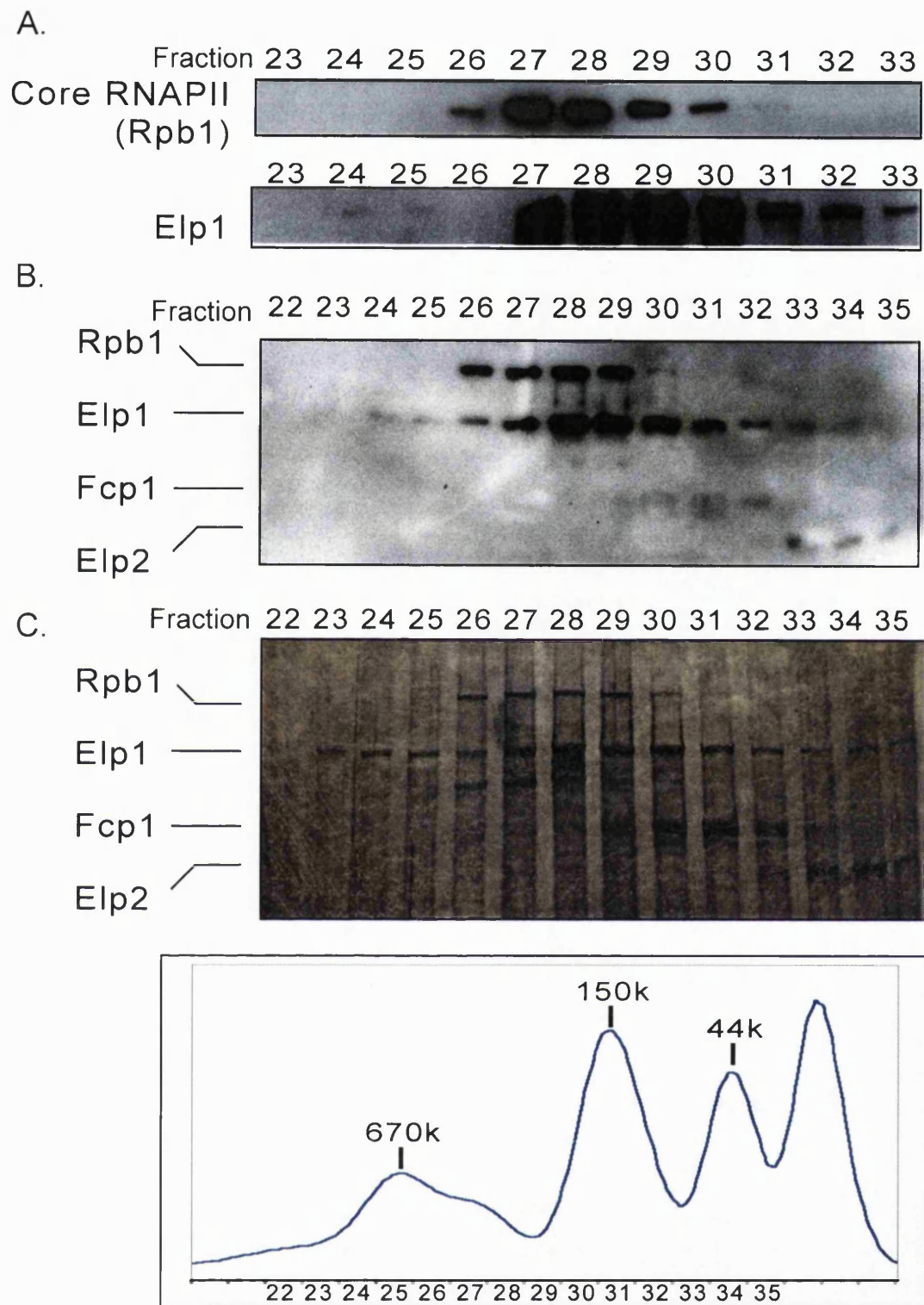
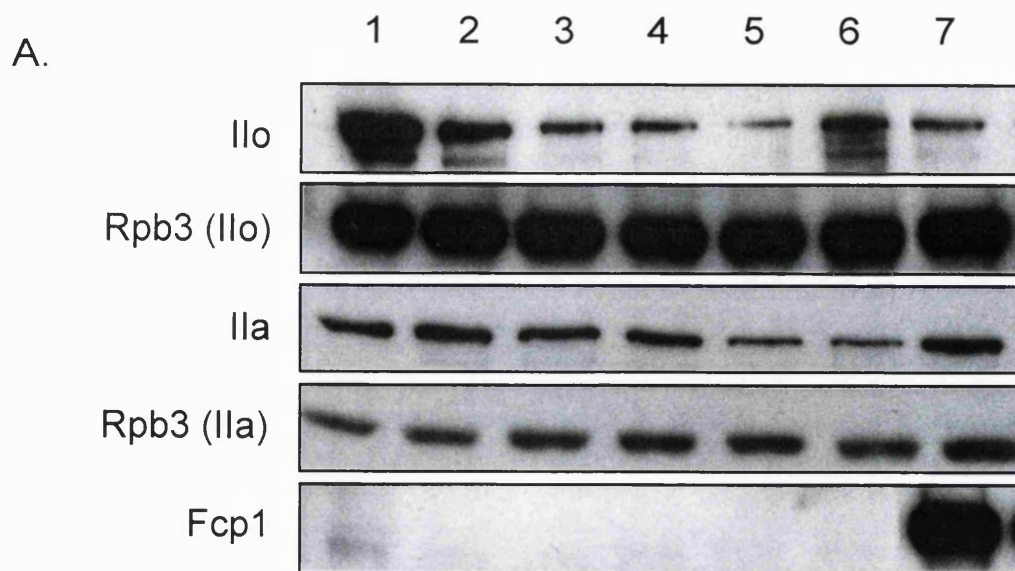


Figure 3.4.1. Possible protein-protein interactions between Core Polymerase, Fcp1, and Elongator subunits Elp1 and Elp2. A. Core polymerase and Elp1 individually resolve as large complexes. Purified RNAPII and Elp1 were subjected to gel filtration through a Superose 6 (Pharmacia) column. Fractions were probed with 8WG16 anti-CTD antibodies and anti-Elp1 antibodies. B. Western blot of incubated RNAPII, Elp1, Fcp1, and Elp2. Proteins were incubated on ice in a buffer of containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 200 mM NaCl and 0.01% NP-40 and resolved as above. Fractions were probed with 8WG16 anti-CTD, anti-Elp1, anti-Fcp1, and anti-Elp2 antibodies. C. Silver stain of incubated proteins. The same fractions as in B were separated on a 7.5% PAGE and silverstained. Elution profile of size markers (BioRad) is shown in diagram.



DNAse	-	-	+	+	+	+	+
MgCl ₂	-	+	+	+	+	+	+
MnCl ₂	-	+	+	-	+	+	-
NTPs	-	-	-	+	+	+	+
Sarkosyl	-	-	-	-	-	+	-
Fcp1	-	-	-	-	-	-	+

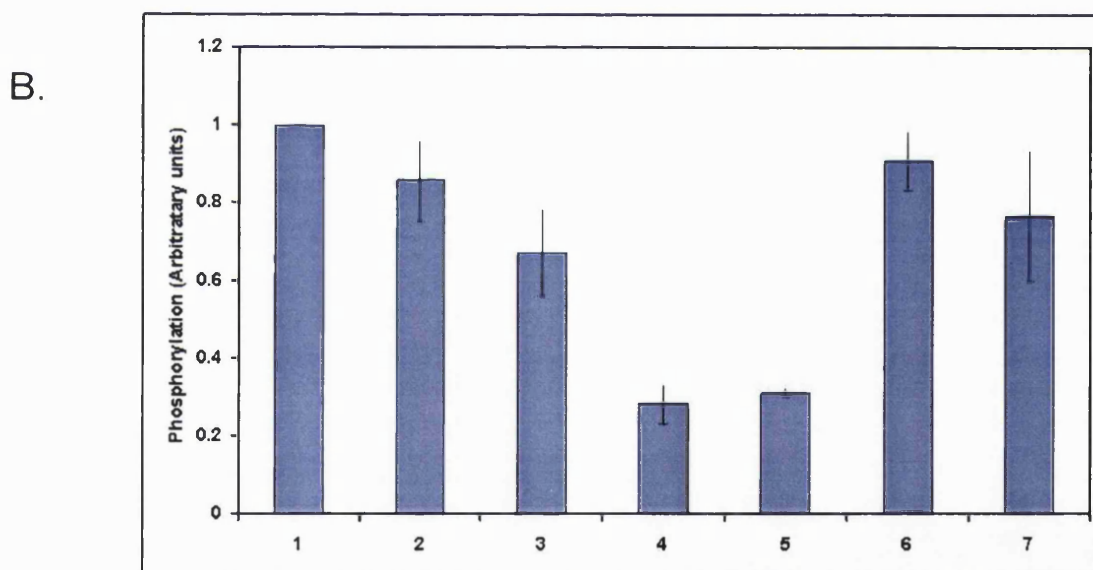


Figure 3.5.1. **Stimulation of Fcp1 activity in chromatin extracts.** A. Levels of phosphorylated CTD were monitored by Western analysis using antibodies against phosphorylated CTD serine-5, hypophosphorylated CTD (8WG16), Rpb3, and Fcp1. All experiments used 2 μ l of chromatin extract. *Lane 1* contains a mock-treated control; *lane 2* - same as lane 1, with 10 mM MgCl₂, and 10 mM MnCl₂; *lane 3* - same as lane 2 with 10 U DNase; *lane 4* - same as lane 1 with 10 mM MgCl₂, 1 mM each NTP and 10 U DNase; *lane 5* - same as lane 2 with 1 mM each NTP and 10 U DNase; *lane 6* - same as lane 4 but with 0.5% Sarkosyl. *Lane 7* contains the same as lane 4 and 100 ng Fcp1. Samples were separated on a 7.5% SDS-PAGE before blotting. Western signals were analysed using NIH Image and are presented in B. Loading variations were normalised using Rpb3 signal, while levels of CTD were normalised using 8WG16 signal.

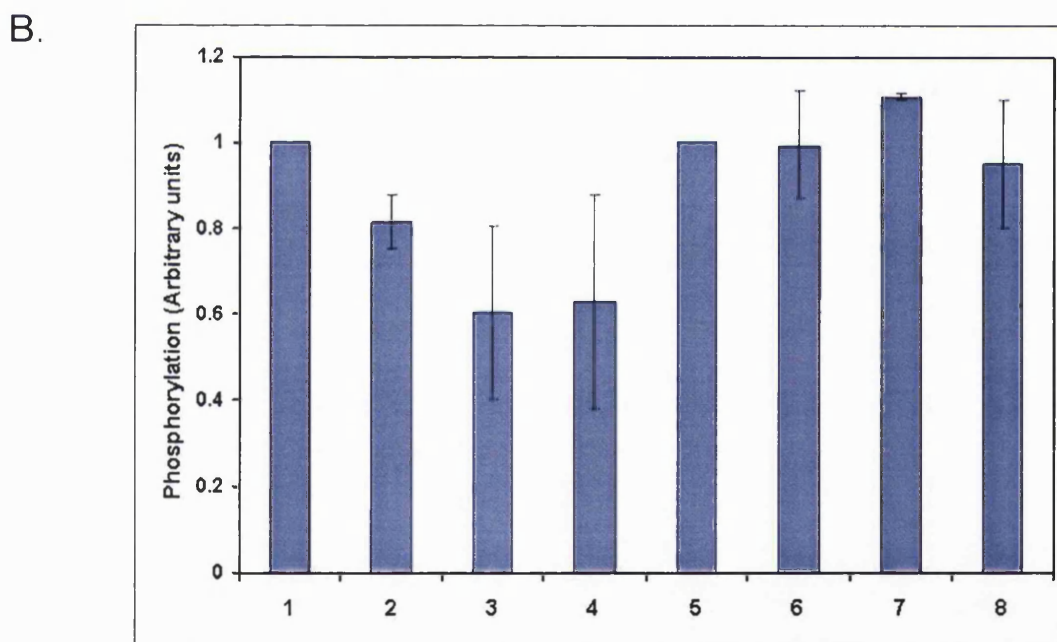
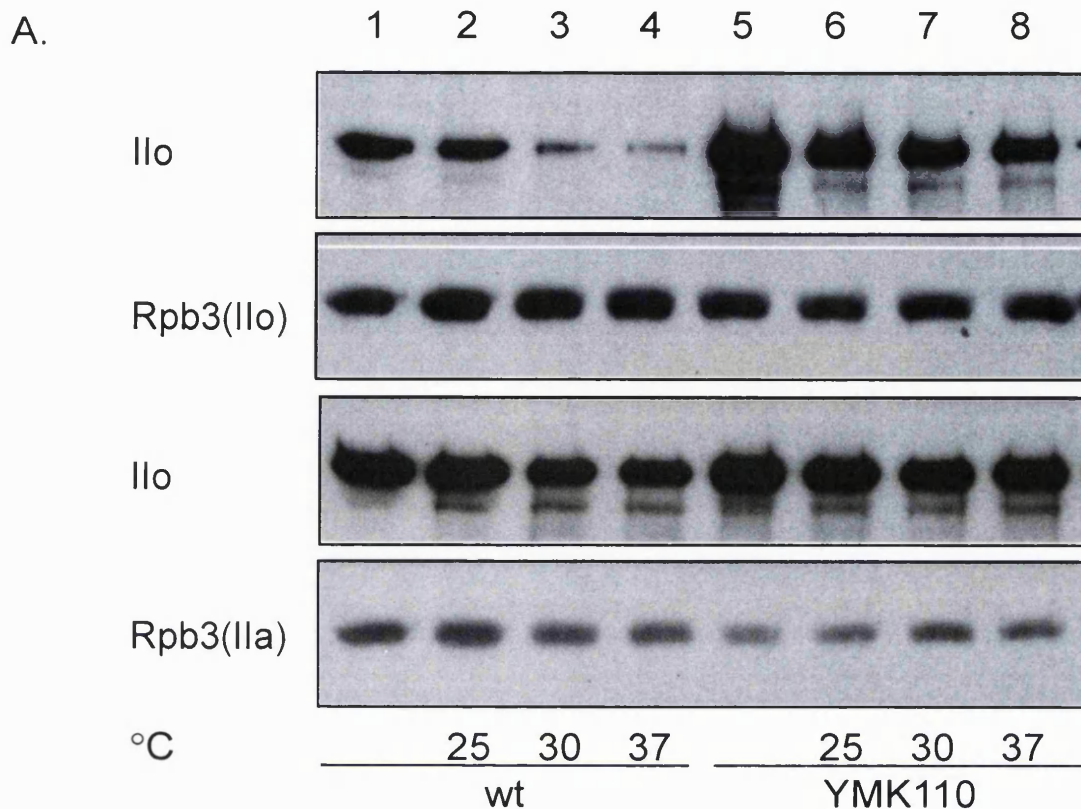


Figure 3.5.2. **Dephosphorylation of CTD is dependent on Fcp1.**

A. Levels of CTD phosphorylation were monitored by Western blot analysis using antibodies specific for CTD phosphorylated at serine-5, CTD, and Rpb3. Chromatin extract from wildtype W303 (*lanes 1-4*) and Fcp1^{ts} strain YMK110 (*lanes 5-8*) were incubated in the presence of 10 U DNase, 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM of each NTP. *Lanes 2 and 6* were treated at 25°C; *lanes 3 and 7* at 30°C; and *lanes 4 and 8* at 37°C. *Lanes 1 and 5* contain untreated controls. Western blots were analysed with NIH image, and quantification is presented in B. Loading variation was normalised by Rpb3 signal, and levels of CTD monitored by 8WG16 signal.

