Interplay between DNA replication, transcription and repair

by

Mag. rer. nat. Brigitte W. Trautinger

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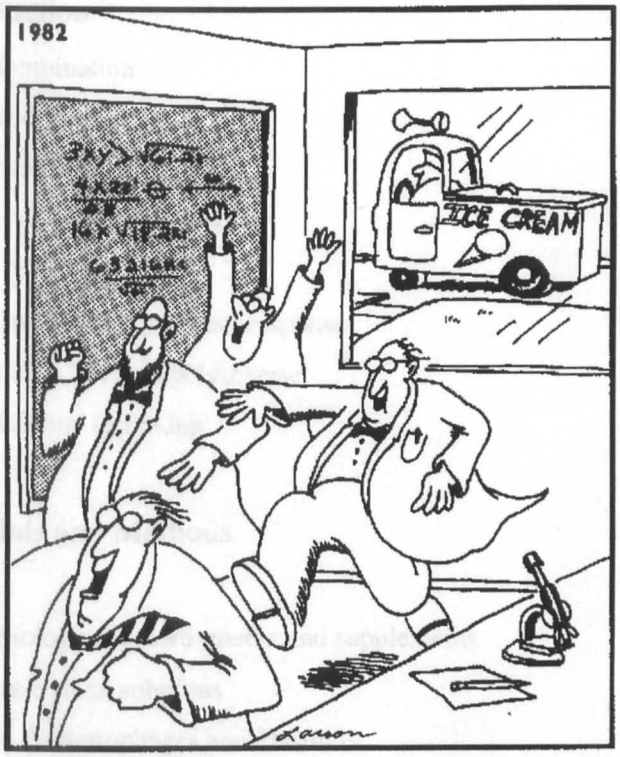
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Contents

Abbreviations

A	adenine
Ac	Acetate
Ap	Ampicillin
ATP	Adenosine 5′-triphosphate
bp	base pair
BSA	Bovine serum albumin
C	cytosine
Cm	Chloramphenicol
DNA	deoxyribose nucleic acid
dNTP	dioxynucleotide
DSB	double-stranded break
dsDNA	double-stranded deoxyribose nucleic acid
DTT	DL - Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	electric mobility shift assay - band shift assay
G	guanine
HPLC	high pressure liquid chromatography
IPTG	Isopropyl β-D-thiogalactoside
Km	Kanamycin
LB broth	Luria-Bertani broth
Mc	mitomycin C
MOPS	3-(N-morpholino)propanesulfonic acid
n/a	not applicable
nt	nucleotide
OC	open complex
OD	optical densitiy
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
(p)ppGpp	guanosinetetraphosphate and guanosinepentaphosphate
^{R}	resistant
Rif	rifampicin
RNA	ribonucleic acid
RNAP	RNA polymerase

s	sensitive
SDS	Sodium dodecyl sulphate
ssDNA	single-stranded deoxyribose nucleic acid
Str	Streptomycin
T	thymine
TBE	Tris-borate/EDTA
Tc	Tetracycline
TE	Tris/EDTA
TEMED	tetramethylene diamine
Tris	tris(hydroxymehtyl)aminomethane
U	uracil
UV	ultra-violet
v/v	volume per volume
wt	wild type
w/v	weight per volume

The standard single-letter and three-letter abbreviations for amino acids were used.

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I specially want to thank my parents for their unwavering encouragement and their support, both moral and not least financial. A big thank you to my whole family, who have always been there for me. Finally, I would like to thank Tim for sympathetic listening.

Statement

The work presented in this thesis was carried out solely by the author during three years from 1st October 1998, in the Division of Genetics, University of Nottingham, whilst registered there as a PhD. Student. This thesis is comprised of original work, which has not been presented for examination in any other form. This research was supported by a grant from the University of Nottingham

Whilst carrying out the work described in this thesis, I was fully aware of the hazards associated with the materials and techniques I was using, as advised in the Control of Substances Hazardous to Health regulations. The guidelines laid down in these regulations and in the departmental rules were strictly adhered to at all times. I was also aware of and followed the regulations and departmental local rules concerning the use and disposal of radioisotopes.

Abstract

The RuvABC and RecBCD protein complexes together can collapse and repair arrested replication forks. With their help a fork structure can be re-established on which replication can be restarted. *ruv* and *recB* mutants are therefore quite sensitive to UV light. Their survival is greatly decreased in the absence of the signalling molecules (p)ppGpp and increased when excess (p)ppGpp is present. (p)ppGpp are the effector molecules of the stringent response, regulating adaptation to starvation and other stressful environmental changes. Absence of (p)ppGpp can be compensated for by mutations in RNA polymerase that are called stringent mutations. Some of those, called *rpo**, also - like excess (p)ppGpp - increase the survival of UV irradiated *ruv* and *recB* cells. A model proposed by McGlynn and Lloyd (*Cell*, Vol. 101, pp35-45, March 31, 2000) suggests that this is achieved by modulation of RNA polymerase, which decreases the incidence of replication fork blocks.

In this work twenty-seven *rpo** mutants were isolated, sequenced and mapped on the 3D structure of *Thermus aquaticus* RNA polymerase. I have found mutants in the β and β' subunits of RNA polymerase. They lie mostly on the inner surface of the protein, well placed to make contact with the DNA substrate or the RNA product. A large number of rifampicin resistant mutations among *rpo** mutations is explained by an overlap between the so-called Rif pocket and the "*rpo** pocket". *rpo** mutations, like stringent mutations, lead to a decrease in cell size, suppress filamentation and increase viability. For in vitro studies I purified wild type and two mutant RNA polymerases with help of a his-tagged α subunit. The experiments confirmed that *rpo** mutant RNA polymerases form less stable open complexes than wild type, just like previously investigated stringent RNA polymerases. In addition I have shown here that (p)ppGpp leads to the destabilisation of RNA polymerase complexes stalled by nucleotide starvation or UV-induced lesions, though there is as yet no indication that *rpo** mutations act in the same way.

Chapter 1.

Introduction

A considerable amount of DNA damage occurs during the life of every cell. The causes for this damage can be both endogenous, such as oxidative damage, and exogenous, such as damage induced by UV-light. DNA damage can contribute to genomic variation and thus drive evolution. But it can also lead to loss of genomic stability, mutations, or cell death, if not repaired. DNA repair systems are therefore vital for the cell. A lot of minor damage is repaired by specific repair mechanisms that deal with certain lesions or classes of lesions, including glycosylases, mismatch repair and excision repair. The SOS response, activated by single-stranded DNA, also plays an important role for DNA repair as it leads to the expression of a number of genes that increase the cell's capacity for DNA repair. That homologous recombination represents another way for the repair of damaged DNA was proposed by Howard-Flanders *et al.* (1969), who showed that repair of single strand gaps in duplex DNA depends on RecA, which promotes homologous pairing and strand exchange (discussed below and for reviews see Kowalczykowski *et al.*, 1994; Lloyd and Low, 1996). The involvement of recombination in DNA repair is also demonstrated by the fact that mutations in most genes involved in homologous recombination increase sensitivity to DNA damage (Lloyd and Low, 1996).

1.1. Homologous recombination

Recombination is an important process in all organisms. Besides its role in DNA repair, which was not immediately recognised, it is responsible for rearranging genes, limiting the divergence of repeated DNA and guiding the proper segregation of chromosomes at cell division. It contributes to both genetic diversity and the conservation of genetic identity. A large number of proteins have been found to take part in recombination, many of them members of the *rec* or *ruv* families in bacteria.

As the topic is so extensive, only the most important players analysed in the *Escherichia coli* model shall be discussed here. Initially, recombination was thought to proceed by one of three different pathways, each defined by their specific set of recombination proteins and called the RecBCD, RecE and RecF pathway respectively. The RecBCD pathway is the major one for conjugational recombination in *E.coli* and most important for this work. SSB (single-strand binding protein), RecA, and RuvABC, among others, participate in this pathway.

RecA protein is the one factor necessary for any kind of strand exchange. It is therefore a vital part of every pathway for any kind of recombination, with the exception of certain events catalysed by RecE (a 5'-3' dsDNA exonuclease) and RecT (a ssDNA binding protein, promoting DNA pairing). A 37.8 kD protein, it binds to single-stranded DNA, much like SSB, but in contrast to SSB it forms a nucleoprotein filament that mediates homologous pairing and strand exchange between single-stranded and double-stranded DNA and promotes renaturation of homologous single-strand DNA molecules. RecA polymerises on DNA in 5' - 3' direction and can drive single-stranded regions into the adjoining duplex. (For reviews see Kowalczykowski and Eggleston, 1994; West, 1992). Single-stranded DNA also activates the coprotease function of RecA that stimulates self-cleavage of the LexA repressor, inducing the SOS response (Shinagawa, 1996).

RecBCD plays two different major roles in the *E.coli* cell. Firstly it is the major exonuclease (Exo V), responsible for degradation of foreign DNA. Both foreign single-stranded and double-stranded DNA are degraded rapidly. Secondly, and somewhat in contrast to its efficient destruction of foreign DNA, the multifunctional enzyme complex plays an important role in recombination (for reviews see Kowalczykowski, 2000; Lloyd and Low, 1996). RecBCD combines (among others) the functions of a 3' - 5' exonuclease, an endonuclease and a helicase. It specifically recognises blunt or nearly blunt DNA double-strand ends and starts to rapidly unwind the DNA while progressively degrading the 3' strand and occasionally nicking the 5' strand (Dixon and Kowalczykowski, 1993). Unwinding by RecBCD is both continuous and processive (Bianco *et al.*, 2001). In the case of foreign DNA this quickly leads to complete degradation. In *E.coli* however, the

presence of certain sequence motifs called χ (Chi) prevents complete degradation. More than 1000 χ sequences are present in the *E.coli* genome, roughly one for every 5 kb (Faulds *et al.*, 1979). χ was initially identified as a sequence that increases the frequency of recombination in its vicinity (for a review see Myers and Stahl, 1994). Upon encountering the octameric χ sequence in the right orientation, RecBCD pauses. Both the 3' and 5' strands are nicked at that point and unwinding resumes, but with downregulated degradation of the 3' strand (Smith *et al.*, 1981) and upregulated degradation of the 5' strand (Anderson and Kowalczykowski, 1997a), leading to the production of a 3' single-stranded tail. RecBCD thus produces 3' tails - ideal for recombination - from blunt ends. Not only can this 3' end then be bound by RecA and stimulate recombination, the binding of RecA is actually promoted by RecBCD upon encountering χ (Fig. 1.1.; Anderson and Kowalczykowski, 1997b). It is also of interest to note that induction of the SOS response leads to inactivation of the nuclease activity of RecBCD, while its recombinagenic activity is stimulated, due to the more efficient generation of single-strand DNA ends (Kogoma, 1997). The RecBCD enzyme complex is therefore a very effective way for processing double-strand ends and stimulating recombination and recombinational repair. It is essential for recombinational repair of double-strand breaks in *E.coli* (Kowalczykowski *et al.*, 1994; Myers and Stahl, 1994)

We have now established how RecBCD can process double-strand DNA ends to form 3' tails and load RecA on them to form nucleoprotein filaments, which can in turn invade homologous duplexes. The invasion of a single-strand into a duplex leads to formation of D-loops. Branch migration then transforms a D-loop into a Holliday junction, the resolving of which requires the RuvABC resolvasome.

RuvABC is another multifunctional enzyme complex. The *ruvA* and *ruvB* genes are SOS inducible (Shurvinton and Lloyd, 1982). RuvA and RuvB were shown to specifically bind Holliday junctions and promote branch migration (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992). RuvA binds Holliday junctions as a tetramer (Rafferty *et al.*, 1996). RuvB is a helicase that forms a hexameric ring around the DNA (Stasiak *et al.*, 1994) and, when targeted to the junction by RuvA, promotes ATP-dependent branch migration (Tsaneva *et al.*, 1992). Two RuvA tetramers together with two

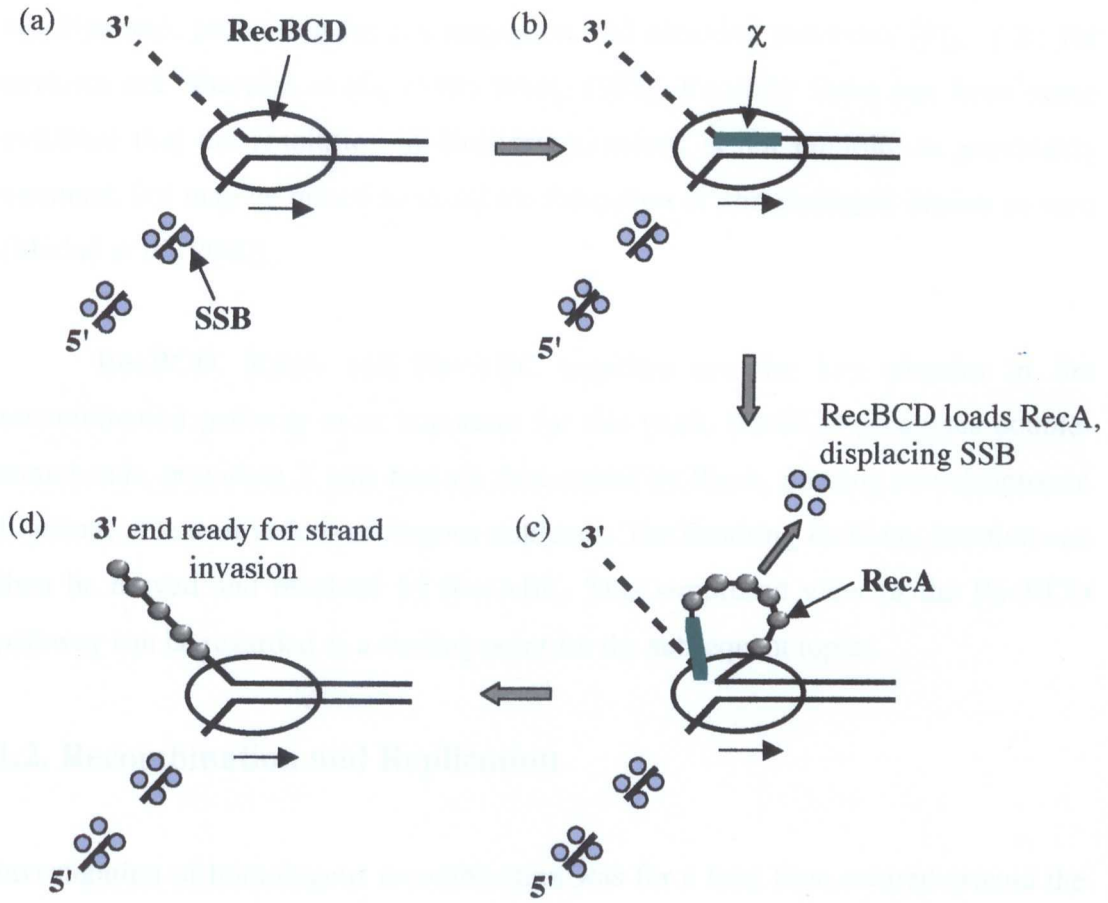

Figure 1.1. Model of the action of RecBCD. The enzyme complex unwinds dsDNA ends, degrading the 3' end and occasionally nicking the 5' end (a). Upon encountering a chi site (b), RecBCD stops degradation of the 3' end and facilitates preferential loading of RecA, while continuing to nick the 5' strand (c). The nucleoprotein filament formed by the 3' strand and RecA is free to invade a homologous duplex (d). Modified and adapted from Eggleston and West (1997).

RuvB hexamers can form an efficient branch migration complex. However, one of the RuvA tetramers can be replaced by a RuvC dimer, which also specifically binds Holliday junctions (Iwasaki *et al.*, 1991). RuvABC together form a Holliday junction resolvasome, promoting branch migration and cleaving junctions (Fig. 1.2.; for reviews see Sharples *et al.*, 1999; West, 1997). Recently there has been some evidence that the resolution of Holliday junctions is not random, as previously assumed, but may be biased to avoid the formation of chromosomal dimers in vivo (Michel *et al.*, 2000).

RecBCD, RecA and RuvABC together are the key players in the recombination pathway most important for this work. RecBCD processes double-strand ends, providing 3' tails that are then coated by RecA, forming a nucleoprotein filament, able to invade homologous duplexes. The resulting Holliday junction can then be moved and resolved by RuvABC. This simplified view of the RecBCD pathway can be regarded as a starting point for the subsequent topics.

1.2. Recombination and Replication

Investigation of homologous recombination was for a long time centred around the model systems of conjugational recombination and recombination of phage λ. Three major models for crossing over had been proposed in the first half of the last century: Firstly, break-join, where homologous chromosomes break at the same point, exchange arms, and are religated (Janssens, 1909). No, or very limited replication is necessary for this model (Fig. 1.3.a.). Secondly, copy-choice, where nascent DNA strands switch templates (Belling, 1931). In this model replication and recombination are linked (Fig. 1.3.b.) Thirdly, break-copy, where a damaged chromosome copies it's missing part from an intact homologous chromosome (Lederberg, 1955), also involving replication (Fig. 1.3.c.). Work on the phage λ system demonstrated that break-join recomb ination can take place in *E. coli* (e.g. Stahl *et al.*, 1985). There were however also hints of an involvement of replication, as λ recombination was found to be dependent on replication in *recD* strains (Thaler *et al.*, 1989). Though some replication was thought to take place during recombination, its extent was unclear and direct proof was missing. Another connection between recombination

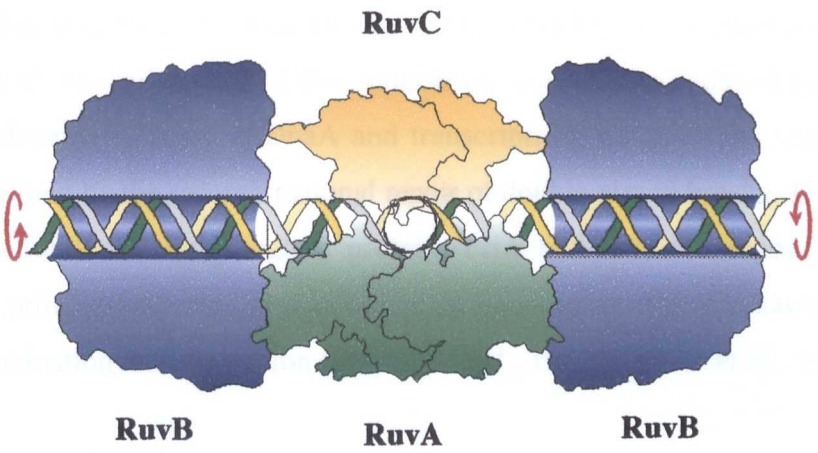

Figure 1.2. Structural model of the RuvABC resolvasome. The drawing is based on the crystal structures of RuvA and RuvC and on electron micrographs of RuvB. The C-terminus of RuvC (lacking in the crystal structure) has been extended slightly so that it appears to form contacts with RuvB. Reproduced with permission from Ingleston, 2000.

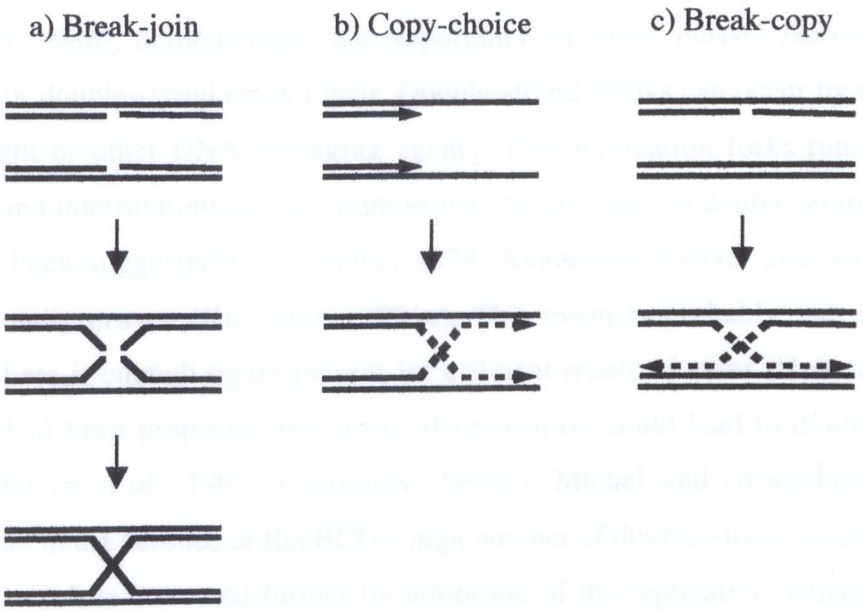

Figure 1.3. The different models for crossing over (modified from Kuzminov and Stahl, 1998). Newly synthesised DNA is shown as dashed line.

and replication was made by Asai and co-workers (1993). They observed that the phenomenon of "inducible stable DNA replication" (replication induced by the SOS response, independent of *oriC*, DnaA and transcription; for a review see Kogoma 1997) was primed by the recombinational repair of double-strand breaks. The finding that mutants for a major replication protein (PriA helicase, a component of the ϕX174-type primosome) were also deficient in homologous recombination further linked recombination and replication (Kogoma *et al.*, 1996; Sandler *et al.*, 1996).

That double-strand breaks might arise by replication running into nicks or gaps in the template was already proposed in 1966 by Hanawalt. A replication fork encountering such a single-strand lesion would collapse and lead to a double-strand break. Skalka (1974) suggested that collapsed replication forks could be repaired by homologous recombination. A model by Resnick, (1976) considered the repair of chromosomal double-strand breaks via invasion of the broken ends into the homologous duplex and restoration by DNA synthesis. Those models were however not further developed for some time. That a single double-strand break in the chromosome is lethal for an *E.coli* cell in the absence of RecA or RecBCD (Murialdo, 1988), demonstrated the importance of those classic recombination proteins for double-strand break repair. Double-strand breaks can occur by exposure to UV light or other DNA damaging agents. That replication forks running into single-strand interruptions in the chromosome can also lead to double-strand breaks had long been suggested (e.g. Skalka, 1974; Kuzminov, 1995a) and was finally experimentally proven (Kuzminov, 2001a). This event is probably rare in *E.coli* cells, as there is enough ligase present for efficient repair of nicks (Heitman *et al.*, 1989). It had been proposed that arrest of replication could lead to double-strand breaks (Bierne *et al.*, 1991; Kuzminov, 1995b). Michel and co-workers (1997) showed that in the absence of RecBCD a high number of double-strand breaks can be detected, which is increased further by inhibition of the replicative helicase. Their work clearly demonstrates that an arrested replication fork is transformed into a double-strand break (Fig. 1.4.) and that this phenomenon occurs quite frequently in the cell, even in the absence of DNA damaging agents. In addition, UV induced lesions represent an effective block to DNA polymerase III (Echols and Goodman, 1990), *E.coli*'s main replicative polymerase. Replication can also be inhibited by

certain DNA sequences (Krasilnikova *et al.*, 1998) and DNA secondary structures, other DNA damage or DNA-bound proteins. Most potential problems for replication are avoided by the action of repair systems, removing lesions from the DNA (Selby and Sancar, 1994). Some lesions can also be simply passed by the replisome, leaving a gap that can be filled in later by recombination or translesion synthesis (Johnson *et al.*, 1999).

As discussed below, most double-strand breaks arise from collapsed replication forks and this work is solely concerned with this type of event. Collapse of replication forks leads to only one double-strand end, contrary to double-strand breaks induced by UV-light, other DNA damaging agents or some endonucleases. In those latter cases, the cell is faced with two double-strand ends and the situation is somewhat different, requiring a different set of recombination proteins for efficient repair (Cromie and Leach, 2001). The model presented by Cromie and Leach (2001) for this type of "ends in" double-strand break repair involves only very limited replication and relies strongly on RecBCD to degrade one DSB arm completely in order to avoid inappropriate replication of the chromosome. Alternatively SbcC, a 3' to 5' exonuclease in this context is speculated to limit replication (Fig. 1.5.).

1.2.1. Collapsed replication forks

From the above findings it can be concluded that, though highly processive, replisomes rarely reach the terminus unhindered (Sandler and Marians, 2000), which in turn leads to the formation of double-strand breaks. An unrepaired double-strand break is lethal for the cell. It has been established that RecBCD is essential for repair of double-strand breaks, which is accomplished by recombination (see above). The mechanism of replication fork collapse was further elucidated by the finding that RuvABC are responsible for the occurrence of double-strand breaks at arrested replication forks (Seigneur *et al.*, 1998). As RuvABC specifically recognise, bind, move and resolve Holliday junctions, Seigneur and co-workers concluded that stalled replication forks must somehow regress to form a Holliday junction, which can then be recognised and processed by RuvABC, giving rise to a double-strand end. Their model proposes that replication is re-established either by RecBCD dependent

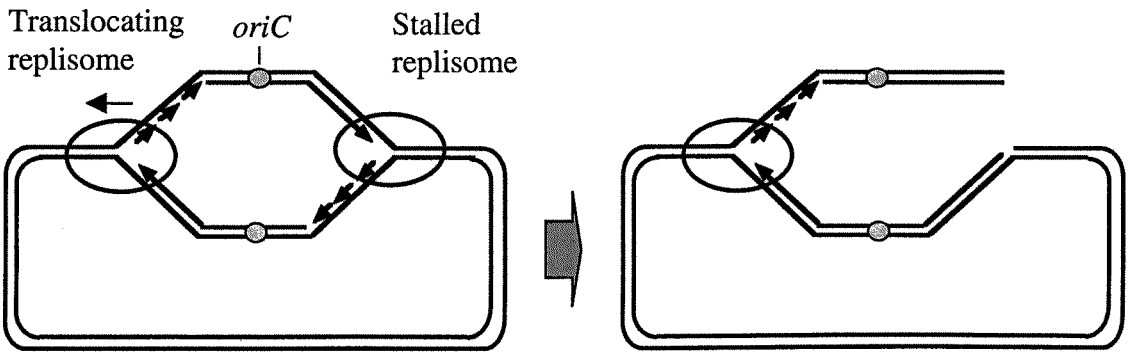

Figure 1.4. A model illustrating the formation of double-strand breaks at locations of replication fork arrest.

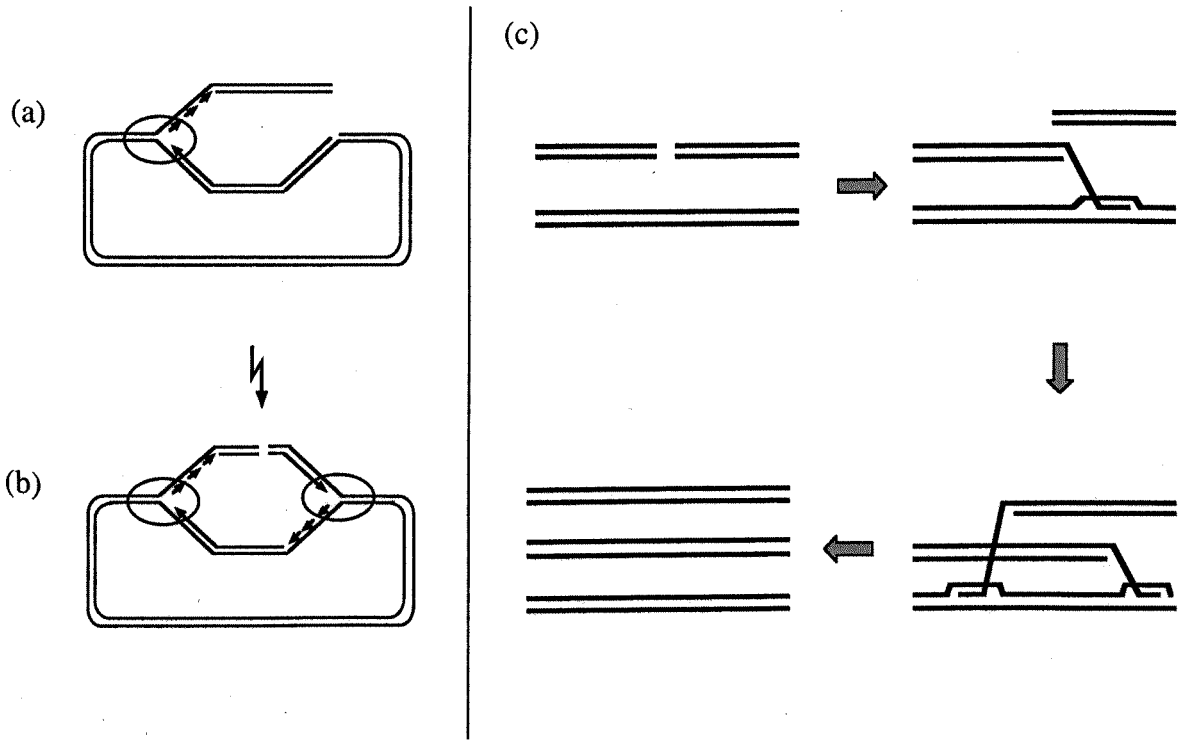

Figure 1.5. Arrest of replication forks leads to a single double-strand end (a). Direct breakage of the chromosome by UV light or other DNA breaking agents leads to two free double-strand ends (b). Recombination in case (a) simply restores replication. In case (b) tighter regulation is necessary to avoid the danger of triplicating the chromosome, which would happen if both ends were repaired by recombination [(c), modified from Cromie and Leach (2001)].

degradation of the double-strand arm, or alternatively by RecBCD and RecA dependent recombination with the intact sister duplex, depending on whether or not RecBCD encounters a χ site (Fig. 1.6.).

1.2.2. Restart of replication forks

It is not yet entirely clear what happens upon arrest of a replication fork. Marians and co-workers (1998) have demonstrated that a stalled replisome only dissociates from the replication fork after 5 - 7 minutes, a very long time for a cell that is trying to replicate its genome. Remaining of the replisome on the DNA would in most cases not allow the removal of the replication block and replication could not be resumed, which would be fatal for the cell. It is therefore likely, that stalled replisomes are displaced in vivo, as suggested by McGlynn *et al.* (2001), to allow removal of the replication hindrance and processing of the stalled fork.

How can a stalled replication fork be restarted? Several models have been proposed. One model involves degradation of the nascent lagging strand and stabilisation of the fork by RecA coating the single-strand region and invading the opposite arm of the fork (Courcelle and Hanawalt, 1999). Whether a stalled fork is likely to regress by pairing of the nascent strands, thus forming a Holliday junction, has been a point of contention. Fork regression was proposed by Seigneur *et al.*, (1998) and supported by data from McGlynn and Lloyd, (2000). Now there is more evidence that this can indeed happen. Apart from the fork reversal via RecG (see below) proposed by McGlynn and Lloyd, (2000), Postow and co-workers (2001) show that positive supercoiling can regress fork structures into Holliday junctions. RecA was discovered to have the same property (Robu *et al.*, 2001), representing a novel RecA activity. It seems therefore likely that regression of replication forks occurs also in vivo.

recG is part of the *spo* operon (Kalman *et al.*, 1992). Other genes in that operon include *rpoZ* (encoding the ω subunit of RNA polymerase) and *spoT* (the major (p)ppGpp degrading activity; see below). RecG has some homology to Mfd (Lloyd and Sharples, 1991), which has been shown to remove RNA polymerase from

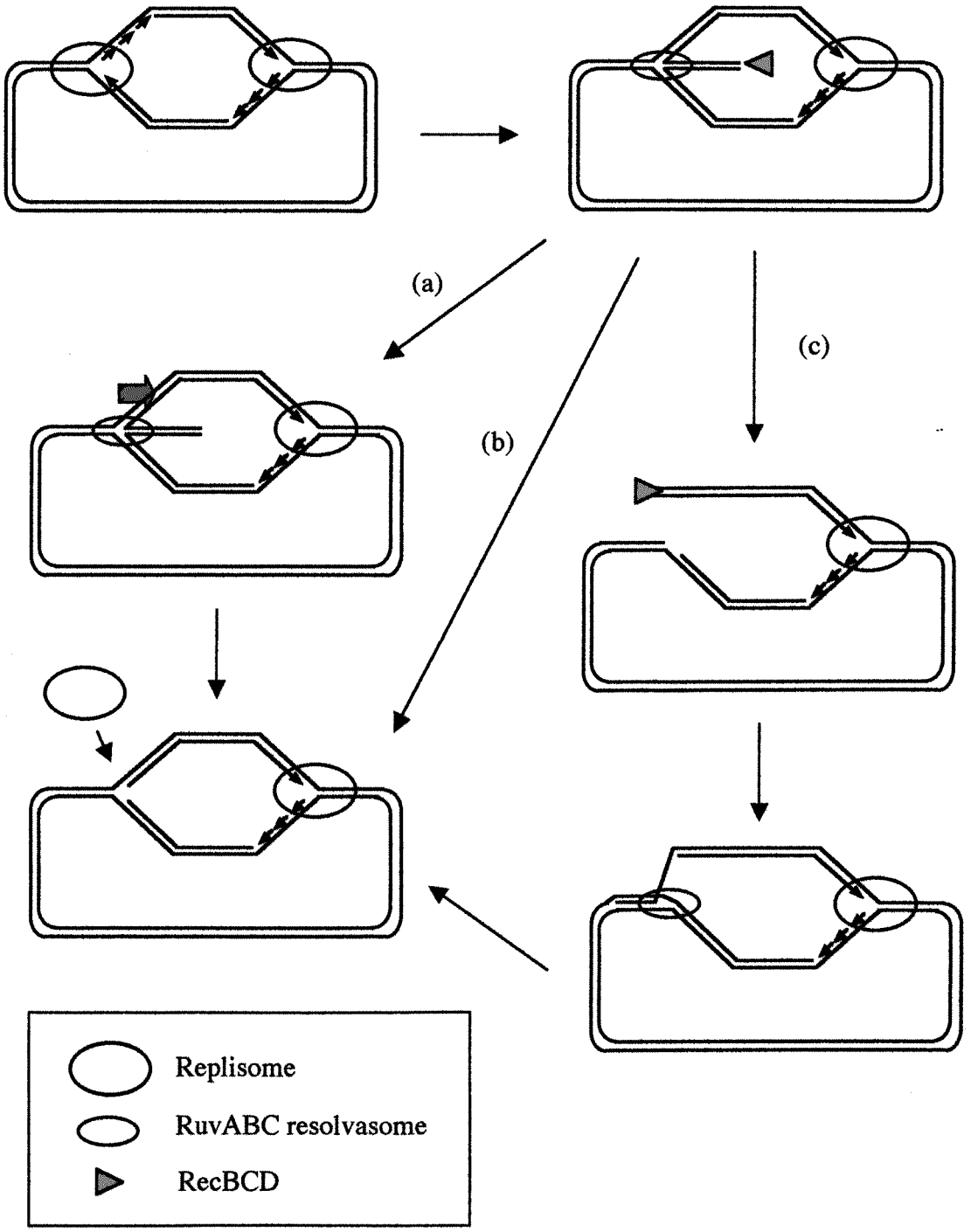

Figure 1.6. The collapse and repair of arrested replication forks. A simplified version of the model proposed by Seigneur *et al.*, 1998. They propose three different pathways. First, RuvABC can move the resulting Holliday junction back into a fork (a). Second, RecBCD could simply degrade the new strands that have annealed during fork regression (b).Third, RuvABC resolves the Holliday junction that arises at a stalled fork and the double-strand end recombines with the intact homologue (c).

the DNA when it is stalled at a lesion and at the same time recruit the UvrAB repair enzymes (Selby and Sancar, 1994). RecG is a structure-specific DNA helicase, able to catalyse branch migration of Holliday junctions and other branched structures (Lloyd and Sharples, 1993). There is a functional overlap between RuvAB and RecG, which explains the remaining recombination activity in *ruv* cells (Lloyd, 1991). RecG has been proposed to form a Holliday junction from a D-loop (Whitby and Lloyd, 1995) and so could play an important role in homologous recombination. Its helicase activity would also allow it to facilitate fork regression, as proposed by McGlynn and Lloyd, (2000). Together with RusA (Sharples *et al.*, 1994), RecG can compensate for the absence of RuvABC (Mandal *et al.*, 1993).

The product of fork regression, a Holliday junction, can be resolved in different ways. RecBCD or another exonuclease could simply digest the short junction arm formed by the two nascent strands, restoring a fork structure (Seigneur *et al.*, 1998). In this model recombination and potentially harmful rearrangements are being avoided. The Holliday junction can also be bound and resolved by RuvABC, resulting in a free double-strand end that can be processed by RecBCD and recombine with the intact chromosome. The so formed D-loop can be used to restart replication (Liu and Marians, 1999) and the junction formed downstream of the new fork can be resolved via the RuvABC proteins. The Holliday junction formed at the arrested replication fork could also simply be branch migrated back into a fork by the RuvAB or RecG helicases (McGlynn and Lloyd, 2000). McGlynn and Lloyd (2000) also propose another model that avoids recombination: If only the leading strand DNA polymerase is stalled and not the replicative helicase, the lagging strand polymerase would continue replication, leading to de-coupling of the polymerases and disassembly of the replisome. The lagging strand would be longer than the leading strand. RecG could unwind it, freeing it for pairing with the leading strand. The lagging strand being longer and having passed the location of the damage on the leading strand template, the lagging strand could then serve as template for continued synthesis of the leading strand. Unwinding of the Holliday junction back into a fork structure (possibly by RecG or RuvAB) would bypass the lesion and allow replication restart (Fig. 1.7.). A later version of this model is presented in Figure 1.8. (from Gregg *et al.*, 2001). The updated model explains in more detail how leading

strand blocks could be circumvented. After fork regression and annealing of the nascent strands, two possible pathways are described: (a) the missing part of the leading strand can be filled in by replication, which, when the Holliday junction is moved back into a fork by RecG leads to bypass of the lesion; (b) the annealed nascent strands can be degraded by an exonuclease or moved back into a fork directly, both of which demands repair of the lesion to allow restart.

Once a fork is re-established, replication can be restarted with the help of PriA helicase (a component of the ϕX174-type primosome) and other proteins. The option of priming replication at locations other than *oriC* and without the need for protein synthesis (Kogoma, 1997) in this context is very important for the cell.

1.3. DNA repair and (p)ppGpp

A connection between the stringent response and DNA damage was observed by Kramer and co-workers (1988), who report an increased sensitivity of a *relA* mutant to near-UV light. McGlynn and Lloyd (2000) discovered that the UV resistance of *ruv* strains was increased by (p)ppGpp, the product of RelA. What is (p)ppGpp, what is it's known role and how does it influence UV resistance? The compounds guanosinetetraphosphate and guanosinepentaphosphate, collectively termed (p)ppGpp, are the effector molecules of the stringent response. "The stringent response is a pleiotropic physiological response elicited by a failure of the capacity for tRNA aminoacylation to keep up with the demands of protein synthesis." (Cashel *et al.*, 1996)

1.3.1. (p)ppGpp and the stringent response

E.coli cells under conditions of amino acid starvation adapt their gene expression to the restricted circumstances. Genes encoding rRNA and proteins which are necessary in abundance for exponential growth, are downregulated, whereas proteins that are useful for the cell in restricted circumstances - like amino acid synthetic operons - are upregulated (Cashel *et al.*, 1996). The same is true for entry

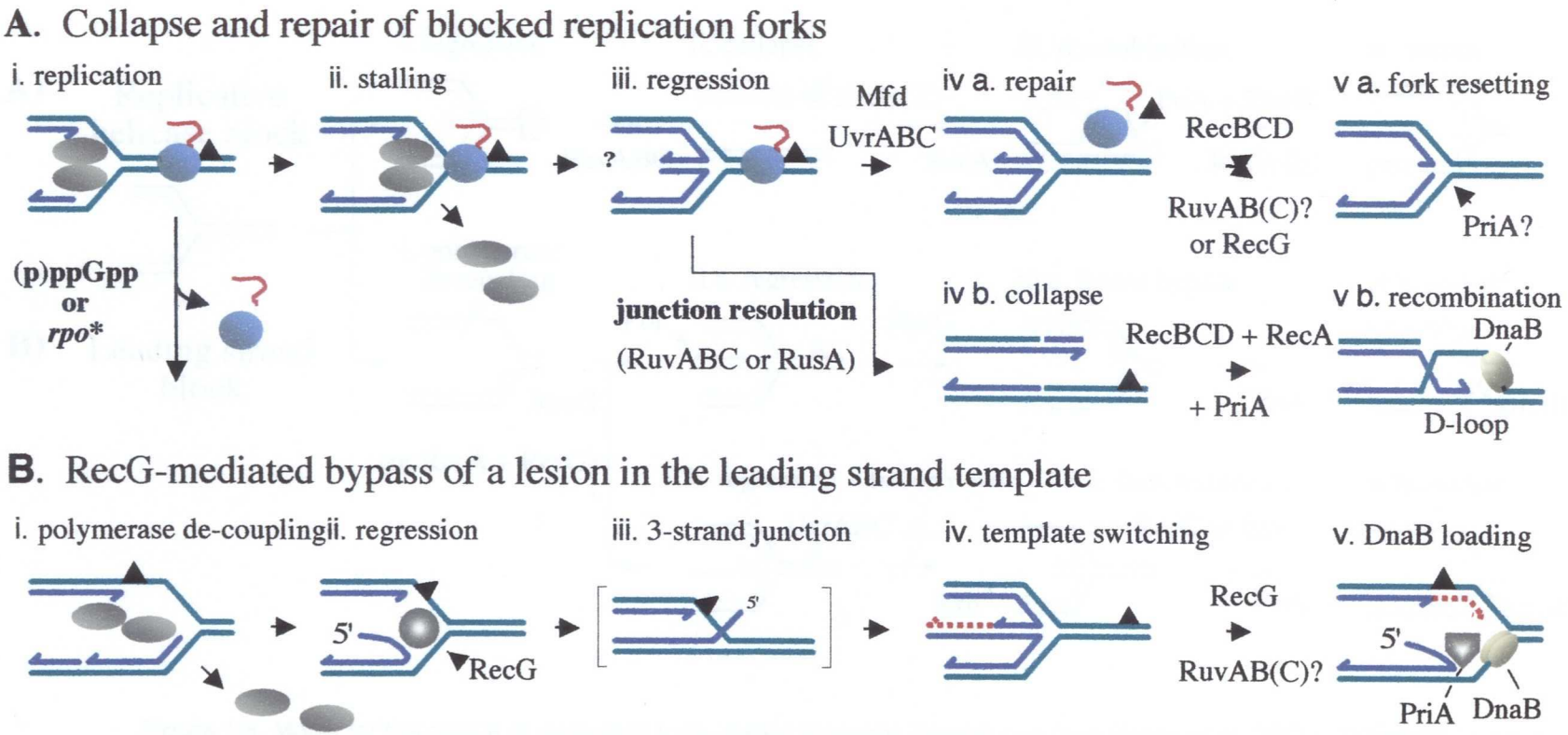

Figure 1.7. A model for the collapse and repair of replication fork, reproduced from McGlynn and Lloyd (2000). **A)** Two possible pathways for the repair of forks where the replicative helicase was arrested. **B)** A RecG-mediated pathway for the repair of forks where only the leading strand polymerase was arrested. See text for more details.