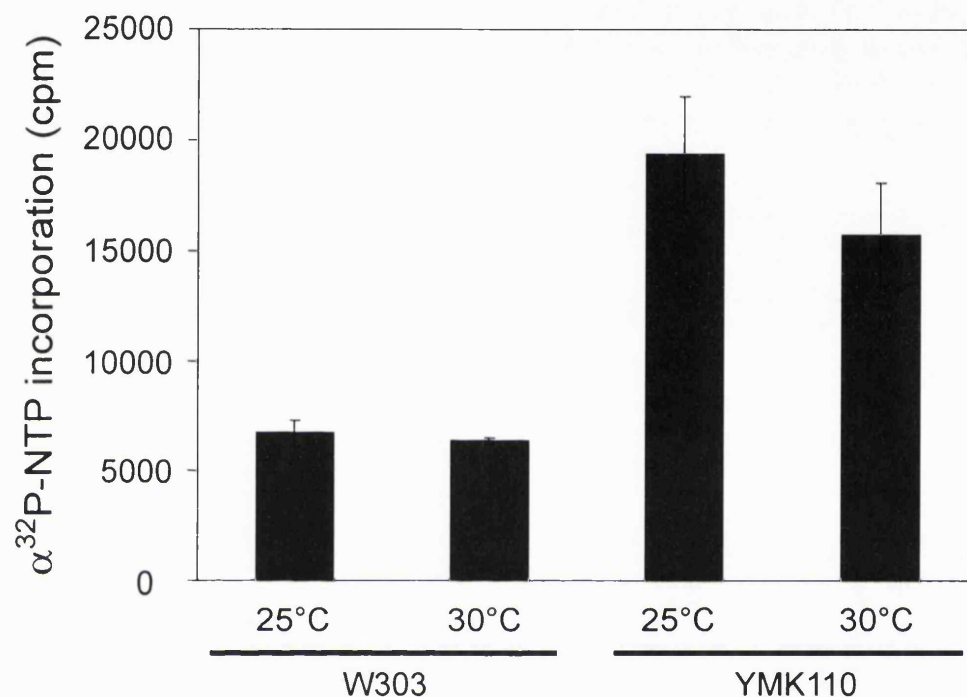


Figure 3.5.3. **Transcriptional activity of the amounts of wildtype W303 and Fcp1^{ts} YMK110 chromatin extracts used in phosphatase experiments.** Extracts were incubated in the presence of $\alpha^{32}\text{P}$ -UTP in transcription conditions for 1 hour at either 25°C or 30°C. Transcripts were applied to DE81 paper, washed three times in 5% (w/v) sodium phosphate, 0.5% (w/v) sodium pyrophosphate-10 H₂O, and 0.1% (w/v) SDS, and counted by a scintillation counter.

Figure 3.6.1:

Phosphorylation of the Rpb1 subunit of RNAPII and removal of TFIID and associated proteins by Ni-Agarose.

100 ng of purified RNAPII was phosphorylated by TFIID as described in Chapter 2.7 (lane 1). TFIID and associated proteins were removed from the RNAPII by incubation with 10 μ l Ni-Agarose beads (Qiagen) in the presence of 10 mM imidazole for 15 minutes at 25°C. Lane 2 represents the supernatant after Ni-Agarose treatment. Both lanes originate from the same gel autoradiograph and extraneous lanes were removed for clarity.

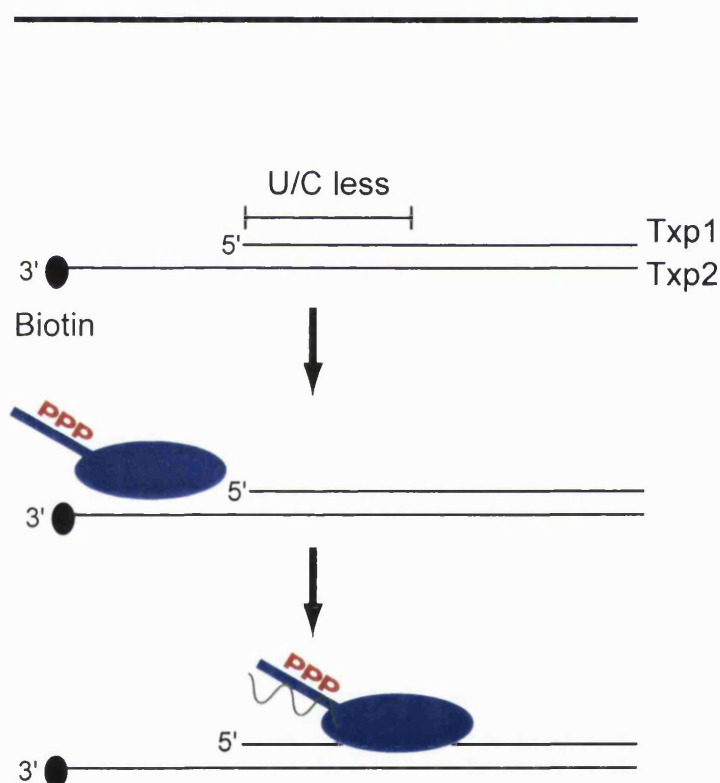
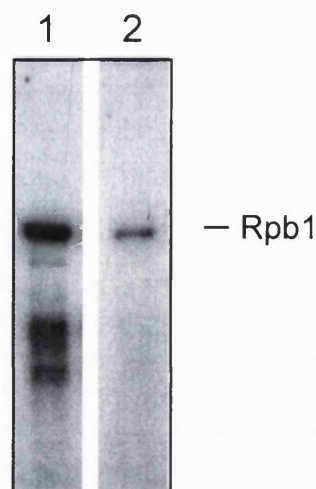


Figure 3.6.2. Formation of ternary complexes in vitro. Two oligonucleotides, Txp1 and Txp2, are annealed together to form a double-stranded DNA substrate with a 3' overhang. Phosphorylated RNA polymerase can be loaded onto this overhang and transcribe the template strand independent of general transcription initiation factors. RNAPII can be stalled on the complex by the omission of UTP and CTP from the transcription reaction.

Figure 3.6.3:

Gel mobility shift of DNA template by elongating RNAPII. 100 ng of purified RNAPII used to transcribe a 25-fold molar excess of template formed by the annealed oligonucleotides Txp1 and Txp2 which were end labelled by T4 polynucleotide kinase in the presence of $\gamma^{32}\text{P}$ -ATP. Forms were separated on a non-denaturing 1xTBE, 29:1 acrylamide:bis-acrylamide gel run at 4°C.

Lane 1 contains the DNA only control; *lane 2* shows the gel-shift caused by an elongating polymerase on the template DNA.

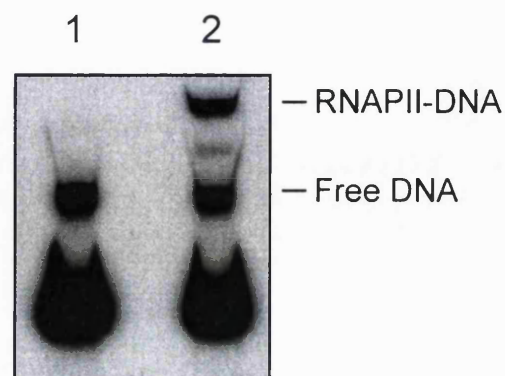
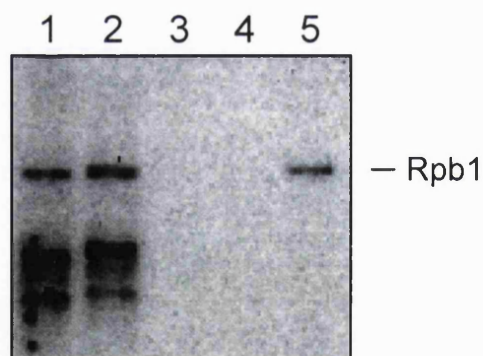


Figure 3.6.4:

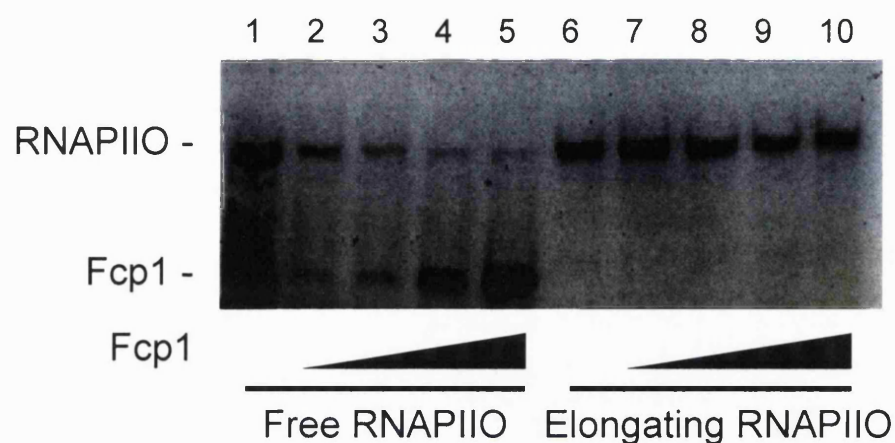
Isolation of RNA polymerase stalled on biotinylated transcription substrate.

RNAPII, the Rpb1 subunit of which is radioactively phosphorylated by TFIIF in the presence of $\gamma^{32}\text{P}$ ATP is allowed to transcribe along biotinylated DNA templates bound to streptavidin Dynal beads. *Lane 1* represents RNAPII before transcription,

lane 2 the supernatant from the beads after transcription, *lanes 3 and 4* two subsequent washes of the beads before the beads were resuspended in 1x SDS-load buffer and boiled for 5 minutes to release phosphorylated RNAPII (*lane 5*).



A.



B.

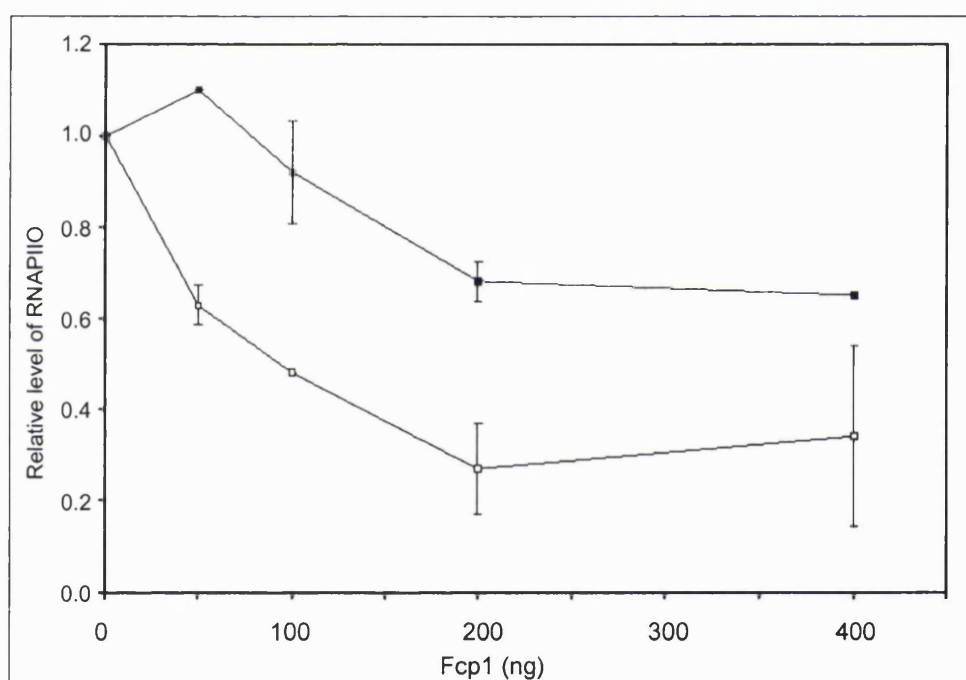


Figure 3.6.5. **Stalled RNA polymerase is resistant to dephosphorylation by FCP1.** 100 ng of RNAPII, which had been phosphorylated with TFIIH in the presence of γ 32 P ATP, either free in solution after nickel and Sephadex G25 chromatography (*lanes 1-5*) or stalled after transcription on bead-bound DNA templates (*lane 6-10*), was incubated with 0, 50, 100, 200, or 400 ng of Fcp1. A. Samples were separated by 7.5% SDS-PAGE and exposed to a phosphorimager screen. B. Autoradiograph signal as quantified by phosphorimager (ImageQuant, Molecular Dynamics). Free RNAPII is represented by - \square -, while RNAPII stalled on the transcription template is represented by - \blacksquare -.

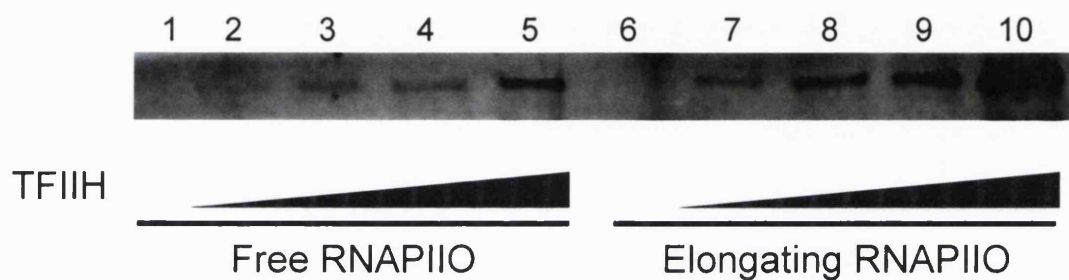


Figure 3.6.6. **The phosphorylation of the CTD tail is not inhibited by the ternary complex.** 100 ng of purified RNAPII, either free in solution (*lanes 1-5*) or stalled after transcription on bead-bound DNA templates (*lane 6-10*), was incubated with 0, 0.06, 0.12, 0.25, and 0.5 μ l of TFIIH for 30 minutes at 30°C. Samples were separated by 7.5% SDS-PAGE before exposure to a phosphoimager screen. Western analysis of the Rpb3 subunit of RNAPII indicates that there was at least four-fold more polymerase in the free RNAPIIO samples than in the Elongating RNAPII samples, making the difference even more significant

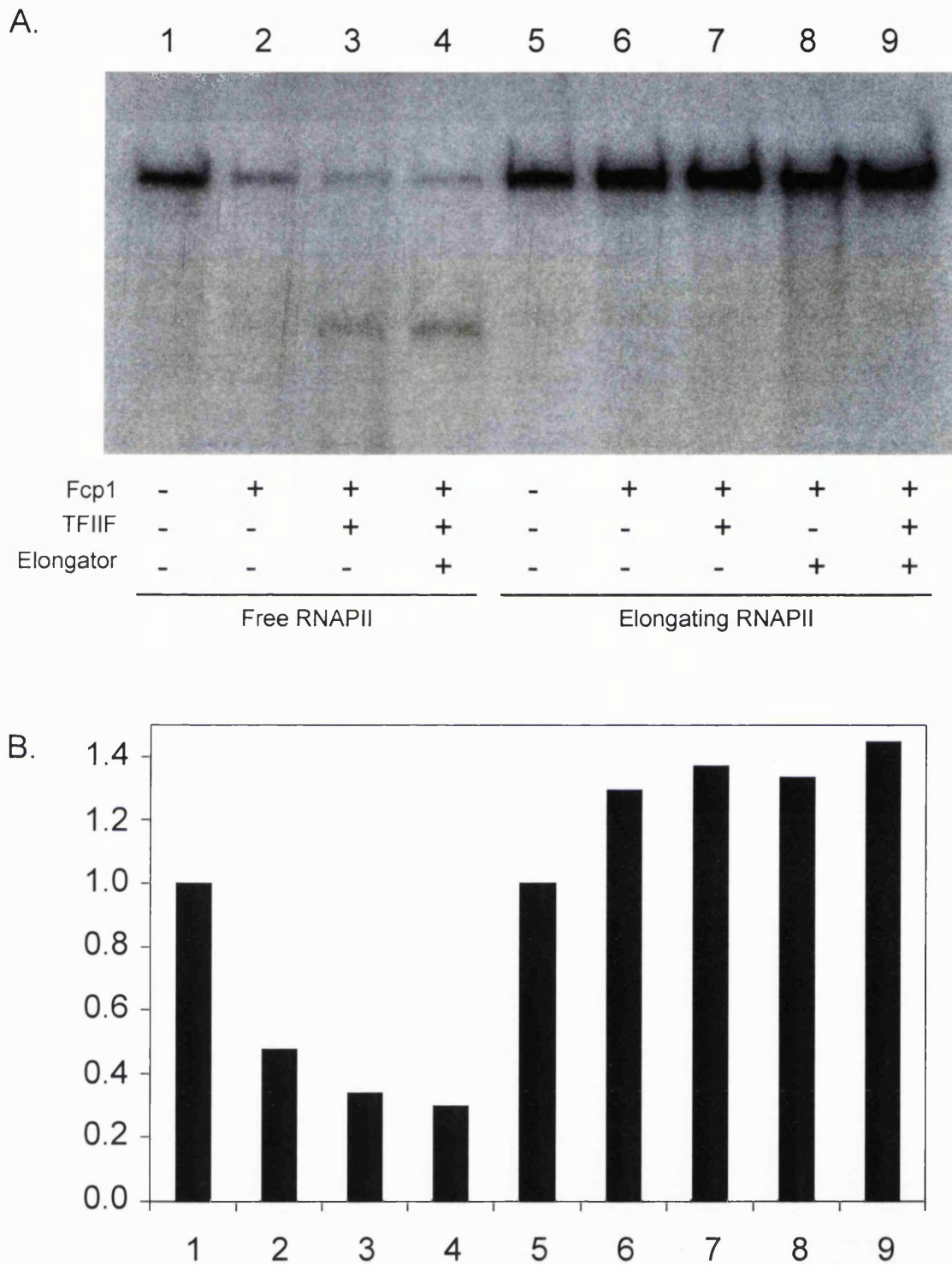


Figure 3.6.7. **Effects of TFIIF and Elongator on Fcp1 activity.** A. Free (*lanes 1-4*) or elongating (*lane 5-9*) ^{32}P -labelled RNAPII that had been phosphorylated by TFIIF was incubated with Fcp1 alone (*lanes 2 and 6*), Fcp1 and TFIIF (*lanes 3 and 7*), Fcp1 and Elongator (*lane 8*), or Fcp1, TFIIF, and Elongator (*lanes 4 and 9*). B. Signal was quantified by NIH image and diagrammed.

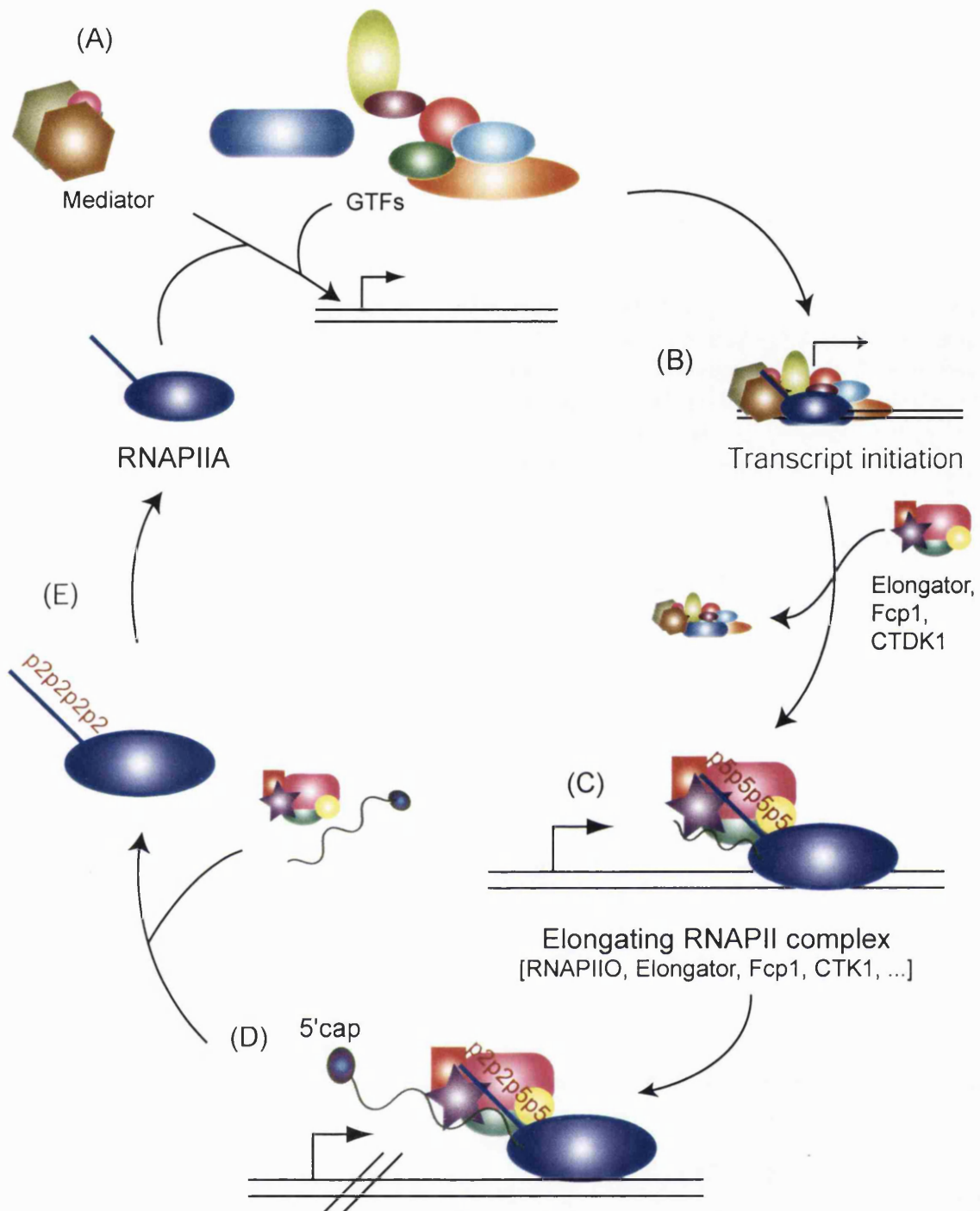


Figure 3.7.1. **Schematic diagram of transcription/phosphorylation cycle model.** A. Dephosphorylated RNAPII (RNAPIIA) is preferentially incorporated into the pre-initiation transcription complex with Mediator and the general transcription factors. B. After transcript initiation, the CTD becomes phosphorylated, most likely by TFIIF, and the hyperphosphorylated RNAPII, RNAPIIO, associates with Elongator, Fcp1, and possibly the serine-2 kinase CTDK-1. C. The elongating polymerase complex transcribes its template. During this process, mRNA processing proteins associate via interactions with the phosphorylated CTD; Fcp1 dephosphorylates serine-5; and serine-2 becomes phosphorylated by the action of a kinase such as CTDK-1. D. This change in serine-2/serine-5 phosphorylation causes a dissociation of capping proteins and the association of polyadenylation and 3'-end formation complexes. Upon transcript termination and RNAPII release, the polymerase becomes dephosphorylated and possibly modified so that it may return to the promoter for another round of transcription.

CHAPTER FOUR: CHARACTERISATION OF NUCLEOTIDE EXCISION REPAIR IN YEAST *SACCHAROMYCES CEREVISIAE* AND ESTABLISHING AN *IN VITRO* TRANSCRIPTION- COUPLED REPAIR SYSTEM

4.1 Background

Nucleotide excision repair removes a broad spectrum of damage through the action of more than 20 proteins. This process has two subpathways -- global genome repair and transcription coupled repair. Although the core mechanism of NER has been elucidated in detail, the mechanistic link between NER and transcription in eukaryotes remains elusive. Genetic evidence implicates a role for CSB/Rad26 in transcription-coupled repair (TCR). Interaction studies suggest CSB/Rad26 act as the transcription-repair coupling factor, as it has been shown that this protein can sequester TFIIH to a DNA-RNA polymerase II-CSB-TFIIH quaternary complex [117].

An *in vitro* system is required in order to establish a mechanism for transcription coupled repair. It would address which factors are involved in transcription coupled nucleotide excision repair (TC-NER) and elucidate the fate of the RNAPII that is stalled at the damage and of the other polymerases which are loaded and transcribing behind it. A model system requires a DNA substrate which can support transcription and contains a single, defined DNA lesion. Core RNAPII can then transcribe along this template and stall at the DNA damage thus presenting the initial platform to study repair in the context of an obstructing polymerase. Whole cell extracts or purified fractions can be added to this stalled polymerase complex leading to the eventual identification of the proteins required for TC-NER.

The studies presented here establish a method for making a whole cell extract that is competent for both transcription and repair. Since excision sites and products vary depending on the type of DNA lesions, initial experiments focus on characterising the repair of a single intrastrand 1,3-(pGpTpG)-cisplatin lesion. The repair of this lesion by yeast whole cell extracts is then compared to that performed by the mammalian NER proteins, which have been studied in

detail [36, 79]. NER can then be studied in the context of transcription and compared to the results obtained with transcription-independent repair.