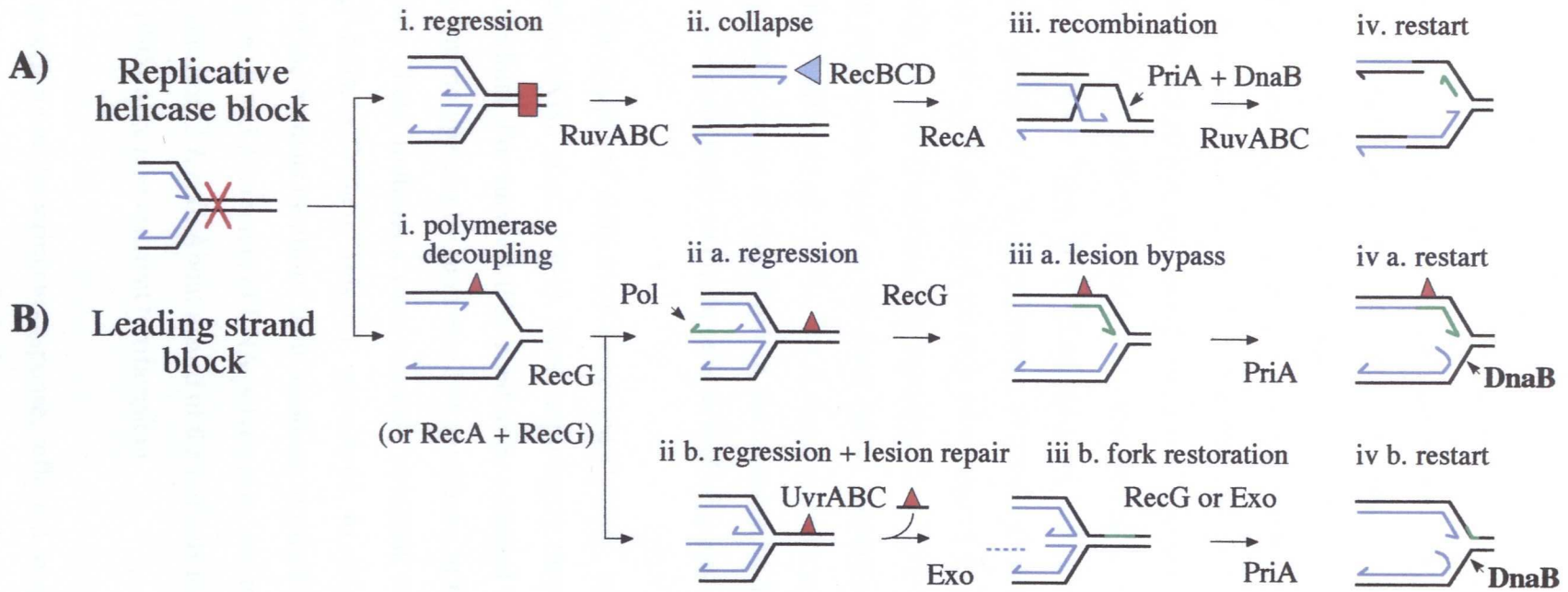

Figure 1.8. Model for the rescue of replication forks stalled at lesions (reproduced from Gregg *et al.*, 2001). **A)** Rescue of a fork stalled at a replicative helicase block (red rectangle). **B)** Rescue of a fork collapsed by a leading strand block. Possible protein functions mediating individual steps are indicated. Newly synthesised DNA is indicated in green.

into stationary phase (which can be regarded as a kind of starvation) and stress. Osmotic shock (Harshman and Yamazaki, 1972), heat shock and oxidative stress (for a review see Hengge-Aronis, 1993) also induce the stringent response. Mutants with unbalanced RNA synthesis were already observed by Borek and co-workers in 1956. Such mutants were called "relaxed" and, in contrast to wild type strains, failed to accumulate (p)ppGpp under stringent conditions (Cashel, 1969). The genes responsible for the stringent response were identified as *relA* (Friesen *et al.*, 1974) and *spoT* (Laffler *et al.*, 1974). Mutants for *relA* were found to continue accumulation of rRNA and tRNA even under stringent conditions (Stent and Brenner, 1961). The RelA protein was identified as a (p)ppGpp synthetic activity. (p)ppGpp are made from GTP, depending on idling ribosomes and binding of uncharged tRNA to a "hungry" codon (Cashel, 1969). Though the fraction of ribosomes that carry the RelA protein is estimated to be only about 1% during exponential growth (Pedersen and Kjeldgaard, 1977), it is sufficient to trigger (p)ppGpp production. SpoT on the other had is primarily responsible for (p)ppGpp degradation (Laffler *et al.*, 1974), but it also possesses a modest synthetic activity, providing basal levels of (p)ppGpp in the absence of RelA (Xiao *et al.*, 1991). Inactivation of both genes results in cells devoid of (p)ppGpp altogether (Fig. 1.9.).

Such (p)ppGpp^0 cells show decreased viability, heterogeneous cell size and filamentation (Xiao *et al.*, 1991). Most importantly they are not able to grow on minimal medium. For survival they need to be supplied with all amino acids. The (p)ppGpp^0 phenotype can be suppressed by mutations in RNA polymerase (Little *et al.*, 1983a). These mutations were termed "stringent mutations" as they restore (p)ppGpp^0 cells, with their "relaxed" phenotype, to stringent, normal behaviour. Mapping of the mutants identified their locations mostly in the *rpoB* and *rpoC* genes, coding for the β and β' subunits of RNA polymerase, and rarely *rpoD*, coding for σ^{70}, the major σ factor of *E.coli.* About a third of the mutants identified by Cashel and co-workers (1996) were also resistant to rifampicin.

The effects of the stringent response, reflected by the levels of (p)ppGpp are manifold, far reaching and complex (for a comprehensive review see Cashel *et al.*, (1996). (p)ppGpp exert their effect by binding to RNA polymerase (Chatterji *et al.*,

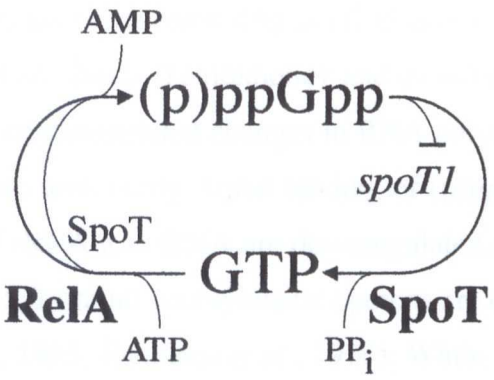

Figure 1.9. Synthesis and degradation of (p)ppGpp in a simplified diagram [reproduced from McGlynn and Lloyd (2000)]. RelA synthesises (p)ppGpp from GTP, SpoT minorly contributes to synthesis while constituting the major degrading activity. *spoT1* mutations abolish degradation.

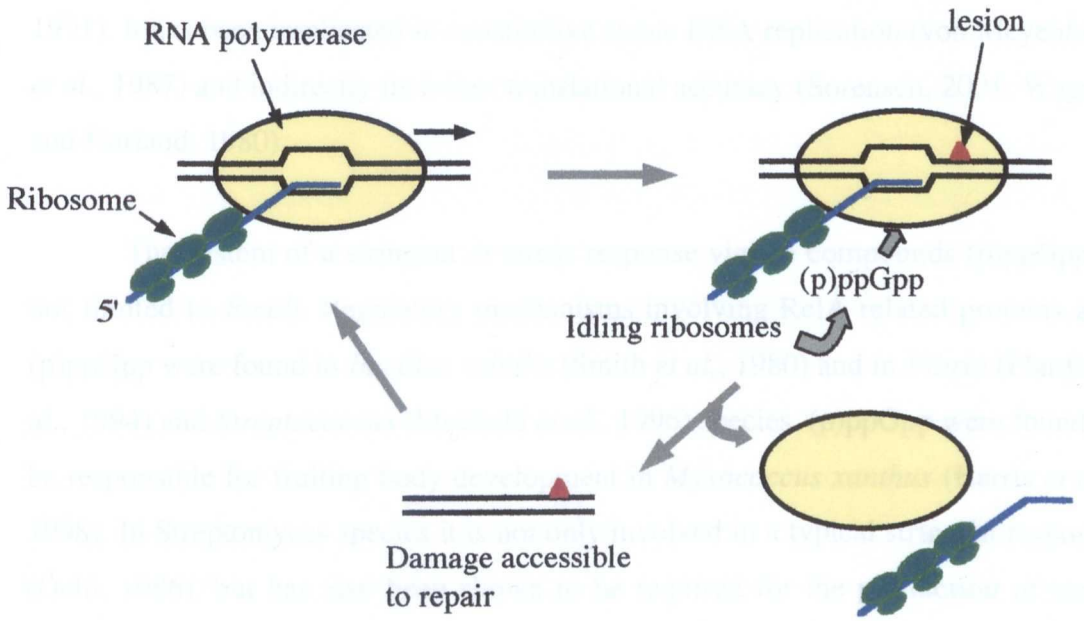

Figure 1.10. Model of a possible way of modulation of RNA polymerase by (p)ppGpp that could promote the survival of UV irradiated *ruv* cells. RNA polymerase stalled at a lesion would also stall translation. Idling ribosomes trigger the production of (p)ppGpp which binds and modulates RNA polymerase. This could lead to facilitated dissociation and exposure of the lesion to repair systems.

1998). It binds to a modular site, formed by the C-terminus of β and the N-terminus of β' (Toulokhonov *et al.*, 2001). Toulokhonov and co-workers found no indication that (p)ppGpp induce conformational changes in RNA polymerase. It does however affect RNA polymerase profoundly. Upon binding of (p)ppGpp gene expression is changed. Synthesis of rRNA and tRNA are downregulated (e.g. Borek *et al.*, 1956; Sands and Roberts, 1952), amino acid synthetic operons are upregulated (Kliachko *et al.*, 1983; Rudd *et al.*, 1985; Stephens *et al.*, 1975). While RNA polymerase genes are under weak negative stringent control (Lideman *et al.*, 1979), Sigma S, the stationary phase sigma factor is upregulated (Gentry *et al.*, 1993), contributing to the expression of a different set of genes. A large number of other genes are under stringent control, including genes encoding proteins involved in carbohydrate metabolism, heat and cold shock, cell wall synthesis, phospholipid metabolism, etc. (for a review see Cashel *et al.*, 1996). The stringent response has also been demonstrated to be involved in replication initiation at *oriC* (Ogawa and Okazaki, 1991). It has been implicated in constitutive stable DNA replication (von Meyenburg *et al.*, 1987) and indirectly increases translational accuracy (Sorensen, 2001; Wagner and Kurland, 1980).

The system of a stringent or stress response via the compounds (p)ppGpp is not limited to *E.coli*. Regulatory mechanisms involving RelA related proteins and (p)ppGpp were found in *Bacillus subtilis* (Smith *et al.*, 1980) and in *Vibrio* (Flardh *et al.*, 1994) and *Streptococcus* (Mechold *et al.*, 1996) species. (p)ppGpp were found to be responsible for fruiting body development in *Myxococcus xanthus* (Harris *et al.*, 1998). In Streptomyces species it is not only involved in a typical stringent response (Ochi, 1986), but has also been shown to be required for the production of some antibiotics (e.g. Chakraburtty and Bibb, 1997; Hoyt and Jones, 1999). *relA* and *spoT* homologues have even been found in *Arabidopsis* (van der Biezen *et al.*, 2000), showing the (p)ppGpp regulatory system to be evolutionarily conserved. A mechanism similar to the stringent response was also found to work in yeast (Warner and Gorenstein, 1978), but using an as yet unknown signalling molecule, different from (p)ppGpp (McEntee *et al.*, 1994).

1.3.2. Modulation of RNA polymerase

Modulation by (p)ppGpp

How can the stringent response influence the UV resistance of *ruv* strains? As pointed out by McGlynn and Lloyd (2000) there are two possible answers. First, RNA polymerase, modified by (p)ppGpp leads to a drastic alteration in gene expression. It is possible that the expression of some gene or genes is induced or stimulated that can compensate for the absence of the RuvABC complex. To date there is no evidence either supporting or excluding this view. Second, the modulation of RNA polymerase itself (Fig. 1.10.) may be the crucial factor for the suppression of the *ruv* phenotype. This theory is supported both by the results of McGlynn and Lloyd (2000) and the data presented here. The possibility that both mechanisms act together cannot be excluded.

How can modulation of RNA polymerase relieve the UV sensitive phenotype of *ruv* strains? In recent years the direct effects of (p)ppGpp on RNA polymerase have become increasingly clearer. Already in 1981 effects of (p)ppGpp on pausing and termination were observed (Kingston and Chamberlin, 1981; Kingston *et al.*, 1981). Krohn and Wagner (1996) found that pausing enhancement by (p)ppGpp was stronger in genes under negative stringent control. This effect was sequence specific, depending on the promoter and adjacent sequences. The presence of (p)ppGpp during initiation was not required. Early studies with RNA polymerase mutants exhibiting an altered response to (p)ppGpp indicated that there might be some direct effect of (p)ppGpp on promoter interactions (Glass *et al.*, 1986; Nomura *et al.*, 1984). Investigation of the effect of (p)ppGpp on different promoters showed downregulation of genes under negative stringent control and upregulation of other genes under positive control, probably due to enhanced decay of open complexes (Kajitani and Ishihama, 1984). Both (p)ppGpp and "stringent mutants", compensating for the absence of (p)ppGpp and mimicking its effect, were found to destabilise open complexes at rRNA promoters (Bartlett *et al.*, 1998; Raghavan and Chatterji, 1998; Zhou and Jin, 1998). Barker and co-workers (2001b) found this to be true for all promoters tested, whether they were negatively or positively regulated by the stringent response. That open complexes have a much shorter half-life at *rrn*

promoters than at amino acid promoters could explain the differential regulation. Decreasing the already short half-life of *rrn* promoters would decrease transcription and so could free enough RNA polymerase to stimulate transcription from amino acid promoters with an also shortened, but still longer open complex half-life.

The above results can provide a possible answer for the mechanism of increase of UV resistance in *ruv* mutants. As proposed by McGlynn and Lloyd (2000), (p)ppGpp or mutants mimicking its effect, could facilitate dissociation of RNA polymerase from the DNA. This could happen at promoters or when RNA polymerase pauses or is stalled at a lesion. Also the enhancement of termination (see above) may play a role. It is not yet clear which of these events is of importance and whether it is sufficient on its own or whether altered gene expression is also involved. Dissociation of paused or stalled RNA polymerase may however confer a crucial advantage to cells that are unable to repair collapsed/stalled replication forks. Under normal circumstances, RNA polymerase does not constitute a block for replication. Whether transcription is taking place in the same or in the opposite direction of replication, transcribing RNA polymerase is simply dislodged and can later faithfully resume transcription (French, 1992; Liu and Alberts, 1995). RNA polymerase stalled at a UV-induced lesion could be a different matter and might constitute a replication fork block. It would certainly delay repair of the lesion, increasing the likelihood of the replisome encountering it. In *ruv* cells, deficient for one important pathway to re-establish stalled replication forks, a decrease in the incidence of fork blocks, mediated by the presence of high concentrations of (p)ppGpp or RNA polymerase mutations mimicking the same effect, may well account for the increase in UV resistance.

Modulation by Mfd

(p)ppGpp is only one possibility for dissociating stalled RNA polymerase. Mfd is the cells major mechanism for removal of RNA polymerase from lesions (Fig. 1.11.A.). It is also called TRCF - transcription-repair coupling factor. Mfd binds directly to RNA polymerase stalled at a lesion (Selby and Sancar, 1995). It then proceeds to displace RNA polymerase from the DNA and recruits the UvrABC excision repair system by binding to UvrA. It stimulates repair of the template strand

when transcription is taking place. (Selby and Sancar, 1993). Why, with the Mfd system in operation, (p)ppGpp should have such a major influence is not quite clear.

Modulation by GreA/B

The GreA and GreB proteins of *E.coli* represent another method of dealing with stalled elongation complexes (Fig. 1.11.B.). When RNA polymerase stalls, be it at a lesion or a regulatory pause site, it can backtrack, extruding the 3' end of the nascent RNA (Lee *et al.*, 1994). Under those circumstances transcription cannot be resumed. GreA and GreB stimulate the intrinsic transcript cleavage activity of RNA polymerase, thereby creating a new RNA 3' end in the right position to allow restart of transcription (Orlova *et al.*, 1995). Up to date there is no evidence supporting an involvement of GreA/B in modulation of RNA polymerase stalled at lesions. They have been shown to act at pause sites. It is however easy to imagine that GreA/B perform the same function at lesions, which would be of great importance in the context of modulating RNA polymerse in order to avoid replication fork blocks.

1.4. RuvABC, RecBCD and (p)ppGpp

McGlynn and Lloyd (2000) have clearly established a connection between the stringent response and its effectors (p)ppGpp, and the repair of stalled replication forks. They find that the UV sensitivity of a *ruv* strain - devoid of Holliday junction resolution activity - is drastically increased in the absence of (p)ppGpp and ameliorated in the presence of excess (p)ppGpp. Also RNA polymerase mutants, mimicking the effect of (p)ppGpp, can relieve the UV sensitivity of a *ruv* strain, in the presence or absence of normal levels of (p)ppGpp. Consequently the increase in resistance to UV induced damage is due to modulation of RNA polymerase activity. As mentioned above, this modulation of activity could act indirectly, by alteration of gene expression. It could also act directly, by decreasing the incidence of stalled RNA polymerase and facilitating the removal of lesions by removing stalled RNA polymerase from them. Both unrepaired lesions and stalled RNA polymerase itself create potential roadblocks for replication. There is also the possibility of direct and indirect mechanisms working together to promote efficient replication. The fact that

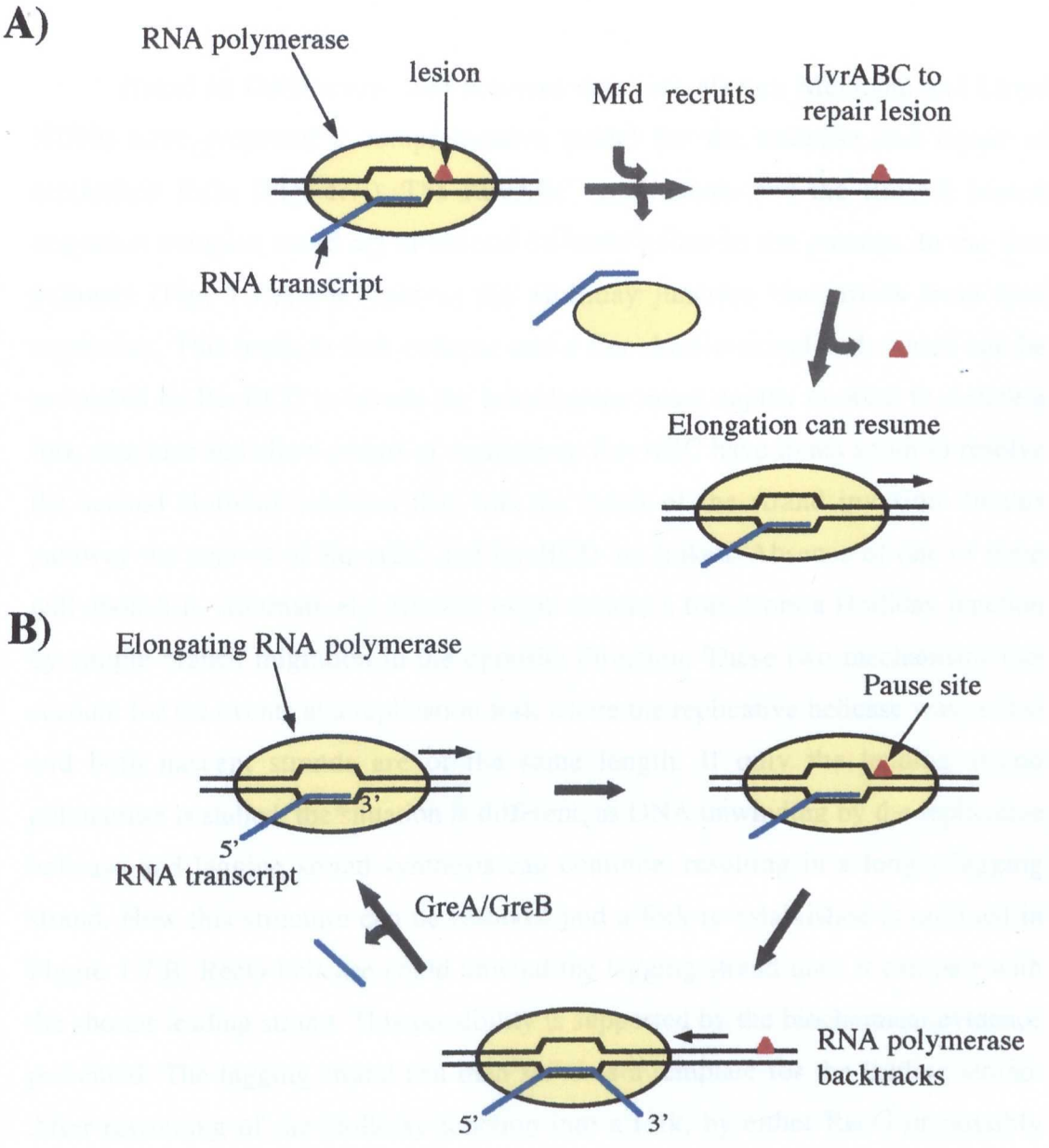

Figure 1.11. A) Cartoon representation of the mechanism of dissociation of RNA polymerase from a lesion and recruitment of the UvrABC excision repair system, both mediated by Mfd. **B)** Promotion of transcript cleavage by GreA/B, facilitating transcription restart.

resistance to mitomycin C or γ rays is not or only very little increased argues against the mechanism of altered gene expression.

Based on these results and previous data (see above), McGlynn and Lloyd (2000) have proposed a comprehensive model for the collapse and repair of replication forks (Fig. 1.7.). The RuvABC resolvasome and the RuvAB branch migration complex could act at several different points in the process. In the first pathway (Fig. 1.7.A.) it resolves the Holliday junction that arises from fork regression. This leads to fork collapse and a free double-strand end, which can be processed by RecBCD to invade the homologous intact duplex in order to restore a fork structure and allow restart of replication. RuvABC have to act again to resolve the second Holliday junction that was the result of the strand invasion. In this pathway the actions of RuvABC and RecBCD are linked. Absence of one of them will abolish it. Alternatively, RuvAB might restore a fork from a Holliday junction by simple branch migration in the opposite direction. These two mechanisms can account for the events at a replication fork where the replicative helicase was stalled and both nascent strands are of the same length. If only the leading strand polymerase is stalled, the situation is different, as DNA unwinding by the replicative helicase and lagging strand synthesis can continue, resulting in a longer lagging strand. How this structure can be resolved and a fork re-established is outlined in Figure 1.7.B. RecG helicase could unwind the lagging strand until it can pair with the shorter leading strand. This possibility is supported by the biochemical evidence presented. The lagging strand can then serve as a template for the leading strand. After rewinding of the Holliday junction into a fork, by either RecG or possibly RuvAB, the site of the lesion on the leading strand template will have been passed, a fork re-established and replication can be restarted.

The work presented in this thesis is based on the above model and further develops its ideas. A number of questions remained to be answered, some of which are going to be addressed here. McGlynn and Lloyd showed that, in the absence of RuvABC, survival of UV irradiated cells depends on RecA, UvrABC, RecG and PriA, but not on RecBCD. *ruv* cells have to rely on excision repair (via UvrABC) to avoid lesions. As they cannot process Holliday junctions, they also have to rely on

RecG to rewind junctions into forks or exonucleases (like RecBCD) to degrade the double- strand fork arm. In the cases where the replicative helicase is not stalled, RuvABC is not required and RecG could well be sufficient to restore a fork structure. PriA is always necessary for restart of replication in places other than oriC and so it is not surprising that it is critical for survival. The role of RecA in the absence of RuvABC is not entirely clear, as it is mostly involved in recombination events, which usually lead to formation of a Holliday junction which could not be resolved in *ruv* cells. It could be connected with the coprotease activity of RecA, promoting self cleavage of the LexA repressor. It could also be due to RecA's ability to regress replication forks (Robu *et al.*, 2001). As RecBCD is necessary for the repair of double strand breaks that are only produced by the activity of RuvABC, it is not essential for the survival of UV irradiated *ruv* cells and in fact makes hardly any difference. RecBCD could also be responsible for degradation of the double strand arms of regressed replication forks, but this task can easily be performed by other exonucleases (e.g. RecJ in conjunction with RecQ helicase).

This work set out firstly to investigate one aspect of the model, namely the connection between RuvABC and RecBCD in this context. Its second aim was to get one step closer to determining the mechanism of the action of both (p)ppGpp and certain RNA polymerase mutants that relieve the UV sensitivity of *ruv* mutants. The model presented by McGlynn and Lloyd (2000) predicts that lack of RecBCD should be equivalent to lack of RuvABC, as they both act in the same pathway and one activity is not much use without the other. Therefore *rpo** mutations (certain RNA polymerase mutants mimicking the effect of (p)ppGpp and increasing the survival of UV irradiated *ruv* cells) should have the same effect in *recB* cells as in *ruv* cells. It should be equally possible to isolate similar RNA polymerase mutants from a *recB* background as the reported *rpo** mutants. This was shown to be the case. *rpo** mutations can be isolated from a *recB* background as well as from a *ruv* background. McGlynn and Lloyd (2000) report one RNA polymerase mutation, isolated from a *ruv* background, that, when transferred into a *recB* background, has a considerably weaker effect, though it still increases survival. In this work a larger number of mutants was isolated from a *recB* background. The mutations are shown to be not inherently different, the same mutations can even be isolated from the different

backgrounds. Studies on those mutants revealed that most mutations have an equal effect in *ruv* and *recB* cells, though there are some mutants that are considerably more effective in *ruv* cells. Also an example for the opposite was found, but with a less marked difference. The isolated mutants were characterised in respect of their phenotype, viability and growth rate. Both viability and phenotypical appearance were improved, but there was no significant change in growth rate compared to the parent.

An attempt was made to ascertain whether UV light would induce the production of (p)ppGpp, as seen with near UV light (Kramer *et al.*, 1988). The chosen method however - thin layer chromatography - turned out to be unsuitable for the task in hand, as UV irradiated samples did not migrate into the gel, trapping a significant amount of material at the point of origin and obscuring the results.

Twenty-seven different mutations could be identified by sequencing and their mutations could be analysed. Most of them are located in *rpoB*, probably reflecting a preference for selection of rifampicin resistant strains. Some are located in *rpoC* and it is likely that *rpoD* (encoding σ^{70}) mutants are among the mutants that have not been identified yet. The mutations mostly concern conserved residues and lie in conserved regions of the genes. Many changed residues are well placed to make contact with the DNA substrate or the RNA product and virtually all of them lie on the inside surface of the enzyme. Two mutant proteins were purified for in vitro studies. The results confirm the previously observed weak open complex formation of RNA polymerase mutants that suppress the relaxed response and the negative effect of (p)ppGpp on open complex formation (Barker *et al.*, 2001a; Barker *et al.*, 2001b; Bartlett *et al.*, 1998). Though under the conditions used there was no indication that mutant RNA polymerases form stalled complexes that dissociate more readily from the DNA, evidence is presented that demonstrates a modest but significant effect of (p)ppGpp on stalled complexes, promoting their dissociation.

Chapter 2.

Materials and Methods

2.1. Materials

2.1.1. Microbiological growth media and supplements

Yeast extract, tryptone and bactoagar were all obtained from Difco. Liquid and solid media used for the growth of *Escherichia coli* strains were prepared by observing standard recipes as follows:

- Mu broth contained 5 g yeast extract, 10 g tryptone, 10 g of NaCl and made up to 1 litre with distilled water (final pH 7.5). Mu agar plates were supplemented with 10 g agar per litre of Mu broth and either 4 g or 6 g of agar per litre for overlays.
- 4 x Mu broth contained 10 g of NaCl and 4 x the amounts of all other ingredients.
- LB (Luria-Burrous) broth contained 5 g yeast extract, 10 g tryptone, 0.5 g NaCl and 0.08 g NaOH in 1 litre of distilled water (final pH 7.5). LB agar plates were supplemented with 15 g of Bactoagar per litre of LB broth.
- Minimal 56/2 salts media (Willetts *et al.*, 1969) contained 2.64 g of KH_2PO_4, 4.3 g of Na_2HPO_4, 1 ml of 10% $MgSO_4.7H_2O$, 10 ml of 10% $(NH_4)_2SO_4$, 0.5 ml of 1% $Ca(NO_3)_2$ and 0.5 ml of 0.05% $FeSO_4.7H_2O$ in 1 litre of distilled water. For minimal salts solid media, 56/2 salts were used at double strength and 15 g of Bactoagar was added per litre. 56/2 salts media was supplemented with thiamine (1 μg/ml), glucose (3.3 mg/ml), and amino acids (50 – 80 μg/ml) as required for the growth of specific strains.
- 10 x carbon sources contained 4% K-acetate (w/v), 4% Glycerol (v/v) and 8% D-glucose (w/v).
- 1000 x phosphate source contained 0.2 M K_2HPO_4.
- 1000 x micronutrients contained 10 μM $ZnCl_2$, 30 μM $CaCl_2$, 3 μM ammonium-molybdate, 10 μM $CuSO_4$, 0.4 mM boric acid, 80 μM $MnCl_2$.

- 10 x MOPS (pH 7.4) contained 0.4 M MOPS, 40 mM Tricine, 95.2 mM NH_4Cl, 0.5 M NaCl, 5 mM $MgSO_4$, 5 μM $CaCl_2$, 0.1 mM $FeSO_4$, 5.52 mM KCl.
- 10 x amino acid/base mix contained 0.5 mg/ml for each amino acid and 2 mM for each of the four bases

All media were sterilised by autoclaving at 121°C, for 15 minutes. Media were supplemented with antibiotics as required. For rich phosphate starvation media, carbon sources, phosphate source, micronutrients, MOPS, amino acids and bases were mixed with sterile dH_2O. For minimal phosphate starvation medium the amino acids were omitted.

2.1.2. Antibiotic stock solutions

Antibiotic stocks were made in sterile distilled water. They were stored at 4°C, with the exception of tetracycline which was stored in 3ml aliquots at -20°C. Solutions were made up to the stock concentrations of 2 mg/ml for Chloramphenicol (Cm) and Tetracycline (Tc), 4 mg/ml for Ampicillin (Ap), Carbenicillin and Kanamycin (Km), and 20 mg/ml for Streptomycin (Str). Antibiotics are used at the following working concentrations: - Chloramphenicol - 20 μg/ml, Ampicillin - 40 μg/ml, Carbenicillin – 25 μg/ml, Tetracycline – 20 μg/ml, Kanamycin – 25 μg/ml and Streptomycin – 100 μg/ml.

2.1.3. Strains, Bacteriophages and Plasmids

The *E. coli* K-12 and B strains used in this work are listed in Table 2.1. Plasmids are listed in Table 2.2. Bacteriophage P1*vir* was used for transductions (Miller 1972).

Table 2.1. E.coli K-12 strains

Strain	Relevant genotype	Source or derivation
a) General		
DH5α	F′ *endA1 hsdR17* (r_K-m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) *U169 deoR* (ϕ*80lac* Δ (*lacZ*) *M15*)	Woodcock *et al.*, (1989); Raleigh *et al.*, (1989)
W3110	F$^-$ IN (*rrnD-rrnE*)	(Bachmann, 1996)
b) MG1665 derivatives		
MG1665	Wild type	(Bachmann 1996)
N4235	Δ*relA251*::*kan* Δ*spoT207*::*cat* $rpoB_{H1244Q}$ (Rif^R)	laboratory strain
N4278	*recB268*::Tn*10*	(McGlynn and Lloyd, 2000)
N4281	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{H1244Q}$	laboratory strain
N4304	Δ*spoT207*::*cat* Δ*relA251*::*kan*	laboratory strain
N4315	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10*	(McGlynn and Lloyd, 2000)
N4583	Δ*ruvABC*::*cat*	laboratory strain
N4355	Δ*ruvA60*::Tn*10* Δ*relA251*::*kan* Δ*spoT207*::*cat* $rpoB_{G534C}$ (Rif^R)	laboratory strain
N4576	Δ*relA251*::*kan* Δ*spoT207*::*cat* Δ*ruvAC65* *eda.51*::Tn*10* $rpoB_{I572S}$ (Rif^R)	laboratory strain
N4600	*recB270*::*kan*	laboratory strain
N5020	*spoT1*	laboratory strain
BT121	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{T563P}$ (Rif^R)	minimal medium selection on N4315

BT124	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{H447R}$ (Rif^R)	minimal medium selection on N4315
BT125	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{V550E}$ (Rif^R)	minimal medium selection on N4315
BT126	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoC_{E1146D}$	minimal medium selection on N4315
BT129	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{G536V}$ (Rif^R)	minimal medium selection on N4315
BT130	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{L571Q}$ (Rif^R)	minimal medium selection on N4315
BT132	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{H551P}$ (Rif^R)	minimal medium selection on N4315
BT133	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoC_{K215E}$	minimal medium selection on N4315
BT134	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* *rpoB*Q148P(Rif^R)	minimal medium selection on N4315
BT139	Δ*ruvABC*::*cat; argE*::Tn*10*	P1.N3794 x N4583 -> Tc^R
BT140	*argE*::Tn*10*	P1.N3794 x MG1665 -> Tc^R
BT142	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{H447P}$ (Rif^R)	minimal medium selection on N4315
BT143	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{S788F}$ (not *rpo**)	minimal medium selection on N4315
BT146	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{G1260D}$ (Rif^R)	minimal medium selection on N4315
BT151	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{L533P}$ (Rif^R)	minimal medium selection on N4315
BT152	Δ*spoT207*::*cat*, Δ*relA251*::*kan*, *recB268*::Tn*10*, *rpoB*L420R(Rif^R)	minimal medium selection on N4315
BT153	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{A532E}$ (Rif^R)	minimal medium selection on N4315

BT163	Δ*ruvABC*::*cat* $rpoB_{L571Q}$	P1.BT130 x BT139 -> *argE*$^+$; *rpo**, Rif^R
BT164	Δ*ruvABC*::*cat* $rpoB_{H551P}$	P1.BT132 x BT139 -> *argE*$^+$; *rpo**, Rif^R
BT165	$rpoB_{L571Q}$	P1.BT130 x BT140 -> *rpo**, Rif^R
BT166	$rpoB_{H551P}$	P1.BT132 x BT140 -> *rpo**, Rif^R
BT175	*recB270*::*kan* $rpoB_{H551P}$	P1.N4600 x BT166 -> Km^R
BT181	*recB270*::*kan* $rpoB_{L571Q}$	P1.N4600 x BT165 -> Km^R
BT230	Δ*ruvABC*::*cat* $rpoB_{Q148P}$	P1.BT134 x BT139 -> *rpo**, Rif^R
BT235	*recB270*::*kan* $rpoB_{T563P}$	P1.N4600 x BT324 -> Km^R
BT236	*recB270*::*kan* $rpoB_{Q148P}$	P1.N4600 x BT325 -> Km^R
BT321	Δ*ruvABC*::*cat* $rpoB_{T563P}$	P1.BT121 x BT139 -> *rpo**, Rif^R
BT324	$rpoB_{T563P}$	P1.BT121 x BT140 -> *rpo**, Rif^R
BT325	$rpoB_{Q148P}$	P1.BT134 x BT140 -> *rpo**, Rif^R
TF2	Δ*spoT207*::*cat* Δ*relA251*::*kan* *recB268*::Tn*10* $rpoB_{L448I}$ (Rif^R)	minimal medium selection on N4315

c) AB1157 derivatives

AB1157	F$^-$ *thi-1 hisG4* Δ(*gpt-proA*)*62 argE3 thr-1 leuB6 kdgK51 rfbD1 ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 supE44 rpsL31 rac*$^-$	(Bachmann 1996)
AM888	Δ*ruvAC65 eda-51*::Tn*10* Δ*rusA*::*kan*	(Mahdi et al., 1996)
N3077	*recB*::Tn*10*	laboratory strain

N4155	*eda51*::Tn*10* Δ*ruvAC65*	laboratory strain
N4287	Δ*relA251*::*kan* Δ*spoT207*::*cat*	laboratory strain
N4293	Δ*relA251*::*kan* Δ*spoT207*::*cat eda-51*::Tn*10* *ruvC65* exP1.AM888	laboratory strain
BT161	$rpoB_{L571Q}$ (Rif^R) $argE^+$	P1.BT130 x AB1157 -> $argE^+$; *rpo**, Rif^R
BT162	*rpoB*H551P (Rif^R) $argE^+$	P1.BT132 x AB1157 -> $argE^+$; *rpo**, Rif^R
BT168	*eda51*::Tn*10*; *ruvAC65* $rpoB_{H551P}$ (Rif^R) $argE^+$	P1.BT132 x N4155 -> $argE^+$; *rpo**, Rif^R
BT170	*eda51*::Tn*10* (Tc^R) *ruvAC65* $rpoB_{L571Q}$ (Rif^R) $argE^+$,	P1.BT130 x N4155 -> $argE^+$; *rpo**, Rif^R
BT171	$rpoB_{H551P}$ (Rif^R) $argE^+$ *recB*::*kan*	P1.4600 x BT162 -> Km^R
BT174	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10*	P1.N4278 x N4287-> Tc^R
BT178	$rpoB_{L571Q}$ (Rif^R) $argE^+$ *recB*::*kan*	P1.4600 x BT161 -> Km^R
BT184	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoB_{Y395D}$ (Rif^R)	minimal medium selection on BT174
BT185	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoB_{R151S}$	minimal medium selection on BT174
BT186	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoB_{P153L}$ (Rif^R)	minimal medium selection on BT174
BT190	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoB_{G181V}$ (Rif^R)	minimal medium selection on BT174
BT195	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoC_{\Delta 312\text{-}314}$	minimal medium selection on BT174
BT199	Δ*relA251*::*kan* Δ*spoT207*::*cat* *recB268*::Tn*10* $rpoB_{G537D}$ (Rif^R)	minimal medium selection on BT174
BT200	Δ*relA251*::*kan* Δ*spoT207*::*cat eda-51*::Tn*10* *ruvC65* $rpoC_{R1330S}$	minimal medium selection on N4293

BT205	Δ*relA251*::*kan*, Δ*spoT207*::*cat eda-51*::Tn*10* *ruvC65* $rpoC_{R1148H}$	minimal medium selection on N4293
BT208	Δ*relA251*::*kan* Δ*spoT207*::*cat eda-51*::Tn*10* *ruvC65* $rpoC_{K789Q}$	minimal medium selection on N4293

Table 2.2. Plasmids

Name	Description	Source
pREII-NHα	Ap^R; ori-pBR322; P_{lpp}-P_{lacUV5}-*rpoA*(NH6)	(Niu *et al.*, 1996)
pRL385	Ap^R; ori-pUC; P_{lacZ}-*rpoB*	(Landick *et al.*, 1990)
pRW208-13b	Ap^R; ss-ori-M13-lacIq-*rpoC*	(Weilbaecher *et al.*, 1994)
pBR322	Ap^R; Tc^R; multicopy cloning vector	(Bolivar *et al.*, 1977)
pCBC1	Ap^R; λ*cro* -188 to +372 segment	(Nowatzke and Richardson, 1995)

Plasmid pREII-NHα was a gift from R. Ebright. pRL385 and pRW208-13b were gifts from R. Gourse. Plasmid pCBC1 was a gift from J. Richardson.

2.1.4. Chemicals and Radiochemicals

Chemicals of analytical research grade were purchased from BDH, Sigma, Fisher and Fisons, with the exception of any other chemicals stated in the methods.

Radiochemicals were purchased from Amersham. Redivue [γ^{32}P] ATP, redivue [α^{32}P] CTP and reidivue [α^{32}P] UTP were supplied at 5000 Ci/mmole. $K_2H[^{32}P]O_4$ was supplied at 200 mCi/mmole.

2.1.5. Biochemicals

Antibiotics, vitamins, amino acids, ATP, DTT and mitomycin C were all purchased from Sigma. Sugars were from Fisher or BDH and nucleotides were from Pharmacia. (p)ppGpp was a kind gift from M.Cashel.

2.1.6. Enzymes

Restriction enzymes, T4 DNA ligase, CIAP and T4 Kinase were from Gibco BRL. They were used with the buffers supplied unless stated otherwise. *Taq* DNA polymerase was purchased from Perkin Elmer Cetus, while Bovine Serum Albumin (BSA) and proteinase K were from Sigma. SDS PAGE molecular weight markers were provided by Biorad. The markers consisted of six proteins of known molecular weight, designed to give consistent molecular weights: 10, 15, 25, 37, 50, 75, 100, 150 and 250 kDa. The 50 kDa protein is of greater intensity, serving as a reference band.