4.2 Transcription and repair competent yeast whole cell extracts

The ideal whole cell extract to study TC-NER had to fulfil two requirements. First, it had to be active for both transcription and repair. Second, the extraction method had to be feasible for large-scale preparations since the cell extract would later be the starting materials for the purification of non-abundant proteins. Previously described NER competent extracts were either extracts from spheroplasts [136] or nuclear extracts that required the addition of whole cell extracts from a Rad2-overexpressing yeast [135]. The spheroplast method proved impractical for cultures over 10 litres, and the latter extract strayed from physiological conditions.

The method I decided to develop further was initially established by Schultz *et al.* to study the activity of RNA polymerase I [103] and later modified by He *et al.* [49]. Briefly, washed cells were frozen in liquid nitrogen, placed into a ceramic mortar, and broken under liquid nitrogen with manual grinding with a pestle until at least 50% lysis had been achieved. The broken cells were then thawed in one cell pellet volume of buffer and cleared of cell debris by a 10,000xg centrifugation followed by a 120,000xg ultracentrifugation. The supernatant was separated from the lower, viscous chromatin layer and the upper lipid layer and concentrated by ammonium sulphate precipitation. The protein precipitate was pelleted, resuspended in a minimal volume of buffer and dialysed overnight. Extracts then were clarified by a 10,000xg centrifugation step. This method was found applicable for cell pellet masses up to 1.5 kg and yielded approximately 1 mg whole cell extract protein per gram of cell pellet. Although Schultz *et al.* have suggested that a Waring blender and dry ice can substitute for the mortar, pestle, and liquid nitrogen [104], I found that this modification results in excess protein degradation and shearing of DNA.

To demonstrate that the extracts made in the above fashion were transcriptionally active, 20 µg of whole cell extract from BJ2168 cells was used to transcribe one of two substrates. One substrate, pGAL4-GC–, contained the *CYC1* promoter followed by a cassette that encodes a 381 nucleotide guanine-

less (G-less) transcript [70]. The other transcription template, pRNR3-G⁻, had the *RNR3* promoter followed by the same G-less cassette. The G-less cassette enables removal of all non-specific transcripts by RNaseT1 digestion. Robust transcription occurred from both the *GAL4-CYC1* and *RNR3* promoters (Figure 4.2.1 and Figure 4.2.5).

The extract was assayed for nucleotide excision repair activity by monitoring levels of repair synthesis in damaged plasmids as measured by the amount of incorporated radiolabelled nucleotide (Figure 4.2.2 A). Upon incubation with NER proficient HeLa or yeast whole cell extracts, more radioactive dCTP is incorporated into the faster migrating, UV-damaged plasmid than its non-damaged control (Figure 4.2.2 B). Similarly, both these extracts were able to repair cis-platin damage (Figure 4.2.2 C).

In addition to transcription and repair, the whole cell extract was capable of assembling nucleosomes upon a naked DNA template as measured by its ability to supercoil relaxed plasmids. When chromatin is assembled on DNA in the presence of topoisomerase activity, the placement of one nucleosome results in one negative supercoil after the removal of protein [42, 56]. As shown in Figure 4.2.3, incubation of a relaxed plasmid with the whole cell extract resulted in such increased level of supercoiling. Chromatin assembly is rapid, and ATP acts as a positive co-factor (Figure 4.2.4). This activity was confirmed to be nucleosome loading since the induction of supercoiling was stimulated by the addition of purified histones (J. Svejstrup, personal communication, [105]).

Preliminary work by J. Murguia and N. Lowndes (ICRF) in collaboration with myself indicate that wildtype whole cell extract is capable of inducing DNA-damaged dependent Rad53 phosphorylation. Rad53 is involved in the DNA-damage-dependent checkpoint pathway in *S. cerevisiae* and via a complex signal cascade becomes phosphorylated after DNA damage [93]. The results of Murguia and Lowndes suggest the existence of a partially intact DNA-damage checkpoint pathway *in vitro* that extends from the sensing of DNA damage and to Rad53 phosphorylation. Other than cell cycle arrest, downstream responses in this pathway include the transcriptional induction of several genes, one of which is *RNR3*. To investigate whether the extract can support a DNA-damage dependent induction of *RNR3* transcription, transcription of the G-less cassette from the pRNR3-G⁻ template as

diagrammed in Figure 4.2.1 was measured with and without the addition of transcriptionally silent UV-damaged pBluescript DNA was then monitored. Upon co-incubation with damaged DNA, transcription levels actually decreased (Figure 4.2.5). This indicates that the whole cell extracts as prepared above are not capable of this DNA-damage dependent transcriptional response. This result, however, has other implications as will be addressed in the Discussion.

4.3 Characterisation of nucleotide excision repair of intrastrand 1,3-(pGpTpG)-cisplatin lesions

Nucleotide excision repair of the intrastrand 1,3-(pGpTpG)-cisplatin lesion by human proteins releases the damage within a 26-30 nucleotide oligonucleotide. These products are the result of incisions at the 9th phosphodiester bond 3' from the lesion and at approximately the 15th phosphodiester bond 5' [79].

The characteristics of the yeast NER products were first studied by a Southern blot method outlined in Figure 4.3.1 A. Whole cell extract from BJ2168 yeast cells was incubated with double-stranded M13 containing a single defined 1,3-(pGpTpG)-cisplatin lesion. The dual incision products were purified, separated on a 12% denaturing acrylamide gel, and analysed by Southern blot using a probe specific for the damaged region. The yeast excision step took 60 minutes to reach completion and yielded products ranging from 23 to 26 nucleotides in length (Figure 4.3.2).

The 3' incision site for yeast NER was mapped by primer extension (Figure 4.3.1 B). As shown in Figure 4.3.3, there are three main incision points starting at the 15th phosphodiester bond from the damage. This suggests that the 5' incision occurs around the 7th phosphodiester bond upstream of the cisplatin lesion.

The sequence of the 3' end of the excised product allowed the dual incision step to be assayed by end-labelling (Figure 4.3.1 C). An oligonucleotide complementary to the excised sequence and carrying a 5' AAA tail was annealed to dual incision products of the repair reaction. The products were then labelled with $\alpha^{32}\text{P}$ -TTP before separation on a 15% denaturing

acrylamide gel (Figure 4.3.4, lane 1). To confirm that the signals were specific to NER, whole cell extracts were made from *rad10* and *rad14* knockout strains, which are repair deficient. These extracts on their own have no activity (Figure 4.3.4, lanes 2 and 3) yet, as expected, could complement each other (Figure 4.3.4, lane 4).

4.4 Purification of a complete Repairosome

To progress further with the *in vitro* TC-NER system, an *in vitro* NER system reconstituted from purified components is needed. Repairosome as defined by Svejstrup *et al.* contains at least the proteins Rad1, Rad2, Rad4, Rad10, Rad14, and core TFIIH since the complex can complement the NER-deficient extracts from strains lacking these respective subunits [115]. However, when this repairosome was assayed on its own, no NER activity was detected (Figure 4.4.1, lanes 7, 9, 11, 13).

Reconstitution studies by Guzder *et al.* indicate that the proteins required for NER include those mentioned above and Rad23 and RPA [44]. Since Rad23 associates tightly with Rad4 [44, 137], which is already present in the repairosome, RPA may be the missing subunit required for activity. The addition of RPA to purified repairosome did not confer activity (Figure 4.4.1, lanes 8, 10, 12, 14). It was clear from these results that active repairosome needed to be isolated in another manner.

The starting material for the previous purification was a whole cell extract made by bead-beater. When these same extracts themselves were assayed for NER activity, none was detected (Figure 4.4.1, lanes 3-6). Therefore, a new scheme began with whole cell extracts from a strain carrying a 6-histidine tagged TFB1 subunit of TFIIH. These extracts were made by the mortar and pestle method outlined in Section 4.2, and purification followed the fractions which were active for NER.

The active whole cell extract was applied to a BioRex70 column, which was eluted with buffers BR300, BR650, and BR1200. None of the fractions alone had NER activity (Figure 4.4.2, lanes 2-5). Activity could be reconstituted, however, when the flowthrough and BR650 fractions were added together

(Figure 4.4.2, lane 7). Additions of the BR300 and/or BR1200 fractions neither inhibited nor stimulated this activity (Figure 4.4.2, lanes 9, 11, 12).

The components in the BioRex70 flowthrough that allow repair activity have not been identified. However, RPA could be easily purified to homogeneity from the BioRex70 flowthrough by separation on a single strand cellulose affinity and MonoQ column (Figure 4.4.3).

To further purify the NER activity that bound to BioRex70, the BR650 peak was then applied to a P11 phosphocellulose (Whatman) column. The P11 column was eluted with buffers A300 and A650. The A650 fraction was active for dual incision when added to the flowthrough of the BioRex70 column (Figure 4.4.4, lane 4).

The histidine-tagged TFB1 subunit of TFIIH was next utilised for affinity purification by loading the P11 peak onto a nickel-agarose column. Surprisingly, and in contrast to what was previously observed by Svejstrup *et al.* [115], most of activity appeared in the flowthrough (Figure 4.4.4, lane 6) and no active complexes were able to bind and elute as a peak (Figure 4.4.4, lanes 7, 15). Western analysis confirmed that the loaded material indeed contained TFB1 (Figure 4.4.5 A), and when the extract was denatured by treatment with 8 M urea, TFB1-6His could be bound and eluted from the nickel resin (Figure 4.4.5 B). Hence, the inability of the repairosome to bind to the nickel column was likely due to the inaccessibility of the histidine tag rather than the absence of the TFB1 protein itself. When the BR650 peak was subjected to size fractionation by a Superose 6 column, activity eluted in fractions corresponding to a molecular mass of 4-8 MDa (Figure 4.4.6). This predicts a repair complex of larger molecular mass than the 750-1000 kDa repairosome purified by Svejstrup *et al.* [115] and the 1000-1500 kDa complex observed by Rodriguez *et al.* [98].

Further purification attempts were thwarted by the instability of this repair complex. Although the whole cell extract maintained activity over several freeze-thaw cycles, fractions from purification steps lost activity after only one freeze-thaw cycle. Hence, without stable activity pursuing an extensive purification scheme was difficult.

4.5 DNA substrates for an *in vitro* transcription-coupled NER system

An *in vitro* transcription-coupled NER system must provide a means to study the repair that occurs only during transcription. Evidence to date suggests that the elongating polymerase stalled at a DNA damage acts as the signal for TCR [77] as exemplified by the mechanism of *E. coli* TC-NER [106].

Two main approaches were made to construct a transcription-repair substrate that would allow RNA polymerase to transcribe up to an intrastrand 1,3-(pGpTpG)-cisplatin. In order that the system would not be limited by the inefficiency of *in vitro* promoter-dependent transcription, all substrate schemes included a 3' tail on the transcribed strand upon which core RNA polymerase II can load and transcribe independent of any other factors. The efficiency of transcription of this type of template is typically better than 50% (cf. Figure 3.6.2). This sort of DNA substrate would also allow the study of NER in the context of the polymerase in its various states, such as the hyperphosphorylated RNAPIIO and RNAPII in association with various defined elongation complexes.

As outlined in Figure 4.5.1 A, the first approach for a TC-NER template used the double-stranded, M13 based cis-platin substrate described in Section 4.3 as a starting material. The substrate was then linearised by digestion with PvuII, a 3' dC tail added by terminal transferase, and the 5' overhang on the non-transcribed strand removed by restriction digest by either XhoI or BanII. However, this method proved unsuitable since purification after the terminal transferase step proved inefficient due to the dispersed nature of the DNA products in agarose gels. Furthermore, the inefficiency of second-strand synthesis of the M13 substrate caused the large scale preparations to be impractical.

The second approach for templates used oligonucleotides that could be annealed and ligated together. The final product would be a 140 basepair double-stranded region with a 20 nucleotide 3' tail on the transcribed strand (Figure 4.5.1B). Synthesis coupling efficiencies for these oligonucleotides were poor and yielded less than 10% of full-length product. Nevertheless, oligonucleotides were gel purified and used in a ligation reaction. Ligation yielded less than 10% full-length product, which might have been due to the presence of n-1 length starting materials. After the successful isolation of a

substrate containing the cis-platin lesion, the template was used in a transcription elongation assay with core RNAPII. Transcription resulted in two major products – one of approximately 67 nucleotides long, the expected length of a damage-stalled polymerase, and the second 140 nucleotides long, which corresponded to a read-through transcription product (Figure 4.5.2). However, when a shorter transcription template was made containing the same sequence surrounding the platination site, it was found that both damaged and non-damaged substrates gave the same transcript products (data not shown). Therefore, the shorter transcript observed earlier was not likely a product of a damage-dependent stalled polymerase but rather of a damage-independent and sequence specific internal transcription stall site.

4.6 Discussion

Although the work presented here did not establish an *in vitro* system for transcription coupled nucleotide excision repair, it has provided important basic information concerning the repair of cis-platin lesions and established methods by which this repair can be monitored. These protocols have been applied to the initial steps of repairosome purification.

Yeast nucleotide excision repair of the intrastrand 1,3-(pGpTpG)-cisplatin lesion differs from that of the human in both length of excision products and sites of incision. This could reflect either differences in the formation of the DNA open complex upon damage recognition or a variance in the repair complex organisation and positioning of the endonucleases Rad1/10 and Rad2. *In vitro* substitution of yeast repair proteins with human ones might identify determining factors for incision site positioning.

Compared to NER by HeLa extracts, repair using yeast whole cell extracts is less robust. Not only are fewer adducts repaired, but yeast NER also requires an hour to reach completion versus the 30 minutes for human whole cell extract. One consideration, though, is that the yeast whole cell extracts used are able to assemble chromatin on the DNA repair substrates, and deposited nucleosomes could have hampered damage recognition. To test this hypothesis, extracts which have been made from cells in which the histone H4 expression has been repressed [71] could be assayed for NER activity.

The DNA-damage dependent phosphorylation of Rad53 demonstrates the biochemical potential of the whole cell extract prepared here (J. Murguia and N. Lowndes, preliminary data). Although the DNA-damage response through this checkpoint pathway did not extend down to the transcription level, my result with *RNR3* corresponds well with data presented by You *et al.* They found that transcription from the *CYC1* promoter was inhibited by co-incubation with UV- or AAF- damaged plasmids [148]. This effect was *RAD26* dependent and could be relieved by the addition of holo-TFIID [148]. TFIID has been proposed to switch between its holoenzyme form, which is used during transcription, and its core state, which is incorporated into the repairosome [115]. You *et al.* propose that Rad26 acts to shift the steady state of TFIID away from transcription complexes and toward integration into the repairosome. The suggested ability of a mammalian quaternary complex containing DNA, RNA polymerase II, RNA, and CSB to recruit TFIID correlates well with this suggestion [117]. However, the interaction studies by Tantin *et al.* [117, 118] used a polymerase which was stalled by nucleotide omission instead of by a DNA damage. Therefore, a DNA-damage dependent recruitment of repair to a stalled eukaryotic polymerase has yet to be demonstrated.

Purification of repair proteins by Svejstrup *et al.* [115] and Rodriguez *et al.* [98] indicated the presence of a pre-assembled multiprotein NER complex. However, the repairosome purified by Svejstrup *et al.* did not have activity on its own, and the active complex isolated by a single-step Rad14-affinity method could represent a group of indirectly interacting proteins. Indeed, Guzder *et al.* conclude from their experiments that a repairosome does not exist prior to DNA damage and that the sequential assembly of four subcomplexes instead occurs at the site of the DNA lesion [43].

The purification presented here confirms the existence of the large multiprotein repair complex independent of DNA damage. Although the BR650 fraction did not have repair activity on its own, it had dual incision activity upon the addition of the BioRex column flowthrough. The fact that size fractionation of the BR650 fraction yielded a very large complex with the same characteristics is certainly indicative of a preassembled repairosome. Further purification is needed to show whether or not a repairosome definitely does exist. It will be necessary to find complex stabilising conditions so that more

chromatography steps can be used. Regardless of whether the complex described here possesses all the proteins required for the global NER pathway, it may be sufficient for studies of transcription-coupled repair.

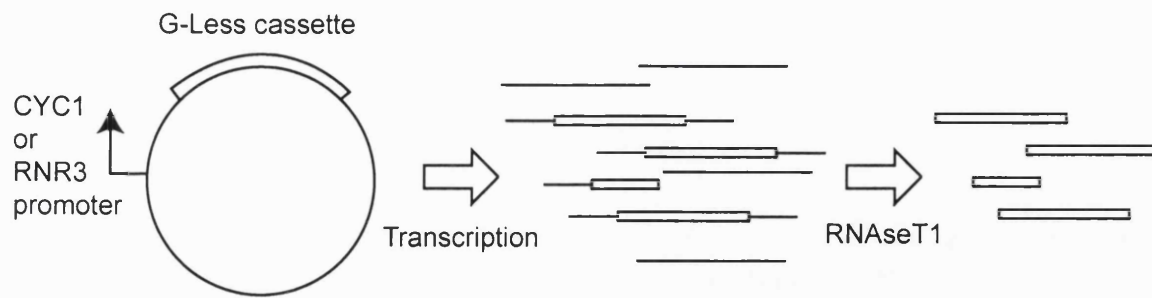
The phenomenon of transcription-coupled repair was first described by Bohr *et al.* in 1985 [10]. However, the dissection of the biochemistry behind TC-NER has been hindered by the lack of an *in vitro* system. Two obstacles were encountered in my attempt to construct a transcription-repair substrate for use in TC-NER. First, technical difficulties arose in the synthesis of substrate DNA. The oligonucleotide-based approach may be feasible once the yield and grade of long oligonucleotide synthesis is improved. Second, the DNA substrate in this study was found to contain an intrinsic block to transcription in the same region as the DNA damage. The sequence of the cis-platin damaged region is comprised of two thymidine-rich regions interrupted by the sequence ACA. The resulting secondary structure of this sequence might contain a rigid kink which retards the progression of RNAPII. Therefore, changes in substrate sequence need to be made to avoid this damage-independent block to transcription.

Once a working DNA substrate is made, TC-NER studies may commence using the RNAPII and partially purified repairosome complex described in this work. It can then be seen whether a cis-platin damage can be repaired in the presence of a stalled polymerase. And if so, how do the repair products differ from those formed by transcription-independent NER? Furthermore, it will be interesting to examine whether RNAPII moves back from the lesion and how this potential movement affects the nascent transcript. By the described methods, one can study if, for example, RNA is released or shortened in response to TC-NER.

The *in vitro* transcription-coupled NER system may well need to incorporate factors in addition to those required for transcription and NER. Active RNAPII elongation is a prerequisite for efficient TCR as the addition of α -amanitin inhibits TCR in hamster and human XP-C cells [130]. Transcript elongation also likely causes the modification of chromatin structure by, for example, the Elongator associated histone acetyltransferase [142]. CSB can also remodel chromatin and does so in an ATP dependent fashion [22]. TCR therefore might be an additive effect between increased DNA accessibility due

to factors acting on the transcribed strand and the increased recruitment of repair enzymes via proteins such as Rad26/CSB. The chromatin assembly capability combined with the transcription and repair activities of the described yeast whole cell extract could be used to investigate this idea. For example, one can measure the differential repair between two damaged chromatinised plasmids, one of which contains a promoter downstream from the specific DNA lesion (Figure 4.6.1). Nucleosomes could first be deposited on the substrates by a repair deficient strain. Upon the addition of nucleotides, RNAPII can elongate up to the damage. The use of a NER-deficient strain would prevent the NER-dependent transcriptional inhibition observed by You *et al.* These packed substrates could then be incubated with either NER competent whole cell extracts or fractions, and repair monitored either by Southern analysis of excised fragments or by agarose gel and measurement of radioactive nucleotides incorporated during repair synthesis.

A.



B.

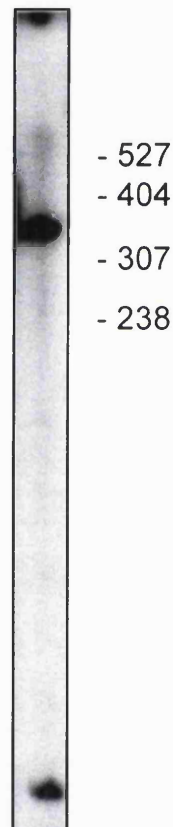


Figure 4.2.1. Transcriptional activity of whole cell extract.

A. Activity was monitored via promoter-specific transcription of a 381 nt G-less cassette, which is resistant to RNase T1 digestion. B. 20 µg of extract was used to transcribe from the RNR3 promoter. Transcripts were treated with RNaseT1 to digest all RNA other than the G-less cassette, and products separated on a 10% denaturing acrylamide gel. Size markers from a pBR322-Msp1 are denoted.

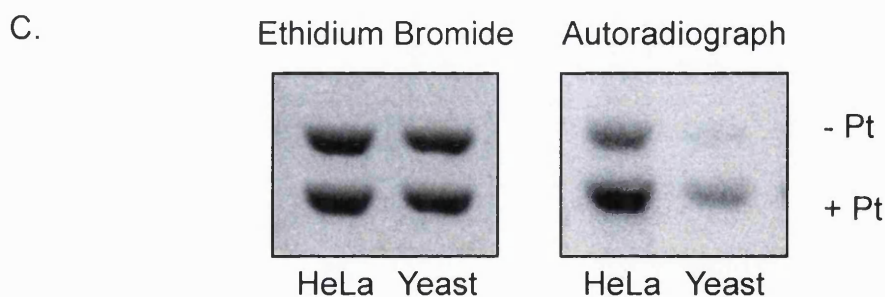
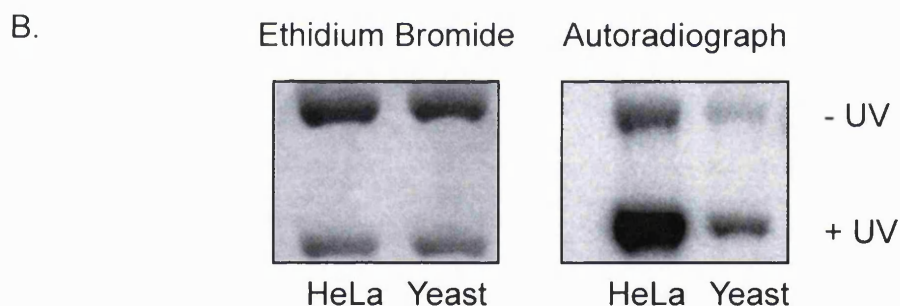
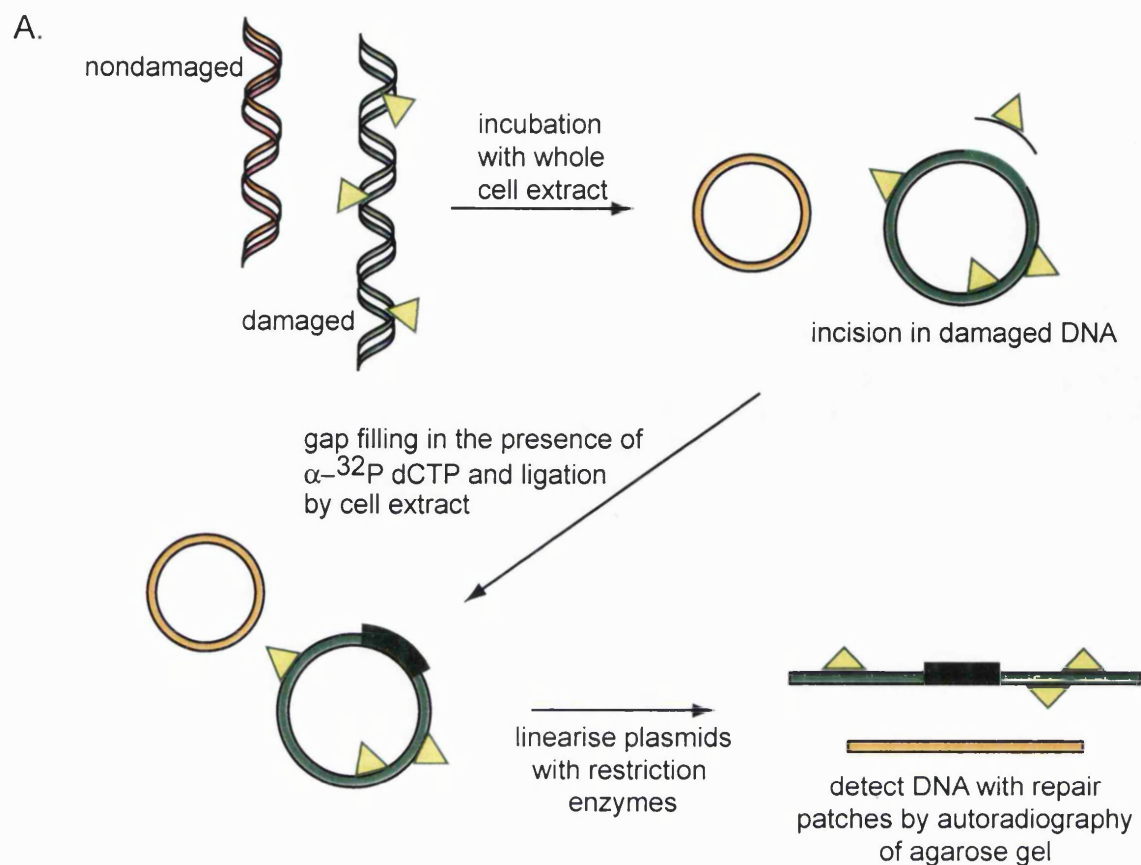


Figure 4.2.2 Nucleotide excision repair activity of yeast whole cell extract.
 A. Scheme for assaying NER activity in which incorporation of radiolabel via repair synthesis is compared between damaged, 3.0 kb pBluescript KS(+) and nondamaged, 3.7 kb pHM14. B. Repair synthesis reactions were carried out as described in Section 2.7 using UV-damaged pBluescript KS(+) and 250 μg of either HeLa or yeast whole cell extracts. C. Repair synthesis reactions with cis-platin damaged pBluescript yielded similar results as in (B) for both HeLa and yeast whole cell extracts.