2.1.7. Proteins and antibodies

Commercial wild type *E.coli* RNA polymerase was obtained from Pharmacia Biotech. Other RNAP's were purified as described in chapter 6. For the determination of protein concentrations BIO-RAD protein assay (BIO-RAD) was used. Primary antibody solution, containing polyclonal antibodies against *E.coli* RNA polymerase (holo enzyme) was a loan from R. Glass.

2.1.8. Oligonucleotides

DNA oligonucleotides were synthesised by phosphoramidite chemistry and prepared by John Keyte, Department of Biochemistry, University of Nottingham. They were supplied in solution and deprotected, but some required further purification by ethanol-precipitation. DNA concentration was measured by absorbance at 260 nm. Primers for PCR and sequencing as listed in Tables 2.3. and 2.4.

Table 2.3. Primers for sequencing *rpoB* and *rpoC*

Name	Oligonucleotide sequence
a)*rpoB*	
RS-A	5'-GACTTGTCAGCGAGCTGAGG-3'
RS-B	5'-GCTTCGATGTCAAAAGATGCGG-3'
RpoE	5'-AGCTGCAGATGGAACTGGTGC-3'

RpoF	5'-CTGCGGAAATCGGCTTGGCG-3'
RpoI	5'-CGTCGTATCCGTTCCGTTGGCG-3'
RpoII	5'-ATACGGAGTCTCAAGGAAGCCG-3'
RS-C	5'-CAACTCTCTGTCCGTGTACGC-3'
RS-D	5'-TGAACAACACGCTCGGATACG-3'
RpoG	5'-TGCGCGTAGCGTTCATGCCG-3'
RpoH	5'-GTTCAGTACGATGTCTACC-3'
RpoJ	5'-GTCGTCACGGTAACAAGG-3'
RpoK	5'-ACCCGACAGCAGTGACCTG-3'
b)rpoC	
RpcA	5'-GCAGCGGATTGTGCTAACTC-3'
RpcB	5'-GTTCAGGTCAGAAGTCGC-3'
RpcC	5'-TCTGCGTCCGCTGGTTCCGC-3'
RpcD	5'-GCCAGACCAGAGCGATAC-3'
RpcE	5'-CGAAGGCATGGTGCTGAC-3'
RpcF	5'-TACCTTCATGGGTACCAC-3'
RpcG	5'-CGTCGTCTGGTTGACGTGGC-3'
RpcH	5'-GGCATATCGGTACCTGGG-3'
RpcJ	5'-GATCTGCGTCCGGCACTG-3'
RpcK	5'-CGGGTTTTTACGTTATTTGCGG-3'

Table 2.4. Primers for PCR of *λ-cro*, *lac-UV5*, and *rrnB-P1* substrates

Name	Oligonucleotide sequence
a) *λ-cro*	
λ-R	5'-TCGTAGAGCCTCGTTGCGTTTG-3'
λ-F	5'-TCCTGGGATAAGCCAAGTTC-3'
b) lac-UV5	
P_{lac}-α	5'-GGCACGACAGGTTTCCCGAC-3'
P_{lac}-β	5'-GTGAGCGAGTAACAACCCG-3'
c) rrnB-P1	
rrnA	5'-GTTAGAACATGAAGCCCC-3'
rrnB	5'-CGTGTTCACTCTTGAGACTTGG-3'

2.1.9. ECL dectection kit

The ECL detection kit was purchased from Amersham Pharmacia Biotech.

2.1.10. Water and buffers

Deionised, filtered water was obtained from a USF ELGA Option 7/15 water purification unit.

a) Buffers for DNA analysis

- TBE – 90 mM Tris-borate, 2 mM EDTA.
- Ficoll loading buffer – 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400.

b) Buffers for protein purification and analysis

- SDS-PAGE loading buffer - 2% SDS, 60 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 100 mM dithiothreitol (DTT), 0.1% bromophenol blue.
- SDS-PAGE running buffer – 0.1% SDS (w/v), 192 mM Glycine, 25 mM Tris-HCl

- Transfer buffer – 192 mM Glycine, 25 mM Tris-HCl, 20% Methanol (v/v)
- T-TBS (pH 7.6) – 20 mM Tris-HCl, 137 mM NaCl, 0.3% Tween-20.
- Grinding buffer – 0.05 M Tris-HCl (pH 7.9), 5% (v/v) Glycerol, 2 mM EDTA, 0.1 mM DTT, 1 mM β-Mercaptoethanol, 0.233M NaCl, 260 μg/μl Lysozyme, 23 μg/μl PMSF.
- TGED – 0.01 M Tris-HCl (pH 7.9), 5% (v/v) Glycerol, 0.1 mM EDTA, 0.1 mM DTT, NaCl as indicated.
- Buffer A – 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5% (v/v) Glycerol.
- Elute buffers/Ni-column - buffer A, 2.5/5/10/20/40 mM Imidazole respectively.
- Storage buffer - 25 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% (v/v) Glycerol.

c) DNA/protein - buffers for the analysis of DNA-protein complexes.

- 5 x TB - 750 mM KCl, 200 mM Tris-HCl (pH 7.9), 20 mM $MgCl_2$, 5 mM DTT, 0.1% IGEPAL, 0.01% acetylated BSA, 5% (v/v) Glycerol.
- 2 x STOP solution - 20mM EDTA, 0.1% SDS, 0.5 mg/ml tRNA, 0.3 mg/ml Proteinase K.
- TG - 0.05 M Tris-HCl, 0.05 M Glycine

2.1.11. Filters and filtration

Millipore filters were used for the sterilisation or clarification of solutions. Buffers for chromatography were filtered through Whatman 0.45 μm filters.

2.1.12. Microscopy

For cell counting a light microscope (Vickers Instruments) was used at a magnification of 400, using a counting chamber (Weber Scientific International). Pictures of cells were taken using an Olympus BX51 microscope under phase contrast at 1000 x magnification. The lens used was a UPlanApo Oil iris Ph3. All pictures were taken at the same magnification and rescaled in an identical way in Adobe Photoshop.

2.1.13. Chromatography

Ni-NTA agarose was purchased from Qiagen, a Mono Q HR 5/5 column from Amersham and Heparin (immobilised on cross-linked 4% beaded agarose) from

Sigma. Thin layer chromatography was performed using 0.1 mm Cellulose MN 300 polyethyleneimine impregnated pre-coated plastic sheets, obtained from POLYGRAM.

2.2 Methods

2.2.1. Microbiological and genetic methods

- **Growth of bacterial strains**

Strains were streaked to single colonies from stock cultures frozen in glycerol at -20°C. Primary overnight cultures were prepared by inoculating 5 ml of liquid broth (LB broth) in 15 ml screw-capped tubes, with a single colony. Cultures were grown at 37°C overnight with gentle rotation. Liquid cultures were stored at 4°C for up to four weeks and were used to inoculate secondary cultures for experiments. For long term storage, 2.5 ml of a fresh overnight culture was mixed with 1.5 ml sterile 80% glycerol and stored at -20°C.

For experimental purposes, such as transduction, transformation, UV survival or viability determination, media were inoculated with approximately one-twentieth (v/v) of an overnight culture and incubated in a shaking water bath (Grants Instruments) with vigorous aeration. Cell density was measured by a Bausch and Lomb Spectronic 20 spectrophotometer at 650nm (with an OD_{650} of 0.4 being approximately equivalent to 2 x 10^8 cells / ml).

For the purification of proteins, 500 ml of 4 x Mu broth in 1 litre baffled flasks, were inoculated with 4 ml of overnight culture. 5 mM IPTG and 200 μg/ml ampicillin were added and the cultures grown up to OD_{600} = 1.5. Cells were harvested by centrifugation.

- **Centrifugation**

Cells were pelleted from cultures using either a microcentrifuge at RT for 1 minute at 13,000 rpm for volumes of less than 1.5 ml; a Sorvall SS-34 rotor at 4 °C for 6

minutes at 6,000 rpm for volumes between 1.5 ml – 30 ml; or a Sorvall GSA rotor at 4 °C for 15 minutes at 5,000 rpm for volumes larger than 30 ml.

- **Bacteriophage P1 transduction**

P1vir phage stocks were prepared using *E. coli* cells grown in Mu broth supplemented with $CaCl_2$ as previously described (Miller, 1972). Transduction of auxotrophic markers was performed as detailed (Lloyd, 1983; Miller, 1972). For the transduction of antibiotic resistance markers, the transduced cells were plated directly on suitable selection plates in a 0.6% Mu overlay agar. The plates were incubated at 37°C for 24 – 48 hours. Transductant colonies were then purified twice on LB plates.

- **Strain constructions**

All strains were constructed by transduction, using bacteriophage P1*vir* grown on the appropriate donor strain. Transductants were selected using auxotrophic or antibiotic resistance markers. Strain genotype was verified by checking the phenotype using diagnostic plate tests, or where necessary by appropriate backcrosses.

- **Transformation**

Bacteria to be transformed with plasmid DNA were grown to an OD_{650} of 0.5 in 8 ml of Mu broth. Cells were harvested by centrifugation and resuspended in 1.5 ml of chilled 50 mM $CaCl_2$. Approximately 1 – 2 µl of plasmid DNA solution was added to 200 µl of competent cells and kept on ice for 20 – 30 minutes. The cells were then heat-shocked at 42°C for 2 minutes and returned to ice. 1 ml of Mu or LB broth was added and the cells incubated at 37°C for 30 minutes to 1 hour. The cells were harvested and resuspended in 200 µl of Mu or LB broth and spread onto LB plates supplemented with the appropriate antibiotic selection. Plates were incubated for 14 – 24 hours at 37°C.

- **Measuring sensitivity to DNA damage**

For semiquantitative UV and mitomycin C plate tests, 10 µl samples of overnight stocks of the strains to be tested were streaked onto agar plates with and without mitomycin C at 0.2 µg or 0.5 µg per ml. UV irradiation of a duplicate set under a

germicidal UV lamp was at a dose rate of 1 J/m^2/sec, at a peak output of 245 nm, for 30 and 60 seconds. In order to test colony phenotypes, single colonies were inoculated in regular arrays (gridded) then incubated for 6 – 10 hours and replica plated onto LB agar plates and exposed to similar levels of UV and MC as described above. Sensitivity to mitomycin C and UV was scored by comparing growth and survival of test strains to the wild type control strains, after 12 – 24 hours incubation at 37°C.

For quantitative measure of UV sensitivity, bacteria were grown to approximately 2 x 10^8 cells / ml (~OD_{650} of 0.4) in LB broth. Serial dilutions decreasing ten-fold down to 10^{-5} dilution were made of the bacteria in 56/2 salts media. 10µl aliquots of each dilution were spotted onto a series of LB plates. After the spots had dried into the agar the plates were UV irradiated at 1 J/m^2/sec for set intervals up to 60 seconds. Irradiated plates were incubated along with an unirradiated control for 18 – 24 hours before colonies of survivors were scored.

- **Isolation of suppressors**

Suppressors of the UV sensitivity of *recB* strains were isolated by plating 100 µl of fresh overnight cultures of strain N4315 (Δ*spoT*, Δ*relA*, *recB268*) on minimal medium plates.

- **Viability determinations**

For the determination of the viability of different strains, bacteria were grown in LB to an OD_{650} of about 0.3, then diluted 10 fold for the purpose of eliminating non-stationary phase cells, and grown up again to an OD_{650} of 0.4, when the cells are still in exponential phase. The number of total cells was then counted, using a counting chamber. The number of viable cells was determined by plating dilutions (in minimal 56/2 salts) on LB plates and counting the colonies.

2.2.2. DNA preparations and analyses

- **Purification of plasmid DNA**

Plasmid DNA was extracted from freshly grown overnight cultures by a modified alkaline lysis method using QIAprep spin column plasmid kits as described by the manufacturer (QIAgen). DNA was eluted from the spin column and resuspended in 20 μl TE buffer or water.

- **PCR amplification of DNA**

The GeneAmp PCR procedure adapted from Mullis and Faloona (1987) and Saiki *et al.* (1988) was used for the amplification of DNA. 100 μl reactions contained ~ 100 pmoles of the 5' and 3' primers, the required DNA template, 20 mM dNTPs, 2.5 units *Taq* DNA polymerase and a final concentration of 1 x of the appropriate buffers supplied with the enzyme (100 mM $MgCl_2$; 100 mMTris-HCl, pH 8.3, 200mM KCl, 0.5% gelatin). A layer of mineral oil was added above the mixtrue to prevent evaporation during the reaction. Amplification of the DNA was performed with a DNA Thermal Cycler (Perkin-Elmer-Cetus) and typically proceeded for 20 cycles. PCR products were analysed by gel electrophoresis and purified by gel extraction. Colony PCR was performed by picking a single colony from a plate, mixing it into 50 μl of dH_2O, vortexing vor 2 - 3 min and incubation at 37°C for 15 min. 2 μl of this mixture were used as DNA template in PCR reactions.

- **Restriction endonuclease digests**

Restriction endonuclease digests were routinely conducted in 20 μl reactions containing 0.2 – 1 μg of DNA in the appropriate buffer with 1 unit of restriction enzyme. Digests were incubated at the required temperature (as recommended by the supplier), for 2 hours or more. Restriction enzymes were inactivated as recommended by the supplier. Products of the digestion reaction were typically analysed by 1% agarose gel electrophoresis.

- **Gel electrophoresis of plasmid DNA, restriction fragments and PCR products**

DNA samples were separated on 0.8% to 1.2% agarose gels, depending on their size. DNA was mixed with approximately 0.2 volumes Ficoll loading buffer, before loading. 2 µl of a 1 kb ladder (BRL) was used as a marker. Gels were run at a constant voltage in TBE buffer, 15 V/cm. Gels were stained with ethidium bromide before casting, included in the gel mix at a concentration of 0.4 µg/ml. DNA fragments were visualised with a UV transilluminator (UVP).

- **Extraction of DNA from agarose gels**

DNA products were extracted by cutting the relevant band out of the gel and purifying it using a QIAprep spin column as described by the manufacturer (QIAgen). DNA was eluted from the spin column and resuspended in 20 µl TE buffer or water.

- **Automated sequencing of PCR products**

PCR products were sequenced by the dideoxy chain termination method of Sanger et al. (1977). 20 µl reactions contained 30 - 90 ng of PCR product, 4 µl "Big Dye" terminator ready reaction mix (PE Applied Biosystems), 4 µl Half Term (Genpak), 0.3 µl 10 µM Primer and dH_2O. The mixtures were overlaid with 40 µl of light mineral oil and sequenced in a DNA thermal cycler for 25 cycles of 96°C - 30sec, 50°C - 15 sec, 60°C - 4 min. The extension products were purified by precipitation with 1/10 vol 3 M NaOAc and 50 µl ethanol. The samples were analysed by denaturing gel elecrophoresis by Ingrid Davies (department of Biomedical Sciences, Nottingham).

2.2.3. Protein analyses

- **Measuring protein concentration**

Protein concentrations were estimated using a Biorad Protein Assay kit, with Bovine Serum Albumin as a standard protein (Bradford, 1976).

- **SDS PAGE**

Proteins were analysed by SDS–polyacrylamide gel electrophoresis, using Miniprotean gel apparatus (Biorad). Resolving gels contained 380 mM Tris-HCl, pH 8.8, 0.1% SDS and acrylamide / bisacrylamide (29:1) at the concentration stated. Stacking gels contained 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 4.2% acrylamide / bisacrylamide (29:1) (Kramel). The gels were polymerised with 0.075% ammonium persulphate and 0.08% tetramethylethylene diamine (TEMED). The protein sample was mixed with 0.5 volumes of 2 x SDS loading dye. Gels were run for 50 to 90 minutes at 200 V (constant) in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS). The gels were stained in Coomassie Blue dye (42% methanol, 17% acetic acid, 0.1% Brilliant Blue) for 15 – 30 minutes and then in destain buffer (20% methanol, 10% acetic acid) for several hours. Stained gels were dried between sheets of GelAir Cellophane support (BioRad) on a vacuum slab dryer for long term storage.

In order to visualise also minor contaminating proteins, silver staining was performed in some cases. All incubations were performed under light shaking. The gel was first fixed for 30 min in acetic acid : methanol : water/10 :40 :50 (Fix 1). This was followed by a 5 min rinse in water and a second fixing step of 15 min in glutardialdehyde : water/ 12.5 : 87.5. The gel was rinsed for 2 x 10 min in water and 15 min in 20% ethanol. Staining was performed for 15 min in freshly prepared staining solution (2 ml 20% AgNi was added to 2 ml 25% ammonia, which was mixed and added to 10 ml 4 % NaOH; the mix was made up to 200 ml with 20% ethanol). Two 5 min rinse steps in 20% ethanol were followed by developing for the required time in 200 ml 20% ethanol containing 200 μl 37% formaldehyde and 50 μl 2.3M citric acid. The stained gels were stored in Fix 1 before drying.

- **Western blotting**

The method was essentially as in Harlow and Lane (1988). The gel and overlaid transfer membrane (Hybond C-extra-Amersham) were sandwiched between three sheets of Whatman 3MM filter paper and two foam sponges in transfer buffer. The sandwich was placed into the transfer tank (LKB Midget MultiBlot Electrophoretic Transfer Unit) and transferred o/n at 200 mA. Successful transfer could be

determined by observing the presence of the prestained molecular weight markers on the membrane. The membrane was incubated for 60 min in T-TBS/7% Marvel, then in the primary antibody solution for another 60 min. The membrane was then washed with T-TBS 3 times for 10 min and incubated with the secondary antibody (Goat Anti-Rabbit/Biorad; 1:5000, in T-TBS/1% Marvel) for 60 min. This was followed by a triple wash step as above. Detection was performed according to the ECL method, using an ECL detection kit.

- **Chromatographic procedures**

Column chromatography was run as Fast Performance Liquid Chromatography (FPLC Pharmacia LKB), which was performed essentially following the manufacturers instructions.

- **3D structure of RNA polymerase**

The 3D structure of RNA polymerase was visualised using RasMol (Sayle and Milner-White, 1995).

2.2.4. Protein-DNA interactions

- **DNA substrates**

DNA substrates intended for 5' end labelling were made by PCR. *λcro* DNA was produced by PCR from the plasmid pCBC1, containing a fragment of the *λcro* gene from residue -188 to +372 with the primers λ-R and λ-F. rrnB-P1 and lac-UV5 fragments were made by colony PCR, using the primers *rrnA* and *rrnB*, and P_{lac}-α and P_{lac}-β respectively. The resulting fragments were ethanol precipitated and gel-purified prior to use.

- **In vitro transcription assays**

In 50 μl volumes, the indicated amounts of RNAP were incubated with indicated amounts of substrate DNA in 1 x transcription buffer for 3 min at 37°C to form open complexes. With the addition of NTPs (final concentration 0.1 mM each) and 0.5 μl [α^{32}P]UTP the transcription reaction was started and allowed to proceed for 15 min at

37°C unless stated otherwise. The reaction was stopped by adding an equal amount of 2 x STOP solution and incubating the samples for 10 min at 37°C. After ethanol precipitation, the pellets were dissolved in formamide loading dye and run on a 6% acrylamide sequencing gel.

- **Labelling of DNA substrates**

For 5' end labelling *λcro* DNA (PCR product) was dephosphorylated. The reaction contained 300nM DNA, 1 x dephosphorylation buffer and 0.6 U/μl calf intestinal alkaline phosphatase (CAIP). After incubation at 37°C for 60 min, CAIP was heat inactivated. The sample was then phenolised, ethanol precipitated and dissolved to give a final DNA concentration of 100 nM. 1 pM of DNA was then labelled by incubation for 45 min at 37°C with 1 μl [γ^{32}P]ATP, 10 U of T4 PNK in 1 x PNK buffer and an end volume of 20 μl. The sample was then phenolised, ethanol precipitated and redissolved in 100 μl to give a final DNA concentration of 10 nM.

- **Hot PCR of DNA substrates**

Hot PCRs were performed like normal colony PCRs, but in 50 μl. 2 to 4 μl of [α^{32}P] CTP were added per reaction. The sample was ethanol precipitated and gel purified (using a QIAgen gel purification kit). PCR conditions were optimised for each substrate.

- **Gel retardation assays**

In a reaction volume of 20 μl, the stated amount of RNA polymerase was incubated with the stated amount of labelled DNA substrate in 1 x TB and an additional 5 % glycerol (giving a final glycerol concentration of 6%) for 3 min at 37°C for open complex formation. Unless stated otherwise, NTP's were added to a final concentration of 2 μM. (p)ppGpp, competitor or heparin were added at this stage as stated. The reactions were then further incubated for 20 min at 37°C in order to allow elongation or stalled complex formation, unless stated otherwise. After addition of heparin to a final conc. of 2.5 μg/μl the samples were loaded on 0.8% agarose gels and run at 100V for about 90 min.

- **Autoradiography and phosphorimaging**

Agarose or acrylamide gels containing radioactively labelled DNA were dried by placing them onto Whatman 3MM filter paper and drying on a vacuum slab drier. The dried gels were exposed to X-ray film or a storage phosphor screen (Molecular Dynamics).

Chapter 3.

Isolation and characterisation of *rpo** mutants

3.1. Introduction

As described in chapter one, *rpo** mutations were first discovered in a *ruv* (p)ppGpp^0 strain. They can be described as a subgroup of stringent mutations. Stringent mutations are defined by their ability to alleviate the relaxed response of (p)ppGpp^0 strains. Wild type cells, under conditions of amino acid starvation and other kinds of stress adapt via the stringent response. rRNA and tRNA production are downregulated and amino acid synthetic operons, among others, are upregulated. At least in part this effect is due to the major regulatory factors of the stringent response, (p)ppGpp, which accumulate rapidly as soon as the cell encounters unfavourable conditions. The levels of (p)ppGpp in the cell are regulated by RelA and SpoT, responsible for its synthesis and degradation. Mutants for *relA* show a relaxed response to starvation. They are deregulated, continuing to accumulate RNA even under conditions of starvation (Stent and Brenner, 1961). Only strains deleted for both *relA* and *spoT* are devoid of (p)ppGpp and termed (p)ppGpp^0. The production of (p)ppGpp is triggered by ribosomes, idling because of the presence of uncharged tRNAs. Both factors, together with RelA, are necessary for (p)ppGpp synthesis (Haseltine and Block, 1973).

In their role in response to starvation and stress, (p)ppGpp affect gene expression in two ways: (i) Directly, by interacting with RNA polymerase (Chatterji *et al.*, 1998), so possibly changing its conformation, promoter binding and specificity (Woody *et al.*, 1987; Bremer and Ehrenberg, 1995, Krohn and Wagner, 1996). (ii) Indirectly by stimulating the expression of *rpoS*, *E.coli*'s main σ-factor during stationary phase. Associated with RNA polymerase, σ^S in turn leads to the transcription of a different set of genes. But (p)ppGpp have also been implicated in

enhanced transcriptional termination and pausing (Kingston and Chamberlin, 1981; Hernandez and Bremer, 1993), translational accuracy (Wagner and Kurland, 1980) and even *oriC* replication (Ogawa and Okazaki, 1991). It may also lower the amounts of σ-factor associated with RNA polymerase (Hernandez and Cashel, 1995). A connection has been made between (p)ppGpp and cell cycle control (Zyskind and Smith, 1992).

McGlynn and Lloyd (2000) observed that some stringent RNA polymerase mutations - termed *rpo** - can increase the survival of UV irradiated *ruv* strains. Their model (discussed in chapter 1) attributes this fact to the modulation of RNA polymerase by (p)ppGpp. They propose that RNA polymerase is less likely to form roadblocks for replication when it is associated with (p)ppGpp or modified by certain mutations, thus relieving requirement for RuvABC, which is a major factor for the repair of collapsed replication forks, as is RecBCD.

The functions of RuvABC and RecBCD are closely linked. There is genetic evidence that RecBCD and RuvABC lie in the same pathway for recombinational repair of damaged DNA (for a review see Taylor, 1992). That they are part of the same repair pathway is also demonstrated by the fact that a double mutant is no more sensitive to UV light than a single mutant (Gregg *et al.*, 2001; McGlynn and Lloyd, 2000). RecBCD processes double-strand (ds) DNA ends into 3' ends (Ponticelli *et al.*, 1985) that can invade the intact sister duplex and so initiate recombinational repair (Fig. 3.1.A.). In this case RuvABC acts after RecBCD. Most double-strand breaks in the *E.coli* cell however are the result of replication fork arrest (Sandler and Marians, 2000), mediated by the action of RuvABC (Seigneur *et al.*, 1998). Thus, in the presence of RuvABC, RecBCD is necessary to process the dsDNA ends produced by RuvABC and initiate recombinational repair (Fig. 3.1.B.; see chapter 1 for details). Here RuvABC acts both before and after RecBCD. In UV irradiated cells, mutation of *ruv* masks a mutation of *recB*. Thus *rpo* ruv recB* is no more sensitive to UV light than *rpo* ruv* alone, which is quite resistant, depending on the *rpo** mutation. It can therefore be concluded that only very few genomic "ends in" double strand breaks occur in UV irradiated cells (McGlynn and Lloyd, 2000), as they would require RecBCD for efficient repair (Cromie and Leach, 2001).

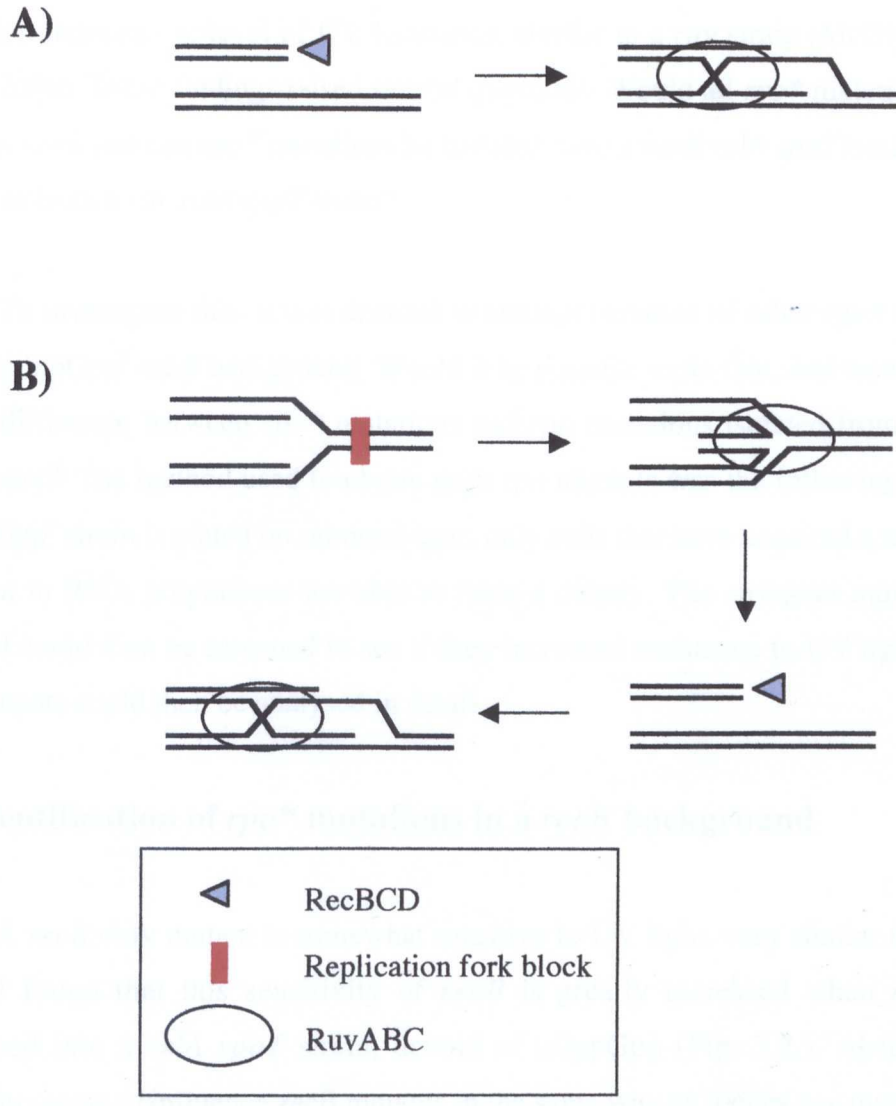

Figure 3.1. A) RecBCD act at a dsDNA end upstream of RuvABC. **B)** At arrested replication forks RuvABC act both upstream and downstream of RecBCD.

As the functions of RuvABC and RecBCD are so closely linked, *rpo** mutations are expected to also increase the survival of a UV irradiated *recB* strain. When an *rpo** mutation was transferred into a *recB* mutant, it was found to somewhat increase the level of UV resistance, similar to a *ruv* strain (McGlynn and Lloyd, 2000). These findings raised several questions. Would all *rpo** mutations also suppress *recB* and can *rpo** mutations be isolated from a *recB relA spoT* background as well as from a *ruv relA spoT* strain?

To investigate this, it was decided to attempt isolation of other *rpo** mutants from a (p)ppGpp^0 *recB* background. Would it be possible to do that, and would there be any difference between *rpo** mutations and *rpo* mutations isolated from a *recB* background? The method used to obtain such *rpo* mutants was the following: When a (p)ppGpp^0 strain is plated on minimal agar, only cells that have acquired a stringent mutation in RNA polymerase are able to form a colony. The stringent mutants so obtained could then be screened to see if they increased resistance to UV light. Any such mutants could then be analysed in detail.

3.2. Identification of *rpo** mutations in a *recB* background

A *recB* only mutant is somewhat sensitive to UV light, very similar to a *ruv* strain. I found that this sensitivity of *recB* is greatly increased when *recB* is introduced into a *relA spoT* strain, devoid of (p)ppGpp (Fig. 3.2.). Absence of (p)ppGpp seems to influence *recB* mutants in the same way as it does *ruv* mutants. A (p)ppGpp^0 *recB* strain is unable to grow on minimal medium, as (p)ppGpp are necessary for the expression of a number of biosynthetic operons (see chapter 1). When such a strain is spread on minimal agar plates however, a number of colonies can be observed. These stringent mutants possess suppressor mutations in either *rpoB*, *rpoC* or, rarely, *rpoD* (Cashel *et al.*, 1996), encoding the β, β' and σ^{70} subunits of RNA polymerase. They allow transcription of the necessary biosynthetic operons in the absence of (p)ppGpp.

As the UV sensitivity of *recB* was decreased by lack of (p)ppGpp, similar to *ruv*, the question was, whether *recB*-induced defects could be partly cured by

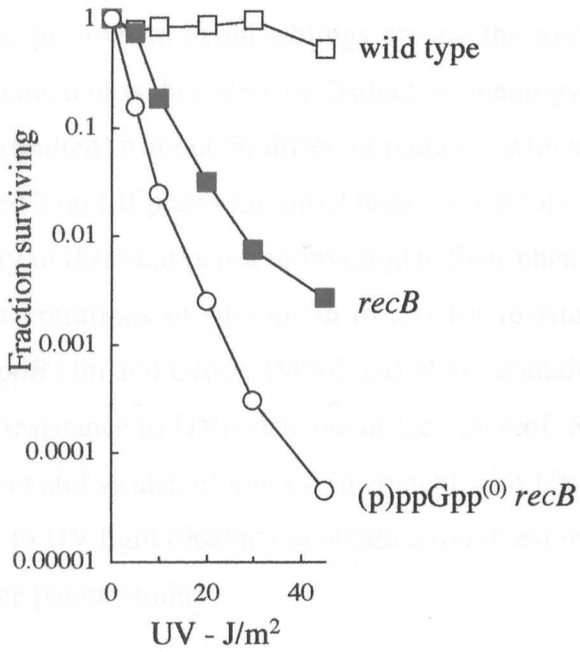

Figure 3.2. The UV resistance of a *recB* strain is considerably decreased in the absence of (p)ppGpp. The strains identified by genotype in the graph are MG1665 (wild type), N4278 (*recB*), N4315 ((p)ppGpp^0, *recB).*

stringent RNA polymerase mutations too. In order to investigate this it was decided to isolate a number of stringent mutants from a (p)ppGpp^0 *recB* strain and screen them for increased UV resistance.

To obtain mutants that would be of interest, 100 μl each of ten different overnight cultures were spread on minimal plates and incubated for 2 days. This process was repeated twice and resulted in 20 to 50 stringent mutant colonies per plate. It was important to use many different overnight cultures, each grown from different single colonies, in order to avoid siblings among the mutants. Only one colony was picked per plate, unless they were of distinct morphology, again to avoid siblings. The selections resulted in about 50 different mutants, which were re-grown in regular arrays ("gridded") on LB plates for initial tests. Those "master" plates were replica-plated on a variety of different plates to investigate their phenotype: (i) plates containing different concentrations of rifampicin to test for resistance [resistance identifies mutations in *rpoB* (Jin and Gross, 1988)]. (ii) plates containing mitomycin C to determine possible resistance to DNA damage or lack thereof. Mytomycin C is a DNA cross-linking agent and so also of interest in context with UV resistance. (iii) plates that were exposed to UV light (90J/m^2) to obtain a rough estimate of their UV resistance compared to the parent strain.

In the initial plate tests a number of strains was observed to have increased UV resistance compared to the parent strain. Those were possible candidates for *rpo** mutants. Rifampicin resistance was also observed in many cases. Resistance to mitomycin C was increased very little or not at all. The effect is not comparable to the significant increase in UV resistance, demonstrating that only the repair of UV induced lesions, but not of cross-links, is markedly improved. No more than two apparently different mutants per initial overnight culture were chosen for showing a greater resistance to UV light than the parent strain. About half of the chosen mutants were Rif^R and the majority showed a very slight increase in resistance to Mitomycin C (data not shown). The UV resistance of the so obtained *rpo* mutants was investigated in more detail by quantitative UV survival tests (Fig.3.3. and Table 3.1.). Most of the mutants obtained show a marked increase in UV survival, though none of the mutants is as UV resistant as wild type. For the purpose of comparison,

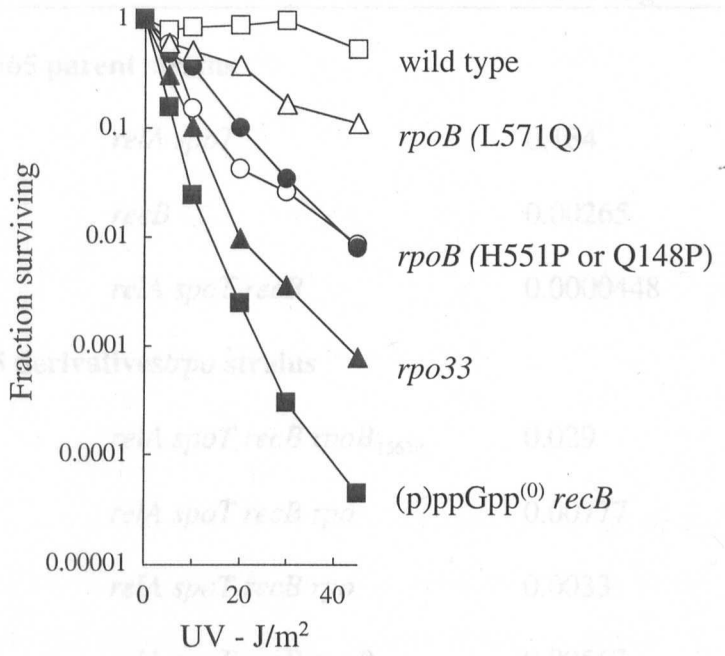

Figure 3.3. Examples of the obtained *rpo* strains. All of them are more resistant than the (p)ppGpp$^{(0)}$ *recB* parent. Most strains fall into the category of *rpo* H551P and Q148P, representing the range of medium suppression of UV sensitivity. Some strains, like *rpoB*$_{L571Q}$ show high resistance, few low, like *rpo33.* The strains identified either by genotype or their respective mutations in the graphs are MG1665 (wild type), N4315 ((p)ppGpp^0, *recB*), BT130 ((p)ppGpp^0, *recB rpoB*$_{L571Q}$), BT132 ((p)ppGpp^0, *recB rpoB*$_{H551P}$), BT133 ((p)ppGpp^0, *recB rpo33)*, BT134 ((p)ppGpp^0, *recB rpoB*$_{Q148P}$). Individual mutations had not yet been identified at this point. They are mentioned in order to avoid a change of nomenclature.

one mutant that did not show any increase in UV resistance was also chosen (BT143).

Table 3.1. UV survival of *rpo* strains. [a)]

Strain	Genotype	Fraction surviving [b)]
a) MG1665 parent strains		
N4304	*relA spoT*	0.184
N4278	*recB*	0.00265
N4315	*relA spoT recB*	0.0000448
b) N4315 derivatives/*rpo* strains		
BT121	*relA spoT recB* $rpoB_{T563P}$	0.029
BT122	*relA spoT recB rpo*	0.00777
BT123	*relA spoT recB rpo*	0.0033
BT124	*relA spoT recB* $rpoB_{H447R}$	0.00567
BT125	*relA spoT recB* $rpoB_{V550}$E	0.022
BT126	*relA spoT recB* $rpoC_{E1146D}$	0.00417
BT128	*relA spoT recB rpo*	0.008
BT129	*relA spoT recB* $rpoB_{G536V}$	0.0063
BT130	*relA spoT recB* $rpoB_{L571Q}$	0.1
BT131	*relA spoT recB rpo*	0.016
BT132	*relA spoT recB* $rpoB_{H551P}$	0.00858
BT133	*relA spoT recB* $rpoC_{K215E}$	0.000775
BT134	*relA spoT recB* $rpoB_{Q148P}$	0.00797
BT135	*relA spoT recB rpo*	0.00493
BT136	*relA spoT recB rpo*	0.032

BT137	*relA spoT recB rpo*	0.005
BT141	*relA spoT recB rpo*	0.00086
BT142	*relA spoT recB* $rpoB_{H447P}$	0.00883
BT143	*relA spoT recB* $rpoB_{S788F}$	0.0000297
BT145	*relA spoT recB rpo*	0.00325
BT146	*relA spoT recB rpo*	0.026
BT147	*relA spoT recB rpo*	0.0045
BT149	*relA spoT recB rpo*	0.00348
BT150	*relA spoT recB rpo*	0.00022
BT151	*relA spoT recB rpo*	0.048
BT152	*relA spoT recB* $rpoB_{L420R}$	0.02
BT153	*relA spoT recB* $rpoB_{A532E}$	0.045

[a] The data represents the means of at least two independent experiments. All strains in category b) are derivatives of N4315. They in all probability also contain a mutation in RNA polymerase, therefore termed *rpo*. Where a mutation has later been identified by sequencing, this is indicated by the amino acid change and number of residue. [b] Strains were irradiated for 45 sec with 1 J/m^2.sec. Survival is given as a fraction of colony forming cells relative to unirradiated controls.

3.3. *rpo* mutants enhance UV resistance of both *recB* and *ruv* strains

Of the several *rpo** mutants obtained by McGlynn and Lloyd (2000), only one was studied in detail. This mutant, *rpo*35*, is an allele of *rpoB* (βH1244Q, see chapter 4). They found that *rpo*35* also increases the survival of a *recB* strain, but to a lesser extent than is seen in a *ruv* strain. This would fit the theory that RecBCD act downstream of RuvABC in the repair pathway for stalled replication forks (see

above and chapter 1). If double-strand ends are produced by RuvABC, RecBCD is needed to process them and thus allow reconstitution of a replication fork. Therefore a *recB* strain that is *ruv*$^+$ and thus proficient for processing of Holliday junctions via RuvABC might be less likely to be rescued by *rpo** than a *ruv*$^-$ strain, that is unable to produce double-strand ends by resolving of Holliday junctions in the first place. It was not clear at that point, whether a *rpo* mutation isolated from a *recB* (p)ppGpp^0 background would be able to suppress *ruv* and could therefore be called *rpo**, or whether it belonged to a different group of *rpo* mutations.

To investigate the question whether *rpo* mutations isolated from (p)ppGpp^0 *recB* were also *rpo**, as defined by their ability to suppress the UV sensitivity of a *ruv* strain, a number of *rpo* mutations were transferred into *recB* and *ruv* only backgrounds respectively (Fig. 3.4.A and B). The mutants were tested for UV sensitivity. Two suppressed *ruv* significantly better than *recB* but one was slightly better at suppressing *recB*. The others suppressed both about equally well. The experiment was repeated for two of the mutants in AB1157 background instead of wild type background, which yielded the same result for the mutants in question (Fig. 3.5.A and B). Interestingly, the two mutants which are significantly better at suppressing *ruv* than *recB* were later found to lie only 12 amino acids apart on the β-subunit of RNA polymerase and were both changes to proline (βH551P and βT563P).

This shows that there is no inherent difference between *rpo** mutants and *rpo* mutants isolated from (p)ppGpp^0 *recB*. The *rpo* mutations isolated here can therefore be termed *rpo**. The data indicates however that different classes of *rpo** mutants do exist as shown by the differential suppression of *ruv* and *recB* by some mutant strains as well as by the varying quality of suppression between different mutants. The results also indicate that even in *ruv*$^+$ strains dsDNA breaks are less common than has been implied in recent models. If breaks arose frequently and were generated by RuvABC, all *rpo** mutants might be expected to suppress *ruv* better than *recB*, which I don't find to be the case in all isolated mutants. However, I cannot rule out the possibility that some of the increase in survival observed in my mutants is due to some factor other than the avoidance of breaks.