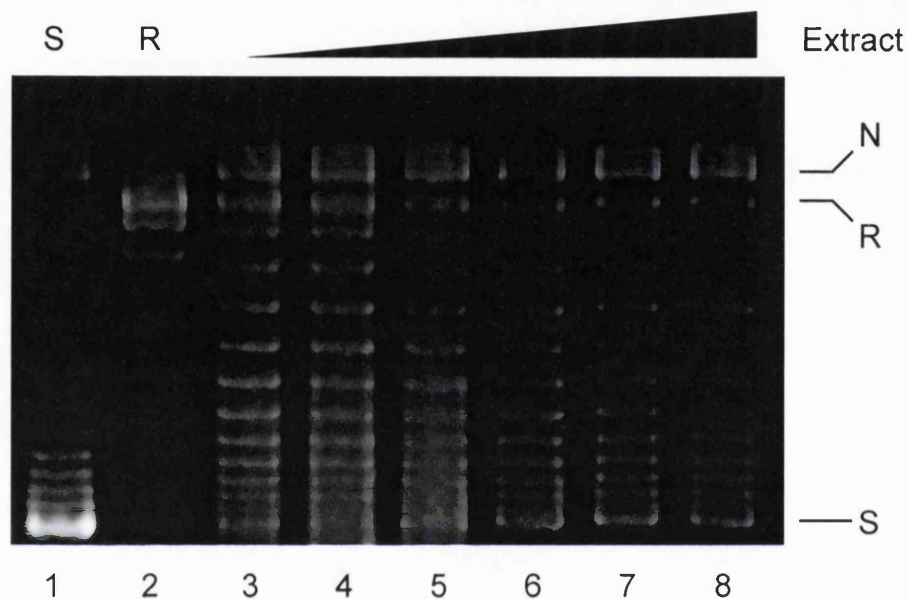


Figure 4.2.3. **In vitro chromatin assembly in yeast whole cell extracts.** 150 ng of relaxed plasmid pGAL4-GC- (R) was incubated with 50 μ g (*lane 3*), 100 μ g (*lane 4*), 150 μ g (*lane 5*), 200 μ g (*lane 6*), 300 μ g (*lane 7*), and 400 μ g (*lane 8*) of yeast whole cell extract for 3 hours at room temperature. Purified DNA products were separated on a 1% 1xTBE agarose gel, stained for 1 h in 3 μ g/ml ethidium bromide, and washed for 10 minutes in H₂O. Relaxed (R), supercoiled (S) and nicked circular (N) forms of DNA are denoted.

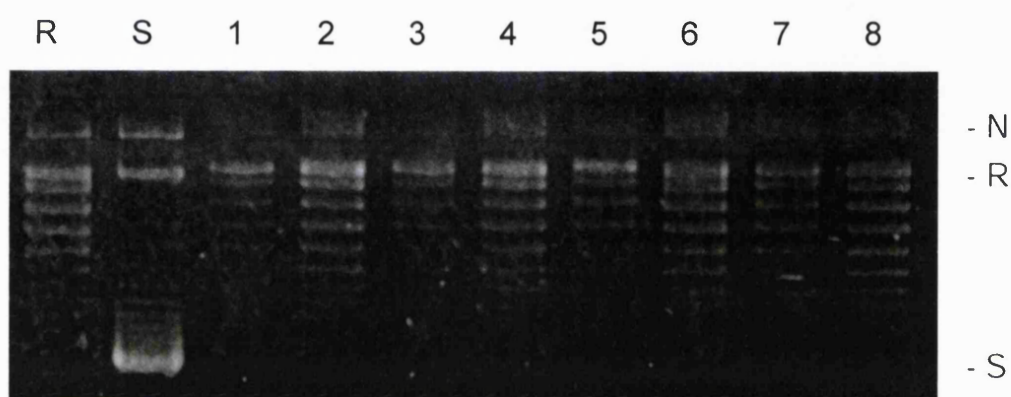


Figure 4.2.4. **ATP is a positive co-factor for chromatin assembly.** 250 ng of relaxed DNA (R) is incubated with 100 μ g of whole cell extracts for 0.5 h (*lanes 1, 2*), 1 h (*lanes 3, 4*), 2 h (*lanes 5, 6*) and 3h (*lanes 7, 8*) at room temperature in the presence (*lanes 2, 4, 6, 8*) or absence (*lanes 1, 3, 5, 7*) of 0.2 mM ATP. The positions of relaxed (R), supercoiled (S) and nicked circular (N) forms of DNA are denoted. Note that the reaction is essentially complete after 0.5h.



Figure 4.2.5. **Extracts not capable of DNA-damage dependent induction of RNR3.** 50 ng of pRNR3-G- and 20 μ g of whole cell extract was used in a transcription assay as described in Section 2.7. Transcripts were treated with RNaseT1 before purification and separation on a 10% denaturing acrylamide gel. *Lane 1* represents transcription products in the absence of damaged DNA while *lanes 2 and 3* contain 50 ng and 100 ng of UV-damaged pBluescript, respectively. Size markers are radiolabelled pBR322-MspI digest fragments.

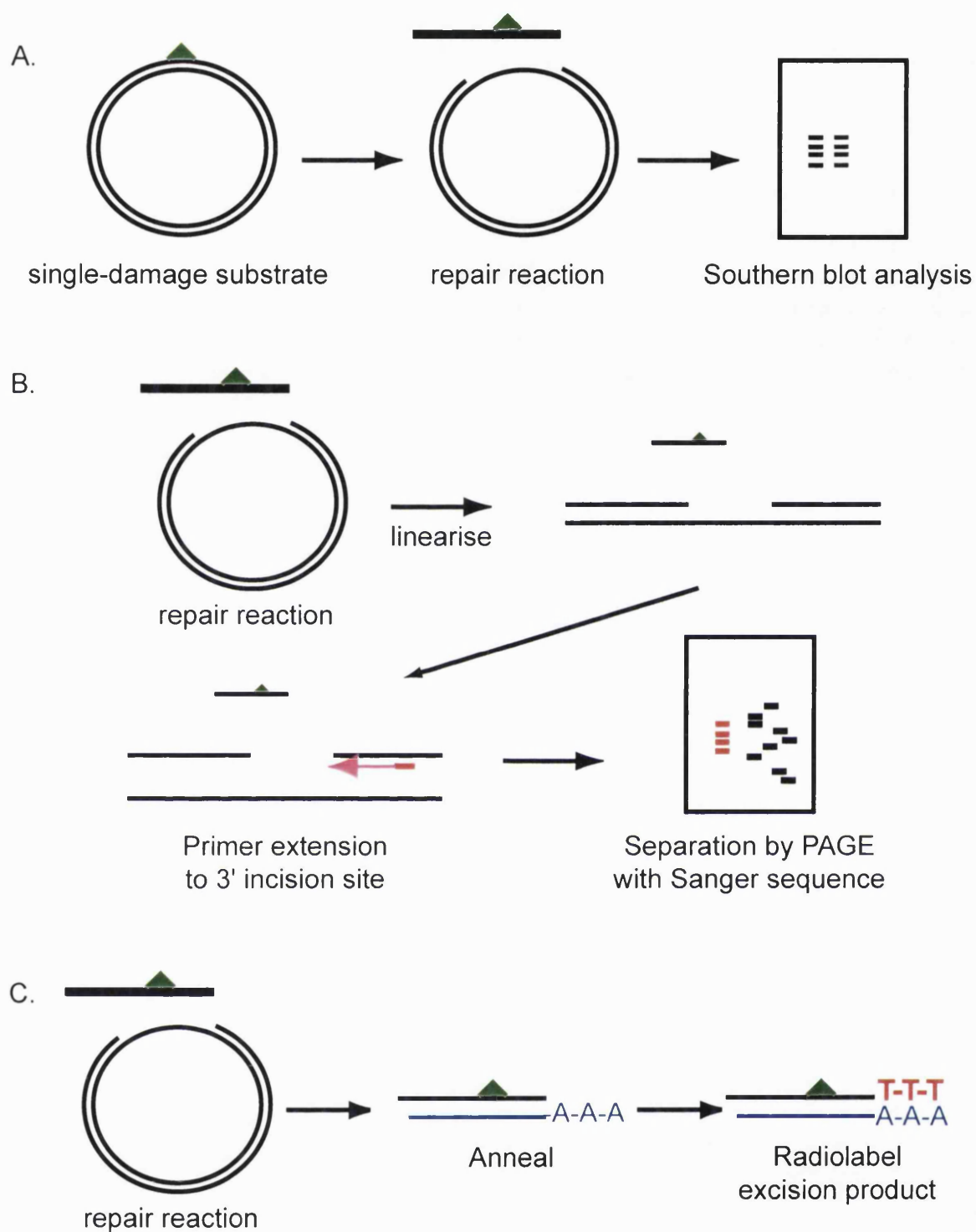


Figure 4.3.1. **Assays used in Section 4.3.** A. Southern blot based NER activity assay. A substrate carrying a single defined damage is incubated with cell extract or protein fractions. NER releases products which can be separated by PAGE and analysed by Southern blot using probes complementary to the damaged region. B. Primer extension for 3' incision mapping. DNA synthesis from primer annealed 3' of the damage site will give products which extend from the primer to the 3' incision site. C. End-labelling. Repair products are annealed with a complementary primer which has a 3' overhang of A-A-A. The repair product can be radiolabelled with Sequenase v 2.0 (Gibco) and $\alpha^{32}\text{P}$ -TTP.

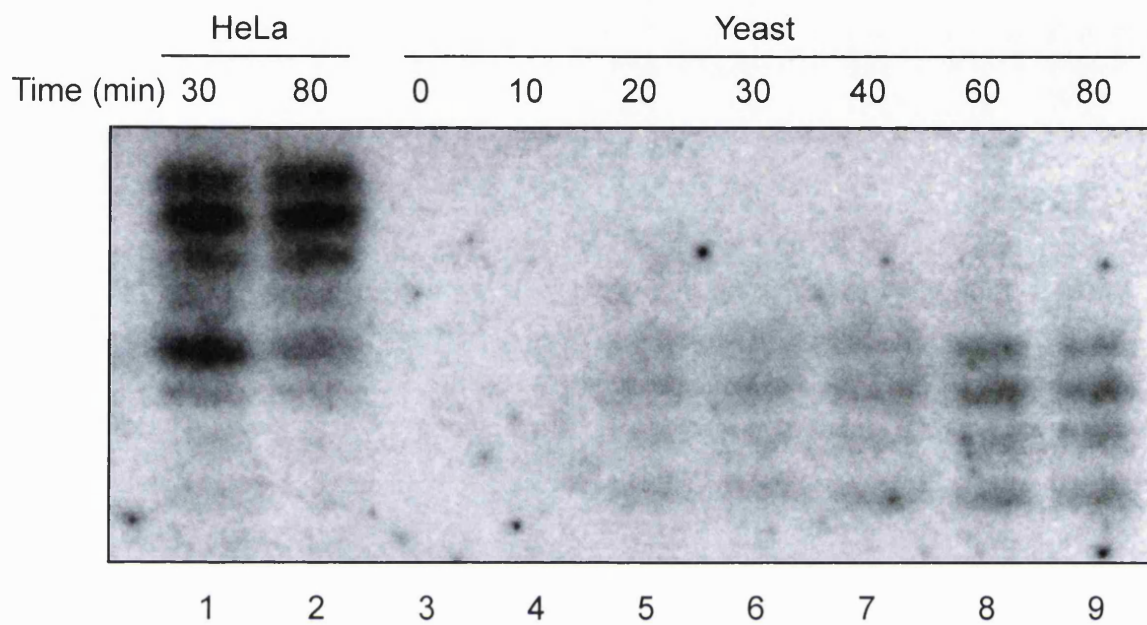


Figure 4.3.2. **Nucleotide excision repair products.** 250 ng of cis-platin damaged M13 substrate was incubated with 100 μ g of either HeLa (lanes 1, 2) or yeast whole cell extract (lanes 3-9) for the times indicated. Products were then separated on a 12% denaturing polyacrylamide gel, Southern blotted, and probed with the oligonucleotide (5'-GAAGAGTGCA CAGAAGAAGAGGCCTGG-3') that had been 32 P-labelled with T4 PNK.

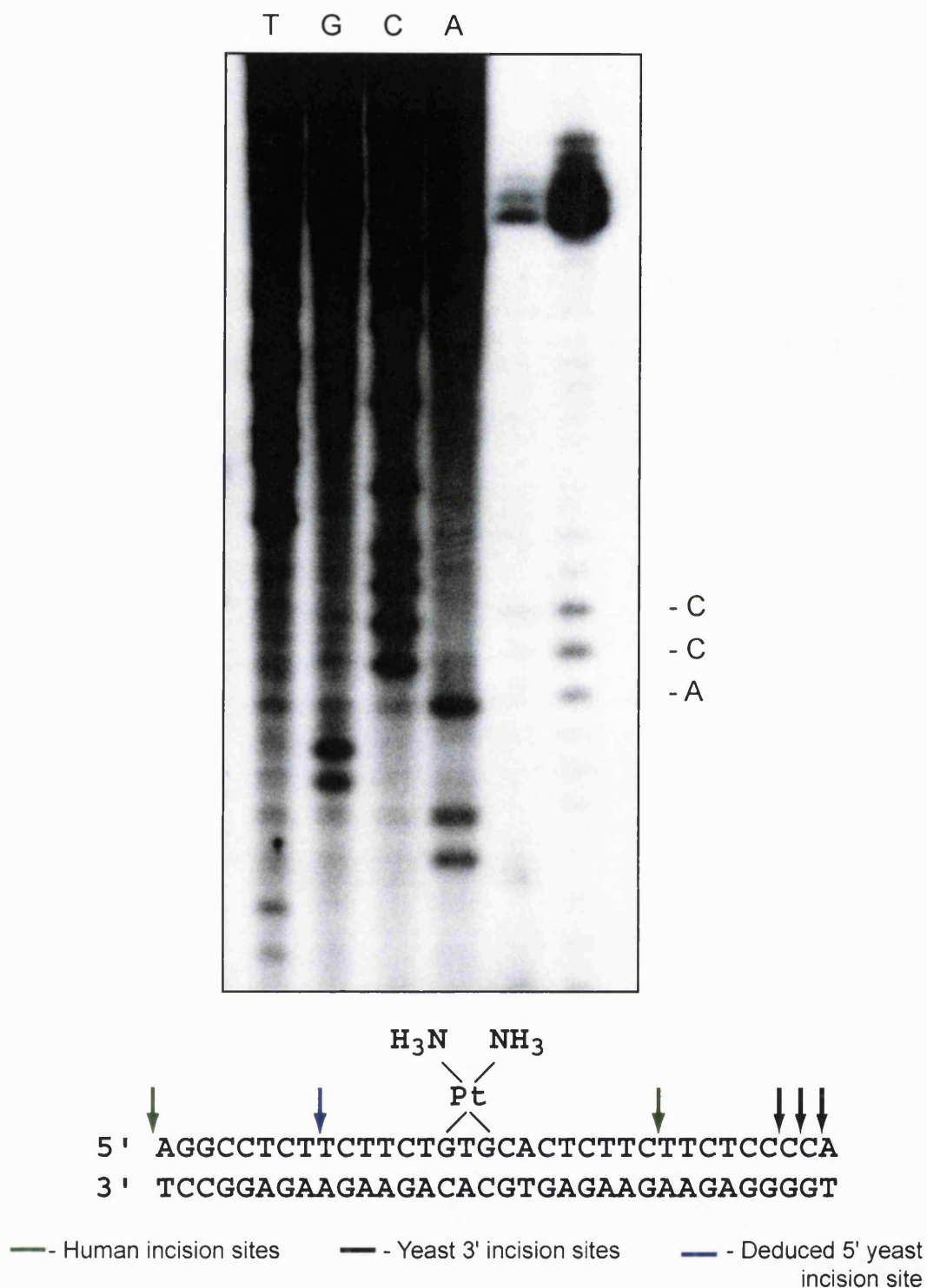


Figure 4.3.3. **Mapping of yeast NER 3' incision sites.** 250 ng of cis-platin damaged M13 substrate was incubated with 100 μ g of yeast whole cell extract for 1 hr at room temperature. Purified products were digested with PvuII and used as templates for primer extension using primer 5'-CAGGAAACAGCTATGAC-3', which was 32 P-labelled with T4PNK. The Sanger sequencing standards used the same primer and undamaged M13-GTG substrate as a template. The major 3' incision sites are shown above as the black arrows, and the deduced 5' incision site with the blue arrow. Green arrows point to the incision sites found for NER by human cell extracts as described by Moggs *et al.* (136).