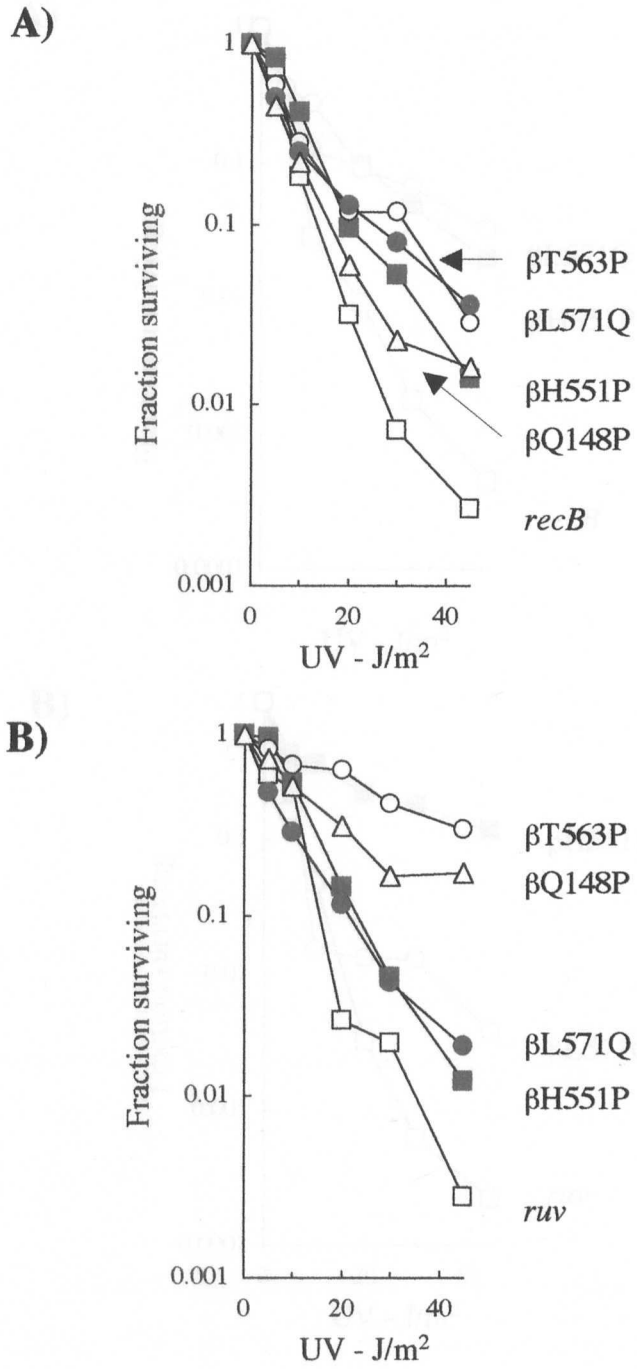

Figure 3.4. A) Effect of four *rpo* mutations on the UV sensitivity of a *recB* only strain (in wild-type background). **B)** Effect of the same mutations on the UV sensitivity of a *ruv* only strain. The strains identified either by genotype or their respective *rpoB* mutations in the graph are N4278 (*recB*), BT175 (*recB* $rpoB_{H551P}$), BT181 (*recB* $rpoB_{L571Q}$), BT235(*recB* $rpoB_{T563P}$), BT236 (*recB* $rpoB_{Q148P}$), N4583 (*ruv*), BT163 (*ruv* $rpoB_{L571Q}$), BT164 (*ruv* $rpoB_{H551P}$), BT230 (*ruv* $rpoB_{Q148P}$), BT321 (*ruv* $rpoB_{T563P}$). The data are means of at least two independent experiments.

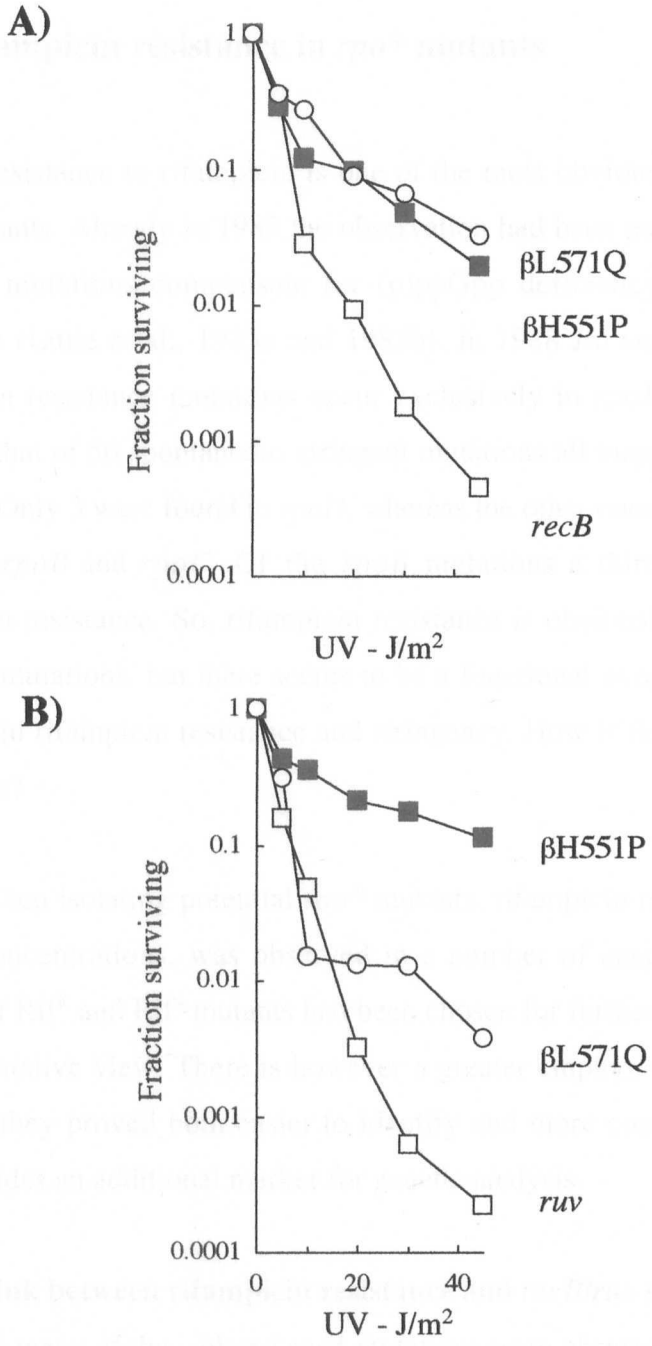

Figure 3.5. Effect of *rpo** mutations on the UV sensitivity of *recB* **(A)** and *ruv* **(B)** strains in AB1157 background. Both recB and ruv strains are about 10 times more sensitive in AB1157 than in MG1665 background. However rpo* mutations suppress UV sensitivity to the same degree as in MG1665 background. H551P is a notable exception, in that it suppresses ruv about 10 times better in AB1157 than in MG1665 background. The strains identified either by genotype or their respective mutations in the graph are N3077 (*recB*), BT171 (*recB* $rpoB_{H551P}$), BT178 (*recB* $rpoB_{L571Q}$), N4155 (*ruv*), BT168 (*ruv* $rpoB_{H551P}$), BT170 (ruv $rpoB_{L571Q}$). The data are means of at least two independent experiments.

3.4. Rifampicin resistance in *rpo** mutants

Resistance to rifampicin is one of the most obvious characteristics of some *rpo** mutants. Already in 1983 the observation had been made that some rifampicin resistant mutations compensate for (p)ppGpp deficiency or alter sensitivity to (p)ppGpp (Little et al., 1983a and 1983b). In 1988 Jin and co-workers found that rifampicin resistance mutations occur exclusively in *rpoB*. In 1996 Cashel *et al.* reported that of 50 spontaneous stringent mutations all mapped to either *rpoB, rpoC* or *rpoD*. Only 3 were found in *rpoD*, whereas the other ones were equally distributed between *rpoB* and *rpoC*. Of the *rpoB* mutations a third was reported to show rifampicin resistance. So, rifampicin resistance is obviously not a requirement for stringent mutations, but there seems to be a functional overlap between the regions involved in rifampicin resistance and stringency. How is this connected to the *rpo** phenotype?

When isolating potential *rpo** mutants, rifampicin resistance, though mostly to low concentrations, was observed in a number of candidates. About an equal amount of Rif^R and Rif^S mutants had been chosen for further investigation to provide a representative view. There is however a greater emphasis on Rif^R mutants in this work, as they proved both easier to identify and more convenient to work with as Rif^R provides an additional marker for genetic analysis.

3.4.1. A link between rifampicin resistance and *recB/ruv* suppression

As many of the isolated *rpo** mutations were observed to confer resistance to rifampicin, I decided to investigate whether there was a correlation between the level of resistance and the quality of suppression of *recB*. The level of rifampicin resistance was assessed by streaking fresh overnight culture on plates containing different concentrations of rifampicin (10, 20, 50 or 100 μg/ml). Each $(p)ppGpp^0$ *recB rpo** mutant was then assigned a rifampicin resistance value from 0 to 100 and the results were compared with the degree of suppression of the UV sensitivity of the $(p)ppGpp^0$ *recB* strain (Fig. 3.6.A and B). There is no clear correlation between rifampicin resistance and suppression. A Rif^{10} or Rif^{20} mutant can be just as good a suppressor as a Rif^{50} or $Rif^{>100}$ mutant. The available data however shows that on

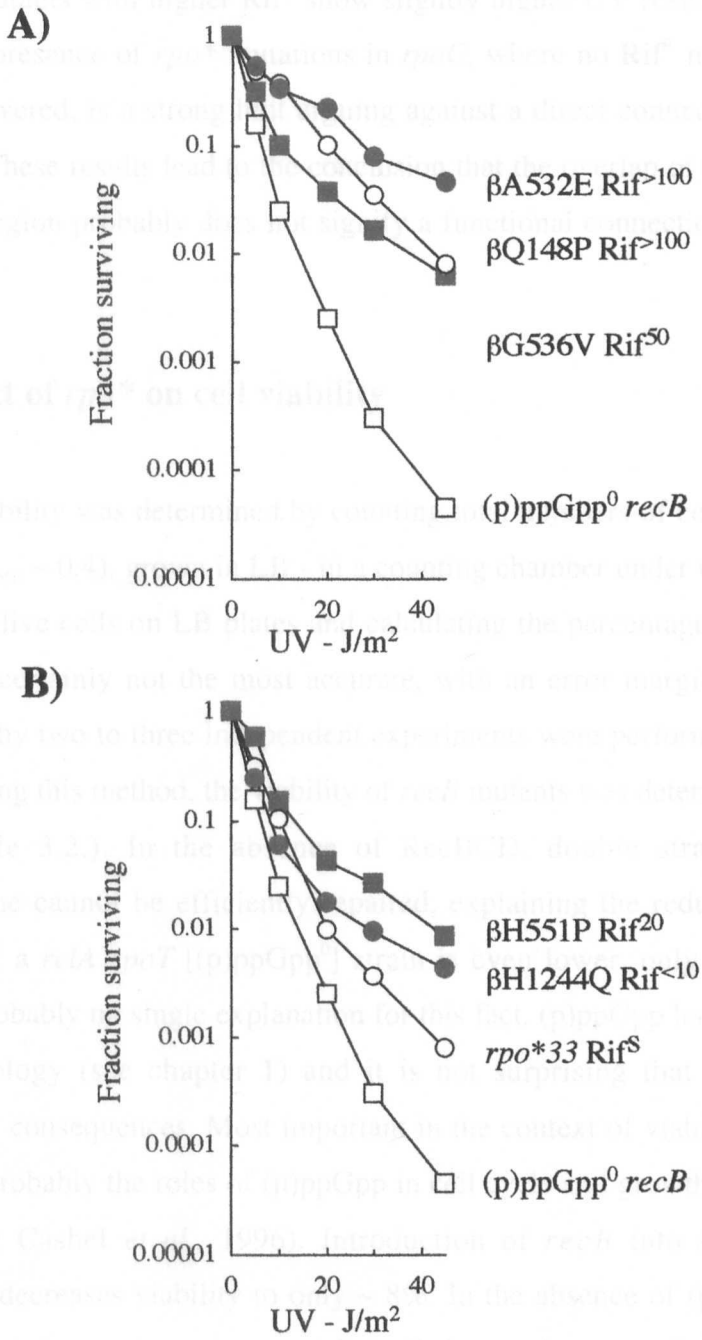

Figure 3.6. Connection between Rif^R and *rpo**. **A)** A selection of *rpo** mutants with high Rif^R. **B)** A selection of *rpo** mutations with low or no Rif^R. There seems to be a slight trend of higher suppression of UV sensitivity in mutants with high Rif^R. There is however a lot of variation of suppression even among mutants with the same Rif^R. The strains identified either by genotype or their respective mutations in the graph are N4315 ((p)ppGpp^0 *recB*), BT129 ((p)ppGpp^0 *recB* $rpoB_{G536V}$), BT132 ((p)ppGpp^0 *recB* $rpoB_{H551P}$), BT133 ((p)ppGpp^0 *recB rpo*33*), BT134 ((p)ppGpp^0 *recB* $rpoB_{Q148P}$), BT153 ((p)ppGpp^0 *recB* $rpoB_{A532E}$). The data are means of several independent experiments.

average mutants with higher Rif^R show slightly higher UV resistance. On the other hand, the presence of *rpo** mutations in *rpoC*, where no Rif^R mutations have ever been discovered, is a strong hint arguing against a direct connection between *rpo** and Rif^R. These results lead to the conclusion that the overlap of the Rif^R region and the *rpo** region probably does not signify a functional connection between the two properties.

3.5. Effect of *rpo** on cell viability

Viability was determined by counting total numbers of cells - in exponential phase (OD_{650} ~ 0.4), grown in LB - in a counting chamber under the microscope, the number of live cells on LB plates and calculating the percentage of live cells. This method is certainly not the most accurate, with an error margin of about ±10 %, which is why two to three independent experiments were performed to obtain mean values. Using this method, the viability of *recB* mutants was determined to be around 65% (Table 3.2.). In the absence of RecBCD, double strand breaks in the chromosome cannot be efficiently repaired, explaining the reduced viability. The viability of a *relA spoT* [(p)ppGpp^0] strain is even lower, only 40% (Table 3.2.). There is probably no single explanation for this fact. (p)ppGpp have many effects on cell physiology (see chapter 1) and it is not surprising that their absence has detrimental consequences. Most important in the context of viability in exponential phase are probably the roles of (p)ppGpp in cell cycle and growth rate control (for a review see Cashel *et al.*, 1996). Introduction of *recB* into a *relA spoT* strain drastically decreases viability to only ~ 8%. In the absence of (p)ppGpp, RecBCD seems to be of more importance to the cell than under normal circumstances. One can speculate that RNA polymerase, not modulated by (p)ppGpp, is more likely to create roadblocks for replication, thus increasing the number of double strand breaks which require RecBCD for efficient repair.

*rpo** mutations increase the viability of a (p)ppGpp^0 *recB* strain considerably. Some representative examples are given in Table 3.2. The viability of (p)ppGpp^0 *recB rpo** strains is usually around 70% in sharp contrast to only 8% in the absence of such a suppressor mutation. *rpo** mutations obviously confer an important

advantage to cells both devoid of (p)ppGpp and the RecBCD restriction complex. This increase in survival suggests that double strand breaks occur less frequently which could well be due to the mutant RNA polymerase decreasing the incidence of replication fork blocks and thus double strand breaks.

Table 3.2. Effect of *rpo** on cell viability.

Strain	**Genotype**	**% viable cells** [a)]
MG1665	wild type	100
N4304	*relA spoT* [(p)ppGpp^0]	40
N4278	*recB*	65
N4315	(p)ppGpp^0 *recB*	7.7
BT132	(p)ppGpp^0 *recB* $rpoB_{H551P}$	70
BT121	(p)ppGpp^0 *recB* $rpoB_{T563P}$	68
BT129	(p)ppGpp^0 *recB* $rpoB_{G536V}$	64
BT130	(p)ppGpp^0 *recB* $rpoB_{L571Q}$	80

[a)] Viability in % of total cells. The number of total cells was counted directly under the microscope in a counting chamber. The number of viable cells was determined by counting colony forming units on LB agar. The values are the means of at least two independent experiments.

3.6. Cell morphology of *rpo** strains

The low viability of *relA spoT recB* strains is correlated with increased cell filamentation (Fig. 3.7.d). Cell filamentation is a feature observed in wild type cells exposed to UV light or other DNA damaging agents. It is caused by induction of the SOS-regulated division inhibitor SulA (Gottesman *et al.*, 1981), which prevents cell division until the DNA damage is repaired. In spite of their reduced viability, *recB* mutant cells have a normal appearance (Fig. 3.7.b). (p)ppGpp^0 cells on the other

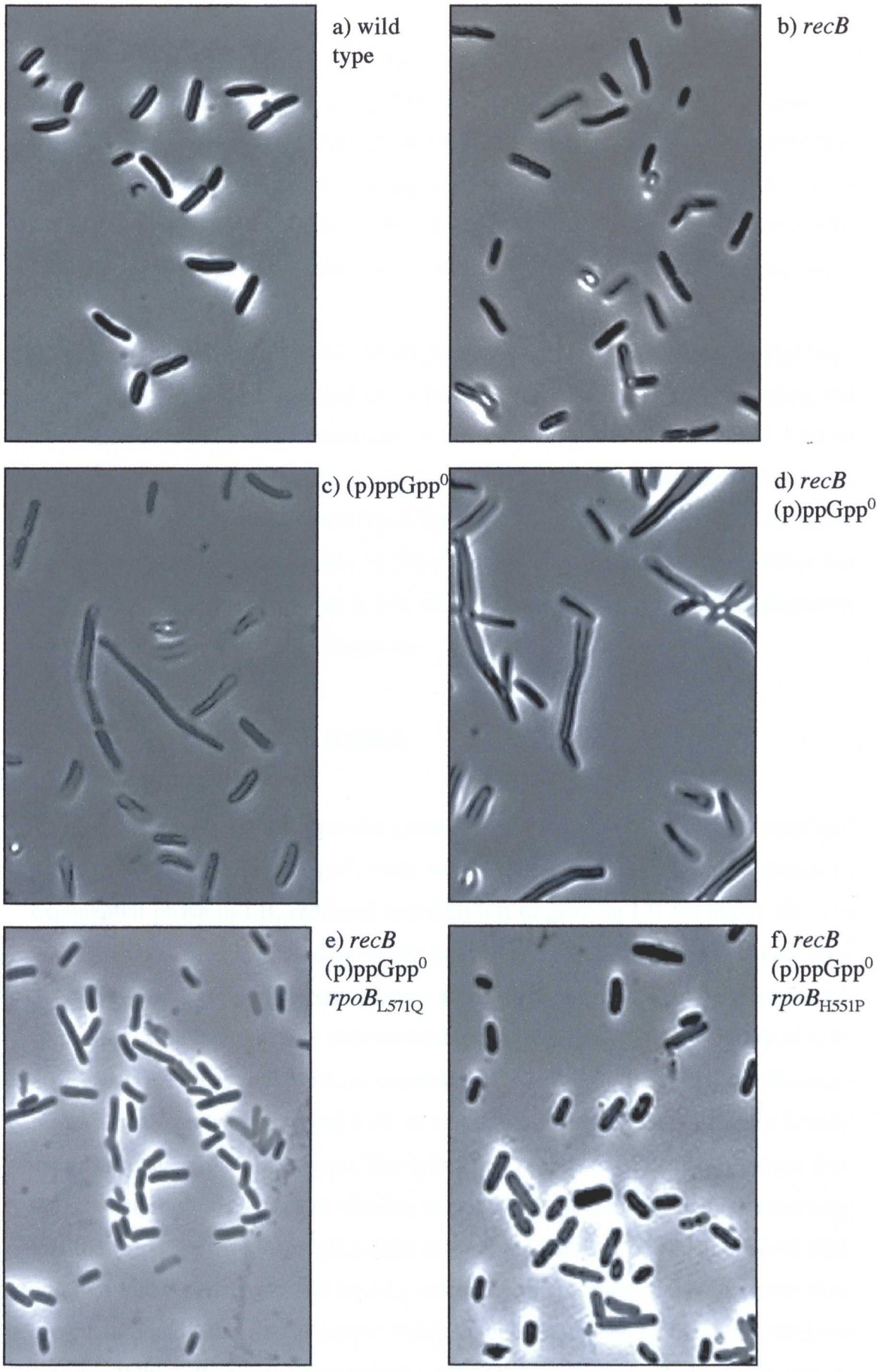

Figure 3.7. Phase microscope images of different strains. Pictures were taken of cells in exponential phase at OD_{650} ~0.4 at 1000 x magnification.

hand show a lot of filamentation (Fig. 3.7.c), possibly suggesting that DNA breaks arise more frequently, inhibiting cell division. This phenotype is enhanced in (p)ppGpp^0 *recB* cells (Fig. 3.7.d), consistent with unrepaired breaks. Again this may be due to RNA polymerase forming roadblocks for replication, which causes RuvABC-mediated fork collapse. As I had found that *rpo** mutations increased viability of (p)ppGpp^0 *recB* strains, I also examined their effect on cell morphology.

As shown in Figures 3.7.e and f, filamentation is nearly absent in (p)ppGpp^0 *recB rpo** cells, suggesting that DNA breaks occur less frequently, obviating the need for RecBCD and/or induction of the SOS response which would lead to inhibition of cell division. Another striking effect was the diminished cell size of some of the *rpo** mutant strains (Fig. 3.7.e). Small cell size has been noted as one of the effects of stringent mutations by Xiao and co-workers (1991). I do however not find it in all of my mutants (Fig. 3.7.f), which may be due to different modulation of RNA polymerase by different mutations.

3.7. Growth rate of *rpo** strains

To estimate and compare the growth rates of wt, *recB*, (p)ppGpp^0, (p)ppGpp^0 *recB*, and (p)ppGpp^0 *recB rpo**, cells were grown up from fresh o/n cultures to exponential phase in LB, rediluted and then left to grow in LB for about 350 min while the OD_{650} was measured in regular intervals. The resulting growth curves (Fig. 3.8) show a slightly different picture from what would have been expected. (p)ppGpp^0, which looks quite sick under the microscope and has a viability of only 40% grows nearly as well as wt (as reported by Cashel *et al.*, 1996). The *recB* mutant grows slightly slower, in accord with its viability of 65% and its reasonably healthy appearance under the microscope. The (p)ppGpp^0 *recB* is quite distinctly slower than the other strains, due to its low viability of only 7.7%. It is therefore quite surprising that the (p)ppGpp^0 *recB rpo** (L571Q) mutant, the viability of which is about 80% and that looks very lively and healthy under the microscope shows the same slow growth in liquid culture as its parent. This cannot be explained by the diminished cell size, as all the cultures are brought to the same OD before the start of the experiment, disregarding the number of cells. It can be speculated that the effect is due to lower

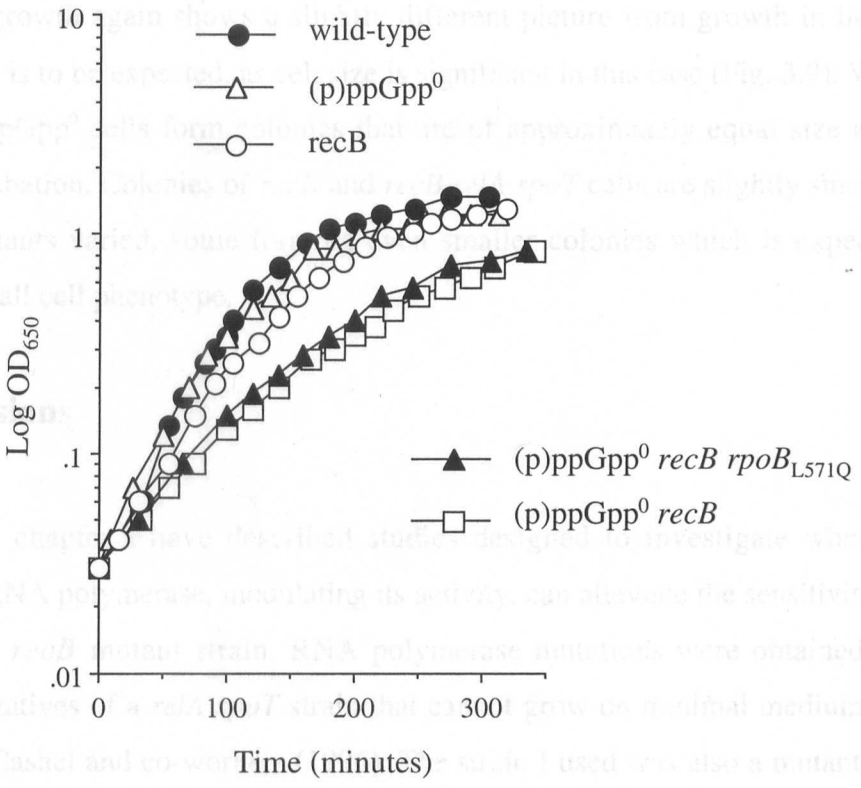

Figure 3.8. Growth of the different parent strains and an *rpo** strain in liquid culture (LB medium). (p)ppGpp^0 is hardly distinguishable from wild-type in growth rate. A *recB* mutant is hardly slower. The (p)ppGpp^0 *recB* double mutant grows significantly slower then wild-type and the single mutants. This characteristic is not alleviated by *rpo** mutations. The strains identified either by genotype or their respective mutations in the graph are MG1665 (wild type), N4304 ((p)ppGpp^0), N4278 (*recB*), N4315 ((p)ppGpp^0 *recB*) and BT130 ((p)ppGpp^0 *recB* *rpoB*$_{L571Q}$). The data are means of two independent experiments.

efficiency of the mutant RNA polymerases compared to wild type enzyme. As (p)ppGpp reduce transcription of rRNA genes and many other household genes strongly transcribed in exponential phase and the mutant RNA polymerases probably mimic the effect of (p)ppGpp, this seems a very likely explanation.

Plate growth again shows a slightly different picture from growth in liquid culture, which is to be expected, as cell size is significant in this case (Fig. 3.9). Wild type and (p)ppGpp^0 cells form colonies that are of approximately equal size after overnight incubation. Colonies of *recB* and *recB relA spoT* cells are slightly smaller. The *rpo** mutants varied, some forming even smaller colonies which is expected given their small cell phenotype.

3.8. Conclusions

In this chapter I have described studies designed to investigate whether mutations in RNA polymerase, modulating its activity, can alleviate the sensitivity to UV light of a *recB* mutant strain. RNA polymerase mutations were obtained by selecting derivatives of a *relA spoT* strain that cannot grow on minimal medium, as described by Cashel and co-workers (1996). The strain I used was also a mutant for *recB*, enabling me to ask directly whether stringent RNA polymerase mutants, obtained from minimal medium selections, also improve UV-survival.

About 30 independent *rpo** mutants were obtained. The extent to which they increase UV-survival varies. I determined that most of them suppress the UV sensitivity of *recB* and *ruv* mutants about equally well, though some show either better suppression of *ruv*, or very slightly better suppression of *recB*. The former can be explained by the requirement for RecBCD in the presence of RuvABC, as double-strand breaks produced by RuvABC from Holliday junctions cannot be efficiently processed, whereas RecBCD is not essential in the absence of RuvABC for this repair pathway. The mutants that are slightly better at suppressing *recB* need to be investigated in more detail. The fact that such mutants exist may indicate that double strand breaks are quite rare even in the presence of RuvABC or can be repaired by a pathway not using RecBCD.

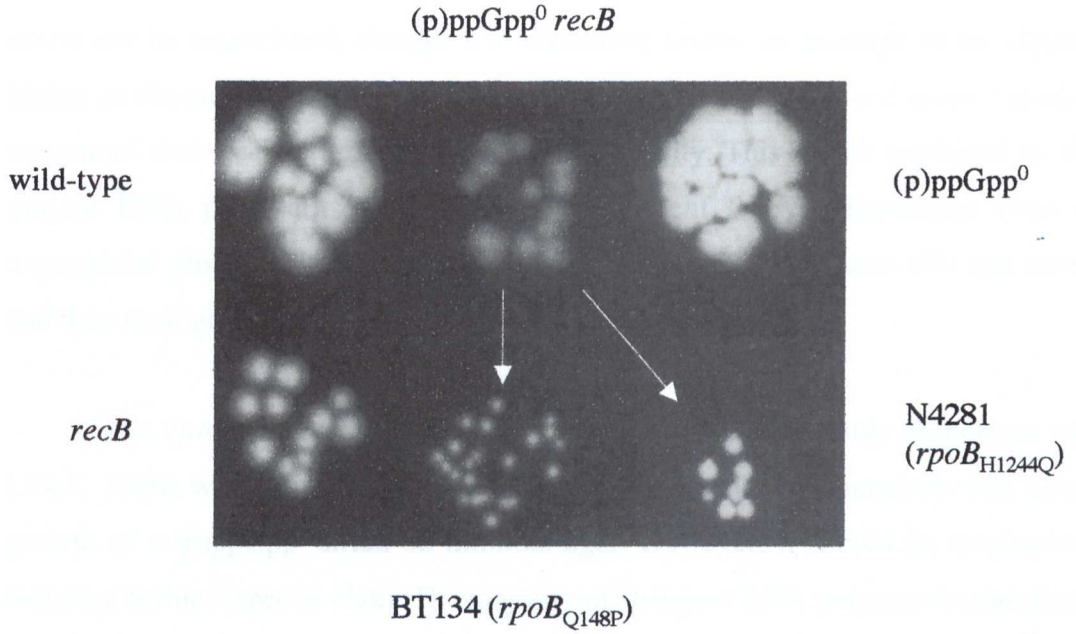

Figure 3.9. Growth of the different parent strains and two *rpo** mutants on solid medium. In contrast to growth in liquid medium, *rpo** mutants form mostly smaller colonies on plates than their (p)ppGpp^0 *recB* parent. It can also be observed, that the *rpo** colonies loose the fuzzy appearance of the parent strain colonies. The strains identified either by genotype or their respective mutations in the graph are MG1665 (wild-type), N4304 ((p)ppGpp^0), N4278 (*recB*), N4315 ((p)ppGpp^0 *recB*), BT134 ((p)ppGpp^0 *recB rpoB*$_{Q148P}$), N4281 ((p)ppGpp^0 *recB rpoB*$_{H1244Q}$). Plates were incubated at 37°C for 22 hours.

All of the examined *rpo** mutations improve cell viability and reduce the extensive filamentation seen in a *relA spoT recB* strain. Resistance to rifampicin occurs in a high number of mutants. The exact proportion could not be determined, as extensive testing would be necessary, including identification by sequencing, which is not practical for large numbers of mutants. The high proportion of Rif^R mutants was chosen for ease of handling. A firm connection between Rif^R and *rpo** could not be established, though UV resistance seems on average to be slightly higher in the mutants with higher Rif^R. Growth rate is not improved in *rpo** strains, in spite of their healthy appearance and high viability. This can be explained by the mutant RNA polymerases behaving like stringent RNA polymerases even in exponential growth. RNA production would be limited to comparatively low levels and thus limit growth.

The *rpo** mutants described here and in a previous study (McGlynn and Lloyd, 2000) were found among a general class of stringent mutations that allow growth of a (p)ppGpp^0 strain on minimal agar. However, it should be emphasised that they define a special class. The majority of stringent RNA polymerase mutations do not suppress the UV sensitivity of *recB* or *ruv* strains. Stringent mutations modulate RNA polymerase, so that it regulates transcription as if under conditions of stress or starvation. This fact allows (p)ppGpp^0 cells to grow on minimal medium, but it is not sufficient on its own to promote survival of UV-irradiated *ruv* or *recB* cells. Only certain, specific modulations of RNA polymerase, termed *rpo**, lead to suppression of UV-sensitivity of *ruv* and *recB* strains.

Chapter 4.

Sequence analysis of *rpo** mutants

4.1. Introduction

The multisubunit DNA-dependent RNA polymerase (RNAP) is essential for transcription and is the principal target for genetic regulatory mechanisms controlling gene expression. The enzyme has been highly conserved through evolution, as shown by the sequence homology of its two largest subunits throughout eubacteria, archaebacteria and eukaryotes (Berghofer *et al.*, 1988; Sweetser *et al.*, 1987). Core RNA polymerase is composed of four subunits: two α (36.511 kD), one β (150.543 kD) and one β' subunit (155.163 kD), encoded by *rpoA*, *rpoB* and *rpoC* respectively. For selective initiation the holo-enzyme is required, which also comprises the σ subunit (70.263 kD), encoded by *rpoD*. RpoD is also called σ^{70} and is the main sigma factor in *Escherichia coli*. RpoA is of little concern for this work, as its main function is the assembly of the other subunits. It has been implicated in proof-reading (Ishihama *et al.*, 1980), implying that it can recognise DNA information, but as yet no connection between α and the stringent response has been noted. Another component of the RNAP holo-enzyme is ω, a 10.105 kD protein, encoded by *rpoZ*. RpoZ's function has long been unclear. It has been implicated in the stringent response (Igarashi *et al.*, 1989), which was later shown to be an artefact, due to the location of *rpoZ* in the same operon as *spoT* (Gentry *et al.*, 1991). Recently ω has been shown to play a role in RNAP assembly (Ghosh *et al.*, 2001). β and β' together form the active centre of the enzyme.

4.2. Mapping *rpo** mutations

According to Cashel *et al.* (1996), mutations influencing the stringent response occur exclusively in the *rpoB*, *rpoC* or *rpoD* genes, encoding the β, β' and σ-subunits of RNA polymerase. Since the *rpo** mutations were obtained on the basis of their stringent phenotype (they allow growth of (p)ppGpp^0 cells on minimal medium), they were assumed to be alleles of one of these three genes. Mutations conferring resistance to rifampicin are most likely alleles of *rpoB* (Jin and Gross, 1988). It was therefore a simple task to sequence the *rpoB* gene in the relevant strains to locate the mutations. However, the location of rifampicin sensitive mutations was not as obvious and another method needed to be employed first to identify which *rpo* gene was affected.

I therefore introduced plasmids, constitutively expressing wild type RpoB or RpoC, into the mutant strains. This should lead to a mixture of RNA polymerases in the cell, the majority of which should contain the wild type subunit, overexpressed, while some would contain the mutant subunit expressed from the chromosome. Assuming that the larger number of RNA polymerases would dominate the phenotype, an *rpo** *recB* mutant with increased UV resistance should become slightly more sensitive again, if the plasmid encoded the wild type of the affected protein. Thus an *rpoB** *recB* mutant should become more sensitive when wild type *rpoB* is introduced and an *rpoC** *recB* mutant with the introduction of wild type *rpoC*. Accordingly, plasmids pRL385 and pRW208-13b, expressing RpoB and RpoC respectively were introduced into the rifampicin sensitive mutants. They were also transformed with pBR322, which acted as a control. The method was mostly successful. With the exception of a few mutants, where all three transformants of a strain appeared to have equal UV resistance, the *rpo** mutation could be assigned to either *rpoB* or *rpoC* (Table 4.1.) The mutations that could not be allocated to either may be alleles of *rpoD*. However, I did not investigate this possibility. As stringent mutations are also sometimes found in *rpoD* and *rpo** mutations are a subclass of stringent mutations, it is quite possible that *rpoD** mutations exist. Until recently it was assumed that the sigma factor dissociates shortly after initiation (e.g. Krummel and Chamberlin, 1989). In this case *rpoD** mutations could not act by decreasing the

half-life of RNA polymerase paused or stalled at a lesion. Recent evidence suggests, that this is not the case, the interactions between σ^{70} and RNA polymerase only being weakened upon elongation, not disrupted (Cromie and Leach, 2001). This scenario would allow for the possibility of *rpoD** mutations to influence pausing and stalling.

4.3. Sequencing of *rpo** mutants

After allocation of a mutation to either *rpoB* or *rpoC*, the relevant gene from the mutant was sequenced. Five to six primer pairs were designed for each gene, each spanning a region of up to 800 base pairs. Sequencing was performed from both primers in order to get independent conformation on any suspected mutation. A single substitution was detected in each of the *rpo** strains identified as carrying alleles of *rpoB* (either Rif^R or Rif^S) or *rpoC*. Each of the sequence changes discovered resulted in an amino acid substitution. The mutations are listed in Table 4.1. Six of the twenty-seven mutations sequenced were changes from or to proline, which is known to generally introduce conformational changes in protein structure (as its side chain is also bonded to the backbone nitrogen, the range of possible conformation is restricted, likely to form a kink in the amino acid chain). The mutations listed here are the only ones found in the respective mutants (in many cases both *rpoB* and *rpoC* were fully sequenced).

Nearly all of the RpoB mutations lead to rifampicin resistance of varying efficiency, though rarely to concentrations of 100 μg/ml or above. However, this predominance of rifampicin resistance among the mutants very likely reflects the screening method used rather than an actual bias in distribution of *rpo** mutations. Resistance is generally low and good suppressors of UV sensitivity can also be found in *rpoC*, which has never been associated with Rif^R. The work of Cashel and others might also lead one to expect similar *rpo** mutations in *rpoD*, encoding σ^{70}, also unconnected with resistance to rifampicin. The Rif^R *rpo** mutations cluster in regions of RpoB previously associated with rifampicin resistance. However, some mutations define new Rif^R alleles. As shown in chapter 3.4.1, resistance to rifampicin is not a prerequisite for *rpo** mutants, but it seems on average to be a slight advantage for suppression of UV sensitivity.

For purposes of comparison one stringent mutation that does not show *rpo** characteristics was also sequenced. It confers stringency, allowing (p)ppGpp^0 cells to grow on minimal medium, but it does not increase the survival of UV irradiated *ruv* or *recB* cells. However, considering only viability, cell size and growth rate, the *rpoB*S788F mutant is indistinguishable from typical *rpo** strains. This observation is important as it indicates that the reduced cell size and increased viability reported in chapter 3 are not directly responsible for the ability of *rpo** to promote survival of UV irradiated *ruv* or *recB* strains.

Table 4.1. *rpo** mutations.

Mutation	**Rif^R (μg/ml)**	**Quality of suppression**	**No. of times isolated**
a) *rpoB* mutations			
Q148P	>100	medium	2
R151S	0	medium	1
P153L	10	medium	2
G181V	10	medium	1
Y395D	10	medium	2
L420R	20	medium	1
H447R	0	medium	1
H447P	<20	medium	2
L448I	>20	medium	1
A532E	>100	high	1
L533P	>100	high	1
G534C	>20	high	1
G536V	50	medium	1
G537D	50	medium	1
V550E	>20	medium (+)	1
H551P	20	high	7
T563P	>100	medium (+)	3
L571Q	50	high	1
I572S	>100	low	1

H1244Q	<10	high	2
G1260D	20	medium	1
b) *rpoC* mutations			
K215E	n/a	low	1
ΔR312, G313,R314	n/a	medium	1
K789Q	n/a	high	1
E1146D	n/a	medium	1
R1148H	n/a	high	1
R1330S	n/a	medium	1
c) stringent non *rpo mutation**			
S788F	20	none	1

4.4. Location of the mutations within RpoB and RpoC

Having identified the above *rpo** mutations by sequencing I investigated their location within the linear protein sequence. RpoB and RpoC contain a number of conserved regions (defined by Jokerst *et al.*, 1989; Sweetser *et al.*, 1987). RpoB also contains three known clusters, where resistance to rifampicin often maps (Rif clusters; Jin and Gross, 1988). Chatterji *et al.* (1998) found that an azido-derivative of ppGpp crosslinks to both the N-terminal and C-terminal domains of the β-subunit of RNAP, though preferentially to the C-terminal part from residue 802 to about 1223. Work using a zero-length crosslinker (6-thio-ppGpp) recently indicated that the N-terminus of β' and the C-terminus of β form a modular ppGpp binding site (Toulokhonov *et al.*, 2001).

Figure 4.1. shows a linear representation of RpoB and RpoC marked with the identified *rpo** mutations. Almost all of the mutations are located in the previously described conserved regions, the major exceptions being E1146D and R1148H (see below). Most of the *rpo** mutations in RpoB are centred in and around Rif clusters I and II and the as yet unnamed Rif region around β146. Whether that is coincidence or a causal connection is not entirely clear (see also chapter 3 and below). The linear map leads to the conclusion that there are four *rpo** clusters on RpoB. There are not

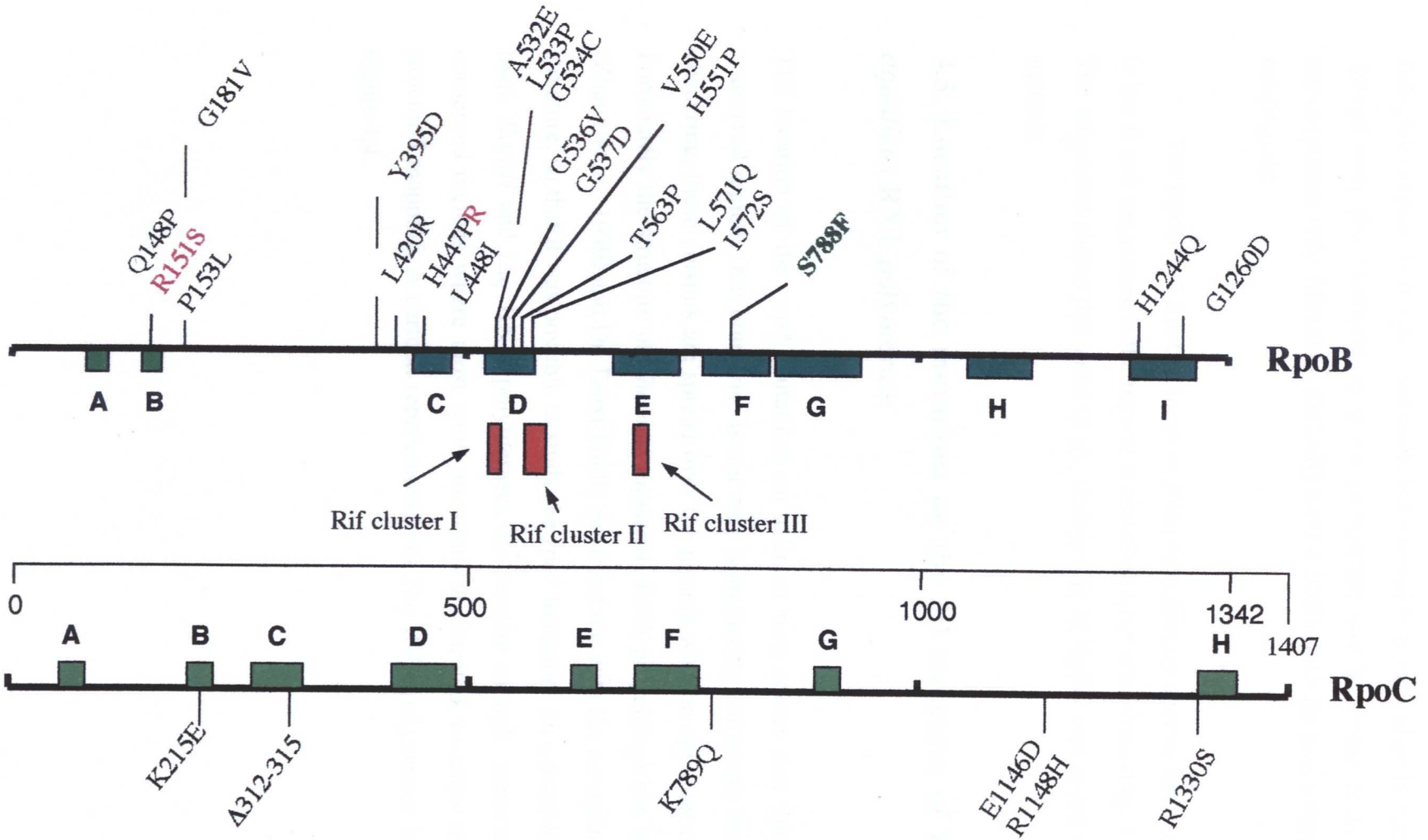

Figure 4.1. Linear map of the RpoB and RpoC proteins respectively. Conserved regions are indicated by green boxes and known regions of Rif^R regions by red boxes. The non *rpo** mutation in RpoB is printed bold. Rif^S mutations in RpoB are coloured red. Note: conserved regions are denoted by green boxes and their identifying letters.

yet enough examples of mutations in RpoC to define clusters. There is every reason though to expect more *rpo** mutations to be found in RpoC. The clusters are widely spread over the molecules and no connection can be made from the linear representation only. Mutations H1244Q and G1260D alone lie near a reputed ppGpp binding site.

The RpoB and RpoC sequences from three diverse species have been aligned to the *E.coli* sequences in the regions harbouring *rpo** mutations (Fig. 4.2 and 4.3). The alignment shows that most of the changes lie in highly conserved stretches of squence.

4.5. Location of the mutations on the 3D structure of *Thermus aquaticus* RNA polymerase

The location of the *rpo** mutations on a linear map shows that they occur in conserved regions, but otherwise is not very illuminating. Although there is some clustering, these clusters are spread over the protein with no apparent connection. Fortunately the structure of *Thermus aquaticus* RNA polymerase has been solved (Zhang *et al.*, 1999) and Dr. Darst kindly provided me with the co-ordinates of the structure, so that it was possible to study the *rpo** mutations on a three-dimensional map. *E.coli* and *Taq* RNA polymerases are similar enough, especially in the conserved regions where most *rpo** mutations are located, to allow this kind of structural mapping. A cartoon representation of *Taq* RNA polymerase is shown in Figure 4.4.

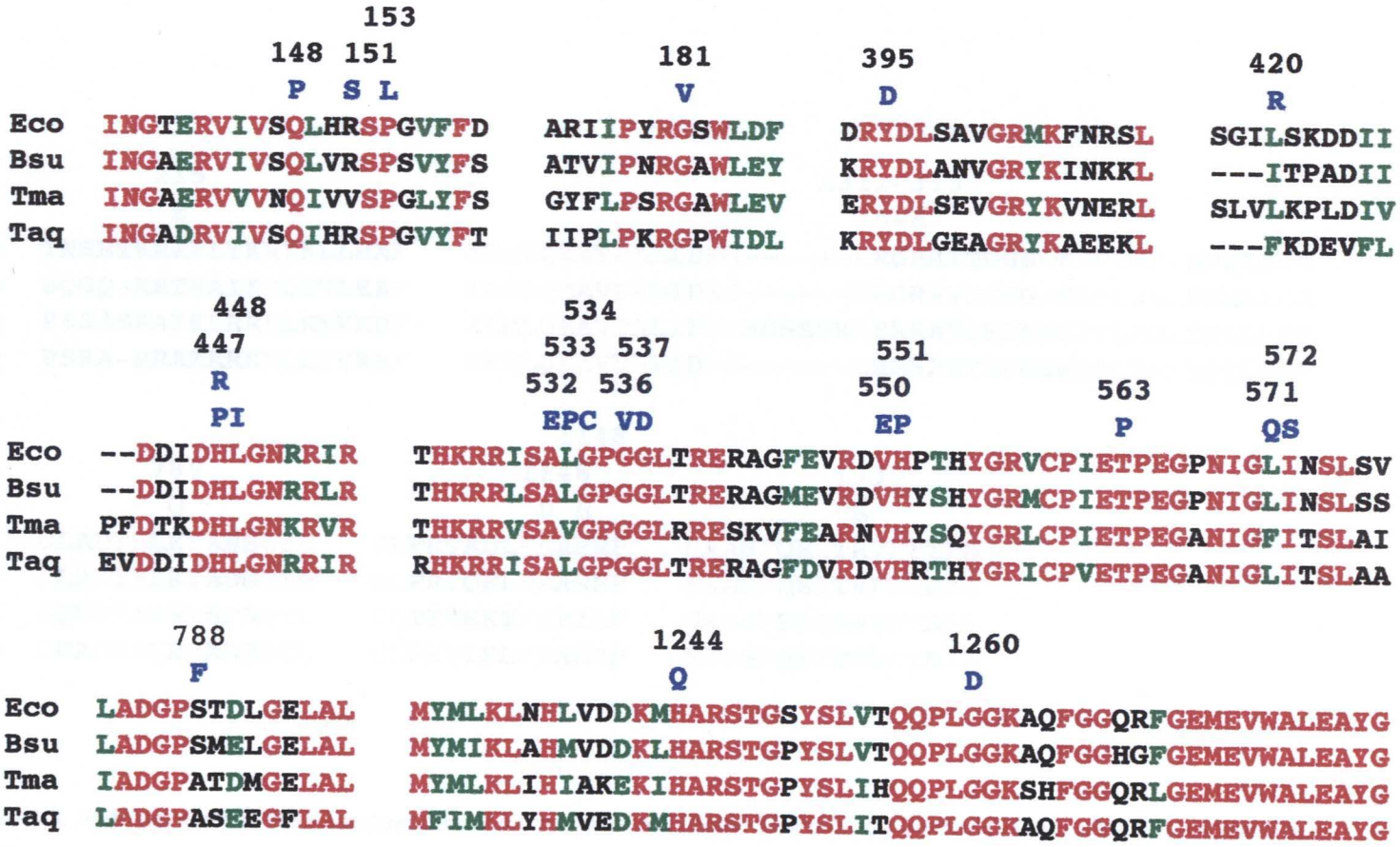

Figure 4.2. Alignment of the RpoB sequences *of Escherichia coli* (Eco), *Thermotoga maritima* (Tma), *Bacillus subtilis* (Bsu) and *Thermus aquaticus* (Taq). Identical amino acids are identified in red, functionally similar ones in green. *E.coli rpo** mutations are shown in blue. Note, S788F is not *rpo**. Functionally related amino acids (V:I:L:M:F:Y:W) (K:R:H) (D:E) (S:T) (G:A) (N:Q).

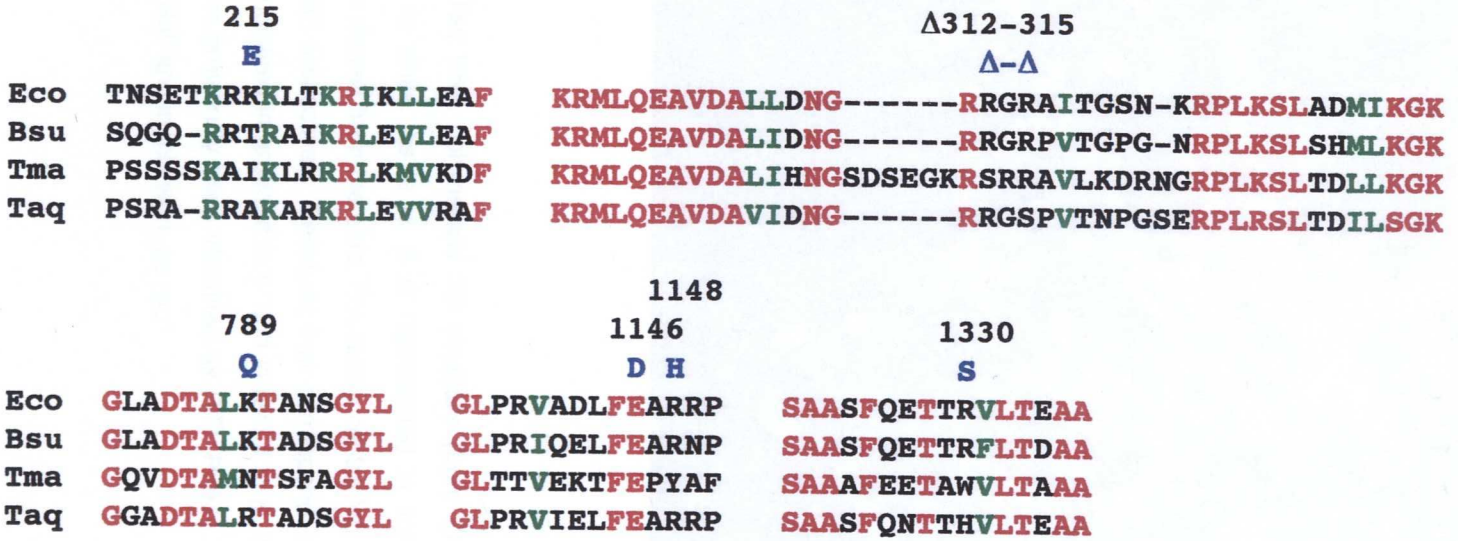

Figure 4.3. Alignment of the RpoC sequences *of Escherichia coli* (Eco), *Thermotoga maritima* (Tma), *Bacillus subtilis* (Bsu) and *Thermus aquaticus* (Taq). Identical amino acids are identified in red, functionally similar ones in green. *E.coli rpo** mutations are shown in blue. Functionally related amino acids (V:I:L:M:F:Y:W) (K:R:H) (D:E) (S:T) (G:A) (N:Q).

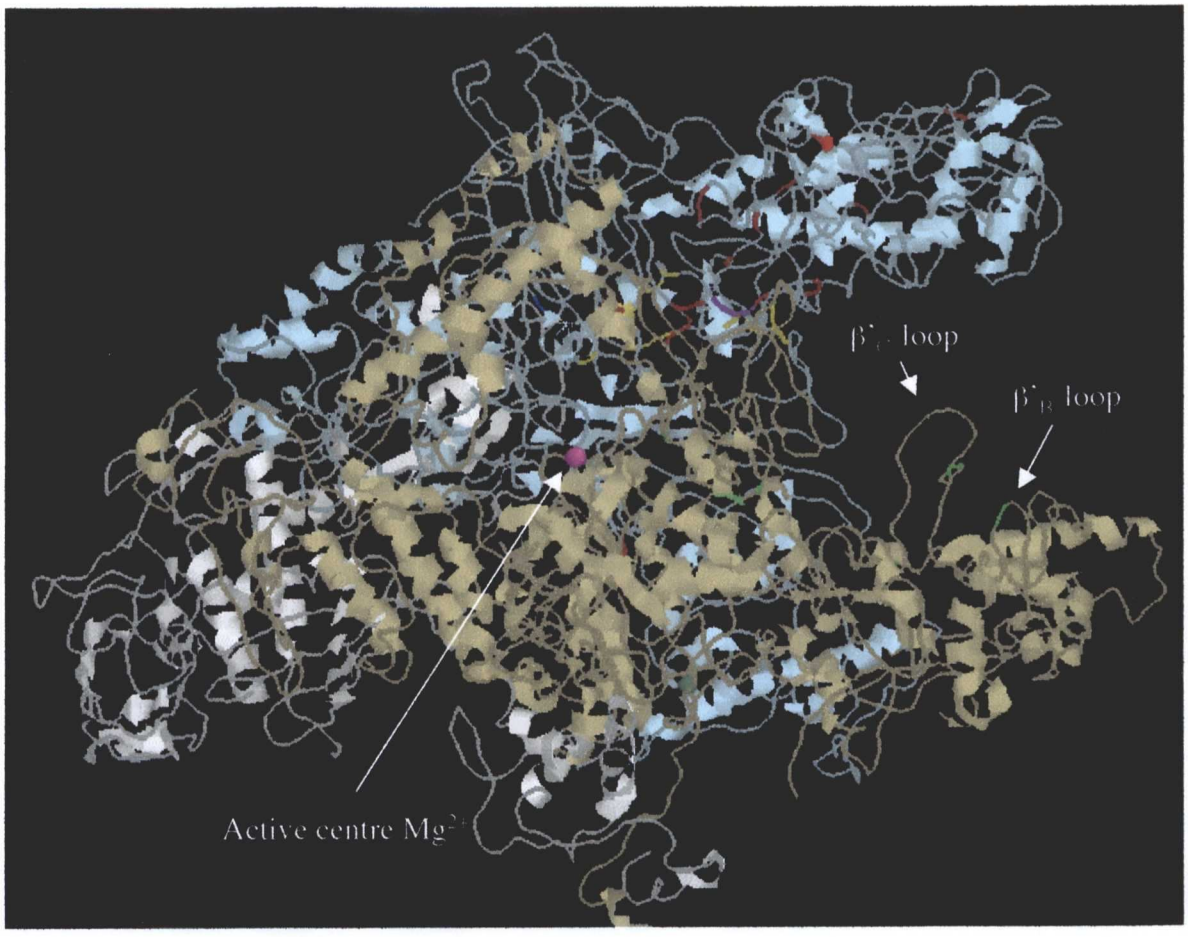

Figure 4.4. *Taq* RNA polymerase 3D structure (from Zhang *et al.*, 1999). The two α-subunits are in white, as is ω. β is represented in light blue, β' in light yellow. The polymerase is viewed from the side. The active centre Mg and the Zn-ions are in magenta and dark green respectively. Residues that correspond to amino acids changed in *rpo** mutants are indicated in green, if in β' and in red, if in β. Yellow residues represent amino acids that have previously been identified as Rif^R. Purple indicates residues isolated both previously as Rif^R and in this work as *rpo**.

4.5.1. RpoC mutants

RpoC is defined as the β' subunit of RNA polymerase. It contains a number of structural features, some of which are modified by the *rpoC** mutations:

The β'_B rudder

The *rpoC** mutation K215E (Fig.4.5, 4.6 and 4.7) affects a region of RpoC that I would like to define as the β'_B rudder (Fig. 4.4) (conserved regions as defined by Jokerst *et al.*, 1989 and Zhang *et al.*,1999). As with the β'_C rudder mentioned below, it defines a region well positioned to steer the DNA into the active site of RNAP. The hairpinlike structure of the β'_B rudder inserts into the major groove of the downstream duplex DNA at about +12/+13 (Korzheva *et al.*, 2000). K215E is a low suppressor of UV sensitivity. Substitution of the conserved hydrophobic and basic lysine at this position with glutamic acid is likely to significantly affect the activity of the β'_B rudder. It might for instance reduce stability of RNA polymerase at promoters, as Bartlett and co-workers (1998) have shown that a deletion of β'215-220 results in an RNA polymerase that forms unstable promoter complexes. However, it should be noted that $rpoC^*_{K215E}$ is a weak suppressor of UV sensitivity.

A deletion on the β'_C rudder

$rpoC^*_{\Delta 312\text{-}314}$ (Fig.4.5, 4.6 and 4.7) deletes most of the "shoulder" of what is called the β'_C rudder (Fig. 4.4; β'298-330, Zhang *et al.*, 1999). This region has been shown to cross-link to positions -6 to -8 on the DNA and it was suggested that the rudder separates the exiting RNA from the DNA template strand (Korzheva *et al.*, 2000). The deleted amino acids themselves are conserved in many species (Fig. 4.3), as is the region preceding it, which constitutes an α helix. It does however not lie in one of the conserved regions of β'. The whole loop constituting the β' rudder is not particularly well conserved between species, although the surrounding areas are very well conserved. The deletion is likely to lead to a shortening in the β'_C rudder and so may produce a conformational change in the region, altering DNA contacts and influencing separation of DNA template and nascent RNA. This may explain the ability of β'Δ312-315 to function reasonably as a suppressor of UV sensitivity.

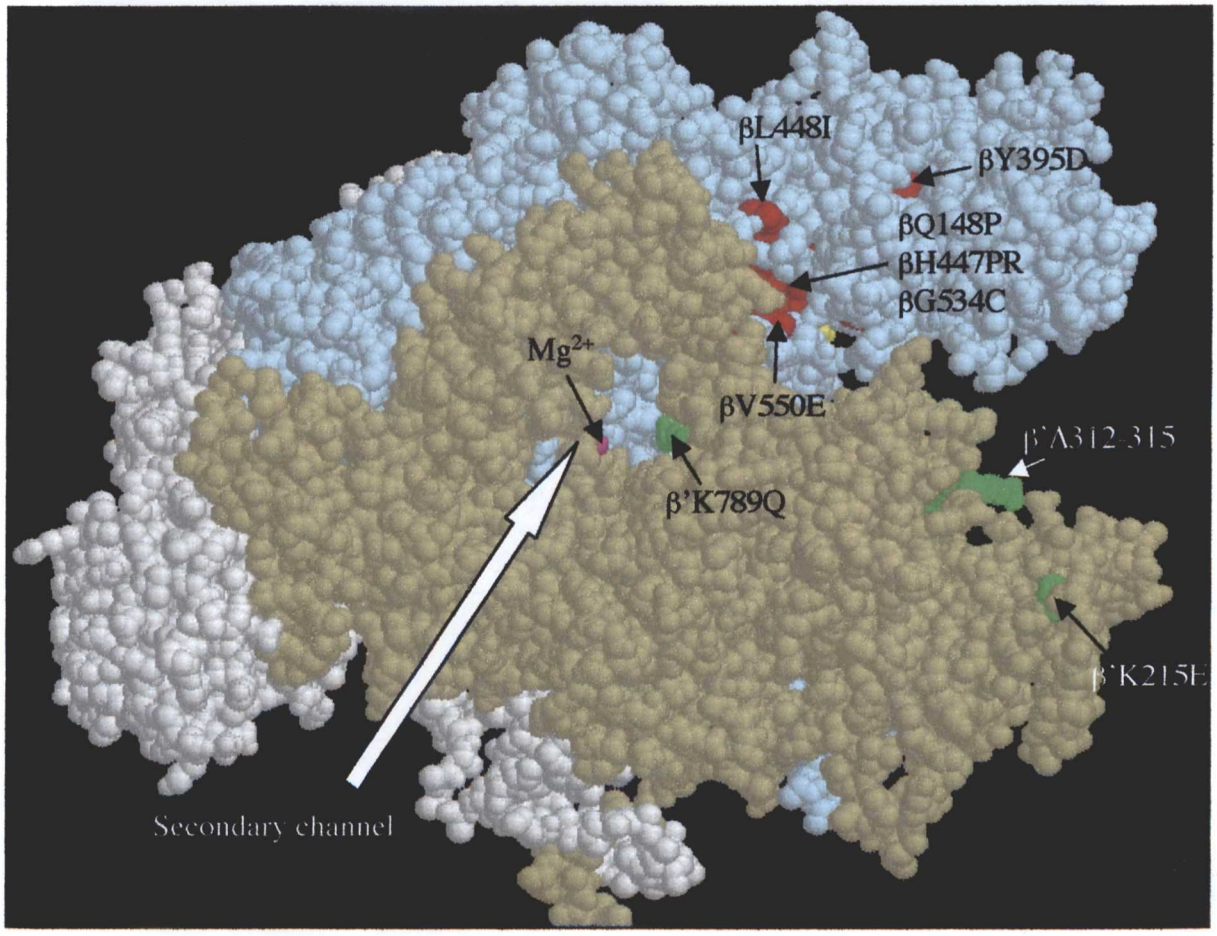

Figure 4.5. The same view of RNA polymerase as in Fig. 4.5., but represented in space fill mode. The secondary channel (where nucleotides enter the active site chamber) is clearly visible and through it the active site Mg^{2+}. The *rpo** mutation β'K789Q near this chamber is identified. Also visible are the positions of the mutations on the two β' rudders, K215E on the β'_B rudder and Δ312-315 on the β'_C rudder.

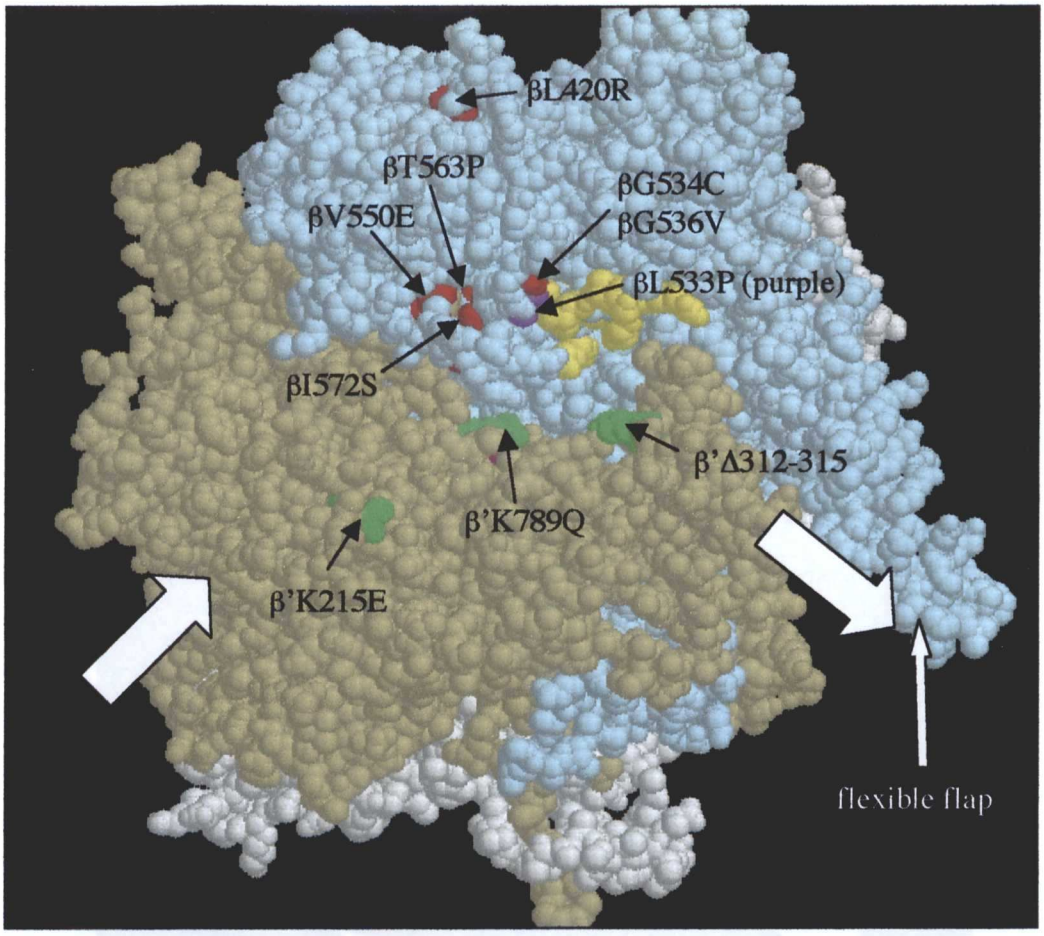

Figure 4.6. RNA polymerase viewed from the front side. DNA entry and exit channels are marked by block arrows. Yellow residues are known Rif^R mutations. Purple residues show an overlap between them and *rpo** muations isolated here. Most of the *rpo** mutations in β from a region adjacent to the Rif pocket. Muations in β' are spread along the path of the DNA.

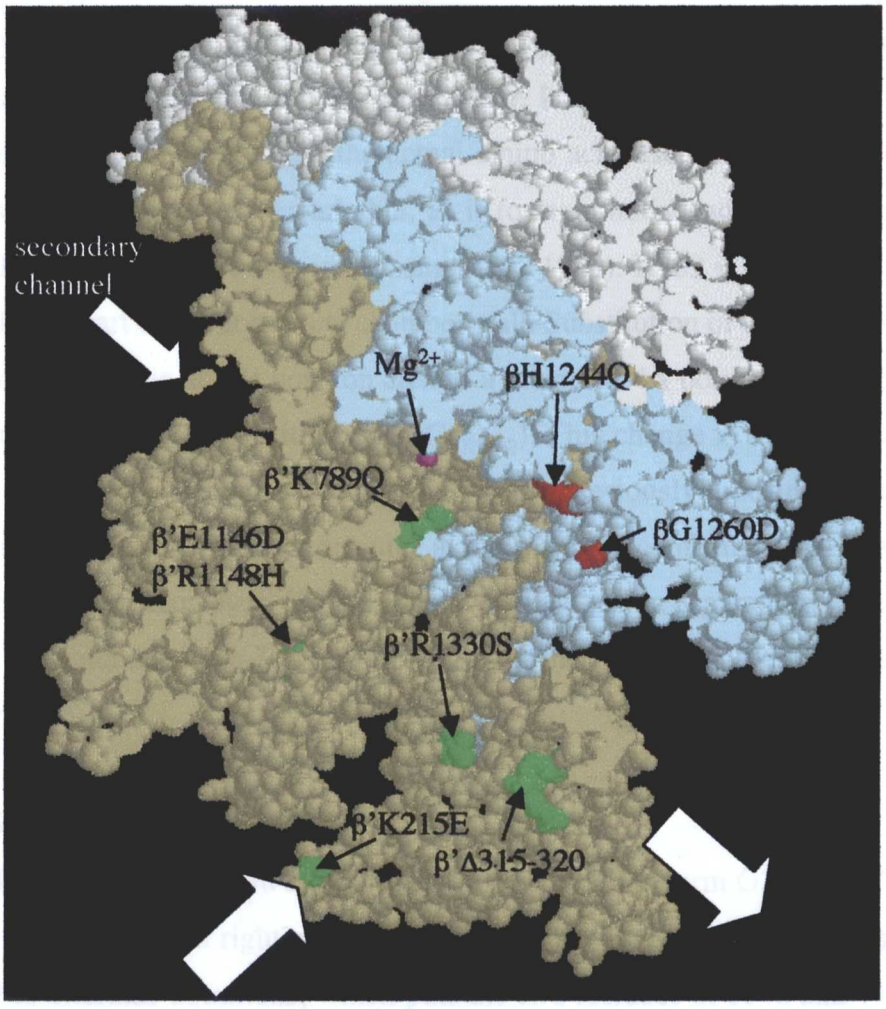

Figure 4.7. RNA polymerase viewed from the top, with 50% of the top sliced off to expose the interior and the inner surface of the β' subunit. All mutations in β' are exposed. The two C-terminal mutations in β are also visible.

A mutation in the active site chamber

β'K789 (Fig. 4.5, 4.6, 4.7, 4.8) forms part of the inside wall of the active site chamber, opposite the active site magnesium. Figure 4.5 shows that lysine 789 actually sticks out into the chamber. It is clearly visible when looking through the secondary channel into the active site chamber. It lies in a well-conserved region of β', termed the F region, though the actual amino acid itself is not conserved in all species. The β'_F region is remarkable, as it forms a helix and loop, traversing the main channel from top to bottom (Zhang *et al.*, 1999). K789 is located on the loop, which forms part of the active site chamber wall. Substitution of the basic lysine with the acidic glutamine at this position creates a mutant RNA polymerase that acts as a good suppressor of UV sensitivity ($rpoC^*_{K789Q}$). This change is likely to have some effect on RNA polymerase complexes without seriously limiting its ability to function. Some species have an arginine at this position, confirming that it is not vital to retain a lysine. However, *Thermotoga maritima* has an asparagine which is very similar to glutamine. The *T.maritima* RNA polymerase may therefore be similar to *E.coli* $rpoC^*_{K789Q}$.

β'E1146D and β'R1148H

Glu1146 and Arg1148 lie in a region of β' I would like to term G2 (Fig. 4.7). *E.coli* carries a long insertion right after the previously defined conserved region G when aligned to *Thermus aquaticus*, which puts the two residues at 1146 and 1148 about 200 amino acids away from the traditional region G. Zhang *et al.* (1999) expanded conserved region G, which, when transferred on the *E.coli* sequence, splits it into what I would like to term β'_{G1} and β'_{G2}. β'_G together with the above mentioned β'_F, forms a wall, bifurcating the main channel into the primary channel, used by the incoming DNA, and the secondary channel, presumably used by the incoming nucleotides (Zhang *et al.*, 1999). Like β'_F, β'_G forms a loop. E1146 is the last amino acid of the helix ending the loop, whereas R1148 lies on the next loop. Both are oriented towards the incoming DNA and well placed to make contact with it. The region β'1095-1189, in which the mutations lie, has been shown to cross-link to the incoming DNA at positions +5 and +15. It forms the side walls of the channel that accommodates the downstream DNA (Korzheva *et al.*, 2000). The amino acids around the affected residues seem not very well conserved, judging from alignments.

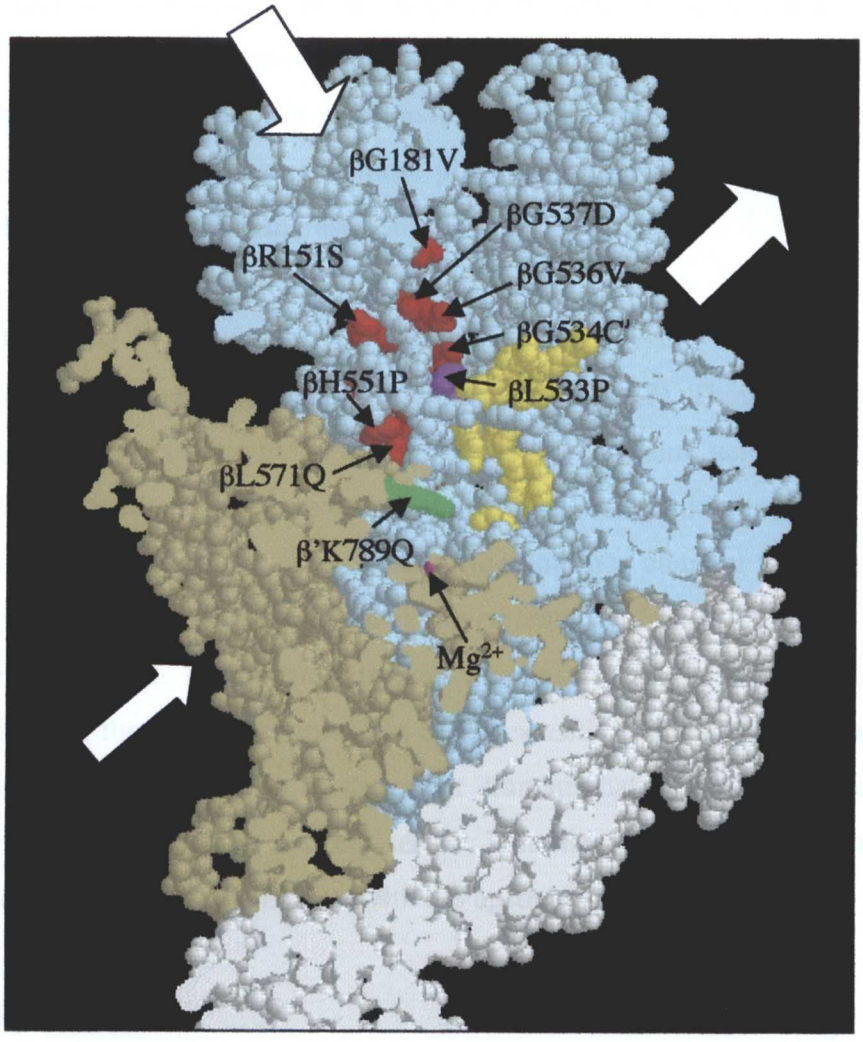

Figure 4.8. RNA polymerase viewed from the bottom, with 50% sliced off to expose the inner surface of the β subunit and the centre. Previously isolated Rif^R mutations, representing the Rif pocket, are coloured yellow. The purple residues are mutations isolated both as Rif^R and as *rpo**. The Rif^R and *rpo** mutations largely form different "pockets". β *rpo** mutations mostly lie in the entry channel of the DNA.

β'E1146 is slightly less accessible than β'R1148. β'E1146D is a medium suppressor and the change is a small one, from glutamic acid to aspartic acid. The more accessible β'R1148H is a good suppressor and the mutation is more radical, from arginine to histidine, which is also basic, but of a very different shape and also hydrophobic. The substitutions at these positions, especially β'R1148H, would be expected to affect RNA polymerase activity.

β'R1330S

Arg1330 lies in conserved region H of β' (Fig. 4.1 and 4.7). Though it is not highly conserved itself, it is surrounded by highly conserved amino acids. Neither R1330 nor any amino acids near it have been implicated in cross-linking studies. It lies just before a helix that participates in forming the entrance channel for the DNA, but facing away from it. It is also too far removed from the transcript exit site to make any contact with it. As a result its lack of conservation is not surprising. The explanation for the average suppression of UV sensitivity by substituting a serine at this position must therefore lie in the rather dramatic nature of the change from the bulky and basic arginine to the tiny serine. This may have some influence on the helix structure, influencing contacts with the upstream DNA.

4.5.2. RpoB mutants

After mapping all of the available *rpo** mutants on the 3D structure it emerged that some of them were clustered in what I would like to term here the *rpo** pocket. This can be seen as an extension of the Rif pocket, as most of the residues in question are at least slightly resistant to rifampicin. The *rpo** cluster also shares some altered residues with the rifampicin cluster and extends from there. (Figure 4.9). The mutations affecting the *rpo** pocket and other residues in RpoB conferring an *rpo** phenotype are discussed individually below:

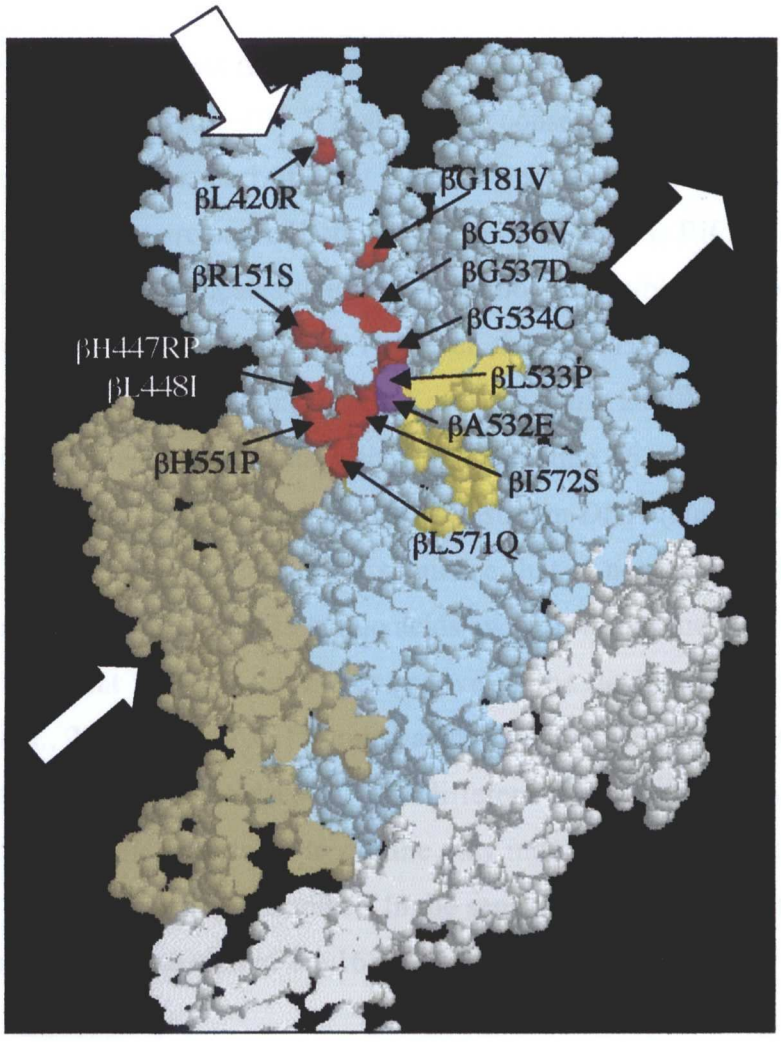

Figure 4.9. RNA polymerase viewed as in Figure 4.9, but with 55% sliced off to expose more residues changed in *rpo** mutants. Most of them lie in a pocket that is separate from the Rif pocket and overlaps slightly with it. DNA entry and exit channels are again marked by block arrows, as is the secondary channel for entry of nucleotides.

4.5.2.1. The *rpo** pocket

βQ148P

Gln148 is part of conserved region β_B (conserved regions as initially defined by Sweetser *et al.*, 1987). (Figure 4.1). It is absolutely conserved, as is a large part of the region surrounding it. It is part of what I define here as the *rpo** pocket and lies adjacent to residues that are part of both the *rpo** pocket and the Rif region (Fig. 4.5, 4.10). The 3D structure indicates that Gln148 is not accessible and so not able to make contact with either DNA or RNA. However, the region β130-183 was shown to cross-link to the nontemplate strand DNA (Korzheva *et al.*, 2000). Region β130-239 had also previously been shown to interact with nucleotide +6 on the template strand (Nudler *et al.*, 1996) and +3 to +15 on the downstream DNA (Nudler *et al.*, 1998). The residue is part of a long stretch of sequence that is not organised into either helices or β-sheets. Q148 is only 15 amino acids away from residue 163, which is the centre of a loop that fits into the major groove of the downstream duplex DNA (Korzheva *et al.*, 2000), but those 15 amino acids span the whole length of structural domain 2 and put the two residues in very different positions. The change at this position from the acidic glutamine to proline may indirectly influence the structure surrounding it, in the acitve site chamber. In the same fashion, by a structural change induced by proline, the position of the 163-loop might be influenced.

βR151S

Though only 3 residues removed from Gln148, Arg151 lies in a quite different position (Fig. 4.8, 4.9 and 4.10), is solvent accessible and forms part of structural domain 2, further towards loop 163. The residue itself does not seem highly conserved, though the region around it is. The basic and bulky arginine is replaced here by the tiny serine. As the residue is in an accessible position in the path of the incoming DNA and cross-linking studies have shown the region to make DNA contacts (see above), this might well have consequences on the interaction of RNA polymerase and DNA. Like βQ148P, βR151S is a suppressor of medium quality.

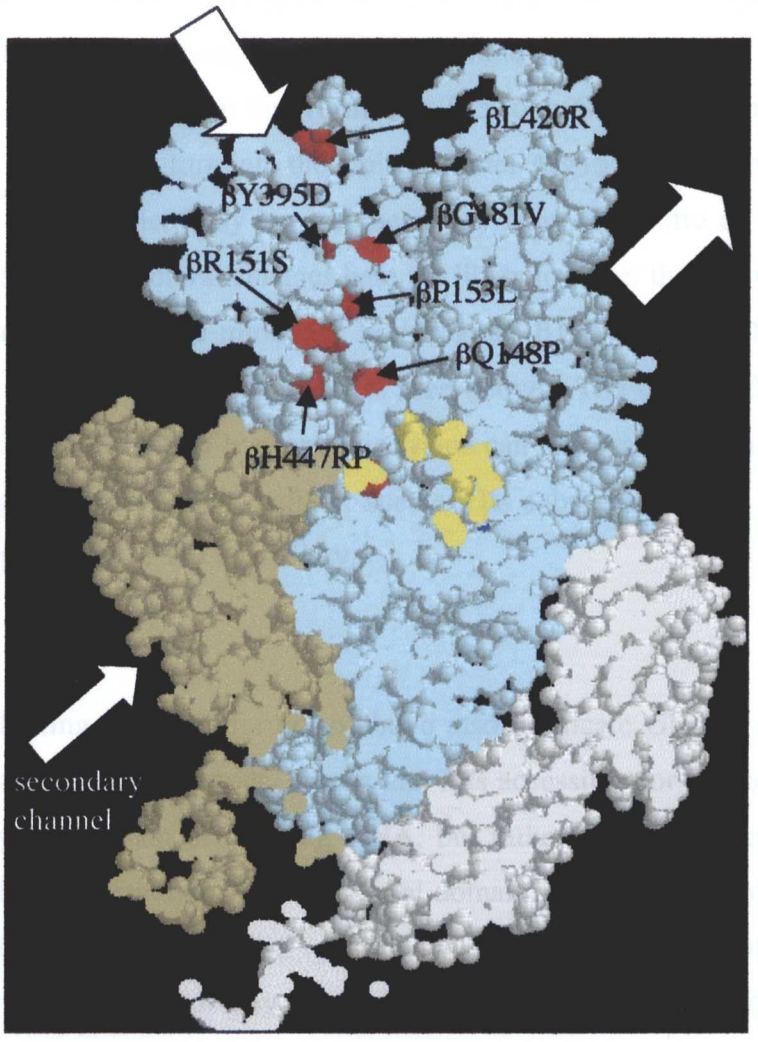

Figure 4.10. RNA polymerase viewed as in Figures 4.9 and 4.10. 60% have been sliced away to expose more residues changed in *rpo** mutants. DNA entry and exit channels are again marked by block arrows, as is the secondary channel for entry of nucleotides.

βP153L

Pro153 is he third in this sequential cluster of residues affected in *rpoB** mutants. It is solvent inaccessible, lying more or less behind βR151S on the same long and seemingly unstructured loop that forms the 163-loop (see above; Fig. 4.10., 4.11., 4.12.). The loop takes a turn into the molecule after Arg151, which positions Pro 153 inside and inaccessible. Only ten residues away from the centre of the 163-loop it lies at its base and structural changes here are likely to affect the whole loop. Also, it is still located right in the middle of both regions β130-183 and β130-239, which have been implicated in DNA contacts (see above). The change from the cyclic proline to the aliphatic and hydrophobic lysine can be regarded as a structural one. P153 is highly conserved throughout species. Its substitution with lysine leads to medium suppression of UV sensitivity.