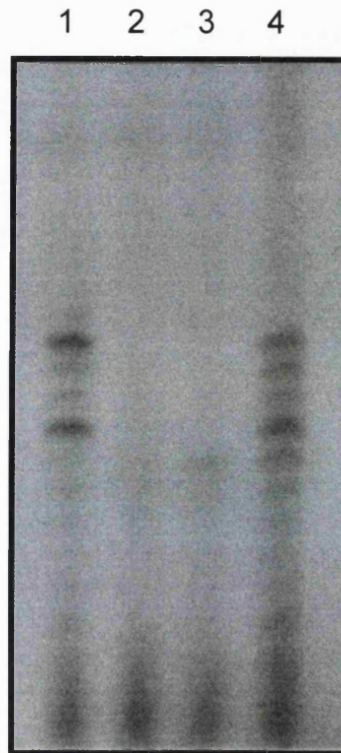


Figure 4.3.4. End-labelling based NER activity assay products and complementation of NER deficient whole cell extracts. 50 ng of cis-platin damaged M13 substrate was incubated with 20 μ g of yeast whole cell extract for 1 hr at room temperature. Reaction products were then annealed to the oligonucleotide 5' -AAATGGGGAGAAGAAGAGTGCA CAGAAGAAGAGGCCTGGTCG-3' before a 5 minute pulse at 37°C with Sequenase v.2 (Gibco) and 32 P-TTP and a 12 minute cold chase with 0.5mM of each dNTP. *Lane 1* contains the labelled NER reaction products from wildtype strain BJ2168, *lane 2* from *rad10* Δ strain, *lane 3* from *rad14* Δ strain, and *lane 4* a mixture of *rad10* Δ and *rad14* Δ strain extracts.

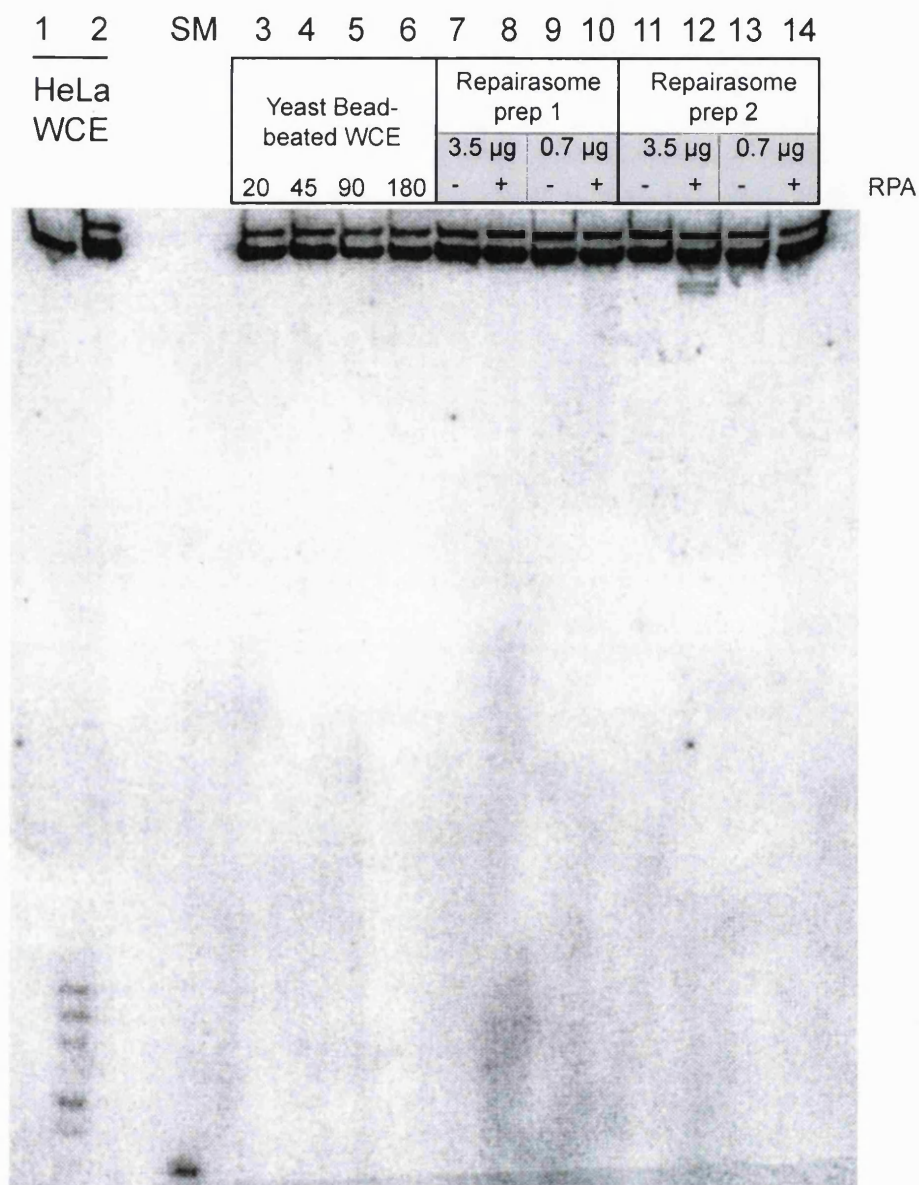


Figure 4.4.1. **Southern of NER products.** 250 ng of cis-platin damaged M13 substrate was incubated with 100 μ g of HeLa whole cell extract for 30 minutes at 30°C (lanes 1,2) or with repair proteins as indicated (lanes 3-14) at room temperature. Purified repair products were prepared, blotted, and probed as described in Section 2.7. *Lane 1* contains a undamaged DNA control after incubation with HeLa extracts. *Lane 2* contains HeLa NER products. *Lanes 3-6* are from incubations with, respectively, 20, 45, 90, and 180 μ g of bead-beated yeast extracts. *Lanes 7-10* and *11-14* are from incubations of two independent preparations of repairasome as described by Svejstrup *et al.* in the amounts denoted. RPA was added to 100 ng/reaction *lanes 8, 10, 12, and 14.*

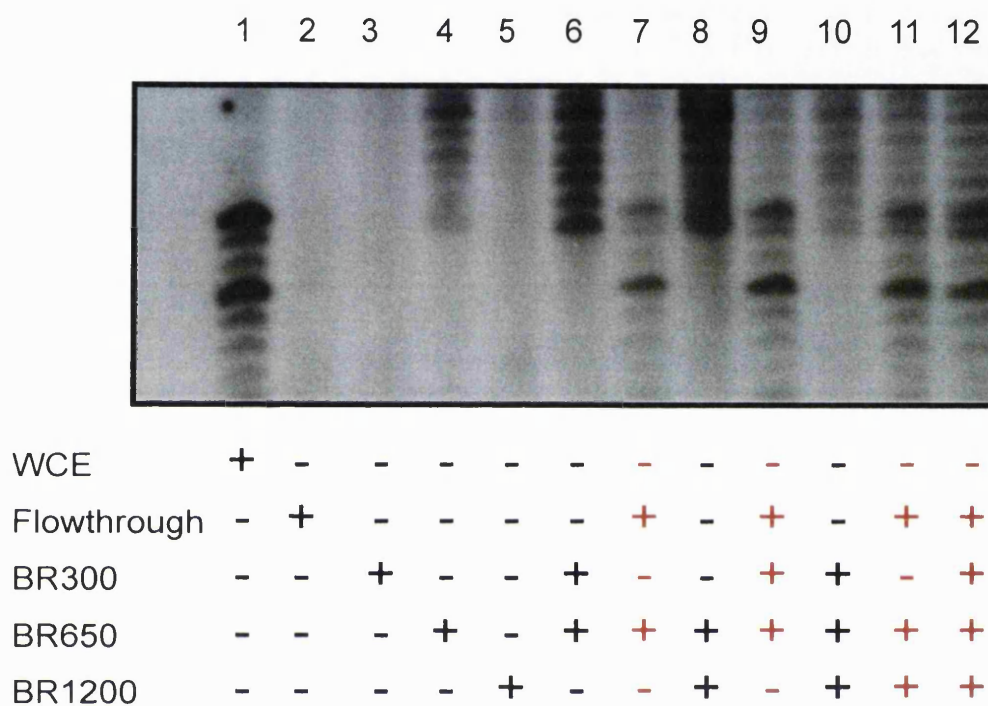


Figure 4.4.2. **NER activity of BioRex fractions.** 50 ng of cis-platin damaged M13 substrate was incubated with 20 μ g of yeast whole cell extract (lane 1) or with 1 μ l of each the BioRex fractions as denoted for 1 hour at room temperature. Repair products were end-labelled as described in Section 2.7. The origin of the non-specific bands above the repair signal is unknown.



Figure 4.4.3. **Silverstain of RPA MonoQ fractions.** Whole cell extract was applied onto a BioRex70 column. The flowthrough was applied to a ssDNA cellulose column and eluted as described in Section 2.5. Peak fractions from the 1.5 M NaCl-ethylene glycol wash were pooled and dialysed against a Buffer D200 containing 20% sucrose (L). After clarification, this material was diluted to 100 mM NaCl with Buffer D and applied to a MonoQ column (HR5/5). The column was washed with Buffer D100 and eluted with a 10 ml gradient from 100 mM to 400 mM NaCl. Fractions were separated by 17% SDS-PAGE and silverstained. The subunits of RPA are indicated on the right.

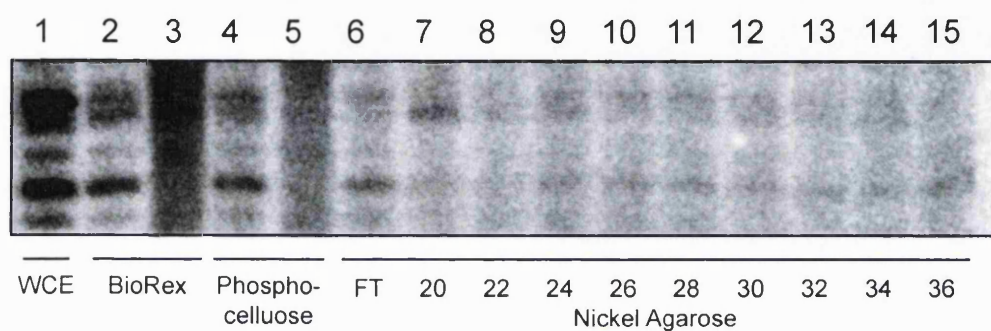
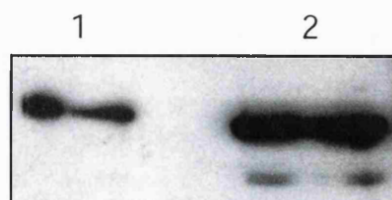


Figure 4.4.4. **NER activity of Phosphocellulose and Nickel Agarose fractions.** 50 ng of cis-platin damaged M13 substrate was incubated for 1 hour at room temperature with 20 μ g of yeast whole cell extract (*lane 1*), 1 μ l of BioRex fractions BR650 and BR-flowthrough (BRFT) each (*lanes 2, 3*), 1 μ l Phosphocellulose fraction A650 and BR (*lanes 4, 5*), or 1 μ l Ni-agarose fractions as denoted, which correspond to fractions of the high imidazole elution. These fractions also have 1 μ l of BRFT. *Lanes 3 and 5* have been supplemented with 10 ng RPA. Dual incision products were then labelled as described in Section 2.7.

A.



B.