βG181V

The cross-linking studies referred to above also implicate Gly181 in downstream DNA interaction. The 3D model shows that it is accessible for interactions, but lies not directly in the path of entering DNA, but rather on the surface of structural domain 2 that is oriented towards structural domain 3, so not directly on the face of the clamp formed by domain 2 (Fig. 4.8., 4.9., 4.10.). The centres of both the 163- and the 191-loop that probably interact with the downstream DNA are about 15 residues away, but on the opposite side of domain 2. Two beta sheets form the surface of domain 2 here and connect the 163- and 191-loops and the loop containing Gly181 on the other side. Gly181 is conserved absolutely through species. In the *rpoB**G181V mutant the glycine is replaced with the also aliphatic valine that only differs in size. Structural changes are therefore unlikely. However, it must affect activity since it leads to medium suppression of UV sensitivity.

βY395D

Tyr395 is deeply buried in domain 2 (Fig. 4.5, 4.10, 4.11, 4.12). It is highly conserved, as are its surroundings. From the 3D model it becomes obvious that, after going back and forth to form the inner surface of domain 2, the chain turns and returns in one loop to the proximal end of the domain. This returning loop harbours Tyr395 in the middle part. The fact that a change from the hydrophobic and aromatic

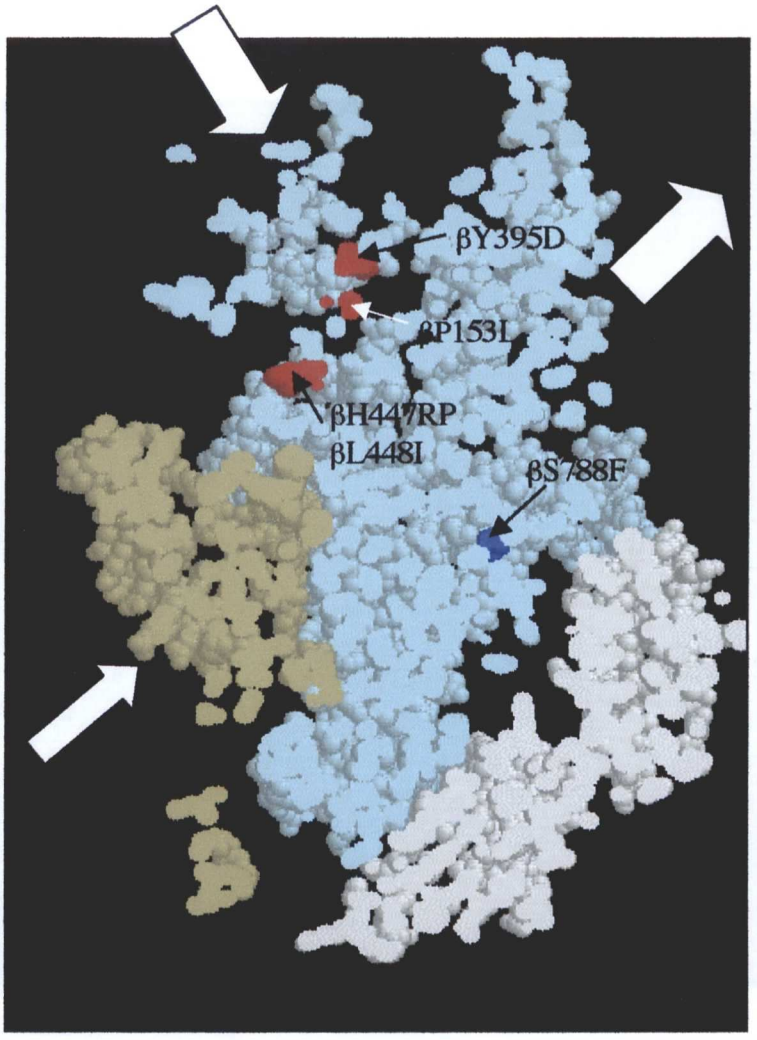

Figure 4.11. RNA polymerase viewed as in Fig's 4.9., 4.10., and 4.11. 65% have been sliced away to expose more residues changed in *rpo** mutants and the residue that confers stringency without increasing UV resistance, shown in blue. It lies buried deeply in the molecule and in a rather different location from any *rpo** residue. DNA entry and exit channels are again marked by block arrows, as is the secondary channel for entry of nucleotides.

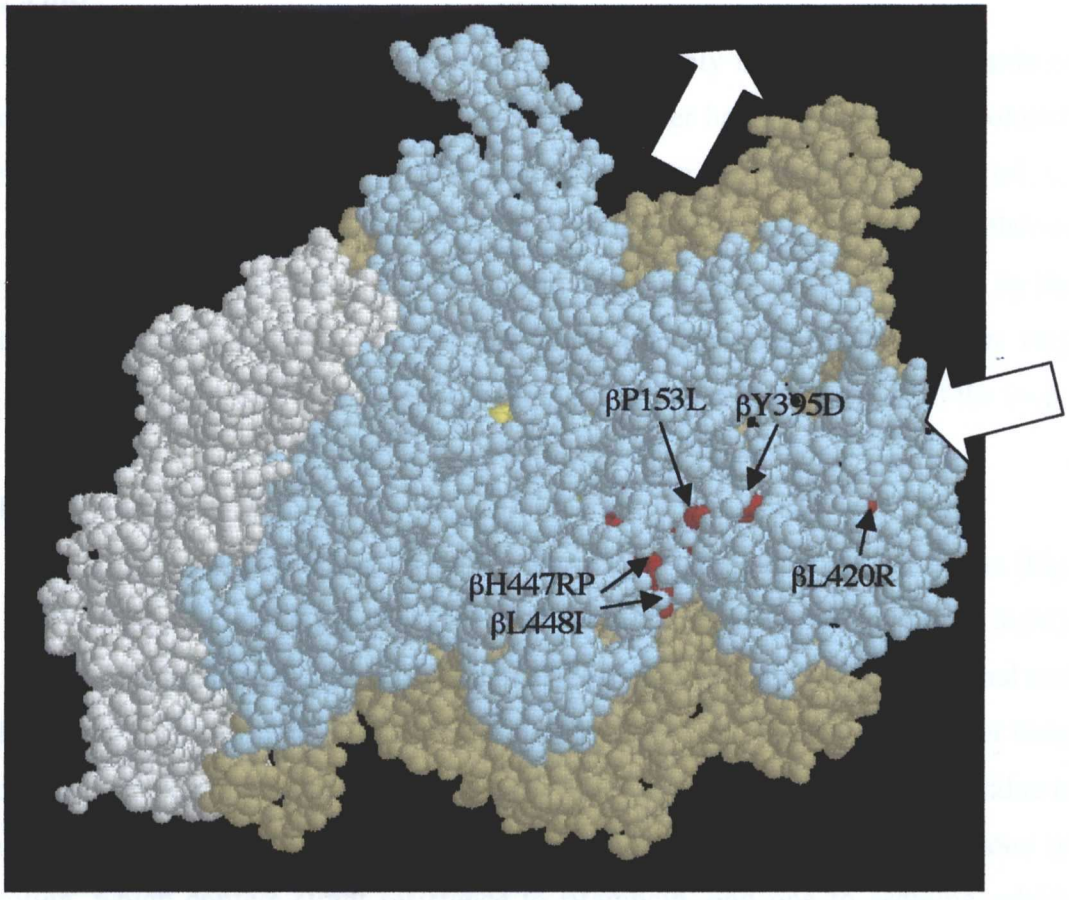

Figure 4.12. View of RNA polymerase from the top, on the β-subunit. The residues shown are more accessible from the outside than from the inside and so unlikely to make substrate contacts.

tyrosine to the small and acidic aspartic acid in that position should lead to medium suppression can only be explained by some structural change.

βL420R

Leu420 is located in a similar position to Tyr395, only more to the distal side of domain 2 (Fig. 4.6., 4.9., 4.10., 4.12.). The chain, after having reached the proximal end of domain 2 turns again to form a long loop, stretching to the distal end, on which lies Leu420. The phenotype of and L420R mutant can again only be explained by a structural change when the aliphatic and hydrophobic leucine is replaced by the bulky and polar arginine. Neither L420 nor the adjacent residues seem very conserved, though there is some conservation further towards the bottom of the loop.

βH447P and βH447R

His447 is again located right at the centre of the *rpo** region near the Rif region (Fig. 4.5, 4.9, 4.10, 4.11 and 4.12). It is part of β_C. His447 and its surroundings are highly conserved through species. It is part of an extensive loop, spanning the proximal end of domain 2 and forms the tip of a hairpin-like structure. The region has not been noticed in cross-linking studies and from the model it seems likely that the residue is buried and inaccessible. Two mutations were identified in that location, one to proline, which confers slight resistance to rifampcin, and one to arginine, which remains sensitive. Both are medium suppressors of UV sensitivity. His447 is part of the centre of RNAP, hidden behind the roof of the active site chamber. In the Rif^S H447R the basic and hydrophobic histidine is replaced by the also basic, but bulkier and polar arginine. The replacement of the histidine by the cyclic and also small proline leads to rifampicin resistance. The proline exchange probably causes a more drastic structural change which might explain its rifampicin resistant phenotype. Their effect on UV sensitivity however is comparable.

βL448I

Leu448 lies right next to the previously discussed mutations (Fig. 4.9, 4.11 and 4.12). In contrast to His447 however, Leu448 is probably accessible from the outside of RNAP, at the left proximal end of domain 2, when seen from below. The change from leucine to isoleucine is a very small one and it is not easy to imagine how such

a minute change in a position that has not been shown to make any substrate contacts could influence the behaviour of RNAP. The high conservation of the region though hints at an important function, structural or otherwise, that might be disturbed by even such a small change as this. The fact that three *rpo** mutants were found in two neighbouring positions makes this hairpin-like structure significant for the *rpo** phenotype. It is interesting, that both the more drastic changes at position 447 and this small change at 448 lead to medium suppression of UV sensitivity.

βG536V

Lying in the spacer region between Rif clusters I and II Gly536 cannot be regarded as part of the Rif pocket (Fig. 4.6., 4.8., 4.9.). It is still in region β516-540, implicated in contacts with the initiating site (Severinov *et al.*, 1995), but from its location on the 3D model it seems unlikely that it actually does so. As with the *rpo** mutants in the Rif pocket, the model favours the cross-linking study of (Markovtsov *et al.*, 1996) that connects it to the 3' RNA terminus. The chain described below, harbouring most Rif^R and several *rpo** mutations, loops to the distal side here where Gly536 is located. The whole region around it is absolutely conserved. Also, Gly536, as well as Gly537, Val550 and His551 (see below) are very near the loop centred round Arg543, which fits into a pocket at the downstream edge of the transcription bubble (Korzheva *et al.*, 2000). The mutation from glycine to valine here is a rather subtle one, only making a small difference in the size of the residue. It is likely that even a small change like this can already influence the protein-RNA interaction and lead to medium suppression of UV sensitivity.

βG537D

Almost everything said about Gly536 also applies to Gly537 (Fig. 4.8 and 4.9). Another glycine in more or less exactly the same position is changed to aspartic acid, which is bigger, acidic and charged. Though this is a more radical change, it still also leads to medium suppression of UV sensitivity.

βV550E

Val550 also lies in the spacer region between Rif cluster I and II (Fig. 4.5 and 4.6). Contrary to the residues above, that consecutively moved out to the distal side of the

clamp on its surface, Val550 is again located right in the centre of the *rpo** region in a kind of pocket, not accessible from the outside, but spatially not far removed from the residues the *rpo** region shares with the Rif region. The study of Markovtsov et al. (1996) connects also this residue with the nascent RNA. The 3D model contradicts this, as the residue is not accessible and seems to lie more in the region of the entering DNA. The spacer region between the Rif clusters returns here more to the centre of the molecule, after having looped into domain 2. The change from the small, hydrophobic and aliphatic valine to the big, acidic and charged glutamic acid is quite a major one and can well be imagined to cause some structural rearrangement to influence substrate contacts. The result of the mutation is medium suppression of UV sensitivity.

βH551P

His551 lies right next to Val550, in the same internal pocket, but more to the proximal side and more exposed and could make interactions with substrate (Fig. 4.8 and 4.9). In this case this would be the 3' end of the nascent RNA (Markovtsov *et al.*, 1996). For more details of the structure and location see V550E. The change from histidine to proline is likely to lead to a structural change. It is associated with high suppression of UV sensitivity. A Rif^S mutation to tyrosine has been identified by Severinov *et al.* (1993) at this position.

βL571Q

Leu571 lies on the same loop as Tyr563 that closes over the base-pair between the incoming nucleotide and position +1 on the template (see below; Fig. 4.8 and 4.9). But it is solvent accessible and nearer the centre of the loop. The change from the aliphatic and hydrophobic leucine that is conserved at this position (functionally) to the acidic and polar glutamine, leads to a strong *rpo** phenotype.

βI572S

Very much the same applies for the neighbouring Ile572 (Fig. 4.6 and 4.9), which is highly conserved. In contrast to L571Q the mutation from the aliphatic and hydrophobic isoleucine to the small and polar serine results in only low suppression of UV sensitivity.

4.5.2.2. *rpo** mutations in the Rif pocket

No mutations conferring resistance to rifampicin have ever been found on RNA polymerase other than those affecting the β subunit (Jin and Gross, 1988). Such mutations are clustered in 3 main regions, termed cluster I (507-534), II (564-574) and III (687). Clusters I and II comprise most of the commonly found mutations, but some additional ones have been found outside them, like R687H (Jin and Gross, 1988) and V146F (Lisitsyn *et al.*, 1984). Those residues are far removed from each other on the linear map of RpoB. On the 3D structure however, they are all part of the roof of the active site chamber in the so called Rif pocket (Zhang *et al.*, 1999). In this work 12 more rifampicin resistant mutations have been identified. Some of them lie in the known Rif^R clusters, but others lie in regions not before associated with resistance to rifampicin. The resistance of the latter is quite low, which probably explains why they have not been described before.

βA532E

The mutation of Arg532 has been linked previously with resistance to rifampicin (Jin and Gross, 1988; Severinov *et al.*, 1993). Jin and Gross (1988) isolated a deletion mutant of Arg532, whereas Severinov *et al.* found the same change as is reported here as *rpo**. Arg532 is located in Rif cluster I (Jin and Gross, 1988), which is highly conserved. Structurally this residue represents the border between the Rif pocket and the *rpo** pocket. As with most other residues associated with giving rise to rifampicin resistance, it forms part of the roof of the active site chamber, where it is accessible (Fig. 4.9). The region β516-540 has been shown to cross-link to the initiating site (Severinov *et al.*, 1995). However, from its position on the 3-D model it seems unlikely that this residue can actually do so. Indeed another cross-linking study by Markovtsov *et al.* (1996) connects region β515 to ~660 with the RNA 3' end, which fits well with the model. Arg532 lies on an extensive loop, with only one short helix and no β-sheets, that also contains most of the residues associated with known Rif^R mutations and many *rpo** residues described here. The change from the tiny, aliphatic and hydrophobic alanine to the bulkier, acidic and charged glutamic acid leads to high suppression of UV sensitivity.

βL533P

Leu533, right next to the previously discussed Arg532, lies in about the same position (Fig. 4.6, 4.8 and 4.9). It is also part of both the Rif^R and the *rpo** pocket and accessible at the roof of the active site chamber. L533P has been found as Rif^R by Jin and Gross (1988), and has been isolated here as *rpo**. The functionally conserved leucine, aliphatic and hydrophobic, is changed to the cyclic proline, probably inducing a structural change. Suppression of UV sensitivity is high.

βG534C

Gly534 (Fig. 4.5, 4.6, 4.8 and 4.9) is the last residue in Rif cluster I. A change from glycine to aspartic acid has been found there by Severinov *et al.* (1993).The G534C substitution has not been described before. Gly534 again lies in nearly the same position as the two previous residues, but slightly more to the distal side. It can be regarded as being the distal border of the Rif pocket, whereas *rpo** mutations are found both more distal and proximal. The change from the tiny and aliphatic glycine to cysteine is not dramatic in size, but might allow the formation of an additional sulfide bridge. It leads to high suppression of UV sensitivity.

βT563P

This mutation has been found by Jin and Gross (1988). Thr563 is part of Rif cluster II and located on an intricately coiled loop (Fig. 4.6), the major part of which is very well conserved. Direct substrate interaction is not possible, as it is inaccessible. Tyr563 also lies at the most proximal part of the *rpo** region, nearest the active site Mg. It is at the base of a loop centred round amino acid 568, which closes over the base pair between the incoming nucleotide and the DNA template at +1 (Korzheva *et al.*, 2000). Changing the hydrophobic threonine to proline is likely to cause structural change, and altering interactions with the substrate. It results in a medium *rpo** phenotype.

4.5.2.3. Other *rpo** mutants

Most of the mutations identified in this work were located either directly in the Rif pocket, or in a region near it that can be seen as an extension of it and which I

have termed *rpo** region or pocket (Fig. 4.9). Of course none of the mutations in RpoC fall into that category, though their effect is very much the same. And even with RpoB there are two mutations quite distanced from the *rpo** region, forming a cluster of their own. This leads to the expectation that there are more *rpo** mutations to be found in other locations.

βH1244Q

The *rpoB**H1244Q (*rpo**35) mutation was described initially by McGlynn and Lloyd (2000). I sequenced the mutation and analysed its possible effect by reference to the 3-D structure of RNA polymerase. Mustaev *et al.* (1991) identified His1237 as a residue that is very close to the binding site of the priming substrate. The regions β1232-1273 and β1232-1304 were later shown to cross-link to the -8 hybrid and +12 duplex, and the -4 hybrid and -23 RNA positions respectively (Nudler *et al.*, 1998). The 3D model also places His1244 right in the active centre and accessible, though a bit removed from the active site Mg^{2+} (Fig. 4.7). It seems certain though that it makes substrate contacts. Predictably the residue itself as well as the surrounding region are well conserved. Like most other residues affected by *rpo** mutation sites, His1244 is part of an extensive loop with no helices or β-sheets. The change from the basic and hydrophobic histidine to the acidic and polar glutamine is certain to have an influence on protein-substrate interactions and probably explains the good suppression of UV sensitivity.

βG1260D

Gly1260 is slightly further removed from the active centre and lies on what one would think of as the DNA exit pathway (Fig. 4.7). It is still encompassed by both regions implicated by cross-linking studies in making contact with the hybrid, the duplex and the RNA. Judging from the 3D model it seems likely that it is involved in making contact with the hybrid, as the latter is only separated by the β'_C-rudder which lies some way after Gly1260 on the exit path of the DNA. The same argument can be used for His1244 (see above). Though 16 amino acids removed from His1244, Gly1260 is still quite close to it on the structure, somewhat moved toward the DNA exit. The loop containing it is highly conserved for a long stretch and contains the GEME motif, implicated in promoter interaction (Cromie *et al.*, 1999).

A change in this position from the small aliphatic and hydrophobic glycine to the bigger acidic, charged and polar aspartic acid is well suited to influence interactions between RNAP and its substrate. Both H1244Q and G1260 lie in the C-terminal fragment of RpoB that has been connected with the modular ppGpp binding site (Toulokhonov *et al.*, 2001), which should also be taken into consideration.

4.5.2.4. A non *rpo** stringent mutation

For purposes of comparison, a stringent mutation was identified by sequencing that does not confer an *rpo** phenotype. It is located at position S788. Even from the linear map it is immediately obvious that there are no other *rpo** mutants in the vicinity, the nearest one being more than 200 residues away. *rpo** mutants all fall into clusters of at least two and Ser788 does not belong to one of them. Also the 3D model shows Ser788 to be in quite a different location from any *rpo** mutations (Fig. 4.11.). *rpo** mutations are located in the *rpo** pocket, the Rif pocket or near the active centre, all of them more or less exposed on the inner surface of the clamp formed by β and β'. S788F on the other hand lies deeply buried in the structure, somewhat on top of the most proximal Rif^R residues, when slicing the molecule in the middle and looking at β from below. The characteristic it shares with *rpo** mutants is its location on an extensive loop without helices or β-sheets. The change from the small and polar serine to the bigger hydrophobic and aromatic phenylalanine is quite likely to cause some structural change. How this could cause stringency in this position seems unclear. Unfortunately the range of mutations causing a stringent phenotype as described by Cashel *et al.* (1996) have not been published yet, so a broader comparison was not possible at this point. It would be very interesting to obtain more stringent mutations for comparison, to ascertain where exactly stringent mutations arise and what pattern they form on the structure of RNA polymerase. Judging from this result alone one would have to conclude that *rpo** mutations are a very special subclass of stringent mutations.

4.6. Discussion

4.6.1. Location of *rpo** mutations

In this work, twenty-seven *rpo** mutations have been sequenced and characterised. With the exception of β'E1146D and β'R1148H they all lie in highly conserved regions of RNAP. Twenty-one of them were found in RpoB, six in RpoC. This distribution is not likely to be representative and more RpoC* mutations are likely to be found, as this study was biased towards rifampicin resistant mutants. If *rpo** mutations are indeed a subclass of stringent mutations, to which the evidence points, then certainly RpoC* mutants remain to be identified. Whether RpoD* mutations exist depends on the mode of action of *rpo** mutations. RpoD mutations are unlikely to be able to decrease the half-life of stalled or paused RNA polymerase molecules, as the sigma subunit dissociates shortly after initiation. So if this is the mechanism of *rpo**, then there will be nor RpoD* mutations. If the *rpo** phenotype is achieved by modulation of promoter interaction only or at least in part, then RpoD* mutants are quite likely to exist.

The location of the *rpo** mutations on the linear map is not very elucidating. The concerned residues are spread all over the genes, though they form clusters in RpoB. Some of the regions had been implicated in substrate contacts by cross-linking studies: β130-239, β1232-1304 (Nudler *et al.*, 1998), β515-660 (Markovtsov *et al.*, 1996), β1237 (Mustaev *et al.*, 1991), β130-183, loop β163, loop β543, loop β568, β'215-220, β'298-330 (Korzheva *et al.*, 2000). But this only covered some of the *rpo** residues and a common feature could not be determined.

Mapping of the residues on the three-dimensional model revealed that all of the *rpo** residues were either in a position to make contact with the DNA substrate and RNA product respectively, or were likely to influence protein-substrate interactions by structural changes. All of the *rpo** residues lie on the inner surface of the clamp structure formed by RpoB and RpoC, or are buried just under the surface and likely to make structural changes. Of the RpoB mutations, some coincide with known rifampicin resistance mutations and some lie in the Rif pocket, but the others

form a separate, though for the most part adjacent region, the *rpo** region. This lies on the roof of the clamp, similar to the Rif pocket, moved to the distal end. There are also *rpo** residues not included in this region, H1244Q and G1260D, which lie near the C-terminus of RpoB, in a region associated by cross linking both with the active centre and the ppGpp binding site (see above). Both lie in a position to make substrate contacts. As the number of RpoC mutations is not as extensive it is more difficult to generalise. But again it can be said that all of them are in a position to either make contact with the substrate or to influence substrate contacts by structural changes. The most obviously important mutations lie on two rudder-like structures at the entrance and exit points of the DNA (β'_B and the β'_C rudder). Another one is right in the active site chamber, very near and opposite the active site Mg.

The one non-*rpo** stringent mutation that has been sequenced can clearly be differentiated by its location. While all *rpo** mutations lie on or at least very near the inner surface of the clamp, S788F is deeply buried within RNAP. The region it lies in is also very distinct from any *rpo** clusters in RpoB, both on the linear map and the three-dimensional structure. The stringency of this mutant can only be explained by structural changes that have far reaching consequences. Unfortunately no other sequence data of stringent mutations has been published as yet, as a wider comparison would be of interest.

4.6.2. Implications of previous work on RNAP mutants

Glass and co-workers (1986) have published two RpoB mutations (V736Q, F906Y) that were thought to render RNA polymerase resistant to (p)ppGpp. This means that in a relaxed strain, the addition of (p)ppGpp will not induce the stringent response. However, Baracchini and co-workers (1988) later found that the apparent insensitivity of those strains was due to reduced accumulation of (p)ppGpp when starved for only one amino acid. The actual property of the mutants was assumed to be increased pausing. First thought to lie in or near the (p)ppGpp binding site, they are both located at the top of loops on the upper outside surface of the β-subunit. How exactly they might influence pausing has not been resolved.

Other mutations conferring (p)ppGpp resistance to RNA polymerase were isolated by Tedin and Bremer (1992). Unfortunately no sequence data has been published for those mutations. Mutations like that could be simple changes to the (p)ppGpp binding site. On the other hand, they might also represent the opposite of *rpo** mutations. We assume that *rpo** mutations decrease the affinity of RNA polymerase for DNA (this is discussed in more detail in chapters 1 and 8). RNA polymerase mutants that don't respond to (p)ppGpp might have an increased affinity for DNA, which may at first sight be indistinguishable from a mutation in the (p)ppGpp binding site. However, they would be expected to show some indication of their genotype even under optimal growth conditions, as is the case for *rpo** mutants. If mutations like that do indeed exist, they would be of great interest in view of *rpo** and the question remains to be investigated in future studies.

Also (p)ppGpp hypersensitive mutants have been found (Little *et al.*, 1983b). Again the most likely explanation would be a mutation in the (p)ppGpp binding site, though the possibility remains, that they might have *rpo** characteristics. Unfortunately no sequence data for either (p)ppGpp resistant or hypersensitive mutations has been published, so it is not possible to resolve the question without further investigation. In addition, wider screens may be necessary to isolate mutations of interest. This leads to the question, whether RNAP*'s are hypersensitive to (p)ppGpp. The matter has not been specifically addressed in this work. What can be said from the available data is that the in vitro data (as presented in chapter 7) up to date does not confirm such a suggestion.

Four of the classic Rif^R mutants (Jin and Gross, 1988) have been studied in vitro in respect to their behaviour at stringently controlled promoters (Zhou and Jin, 1998). Fortuitously two of them are identical to mutations isolated also in this work as *rpo** (*rpoB*L533P and *rpoB*T563P) and the other two are very close (*rpoB*S531F and *rpoB*Δ532), which makes the results of Zhou's and Jin's experiments directly applicable. They found that the mutant RNA polymerases specifically reduce transcription from the two major promoters of the *rpoD* gene, encoding σ^{70}. As those promoters are under negative stringent control this result supports the hypothesis that

the RNAP mutants act as if in the presence of (p)ppGpp. The mechanism for this effect is the destabilising of mutant RNAP-stringent promoter complexes.

Bartlett and co-workers (1998) have studied other RNA polymerase mutants that are very closely related to *rpo** mutants described here. They report a mutant with the genotype *rpoC*Δ215-220, in contrast to *rpoC*K215E; *rpoC*R780C, close to *rpoC*K789Q described here and *rpoB*R454H, near *rpoB*L448I The mutations are close enough to make their studies relevant. Their findings are very similar to those of Zhou and Jin, (1998), showing instability of mutant RNAP-stringent promoter complexes. In addition they investigated the necessary concentrations of the initiating NTP. Much higher concentrations were required for efficient transcription by mutant RNA polymerase than wild type.

This intrinsic instability of stringent promoter complexes with two of the identified RNAP*s raises the possibility that also the stability of stalled complexes might be influenced. This question is of major interest for this work and will be addressed in chapter 7. RNAP*s forming less stable stalled complexes might explain the *rpo** phenotype of increasing UV-resistance in *rec* and *ruv* strains. If RNA polymerase stalled at lesions or pause sites would dissociate more readily, it would be more likely to allow repair of the lesion before the replisome can run into it and thus obviate the need for recombinational repair, involving the *rec* and *ruv* gene products.

Chapter 5.

UV light and induction of (p)ppGpp

5.1. Introduction

Lack of (p)ppGpp increases the severity of the UV sensitivity of *rec* and *ruv* strains. *rpo** mutations reverse this effect and even enhance survival by acting as if in the presence of excess (p)ppGpp (McGlynn and Lloyd, 2000). In some cases this works so well that the introduction of such a mutation into a *rec* or *ruv* only strain (where normal amounts of (p)ppGpp are present) increases its UV survival (see section 3.3). This leads to the conclusion that high levels of (p)ppGpp either somehow help with recombination and repair or avoid the necessity for RecBCD or RuvABC by helping to avoid DNA damage. McGlynn and Lloyd (2000) have shown that introduction of a *spoT1* mutation (which increases the amount of (p)ppGpp in the cell by abolishing degradation) greatly increases the UV survival rate of a *ruv* strain. Seeing that (p)ppGpp can perform this function, it would be logical for the cell to induce (p)ppGpp upon exposure to UV light.

There has been some indication that it might do so. Ramabhadran and Jagger (1976) showed that near-UV light affects the regulation of RNA synthesis, similar to amino-acid starvation. The effect depends on 4-thiouridine, found in 65% of tRNA species, which has an absorption maximum in the near-UV region. Exposure of 4-thiouridine leads to cross-linking, which makes the tRNAs poor substrates for aminoacylation (Yaniv *et al.*, 1971). The effect would be the same as amino-acid starvation. That (p)ppGpp levels actually rise in near-UV irradiated *Salmonella typhimurium* was demonstrated by Kramer and co-workers (1988). Could a similar scenario be true for UV light?

To test this probability, it was decided to investigate (p)ppGpp levels under conditions of starvation, UV light or with no stress, using wild type, *relA spoT*, *spoT1* and *rpo** strains. Kramer and co-workers (1988) had measured (p)ppGpp levels by purification of (p)ppGpp via a quaternary-amine solid-phase column and high-pressure liquid chromatography. Originally (p)ppGpp was discovered using polyethyleneimine-cellulose thin layer chromatography (Cashel, 1969). As the latter method seemed far more accessible it was used in these experiments. It consists of starving the cells for phosphate and then labelling the cultures with $K_2H[^{32}P]O_4$. Acid extraction is performed on the cells and the supernatant dropped onto a thin layer chromatography (TLC) plate and developed in one dimension. The result are a number of spots, representing U/C/A/GTP as well as pppGpp and ppGpp. The amounts of (p)ppGpp can then be compared between different strains and conditions and should answer the question whether UV has an effect on (p)ppGpp synthesis or not.

5.2. Starvation induces (p)ppGpp synthesis

First of all the method had to be established and it had to be demonstrated that it worked and showed recognisable differences between different strains and conditions. The protocol of Cashel (1969) was followed for the most part, with a few exceptions. Originally cultures were grown in synthetic low phosphate medium to allow labelling of (p)ppGpp with [^{32}P]. This is very slow and laborious however, and I found that it is sufficient to grow the cells in LB to the required OD, then spin them down, wash the pellet three times in the synthetic low phosphate medium and then resuspend them in it for further treatment. Instead of the mentioned Tris-glucose minimal medium MOPS medium was used, with the same concentrations of micronutrients, carbon sources, KH_2PO_4, bases, and for rich medium amino acids.

To ascertain that the procedure shows the increase of (p)ppGpp in starved cells, the following experiment was performed: wild type, *ΔrelA ΔspoT*, wt *rpo**, and *spoT1* strains were grown to OD_{650} 0.3 in LB, corresponding to a point in exponential phase. 50 µl were removed from the culture, the cells spun down in a microfuge and the pellet washed (redissolved and respun) in 0.3 ml of synthetic rich medium for

one batch and synthetic minimal medium for another. The washing procedure was repeated three times to remove as much phosphate as possible. The pellets were resuspended in 20 μl of the respective synthetic medium, 2 μl of $K_2H[^{32}P]O_4$ were added and the samples were incubated at 37°C for 30 min to allow labelling. Acid extraction was performed by addition of 1/10 volume of 11 M formic acid and 15 min incubation in ice-water. After a 1 min spin in a microcentrifuge, the supernatants were frozen and later used for chromatography. Scintillation counting was used to attempt the loading of equal amounts of radioactivity for each sample, when dropped on the filter. Filters were developed in one dimension with 1.5 M KH_2PO_4 (pH 3.4) to 17 cm above the origin and phospho-imager cassettes were used for visualisation.

Figure 5.1 shows the result. Under normal conditions, only the *spoT1* sample shows a trace of ppGpp (lane 4). This is the expected result, as a *spoT1* strain has a (p)ppGpp degradation deficiency. In the other lanes neither ppGpp nor pppGpp are detectable. Under conditions of starvation wild type (lane 5), *rpo** (lane 7) and *spoT1* (lane 8) samples show both ppGpp and pppGpp, as expected. The fact that the *spoT1* sample does not have more (p)ppGpp can not be satisfactorily explained. Wild type, *rpo** and Δ*relA* Δ*spoT* however conform to expectations and, the assay being established, experiments involving UV light were attempted.

5.3. Does UV light induce (p)ppGpp?

In order to answer this question, the same assay as in section 5.2 was used, with the modification that a range of samples was exposed to UV light. In order to achieve irradiation in liquid culture, 500 μl of culture (washed and resuspended in synthetic medium) were put on a small petri dish and irradiated for 20 sec under constant stirring. The result (Fig. 5.2) was disappointing. In rich medium (Fig. 5.2.A) only *spoT1* showed a slight and probably not significant increase in ppGpp (compare lanes 4 and 8). Under starvation conditions there is no discernible difference, if not a decrease of (p)ppGpp, between non irradiated and irradiated samples (Fig. 5.2.B). The experiment was repeated, irradiating for 60 sec. Again the result was tantalisingly inconclusive (Fig. 5.3.A). After 60 sec irradiation, there was

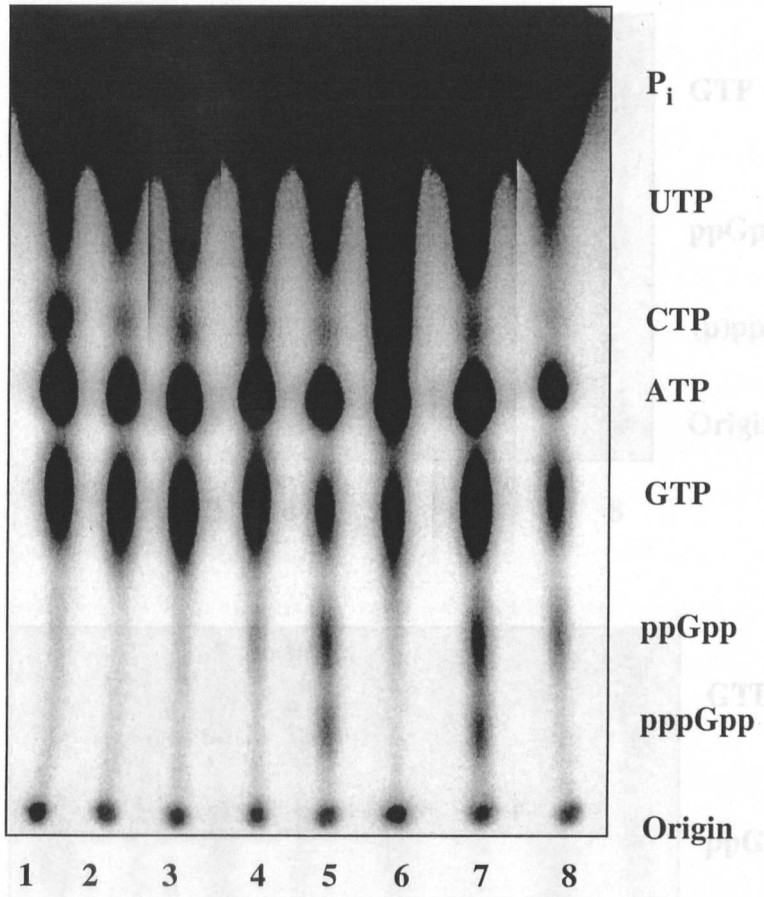

Figure 5.1. Thin layer chromatography (TLC) for the detection of ppGpp and pppGpp. Samples were labelled with $K_2H[^{32}P]O_4$. Lanes 1 and 5: wild type, lanes 2 and 6: (p)ppGpp^0 (*relA spoT*), lanes 3 and 7: *rpo**, lanes 4 and 8: *spoT1*. Cultures were starved for amino acids in lanes 5 to 8. Inorganic phosphate (P_i) - unincorporated label - travels fastest and forms the broad band on top of the TLC plate. This is followed by more or less well defined spots of UTP, CTP, ATP and GTP. ppGpp and pppGpp, being the biggest compounds, only travel a little way on the TLC plate and are found near the bottom (Cashel, 1969). The strains, identified by their genotype, are MG1665 (wild type), N4304 ((p)ppGpp^0), BT165 (*rpoB*$_{L571Q}$), N5020 (*spoT1*).

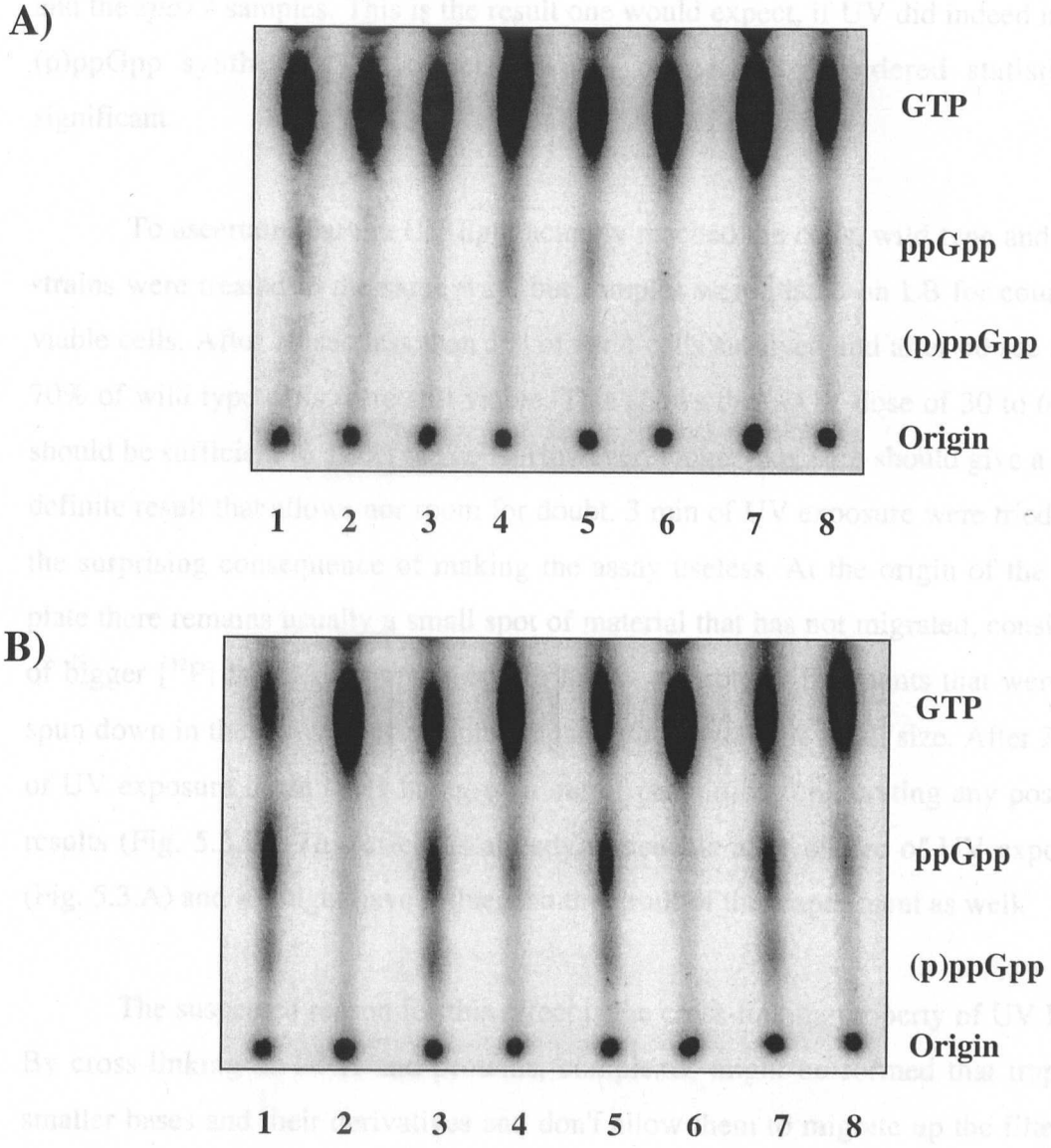

Figure 5.2. TLC showing samples that had been exposed to UV light. Lanes 1 and 5: wild type, lanes 2 and 6: (p)ppGpp^0 *(relA spoT)*, lanes 3 and 7:*rpo**, lanes 4 and 8: *spoT1*. **A)** Cells were incubated in rich medium. Samples for lanes 5 to 8 were exposed to 20 sec of UV light. **B)** Cells were starved for amino acids. Samples for lanes 5 to 8 were exposed to 20 sec of UV light. The strains, identified by their genotype, are MG1665 (wild type), N4304 (*relA spoT*), BT166 ($rpoB_{H551P}$), N5020 (*spoT1*).

indeed slightly more ppGpp than in the unirradiated samples, both in the wild type and the *spoT1* samples. This is the result one would expect, if UV did indeed induce (p)ppGpp synthesis. The effect however cannot be considered statistically significant.

To ascertain that the UV light actually reached the cells, wild type and *recA* strains were treated in the same way, but samples were plated on LB for counts of viable cells. After 30 sec less than 1% of *recA* cells survived and after 60 sec about 70% of wild type cells were still viable. This shows that a UV dose of 30 to 60 sec should be sufficient to affect the cells. However, longer exposure should give a more definite result that allows nor room for doubt. 3 min of UV exposure were tried with the surprising consequence of making the assay useless. At the origin of the TLC plate there remains usually a small spot of material that has not migrated, consisting of bigger [^{32}P] labelled compounds like DNA- or protein- fragments that were not spun down in the last step of sample preparation due to their small size. After 3 min of UV exposure those spots had grown out of proportion, obliterating any possible results (Fig. 5.3.B). This effect is already noticeable after 60 sec of UV exposure (Fig. 5.3.A) and so might have influenced the result of this experiment as well.

The suspected reason for this effect is the cross-linking property of UV light. By cross-linking of DNA and proteins, complexes might be formed that trap the smaller bases and their derivatives and don't allow them to migrate up the filter. In order to circumvent this problem several approaches were used on an irradiated wild type sample: It was incubated with the following chemicals/proteins/combinations: Proteinase K, Triton X-100, NaOH, Proteinase K and Triton X-100, Proteinase K and NaOH, Triton X-100 and NaOH, DNase, RNase, DNase and RNase, DNase and Proteinase K, RNase and Proteinase K. Some of the treatments reduced the origin-spot somewhat, especially combinations of Proteinase K and NaOH. None however were efficient enough to allow use in an assay with a negligible difference between origin-spots of irradiated and unirradiated samples. It has to be concluded that this method of determining variations in (p)ppGpp concentration is unsuitable for use in conjunction with UV irradiation.

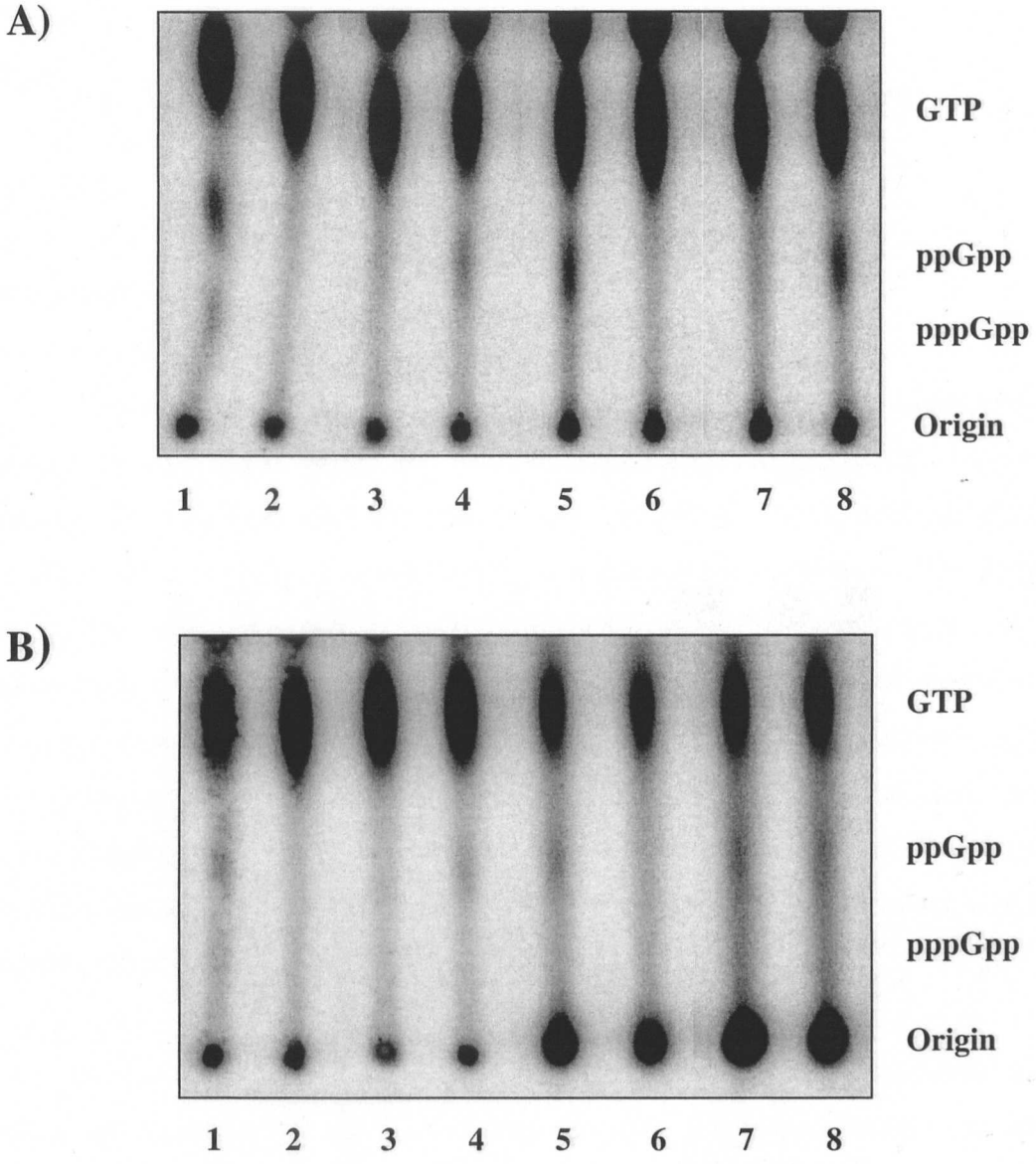

Figure 5.3. TLC, showing the effect of increased doses of UV light. Lanes 1 and 5: wild type, lanes 2 and 6: (p)ppGpp^0 (*relA spoT*), lanes 3 and 7: *rpo**, lanes 4 and 8: *spoT1*. Cells were grown in rich medium. **A)** Lanes 5 to 8 were exposed to 60 sec of UV light. **B)** Lanes 5 to 8 were exposed to 3 min of UV light. The strains, identified by their genotype, are MG1665 (wild type), N4304*(relA spoT)*, BT166 ($rpoB_{H551P}$), N5020 (*spoT1*).

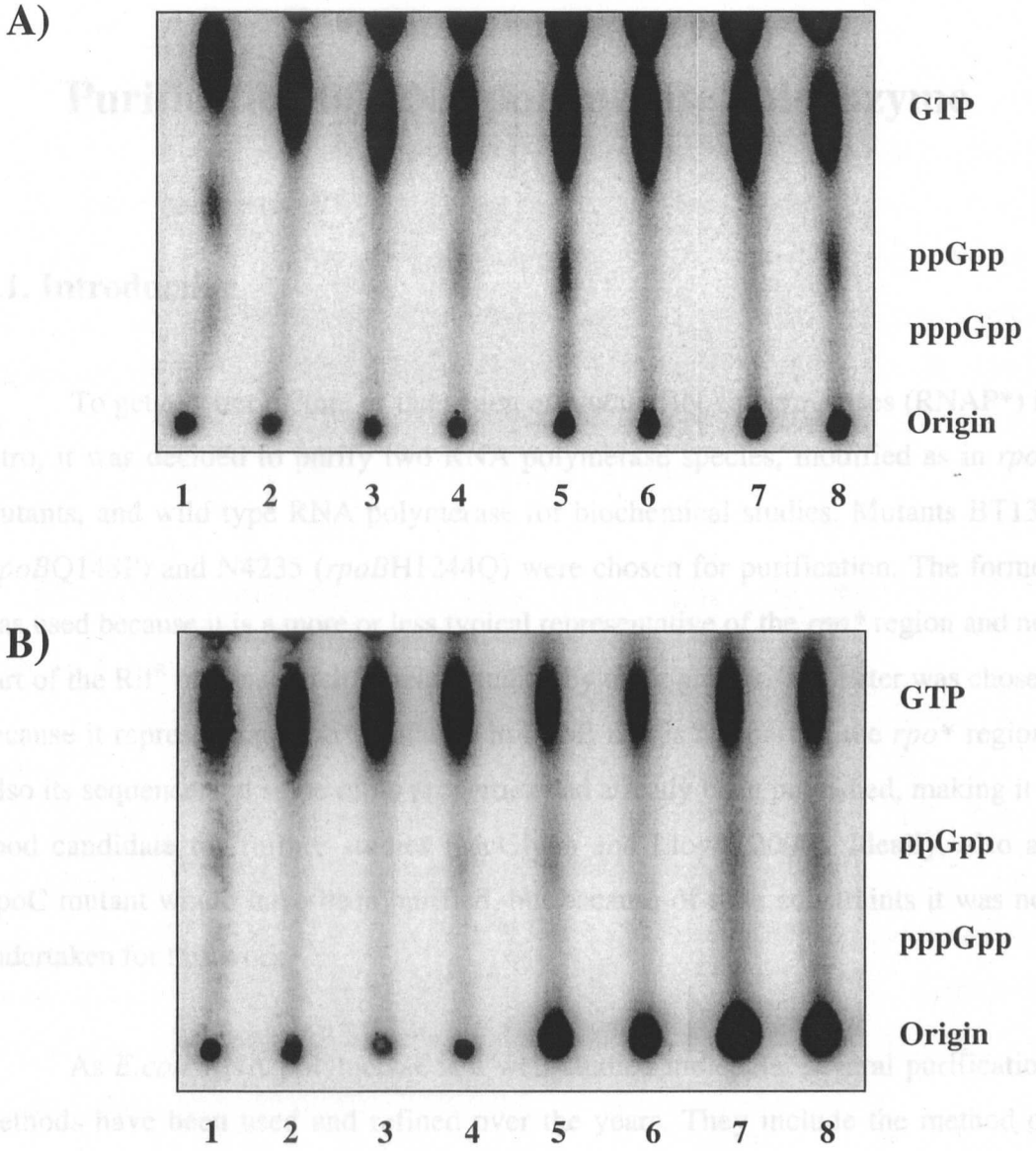

Figure 5.3. TLC, showing the effect of increased doses of UV light. Lanes 1 and 5: wild type, lanes 2 and 6: (p)ppGpp^0 (*relA spoT*), lanes 3 and 7: *rpo**, lanes 4 and 8: *spoT1*. Cells were grown in rich medium. **A)** Lanes 5 to 8 were exposed to 60 sec of UV light. **B)** Lanes 5 to 8 were exposed to 3 min of UV light. The strains, identified by their genotype, are MG1665 (wild type), N4304*(relA spoT)*, BT166 ($rpoB_{H551P}$), N5020 (*spoT1*).

Chapter 6.

Purification of RNA polymerase holoenzyme

6.1. Introduction

To get a better picture of the action of mutant RNA polymerases (RNAP*) in vitro, it was decided to purify two RNA polymerase species, modified as in *rpo** mutants, and wild type RNA polymerase for biochemical studies. Mutants BT134 (*rpoB*Q148P) and N4235 (*rpoB*H1244Q) were chosen for purification. The former was used because it is a more or less typical representative of the *rpo** region and not part of the Rif^R region, which is being studied by other groups. The latter was chosen because it represents an *rpo** mutation in RpoB that is not part of the *rpo** region. Also its sequence and some of its properties had already been published, making it a good candidate for further studies (McGlynn and Lloyd, 2000). Ideally, also an RpoC mutant would have been purified, but because of time constraints it was not undertaken for this work.

As *E.coli* RNA polymerase is a well-studied molecule, several purification methods have been used and refined over the years. They include the method of Burgess and Jendrisak (1975), involving polymin P precipitation and a DNA-Cellulose column. Zalenskaya and co-workers (1990) overexpressed the subunits singly and assembled them into recombinant RNA polymerase. Polyol-responsive monoclonal antibodies against RNA polymerase were used by Thompson and co-workers (1992). Niu and co-workers (1996) used a his-tagged α-subunit to affinity purify RNA polymerase holo-enzyme via a Ni-agarose column. After careful consideration the latter method was chosen for use with the *rpo** mutants. It provides a relatively simple way to purify mutant enzyme from the cells actually carrying the mutation on their chromosome. As the his-tag is on the α-subunit, it is unlikely to interfere with the effect of *rpo** mutations. The plasmid can be introduced into any

without the risk of contamination by wild type β-subunit, as the only β present in the cell will be *rpo**.

The protocol includes introduction of a plasmid (pREII-NHα) into wild type and the two mutant strains respectively. The plasmid overexpresses his-tagged RpoA, when induced with IPTG. As there is then an excess of his-tagged RpoA over wild type RpoA present in the cell, a majority of RNA polymerases will have incorporated at least one his-tagged α-subunit. Cell lysis, polymin P precipitation and ammonium sulfate precipitation are performed as in Burgess and Jendrisak (1975). Further purification is achieved with help of a Nickel-agarose column, followed by Mono-Q chromatography (Borukhov and Goldfarb, 1993).

6.2. Overexpression of a his-tagged α-subunit in the *rpo** strain(s)

To allow purification of RNA polymerase holo enzyme, a his-tagged α-subunit was overexpressed in either wild type or the respective strain containing an *rpo** mutation. The respective strain was transformed with pREII-NHα, which makes them resistant to ampicillin. Fresh overnight cultures were used to inoculate 4 x Mu broth. 200 μg/ml Ap and 5 mM IPTG were added immediately and the culture grown up to an OD_{650} of about 1.5 in a shaking incubator at 37°C. Cells were harvested by centrifugation and pellets stored at -20 °C until used. Two litres of culture yielded about six grams of wet cells.

6.3. Purification

To purify the RNA polymerases with help of the his-tagged α subunit the method of Niu and co-workers (1996) was used, with some modifications as stated. This method is a synthesis of cell lysis and ammonium sulfate precipitation as in Burgess and Jendrisak (1975), a Nickel-column and Mono-Q column (Borukhov and Goldfarb, 1993). Polymin P precipitation was discarded after a few failed attempts, as it seemed to achieve the opposite of the desired result and behaved very differently from how it is described. This eliminates a major purification step, as

stated by Burgess and Jendrisak (1975), but it critically improved the yield and did not seem to have adverse effects on purity.

6.3.1. Preparation of crude extract

Two to three cell pellets, collected from 1 l of induced cultures were used per purification. They were resuspended in 9 ml of grinding buffer each, on ice. 150 μl of 16% Na-deoxycholate per pellet were added and incubated at room temperature for 20 min. 11 ml of TGED (0.2 M NaCl) were added before thorough sonication. The sample was centrifuged at 10,000 rpm for 45 min and the supernatant collected. After addition of 35 g/100 ml ammonium sulfate the sample was incubated at 4°C for 20 min and then centrifuged for 20 min at 15,000 rpm. The pellet (PI) was resuspended in 10 ml buffer A per initial cell pellet and run through a 5 μm filter for clarification of the solution. The supernatant of the ammonium sulfate precipitation contains a comparatively large amount of his-tagged α-subunit. Judging from the absence of β and β' it is probably free excess his-tagged α that has not been incorporated into RNA polymerae holo enzyme (Fig. 6.1, lane 1). The crude PI extract still contained a vast number of proteins. The his-tagged α-subunit is already visible as a major band and in addition also β and β' are present (Fig. 6.1, lane 2).

6.3.2. Nickel-NTA batch purification

2 ml Ni-NTA agarose were equilibrated with 6 ml buffer A. The crude, clarified sample from 6.3.1. was applied to the agarose and incubated over night under gentle shaking. The Ni-agarose was then "harvested" by centrifugation (supernatant = flow-through). This procedure was repeated for the wash and elution steps, using 20 min incubations for each step. The wash consisted of 20 ml buffer A. Bound protein was eluted in 5 steps with 6 ml buffer A and 2.5, 5, 10, 20 and 40 mM imidazole respectively. In both the flow-through and wash fractions hardly any his-tagged α-subunit, nor β or β' are present, as judged from the protein gel (Fig. 6.1, lanes 9 and 10). All of the elution fractions contain some RNA polymerase holo enzyme (as shown by the presence of α, β, β' and σ; Fig. 6.1, lanes 3 - 5, 7 and 8), but also still an array of other proteins. The fractions containing 10 and 20 mM

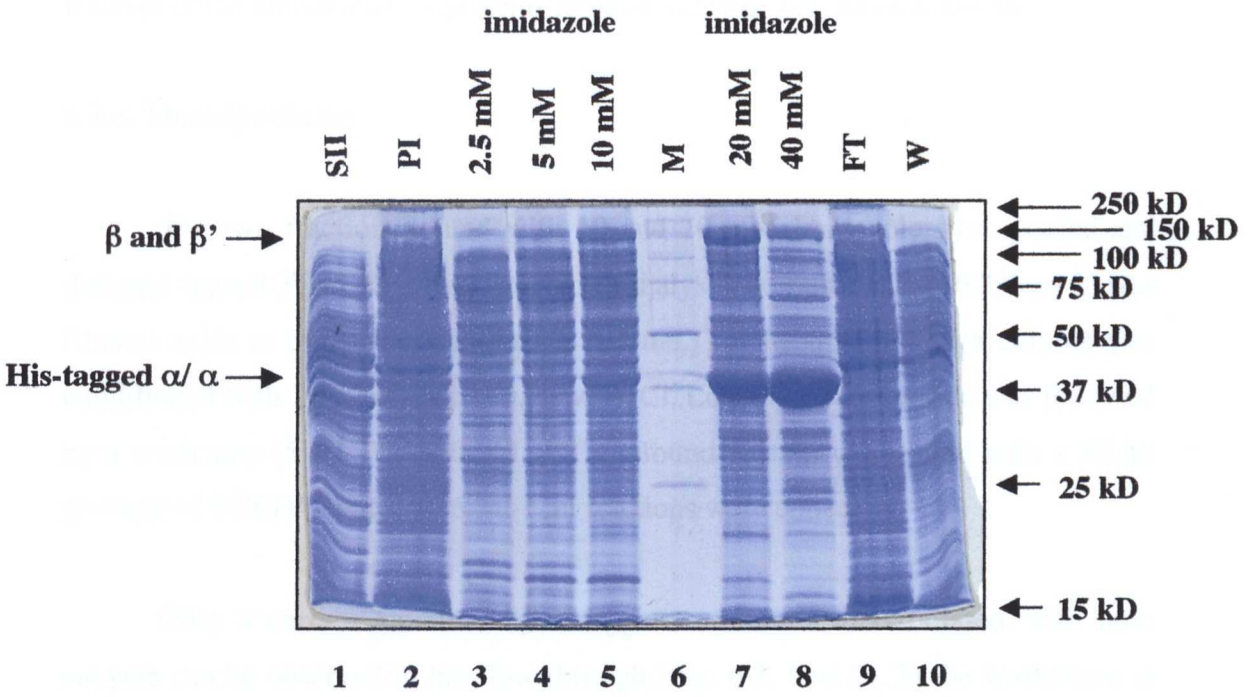

Figure 6.1. 12.5 % SDS protein gel showing the results of the initial purification steps. Lane 1: SII extract - the supernatant of the ammonium sulfate precipitation step. Lane 2: PI - the ammonium sulfate precipitation pellet. Lanes 3 - 5, 7 and 8: fractions from the Ni-agarose column, eluted with the indicated amounts of imidazole. Lane 9: flow through (Ni-agarose column). Lane 10: wash (Ni-agarose column). About 10 μg of protein were loaded for each lane. Lane 6 is the molecular weight marker.

imidazole were chosen for further purification, containing enough of all 3 different subunits to make further purification worth while (Fig. 6.1, lanes 5 and 7). Fraction "40" contained a vast excess of his-tagged α-subunit that is not part of a holo enzyme (Fig. 6.1, lane 8). Fractions "2.5" and "5" contained too little RNA polymerase as a fraction of the contaminating proteins to be of use (Fig. 6.1, lanes 3 and 4).

6.3.3. MonoQ column

The two fractions eluted with 10 and 20 mM imidazole were pooled and dialysed against TGED/0.25 M NaCl. After dialysis the sample (Fig. 6.2, lane 1) was filtered again to avoid blocking of the column. The Mono Q HR 5/5 column was equilibrated with 10 ml of TGED/0.25 M NaCl. Loading of the sample was followed by a wash step (5 ml TGED/0.3M NaCl). Bound protein was eluted with a 50 ml gradient of TGED/0.35 M – 0.5 M. 1 ml fractions were collected.

Only a certain amount of his-tagged α-subunit unassociated with holo enzyme can be observed in the flow-through (Fig. 6.2, lane 2). In the wash there is again a small fraction of holo enzyme (as indicated by the presence of some β and β' subunit), but again mostly unassociated his-tagged α-subunit (Fig. 6.2, lane 3). The first peak, spanning fractions 2 – 4 and eluting at 0.353 – 0.359 M NaCl contained α, β, and β', but no or very little σ-subunit, which represents the core enzyme (Fig. 6.2, lanes 3 and 4). The second peak, however, represented by fractions 5 and 6 and eluting at a NaCl concentration of 0.362 M – 0.365 M contained also a significant amount of σ-factor besides the core enzyme (Fig. 6.2, lanes 7 and 8), justifying the assumption that the majority of the RNA polymerase in those fractions would be holo enzyme. Fraction 10 and 11, representing a third low peak, also contain holo enzyme (Fig. 6.2, lanes 9 and 10). The protein concentration however is very low in those fractions, and they contain additional impurities, making them unsuitable for direct use.

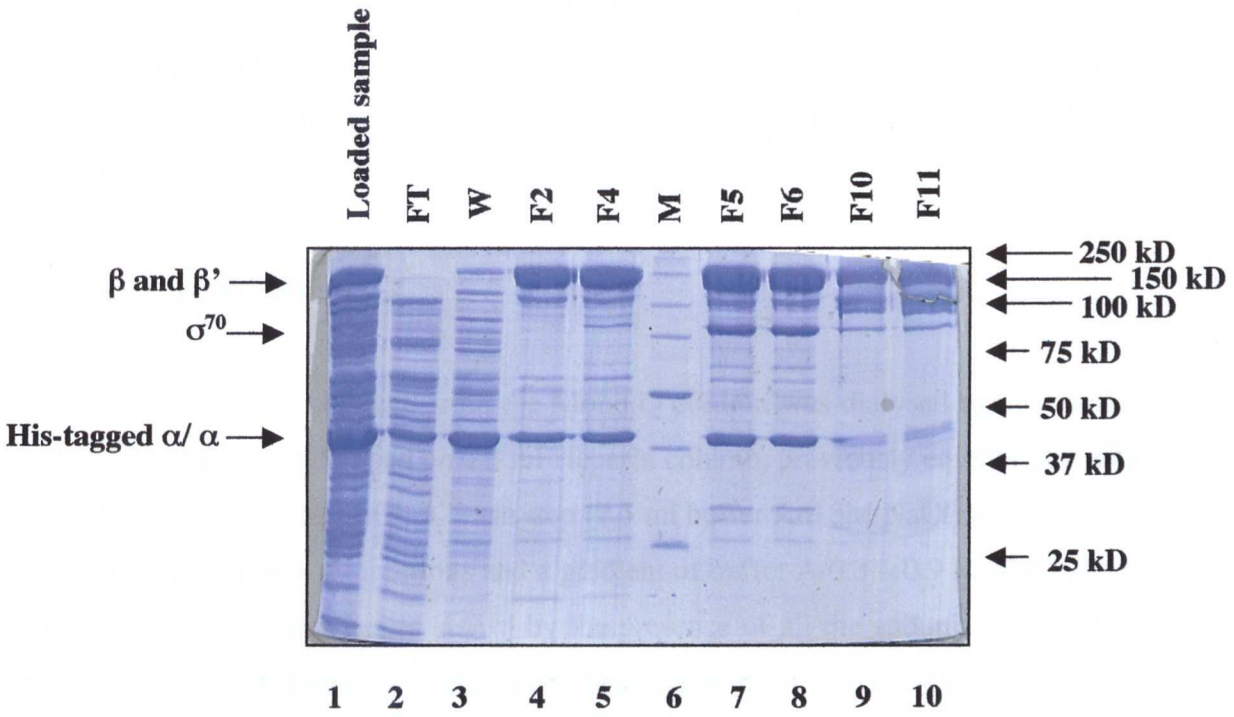

Figure 6.2. 12.5% protein gel, demonstrating the result of the Mono Q column purification step. About 10 μg of protein were loaded in each lane. Lane 1: the pooled fractions from the Ni-agarose column, for loading on the Mono Q column. Lanes 2 and 3: flowthrough and wash respectively. Lanes 4 - 5 and 7 - 10: fractions from the Mono Q column that contained protein peaks. Lane 6: molecular weight marker.

In two out of three cases RNA polymerase obtained from the Mono Q column contained only minor impurities (as shown in Fig. 6.2 for wild type RNA polymerase) and was suitable to be used in band shifts and in vitro transcription assays. In those cases the sample was dialysed against storage buffer and kept at –20°C. One mutant however (RNAPβH1244Q) was not pure enough at that stage, containing a DNase contaminant and requiring further purification. The method of choice was a Heparin column, roughly following the protocol of Kashlev and co-workers (1993).

6.3.4. Heparin column

The sample obtained from the Mono Q column was dialysed against buffer A/0.3 M NaCl and adsorbed on a 3 ml Heparin column, previously equilibrated with 10 ml buffer A/0.3 M NaCl. A wash step (7.5 ml buffer A/0.3M NaCl) was followed by elution with 30 1 ml fractions and a gradient of buffer A/0.3 – 0.9 M NaCl. RNA polymerase holo enzyme (as shown by the presence of all the subunits, Fig. 6.3.A, lanes 5 - 8 and B, lanes 2 - 7) eluted in a broad peak from fraction 8 to 17 at a NaCl concentration of 0.46 M to 0.64 M. Protein gels were silver stained in this case, as this method is more sensitive, and the purification step as well as remaining impurities could be more precisely assessed. Contamination is visibly diminished in all the fractions of the peak, compared to the loaded sample (Fig. 6.3.A, lane 1). The remaining DNase contamination was negligible. The peak fractions were pooled, again dialysed against storage buffer and kept at –20 °C.

6.4. Identification with anti-holo enzyme antibodies

To prove that the bands on the protein gel, migrating at the position that the RNA polymerase subunits do, were actually the α, β, β' and σ-subunits, as suspected, antibodies were used. Commercial *E.coli* RNA polymerase was run on a SDS PAGE side by side with a sample of the his-tag purified RNA polymerase. A Western blot was performed, using anti-holo enzyme antibodies (Fig. 6.4). Apart from a slightly higher amount of commercial polymerase loaded and therefore stronger bands, there is no discernible difference between the two enzymes. It can therefore be concluded

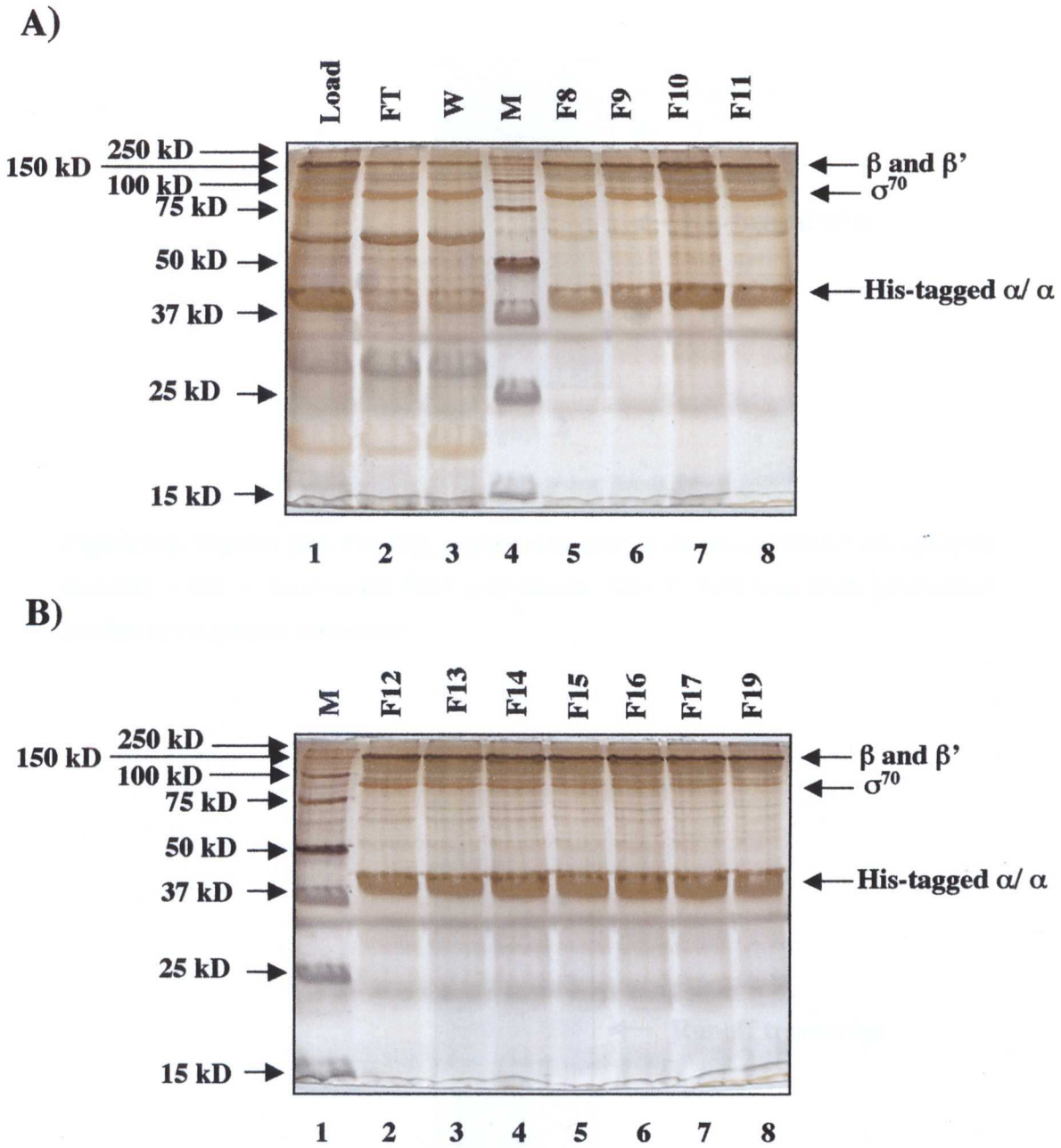

Figure 6.3. Key fractions from the heparin column purification step on a 12.5% protein gel. Proteins were visualised by silver staining. About 10 μg of protein were loaded per lane. **A)** Lane 1: loaded sample (pooled peak fractions from the Mono Q column). Lanes 2 and 3: flowthrough and wash respectively. Lane 4: molecular weight marker. Lanes 5 - 8: fractions from the broad but low peak that eluted from the heparin column. **B)** Lane 1: molecular weight marker; lanes 2 - 8: fractions from the broad but low peak that eluted from the heparin column.

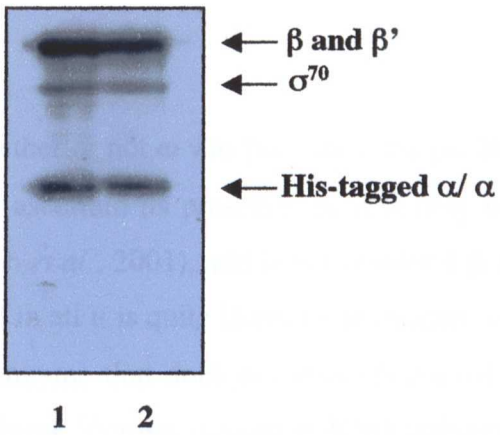

Figure 6.4. Western blot. The ECL system was used to detect anti-RNAP holo enzyme antibody. Lane 1: commercial RNA polymerase; lane 2: wild type RNA polymerase purified in the manner described.

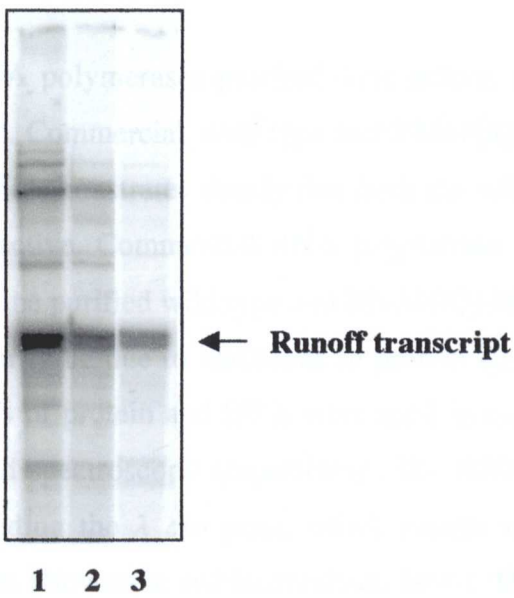

Figure 6.5. In vitro transcription assay. Each reaction contained about 1.37 pM [^{32}P] end-labelled *λcro* DNA and 3.3 pM of protein in a reaction volume of 50 μl. (Final DNA and protein concentrations were 25 nM and 65 nM respectively.) Lane 1: commercial RNAP, lane 2: wild type RNAP, lane 3: RNAPβQ148P.

that all the subunits are present and the suspected bands are indeed the RNA polymerase subunits.

It was not determined, whether or not ω was present in the purified complex. No particular effort was made to ascertain its presence, as it is only necessary for RNA polymerase assembly (Ghosh *et al.*, 2001), and is not involved in the stringent response (Gentry *et al.*, 1991). All in all it is quite likely to be present, as it seems to be an integral part of the holo enzyme that does not dissociate easily [eg it was fortuitously present in the crystallised *Thermus aquaticus* RNA polymerase (Zhang *et al.*, 1999)]. If it is present, the failure of its detection by the anti-holo enzyme antibodies is due to its small size of about 10 kD, which would lead to its running off the protein gel.

6.5. The purified RNA polymerases are active

To show whether the RNA polymerases purified were active, an in vitro transcription assay was performed. Commercial, wild type and RNAPβQ148P were compared side by side. Figure 6.5 demonstrates clearly that both the wild type and RNAPβQ148P polymerases are active. Commercial RNA polymerase appears to have the highest specific activity, the purified wild type and RNAPβQ148P showing less activity. This may at least partly be due to variations in protein concentration measurements. The same amounts of protein and DNA were used in each lane, as estimated by Bradford assays and spectroscopy respectively. The DNA substrate used was a DNA fragment encoding the λ *cro* gene, which results in a runoff transcript length of 372 nucleotides (Nowatzke and Richardson, 1996). The fact that RNAPβQ148P is less active than wild type enzyme that was purified in the same manner may be significant and should be kept in mind with regard to later experiments. An RNAP* would be expected to produce less transcript from most promoters, as it acts as if in the presence of (p)ppGpp, which downregulates most genes.

As RNAPβH1244Q was purified at a later time it is not included in this activity assay. The activity of this mutant was demonstrated directly on a band shift assay (see chapter 7).

As RNAPβH1244Q was purified at a later time it is not included in this activity assay. The activity of this mutant was demonstrated directly on a band shift assay (see chapter 7).

6.6. Summary

Three different RNA polymerases (the wild type, *rpo**Q148P and *rpo**H1244Q) were purified. The method used was composed from different protocols. It relied principally on a his-tagged α-subunit, with help of which holo enzyme, wild type and *rpo** alike, could be fished out via Nickel agarose. Further purification steps included a MonoQ and in one case a Heparin column. The resulting sample contained the pooled and dialysed fractions from the holo enzyme peak of the MonoQ column and the peak of the Heparin column resprectively. When run on a protein gel, only very few secondary bands are present, representing a negligible amount of protein, comparable to commercially obtained *E.coli* RNA polymerase. The major bands clearly are the α-, β-, β'- and σ-subunits, migrating at their reported molecular weights (σ^{70} is known to migrate at a molecular weight of around 80 kD). As additional proof, immunoblotting with anti-holo enzyme antibodies was performed, confirming the proteins as the RNA polymerase subunits.

This method proved to be a reliable and reproducible way of purifying both wild type and different RNAP*'s as holo enzymes and in an active form. Due to the his-tagged α-subunit, RNAP*'s with mutations in either RpoB, RpoC or RpoD can be easily purified without having to modify the procedure.

Chapter 7.

Activity of wild type and mutant RNA polymerases and effect of (p)ppGpp

7.1. Introduction

The role of (p)ppGpp and their mode of action have been a topic of lively debate for more than 20 years. As the effector molecules of the stringent response (Cashel, 1969) they were known to be induced upon amino acid starvation and other kinds of stress and to at least participate in the downregulation of mRNA and rRNA genes (Borek *et al.*, 1956). The results of early investigations indicated that (p)ppGpp upregulate amino acid biosynthetic operons via the amino acid pool (Messenguy, 1979), lower transcription elongation rate by enhanced pausing (Kingston *et al.*, 1981) and enhance termination by NusA from the *rrnB P1* promoter (Kingston and Chamberlin, 1981). (p)ppGpp was soon found to have some connection to RpoB, as suggested by several studies (e.g. Kingston and Chamberlin, 1981; van Ooyen *et al.*, 1976) and the mapping of (p)ppGpp "resistant" mutations to the *rpoB* gene (e.g. Nene and Glass, 1983; Tedin and Bremer, 1992). (p)ppGpp was shown to bind both the N- and C-terminal domains of the β-subunit of RNA polymerase (Chatterji *et al.*, 1998). Their result was refined by Toulokhonov *et al.* (2001), who showed that the (p)ppGpp binding site on RNA polymerase is allosteric, modular, and involves the β' N-terminus together with the β C-terminus. This data suggests a direct effect of (p)ppGpp on regulation of transcription via RNA polymerase. There is strong evidence supporting this view at least for genes that are negatively controlled by the stringent response, like *rrn* genes (Barker *et al.*, 2001a; Barker *et al.*, 2001b). Evidence whether or not (p)ppGpp is involved in growth-rate control is conflicting (Gaal *et al.*, 1997; Liang *et al.*, 1999) and the question is as yet unresolved.

A number of mutants have been identified that mimic the effect of (p)ppGpp (Bartlett *et al.*, 1998; McGlynn and Lloyd, 2000; Zhou and Jin, 1998). Some of them have been well studied in vivo and/or in vitro with respect to their behaviour on different promoters and substrates in comparison to wild type enzyme. As most of the mutants can be said to be at least somewhat related if not identical to the RNA polymerase mutations discussed here, the results of these experiments are of great interest and may, with limitations, be true for them as well. Zhou and Jin (1998) have performed experiments on four mutants: *rpoB*S531F, *rpoB*Δ532, *rpoB*L533P and *rpoB*T563P, the latter two of which are identical to mutations isolated here independently. All four mutants were initially identified as Rif^R alleles (Jin and Gross, 1988) and had been noted for their enhancement of the temperature sensitivity of a *rpoD* mutant (Jin and Gross, 1989), which was found to be due to decreased transcription from the *rpoD* operon (Zhou and Jin, 1997). The underlying mechanism of this effect was identified as unstable interactions between promoters under negative stringent control and the mutant RNA polymerase (Zhou and Jin, 1998). Mutant polymerases with the genotypes *rpoB*R454H, *rpoC*Δ215-220, *rpoC*R780C were investigated by Bartlett and co-workers (1998). The mutations concern residues in the vicinity of mutations described in this work. They also found their mutants to form very unstable complexes with stringent promoters. More detailed investigations revealed, that (p)ppGpp or RNA polymerase mutants decreased the half-lives of open complexes at all promoters tested, whether they were regulated negatively, positively or not at all (Barker *et al.*, 2001b).

Most studies so far concentrated on the effects of (p)ppGpp or RNA polymerase mutants on the initiation steps of transcription. It was however also shown that at least some (p)ppGpp-mediated inhibition can take place after open complex formation (Kajitani and Ishihama, 1984). Observed effects of (p)ppGpp on transcription elongation rates were found to be due to enhanced pausing that was strongest when transcription had been initiated from promoters under negative stringent control (Krohn and Wagner, 1996; Sorensen *et al.*, 1994). This led to the conclusion that the promoter discriminator sequence can influence pausing later on.

7.1.1. Objectives

Taken together, those results do not provide a satisfactory explanation for the mechanism of *rpo**. How do mutant RNA polymerases increase the UV sensitivity of *rec* and *ruv* strains? A possible clue was provided by the work of McGlynn and Lloyd, (2000). Their model (discussed in more detail in chapters 1 and 8) proposed that both RuvABC and RecBCD were part of a repair pathway for stalled replication forks. In brief, RNA polymerase stalled at a lesion would both create a possible block for the replisome and delay repair of the lesion, which in turn could then create an obstacle for the replisome. The latter would dissociate, leaving the fork to regress and form a Holliday junction, the resolving of which requires the RuvABC resolvasome and the RecBCD complex. One of them being absent, this repair pathway is blocked. Forcing the cell to use other, less effective means of dealing with the problem might explain those mutants increased UV sensitivity. One can speculate now that lower affinity of RNA polymerase for DNA and so quicker dissociation from the DNA when stalled at a lesion would make the latter accessible for repair. This in turn would reduce the number of obstacles for the replisome, relieving the need for RuvABC and RecBCD and partly restoring UV tolerance.

I wanted to investigate whether this was indeed the mechanism of increase of UV resistance of *rec* and *ruv* strains by RNA polymerase mutants. It had to be determined, whether or not RNAP*'s have a lower affinity to DNA in general and whether they would dissociate more easily when stalled on the template. In order to answer those questions, electrophoretic mobility shift assays (EMSA, band shift assays) were used. Radiolabelled DNA was incubated with RNA polymerase under varying conditions, yielding open complexes that could be separated on agarose gels. The formation of stalled complexes was achieved either by omitting one nucleotide or by UV irradiation of the DNA substrate to introduce lesions blocking transcription. Open and stalled complexes can readily be separated on agarose gels. As a basic, general DNA substrate a piece of the λ *cro* gene was chosen. It is well established as a substrate for in vitro transcription (Nowatzke and Richardson, 1996). λ *cro* also provides in all probability a neutral substrate with respect to (p)ppGpp regulation that still has a strong promoter, in contrast to the also relatively neutral *lacUV5*, with its a priori weak constitutive promoter. The λ *cro* fragment can easily

be amplified by PCR from a plasmid and also contains a mutation of C to G at position +7 of the *cro* gene, which allows the formation of complexes stalled at position +10 by omitting CTP from the assay. This is important, as RNA polymerase is only finally committed to elongation after about 10 nucleotides and retains the σ-factor for the first 5 - 8 nucleotides (Krummel and Chamberlin, 1989). My findings agree with this assumption, as I have been routinely able to separate open and stalled complexes by virtue of the faster migration of the smaller elongation complexes. In contrast, Mukhopadhyay and co-workers (2001) report that the σ-factor actually translocates with RNA polymerase during elongation. They explain previous findings by proposing decreased stability of the σ^{70}-RNA polymerase complex at the transition from initiation to elongation. Decreased stability of the connection of σ^{70} with RNA polymerase could also explain that complexes of different size are observed in band shift assays.