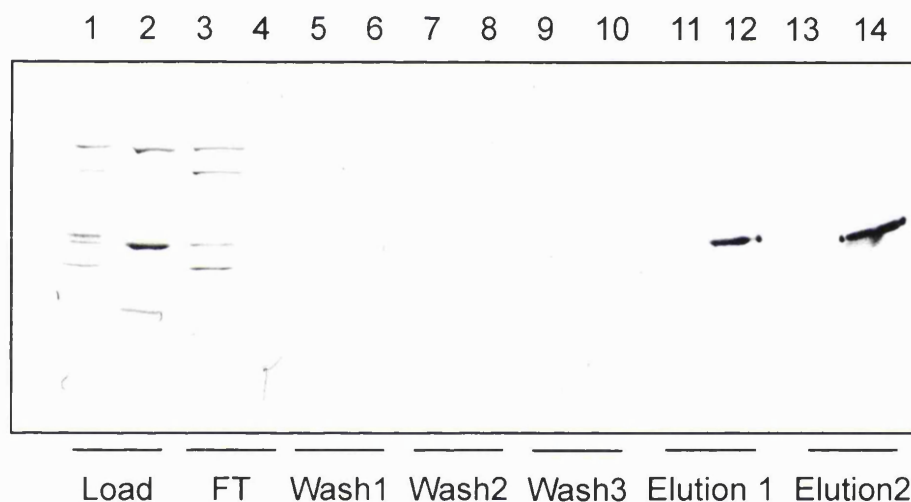


Figure 4.4.5. Phosphocellulose A650 fraction and whole cell extracts contain histidine-tagged TFB1. A. Western analysis of Phosphocellulose A650 fraction probed with anti-TFB1 antibodies. *Lane 1* contains core TFIIF while *lane 2* contains P11 A650. B. Whole cell extracts from wildtype control W303 (*lane 1*) and TFB1-6His (*lane 2*) were denatured by treatment with 8 M urea and applied to Ni-agarose resin. *Lanes 3* and *4* contain the flowthrough from the Ni-columns for W303 and TFB1-6His respectively. Samples were washed thrice with Buffer I, 50 mM KOAc, 5 mM imidazole (W303 - *lanes 5, 7, 9*; TFB1-6His - *lanes 6, 8, 10*) and eluted with Buffer I, 100 mM KOAc, 100 mM imidazole (W303 - *lanes 11, 13*; TFB1-6His - *lanes 12, 14*). Fractions were run on a 7.5% SDS page and analysed by Western blot with anti-TFB1 antibodies.

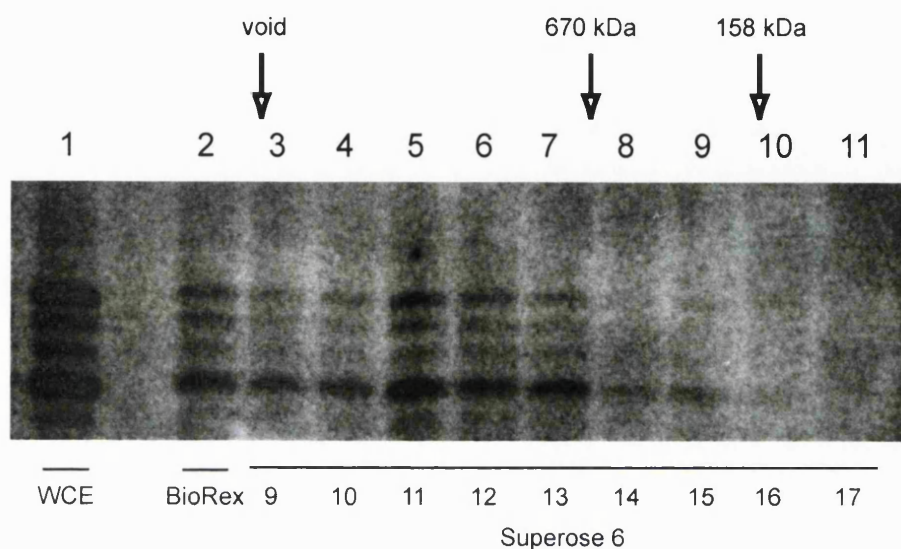


Figure 4.4.6. **NER activity of Superose 6 fractions.** BioRex70 BR650 fraction (lane 2) was applied onto a Superose 6 PC 3.2/30 (Pharmacia) and fractions 9-17 (lanes 3-11) were assayed for NER activity. 50 ng of cis-platin damaged M13 substrate was incubated for 1 hour at room temperature each fraction and 1 μ l BioRex flowthrough or 20 μ g of WCE (lane 1). Repair products were radiolabelled as detailed in Section 2.7 and separated on a 15% denaturing acrylamide gel. Elution of protein molecular weight standards are indicated by the arrows.

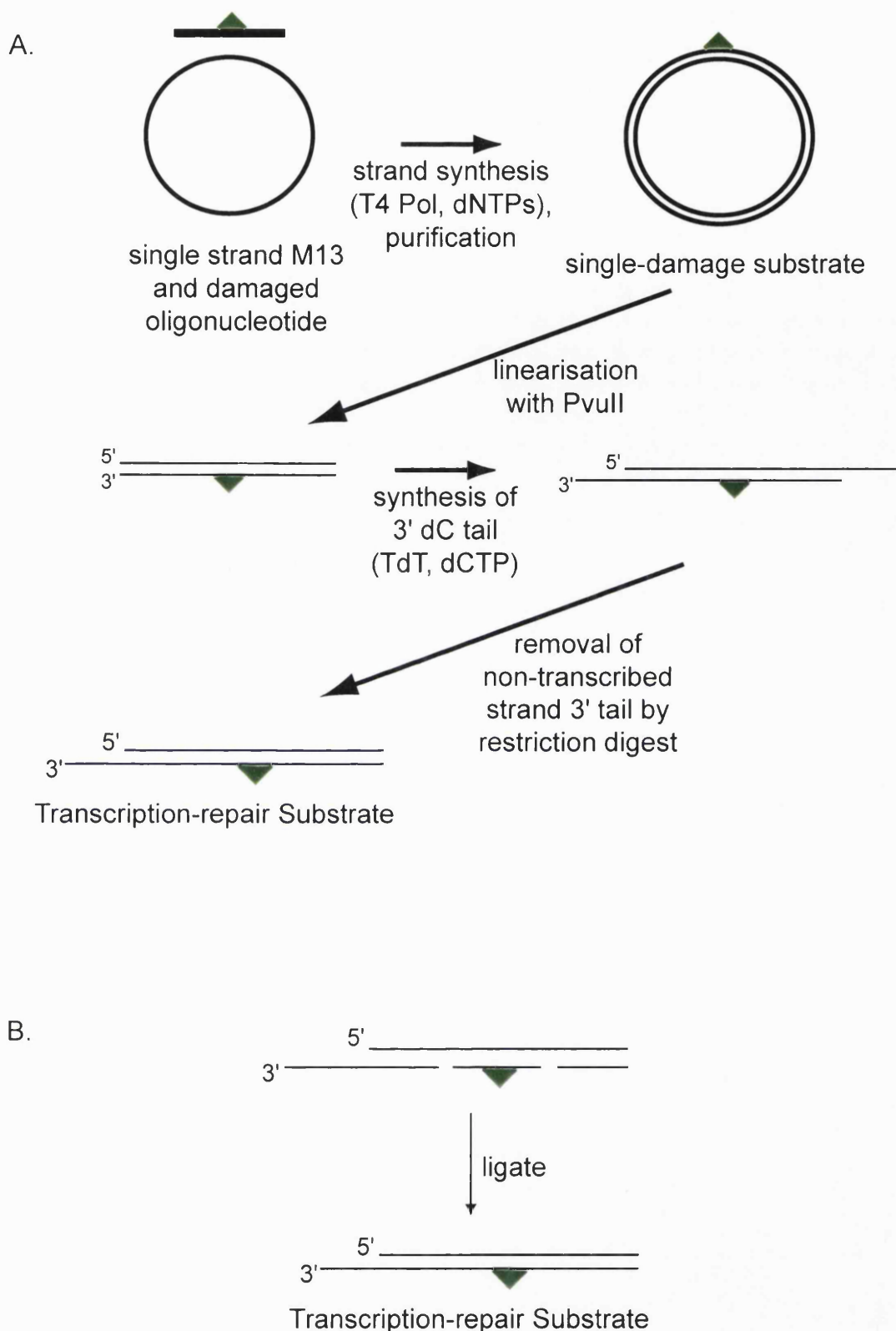


Figure 4.5.1. **Schemes for transcription-repair substrates.** A. The NER M13 derived double-strand substrate is synthesised from primer extension of a cis-platin damaged oligonucleotide around the single-stranded M13 template. This DNA was then digested with PvuII to yield a linear fragment. Terminal transferase was used to extend each of the 3' ends with a dC tail, and lastly the 3' tail on the non-transcribed strand was removed by digest by BanII or XhoI. B. Four oligonucleotides, one of which is damaged with cis-platin, was annealed together and ligated to form the transcription-repair substrate

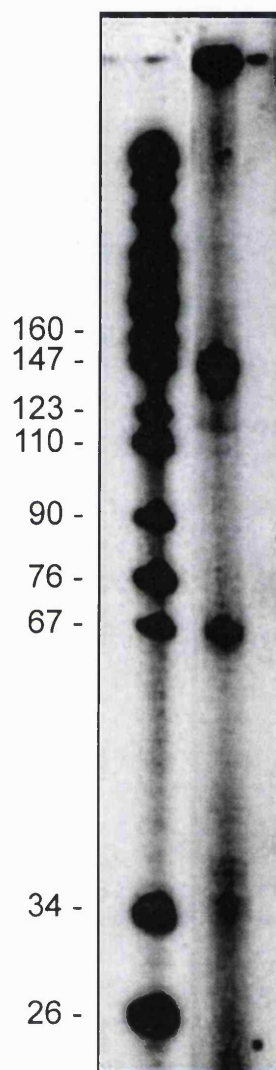


Figure 4.5.2. **Transcription products of damaged substrate.** Approximately 25 ng oligonucleotide-derived substrate was used as a transcription template that was incubated with 100 ng core RNAPII for 30 minutes at room temperature in the transcription conditions described in Section 2.7. Products were then purified by Protease K digestion, precipitated, and resuspended in formamide loading buffer before separation on a 10% denaturing acrylamide gel.

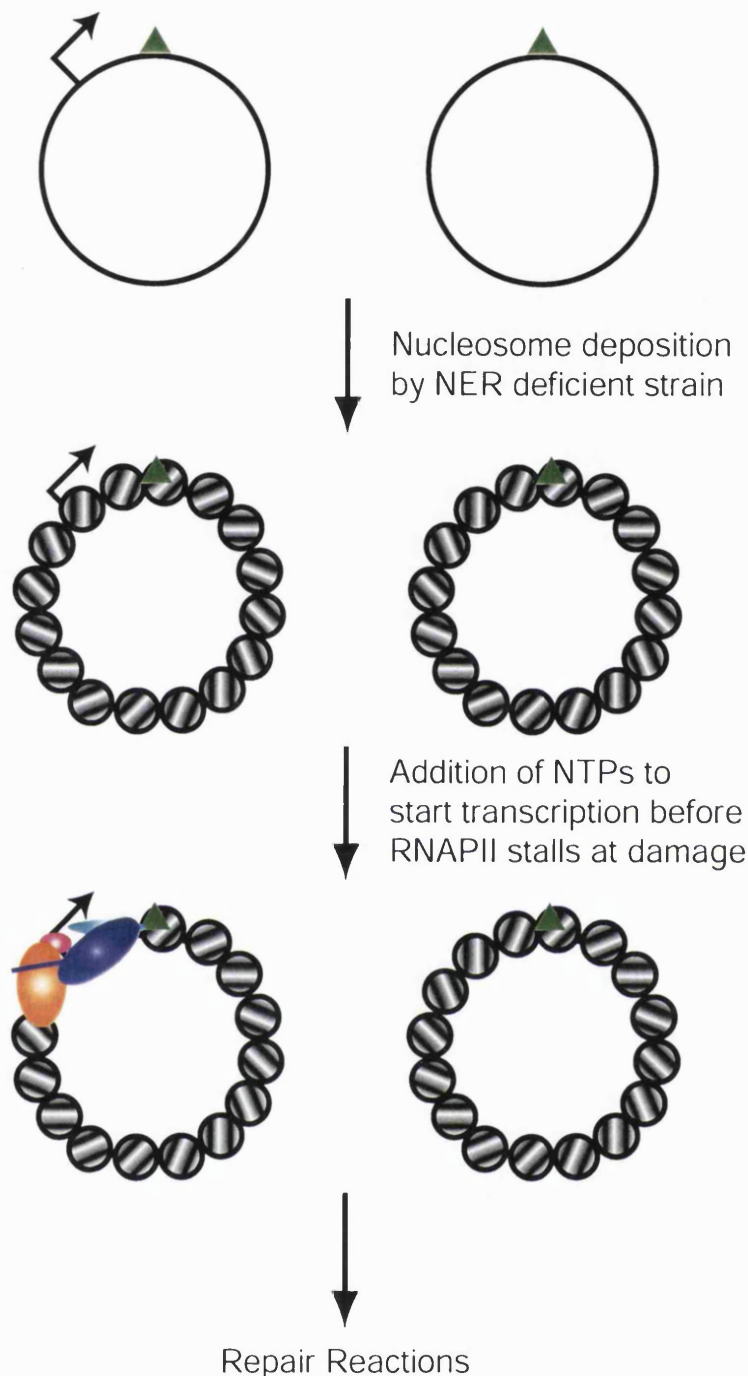


Figure 4.6.1. **Scheme for TC-NER substrate.** The following scheme incorporates aspects of chromatin remodeling to those of transcription and repair and consists of two plasmids, both of which would have a specific DNA adduct within regions that can be distinguished apart by Southern analysis. Furthermore, one plasmid would contain a promoter from which transcription can start. Both DNAs would be packed in chromatin via incubation with whole cell extract from a NER deficient strain. Upon subsequent addition of NTPs, transcription could then be initiated and the polymerase allowed to elongate until it stalled at the DNA damage. These substrates can then be incubated with either whole cell extracts or purified fractions, and repair products examined by Southern analysis.

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