7.1.2. Establishing λ *cro* band shift assays for measuring RNAP transcription complexes

The aim of these experiments was to visualise DNA substrate and stalled complexes in order to compare the stabilities of the latter with different RNA polymerases and under different conditions. The preparation of samples for band shifts differs only very little from in vitro transcription assays. For both procedures well-established protocols with only minor differences are available, which were adapted for this purpose. DNA substrate is incubated with RNA polymerase for open complex (OC) formation. To produce run-off transcripts, all four nucleotides are added. To produce stalled complexes, either only three nucleotides are added, halting the RNA polymerase, or the DNA substrate is UV-irradiated prior to use, which introduces various lesions that also lead to stalling of the transcription complex. DNA and the different complexes were resolved on agarose gels. In Figure 7.1, lane 1, substrate only has been loaded and forms a sharply defined band. This is not influenced by the addition of heparin (lane 2). Upon addition of RNA polymerase, one sharp band appears (assumed to represent open complex), along with a smear of bigger complexes. When heparin is added at the end of this same reaction (lane 3), the unspecific bands disappear, the sharp band remaining, confirming the assumption that it represents open complex. Upon addition of heparin along with RNA

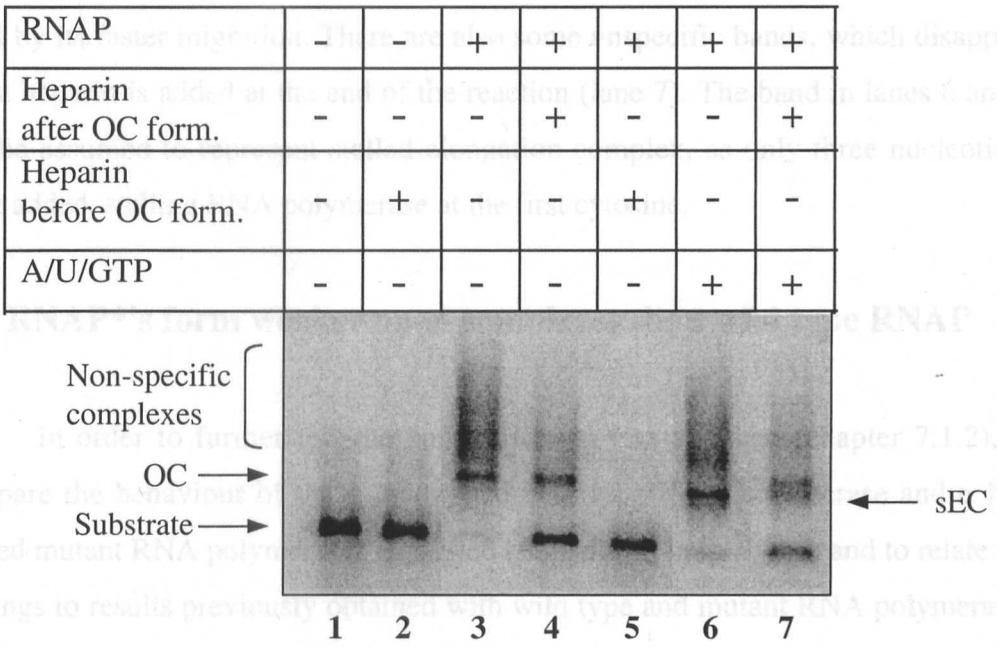

Figure 7.1. Band shift assay showing formation of RNAP complexes. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled *λcro* DNA. *E.coli* RNA polymerase (obtained commercially), nucleotides and heparin were added as indicated to a final concentration of 10 nM, 2 μM and 2.5 μg/μl respectively. Open complexes were formed in the presence (lane 5) or absence (lanes 3 and 4) of heparin by incubation at 37°C for 3 min. The reactions were then kept on ice during the addition of nucleotides (as indicated). All samples were then transferred to 37°C for 20 min. Lane 1: DNA substrate only. Lane 2: as lane 1, heparin added at the start. Lane 3: RNA polymerase and DNA. Lane 4: as lane 3; heparin was added after the 20 min incubation step. Lane 5: as lane 3; heparin was added at the very start, before the 3 min incubation. Lane 6: as lane 3; nucleotides were added. Lane 7: as lane 6; heparin was added after the 20 min elongation step. OC - open complex. sEC - stalled elongation complex.

polymerase before the formation of open complexes is initiated by transferring the reaction to 37°C, no band appears. Open complex formation is inhibited. When, after the formation of open complexes, three nucleotides are added and incubated for another 20 min, another sharp band appears (lane 6), distinct from the open complex band by its faster migration. There are also some unspecific bands, which disappear when heparin is added at the end of the reaction (lane 7). The band in lanes 6 and 7 can be assumed to represent stalled elongation complex, as only three nucleotides were added, stalling RNA polymerase at the first cytosine.

7.2. RNAP*'s form weaker open complexes than wild type RNAP

In order to further test the band shift assay established (chapter 7.1.2), to compare the behaviour of the α his-tagged wild type RNA polymerase and α his-tagged mutant RNA polymerases identified and isolated in this work and to relate the findings to results previously obtained with wild type and mutant RNA polymerases (Barker *et al.*, 2001a; Barker *et al.*, 2001b; Zhou and Jin, 1998), open complex formation was investigated. The mutant RNA polymerases used in the in vitro studies were *rpoB*Q148P and *rpoB*H1244Q. Both mutations lie in well defined conserved regions of RpoB (see chapter 4 for details), but no in vitro studies had been done on such mutants to date. Would they behave like the previously investigated stringent RNA polymerase mutants and decrease stability of open complexes? Figure 7.2 shows that this is indeed the case. While the concentration of RNA polymerase was the same for wild type and mutants and it was added in 20-fold molar excess over DNA, both mutant enzymes hardly show any sign of open complexes (lanes 3 and 4), whereas the wild type RNAP gives a strong signal (lane 2). It can be concluded that, as suggested by in vitro data from stringent mutants similar or identical to RNA polymerase mutants identified here, RNAP*'s also decrease stability of open complexes.

Run-off transcription and ternary complexes stalled by amino acid starvation were also examined, as described by Nowatzke and Richardson (1996). As demonstrated already in Figure 7.1, wild type RNAP open complexes are far more stable with the wild type protein than with the mutant RNA polymerases (Fig. 7.2).

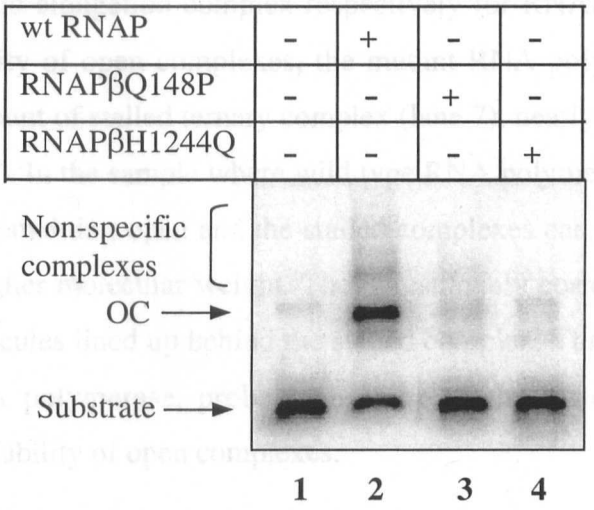

Figure 7.2. Open complex formation by wild type and mutant RNA polymerases. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA. RNA polymerases were added as indicated to a final concentration of 10 nM. 2.5 μg/μl heparin was added to all reactions after the 20 min incubation step. Lane 1: substrate only. Lane 2: wild type RNA polymerase added for open complex (OC) formation. Lane 3: as lane 2, but with RNAPβQ148P. Lane 4: as lane 2, but with RNAPβH1244Q.

For stalled ternary complexes the situation is slightly different (Fig. 7.3). Lanes 2 to 4 show the results of reactions performed in order to achieve open complex formation, run-off transcription and stalled elongation complexes respectively. Wild type RNA polymerase was used. In the run-off transcription (lane 2) weak signals of both open and elongation complexes are present. Lanes 5 to 7 show open complex, run-off and stalled elongation complex respectively for RNAPβQ148P. In spite of the high instability of open complexes, the mutant RNA polymerase still forms a considerable amount of stalled ternary complex (lane 7), nearly to the same extent as wild type (lane 4). In the sample where wild type RNA polymerase was used, a faint band above the remaining open and the stalled complexes can be seen, representing complexes of higher molecular weight. They presumably correspond to other RNA polymerase molecules lined up behind the stalled complex. This does not occur with the mutant RNA polymerase, probably because of the lower rate of successful initiation and instability of open complexes.

Both in Figure 7.2, lanes 3 and 4, and 7.3, lane 5 a second faint band, apart from the trace of open complex, is present in the lanes where open complexes have been formed with mutant RNA polymerases. No nucleotides were added to the samples. A conclusive explanation for this has yet to be found. I can only speculate that, as proposed by Hernandez and Cashel (1995), the mutant RNA polymerases - by mimicking the effect of (p)ppGpp - have indeed a lower affinity to the σ-factor, allowing it to dissociate even at the stage of open complex formation in some cases.

7.3. UV light stalls wild type RNAP and RNAP*

Since *rpo** mutations are defined by their effects on the survival of UV-irradiated cells, it was important to determine whether RNA polymerase could in fact be stalled in vitro by lesions induced by UV-irradiation of the template. If RNA polymerase does stall at lesions, this would delay repair by making them inaccessible to repair enzymes. Either the lesions themselves or RNA polymerase stalled at a lesion might then create a roadblock to replication. To demonstrate whether irradiation with UV light stalls RNA polymerase, the DNA template was exposed to a dose of UV light prior to use in a band shift assay. Figure 7.4.A shows the results

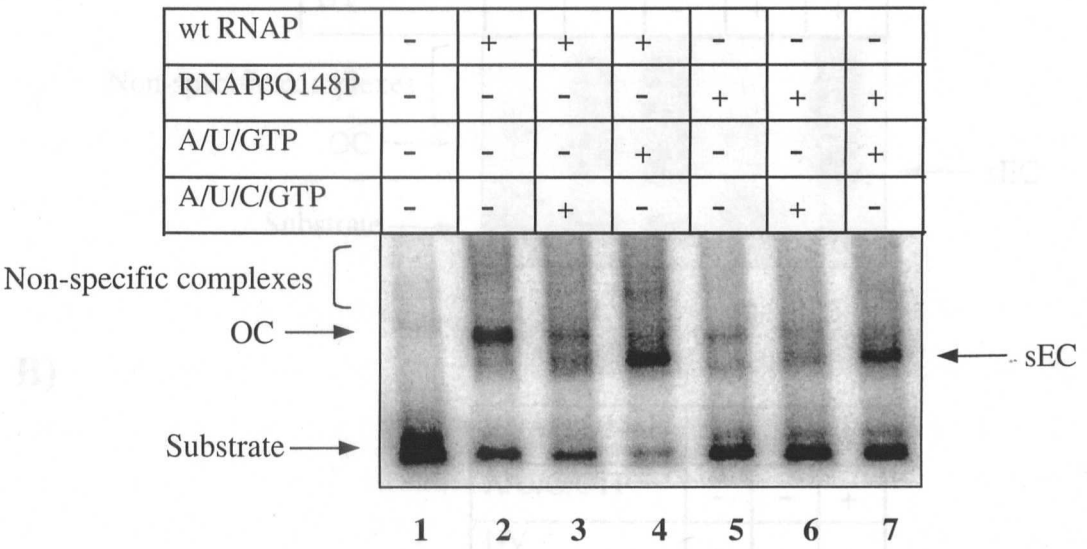

Figure 7.3. Open and ternary complex formation by RNAPβQ148P compared to wild type RNAP. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled *λcro* DNA. RNA polymerases and nucleotides were added as indicated to a final concentration of 10 nM and 2 μM respectively. 2.5 μg/μl heparin was added after the 20 min incubation step. Lane 1: substrate only. Lane 2: wild type RNAP was added at the start in order to form open complexes. Lane 3: as lane 2, but all four nucleotides were added after the 3 min incubation step. Lane 4: as lane 2, but three nucleotides were added after the 3 min incubation step. Lanes 5 - 7: as lanes 2 - 4, but with RNAPβQ148P instead of wild type RNAP.

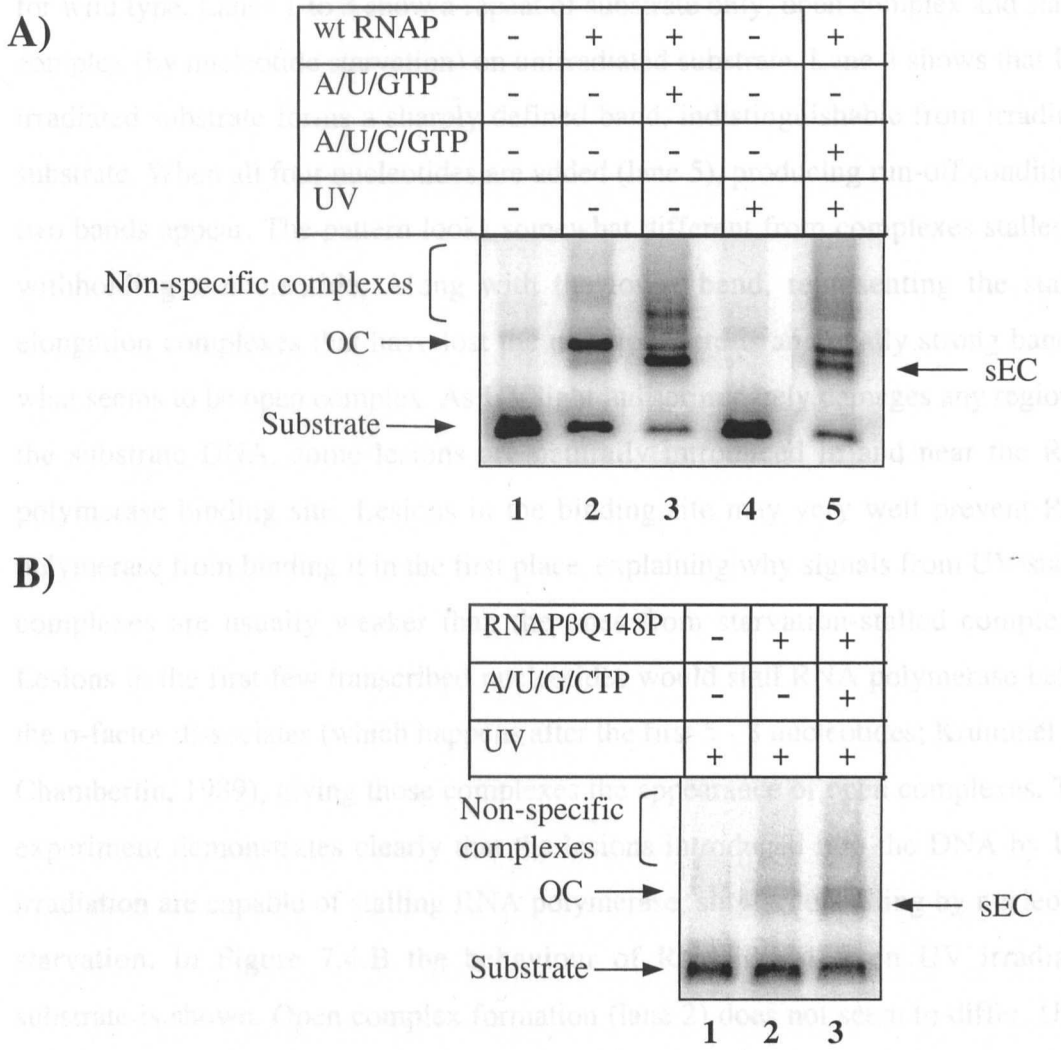

Figure 7.4. Band shift assay, demonstrating the effects of UV light. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled *λcro* DNA (UV-irradiated where indicated). RNA polymerases and nucleotides were added as indicated to a final concentration of 10 nM and 2 μM respectively. 2.5 μg/μl heparin was added after the 20 min incubation step. **A)** Lane 1: substrate only. Lane 2: wild type RNAP was added for open complex formation. Lane 3: as lane 2, but 3 nucleotides were added after the 3 min incubation step. Lane 4: UV-irradiated substrate only. Lane 5: as lane 4, but with RNA polymerase added at the start and all 4 nucleotides added after the 3 min incubation step to produce run-off transcription conditions. **B)** Lane 1: UV-irradiated substrate only. Lane 2: as lane 1, but with RNAPβQ148P added at the start. Lane 3: as lane 2, but all four nucleotides were added after the 3 min incubation step to produce run-off transcription conditions.

for wild type. Lanes 1 to 3 show a repeat of substrate only, open complex and stalled complex (by nucleotide starvation) on unirradiated substrate. Lane 4 shows that UV-irradiated substrate forms a sharply defined band, indistinguishable from irradiated substrate. When all four nucleotides are added (lane 5), producing run-off conditions, two bands appear. The pattern looks somewhat different from complexes stalled by withholding a nucleotide. Along with the lower band, representing the stalled elongation complexes that have lost the σ-factor there is an equally strong band of what seems to be open complex. As UV light indiscriminately damages any region of the substrate DNA, some lesions are naturally introduced in and near the RNA polymerase binding site. Lesions in the binding site may very well prevent RNA polymerase from binding it in the first place, explaining why signals from UV-stalled complexes are usually weaker than the ones from starvation-stalled complexes. Lesions in the first few transcribed nucleotides would stall RNA polymerase before the σ-factor dissociates (which happens after the first 5 - 8 nucleotides; Krummel and Chamberlin, 1989), giving those complexes the appearance of open complexes. This experiment demonstrates clearly that the lesions introduced into the DNA by UV-irradiation are capable of stalling RNA polymerase, similar to stalling by nucleotide starvation. In Figure 7.4.B the behaviour of RNAPβQ148P on UV irradiated substrate is shown. Open complex formation (lane 2) does not seem to differ. Upon addition of all four nucleotides (lane 3), the result is very much the same as with wild type RNAP (Fig. 7.4.A, lane 5). Also mutant RNAP is stalled by UV-induced lesions in the template. The fact that the bands are considerably weaker (demonstrated also in Fig. 7.5.B) may be of interest, as this is not the case when the RNA polymerases are stalled by nucleotide starvation, when the mutant polymerase gives a signal that is nearly as strong. The effect observed here may be due to a diminished rate of promoter escape when the template is damaged. There is also the possibility that RNAPβQ148P does not form stable stalled complexes at sites of UV-induced lesions.

7.4. Are Stalled RNAP* complexes unstable compared to stalled wild type RNAP complexes?

One of the crucial questions I then set out to answer was, whether the mutant RNA polymerases stalled at UV induced lesions would dissociate from the DNA more easily than wild type RNA polymerase. If this were the case, it would support the theory that mutant RNA polymerases - by the decreased stability of their stalled complexes - allow repair to take place before the lesion or the stalled complex can create an obstacle for replication. As the RNA polymerase mutations mimic the effect of (p)ppGpp, this compound should have the same effect, which has been shown in vitro for open complexes (Barker *et al.*, 2001b).

A first step towards answering the question has incidentally already been made by the data in Figure 7.3. In all band shift experiments (except Fig. 7.1, where the method was established) a high concentration of heparin (2.5 $\mu g/\mu l$) was added to every sample at the end of the experiment before loading on the gel. Heparin is a strong competitor that can prevent open complex formation when added together with RNA polymerase to the DNA (Fig. 7.1, lane 5). Any polymerases that are not in a stable complex with template DNA after the 20 min incubation period would not be able to rebind the substrate, but be prevented from it by heparin and not contribute to the band shift. Mutant RNA polymerase does form fewer stable stalled complexes both when stalled by nucleotide starvation (Fig. 7.3) and UV induced lesions (Fig. 7.4.B). But (at least where complexes are stalled by nucleotide starvation) this is likely to be caused by its more unstable promoter complexes, initiating transcription less frequently in the incubation period than wild type.

An unfortunately flawed (see below) attempt was made to further clarify the matter. Figure 7.5.A shows the usual formation of open and stalled complexes (for wild type RNAP lanes 2 and 3, for RNAPβQ148P lanes 5 and 6). In lanes 4 and 7 competitor (unlabelled template) was added during the formation of stalled complexes. A decrease in mutant RNAP stalled complexes, but not in wild type RNAP stalled complexes was observed. The experiment was also performed using

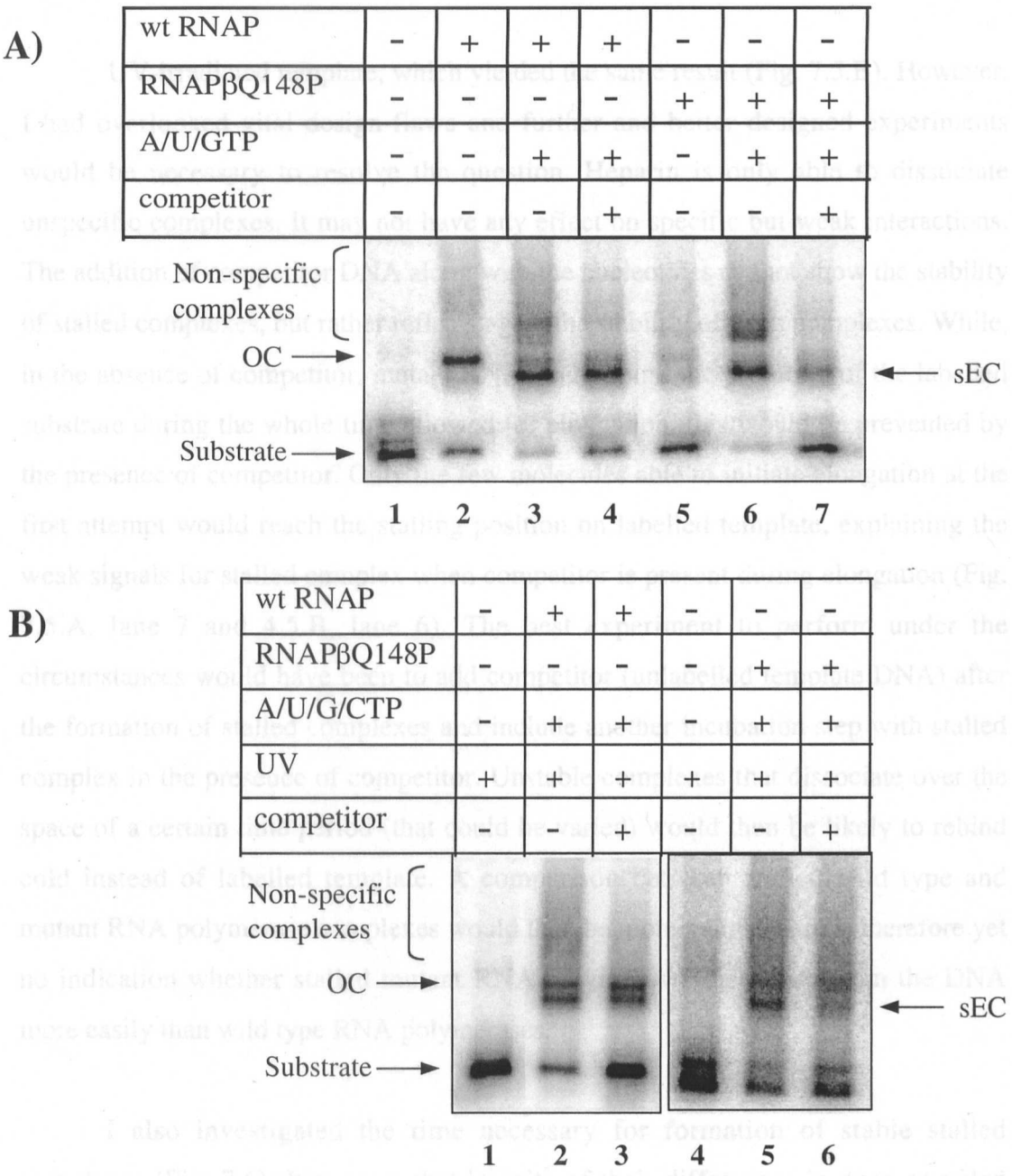

Figure 7.5. Effect of competitor DNA on the stability of RNAP complexes. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled *λcro* DNA (UV-irradiated where indicated). RNA polymerases and nucleotides were added as indicated to a final concentration of 10 nM and 2 μM respectively. 2.5 μg/μl heparin was added after the 20 min incubation step. Unlabelled *λ cro* DNA to 50 - 70 fold excess over the labelled substrate was used as competitor. Competitor was added as stated after the 3 min incubation step, at the same time as nucleotides. **A)** Lane 1: substrate only. Lane 2: wild type RNAP was added at the start to form open complexes. Lane 3: as lane 2, but with three nucleotides added after the 3 min incubation step. Lane 4: as lane 3, but with competitor. Lanes 5 - 7: as lanes 2 - 4, but with RNAPβQ148P. **B)** Lane 1: UV-irradiated substrate only. Lane 2: wild RNAP was added at the start for open complex formation and all four nucleotides were added after the 3 min incubation step to produce run-off transcription conditions. Lane 3: as lane 2, but with added competitor. Lanes 4 - 6: as lanes 1 - 3, but with RNAPβQ148P.

UV-irradiated template, which yielded the same result (Fig. 7.5.B). However, I had overlooked vital design-flaws and further and better designed experiments would be necessary to resolve the question. Heparin is only able to dissociate unspecific complexes. It may not have any effect on specific but weak interactions. The addition of competitor DNA along with the nucleotides cannot show the stability of stalled complexes, but rather reflects again the stability of open complexes. While, in the absence of competitor, mutant RNAP can rebind the promoter of the labelled substrate during the whole time allowed for elongation, this would be prevented by the presence of competitor. Only the few molecules able to initiate elongation at the first attempt would reach the stalling position on labelled template, explaining the weak signals for stalled complex when competitor is present during elongation (Fig. 7.5.A, lane 7 and 4.5.B, lane 6). The best experiment to perform under the circumstances would have been to add competitor (unlabelled template DNA) after the formation of stalled complexes and include another incubation step with stalled complex in the presence of competitor. Unstable complexes that dissociate over the space of a certain time period (that could be varied) would then be likely to rebind cold instead of labelled template. A comparison between stalled wild type and mutant RNA polymerase complexes would then be more valid. There is therefore yet no indication whether stalled mutant RNA polymerases dissociate from the DNA more easily than wild type RNA polymerases.

I also investigated the time necessary for formation of stable stalled complexes (Fig. 7.6). It appears that in spite of their differences in open complex formation (lanes 2 in A and B), both wild type (A) and mutant (B) RNA polymerases seem to form stalled complexes at a comparable rate (lanes 3 to 8 respectively). Samples were incubated with three nucleotides (missing CTP and so stalling elongation at the first C) for 30 sec to 20 min. Stalled mutant RNAP- and stalled wild type RNAP-signals are of similar strength for every time point. In spite of its unstable promoter complexes mutant RNA polymerase seems to be equally efficient at initiating transcription, at least on the λ *cro* promoter.

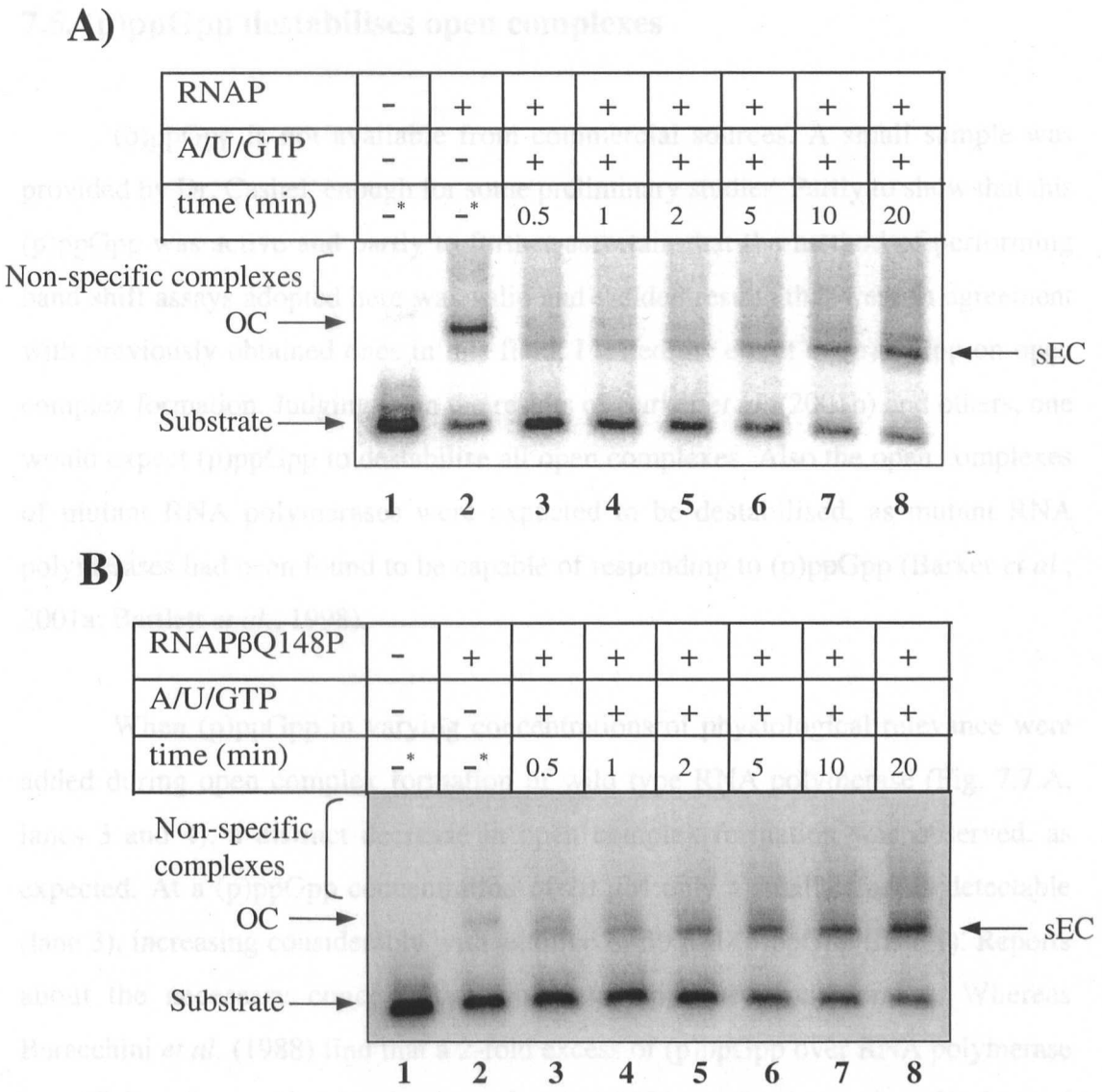

Figure 7.6. Stalled complex formation over time. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA . RNA polymerases and nucleotides were added as indicated to a final concentration of 10 nM and 2 μM respectively. 2.5 μg/μl heparin was added after the 20 min incubation step. **A)** Lane 1: substrate only. Lane 2: RNAP was added for open complex formation. Lanes 3 to 8: as lane 2, but with three nucleotides added after the 3 min incubation step. Samples were incubated for the indicated times instead of the usual 20 min incubation step. **B)** Lanes 1 - 8: as lanes 1 -8 of A), but with RNAPβQ148P. * Time is not applicable here, as no nucleotides were present. The samples were incubated for the usual 3 and 20 min periods.

7.5. (p)ppGpp destabilises open complexes

(p)ppGpp is not available from commercial sources. A small sample was provided by Dr. Cashel, enough for some preliminary studies. Partly to show that this (p)ppGpp was active and partly to further ascertain that the method of performing band shift assays adopted here was valid and yielded results that were in agreement with previously obtained ones in this field, I tested the effect of (p)ppGpp on open complex formation. Judging from the results of Barker *et al.* (2001b) and others, one would expect (p)ppGpp to destabilise all open complexes. Also the open complexes of mutant RNA polymerases were expected to be destabilised, as mutant RNA polymerases had been found to be capable of responding to (p)ppGpp (Barker *et al.*, 2001a; Bartlett *et al.*, 1998).

When (p)ppGpp in varying concentrations of physiological relevance were added during open complex formation of wild type RNA polymerase (Fig. 7.7.A, lanes 3 and 4), a distinct decrease in open complex formation was observed, as expected. At a (p)ppGpp concentration of 20 μM only a small effect is detectable (lane 3), increasing considerably with addition of 50 μM (p)ppGpp (lane 4). Reports about the necessary concentrations of (p)ppGpp vary considerably. Whereas Baracchini *et al.* (1988) find that a 2-fold excess of (p)ppGpp over RNA polymerase is sufficient for maximal reduction of stable RNA synthesis in vivo, Krohn and Wagner (1996) consider as much as 350 μM as stringent conditions. In vivo and in vitro conditions seem to differ significantly in that respect, as probably do the requirements of different promoters. Therefore a wide range of (p)ppGpp concentration was initially used in trial experiments. Concentrations of 20 to 200 μM were found to be most effective under the conditions (see section 7.6).

The open complex formation of the two mutant RNA polymerases in the presence of (p)ppGpp was also investigated (Fig. 7.6.B). Wild type RNA polymerase is shown again, as a control (lanes 2 -4), and responds to the given concentrations of (p)ppGpp as before. It is worth noting that increasing the concentration of (p)ppGpp from 50 μM to 200 μM has no additional effect. About 2000 to 5000-fold the amount of (p)ppGpp over RNAP seems to be necessary to saturate RNA polymerase in my

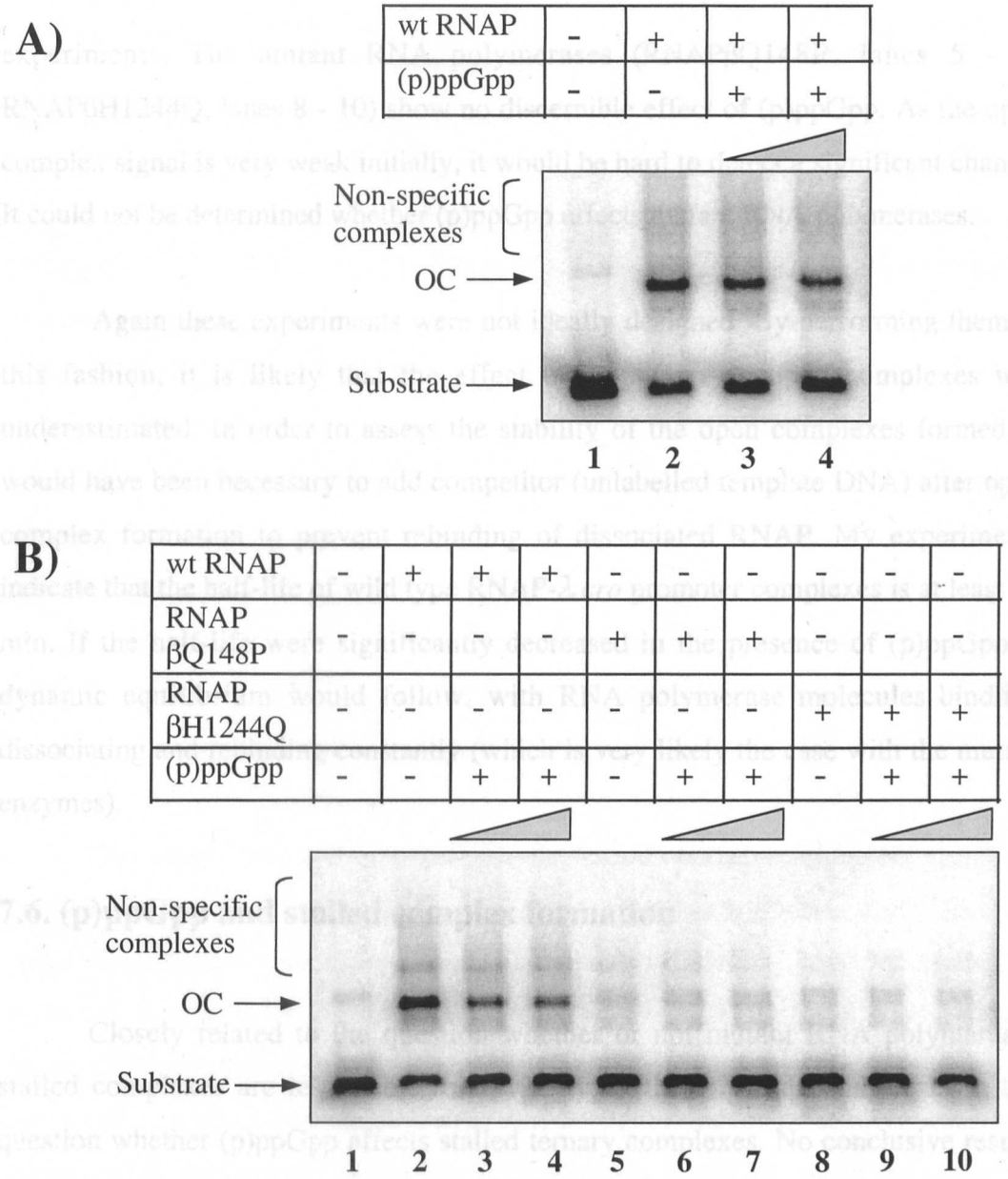

Figure 7.7. Influence of (p)ppGpp on open complex formation. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA . RNA polymerases were added as indicated to a final concentration of 10 nM. (p)ppGpp (20 ◢ 50 μM final concentration) were added where indicated at the start. 3 min open complex formation were followed by another incubation of 20 min. **A)** Lane 1: substrate only. Lane 2: wild type RNAP was added at the start to allow open complex formation. Lane 3: as lane 2, but with 20 μM (p)ppGpp. Lane 4: as lane 3, but with 50μM (p)ppGpp. **B)** The reactions contained 0.5 nM internally [32P]-labelled λ*cro* DNA. Lanes 1 - 4: as lanes 1 - 4 of A). Lanes 5 - 7: as lanes 2 - 4, but with RNAPβQ148P. Lanes 8 - 10: as lanes 2 - 4, but with RNAPβH1244Q.

experiments. The mutant RNA polymerases (RNAPβQ148P, lanes 5 - 7; RNAPβH1244Q, lanes 8 - 10) show no discernible effect of (p)ppGpp. As the open complex signal is very weak initially, it would be hard to detect a significant change. It could not be determined whether (p)ppGpp affects mutant RNA polymerases.

Again these experiments were not ideally designed. By performing them in this fashion, it is likely that the effect of (p)ppGpp on open complexes was underestimated. In order to assess the stability of the open complexes formed, it would have been necessary to add competitor (unlabelled template DNA) after open complex formation to prevent rebinding of dissociated RNAP. My experiments indicate that the half-life of wild type RNAP-λ *cro* promoter complexes is at least 20 min. If the half-life were significantly decreased in the presence of (p)ppGpp, a dynamic equilibrium would follow, with RNA polymerase molecules binding, dissociating and rebinding constantly (which is very likely the case with the mutant enzymes).

7.6. (p)ppGpp and stalled complex formation

Closely related to the question whether or not mutant RNA polymerases stalled complexes are less stable than wild type RNAP stalled complexes is the question whether (p)ppGpp affects stalled ternary complexes. No conclusive result, elucidating the stability of stalled mutant RNA polymerase complexes could be obtained by the experiments presented in section 7.4. Mutant RNA polymerases mimic the effect of (p)ppGpp, so both - an *rpo** mutation and (p)ppGpp - should have the same effect and the use of (p)ppGpp in band shift assays should provide further insight into the problem at hand.

To visualise possible effects of (p)ppGpp on stalled wild type RNAP complexes, excess competitor was added along with it to prohibit rebinding of RNA polymerases. Again the mistake of adding (p)ppGpp and competitor only at the same time as the nucleotides has been made. Ideally stalled complexes should have been allowed to form before addition of (p)ppGpp and competitor at least in one set of

reactions. Figure 7.8 shows the result. In lane 4, stalled complex made in the absence of competitor is shown. As was demonstrated in Figure 7.5.A, the addition of competitor after open complex formation does not lead to significant decrease in the amount of stalled complex that can be made. In Figure 7.8, lane 5, some decrease can be observed. This is untypical however. Nevertheless, upon addition of increasing amounts of (p)ppGpp (lanes 6 - 9) there is a distinct decrease of the stalled complex signal while the unbound template signal increases. As observed in Figure 7.7.B, concentrations of (p)ppGpp above 50 μM lead to no detectable further change (Fig. 7.8, lanes 7 - 9). For wild type RNA polymerase stalled at UV induced lesions (lanes 10 to 15) the picture is not quite as clear. Though a slight increase in free substrate can be observed with addition of (p)ppGpp, no significant decrease in stalled complex signal occurs. It is probably harder to observe changes in RNAP complexes stalled by UV induced lesions than by nucleotide starvation, as the signals are rather weak and changes are too slight, if they occur.

The experiment was repeated with the additional feature of adding (p)ppGpp before open complex formation (Fig. 7.9). (p)ppGpp concentrations in the observed range of maximum effect were used. For wild type RNA polymerase stalled by nucleotide starvation the result was very much as the one of the previous experiment (in lanes 5 and 6 (p)ppGpp was again added after open complex formation with the nucleotides). (p)ppGpp reduces the band shift signal and increases free substrate. When added before open complex formation (lanes 7 and 8), (p)ppGpp can act at both steps, open and stalled complex formation and the band shift signal is further decreased, just as expected. For wild type RNA polymerase stalled at UV induced lesions the picture is again slightly different. In contrast to Figure 7.8, a definite effect of (p)ppGpp can be observed, probably because the gel is clearer and shows stronger stalled complex signals. In this case there is no discernible difference whether (p)ppGpp is added before or after open complex formation. This may be due to the more uniform distribution of UV induced lesions compared to stalling by nucleotide starvation. When stalled by UV-light, RNA polymerase stalls at many different locations on the substrate, including the promoter region (as discussed above). This in turn leads to the observed mixture of open complex and elongation complex signals. (p)ppGpp can act at both, whether it is added before open complex

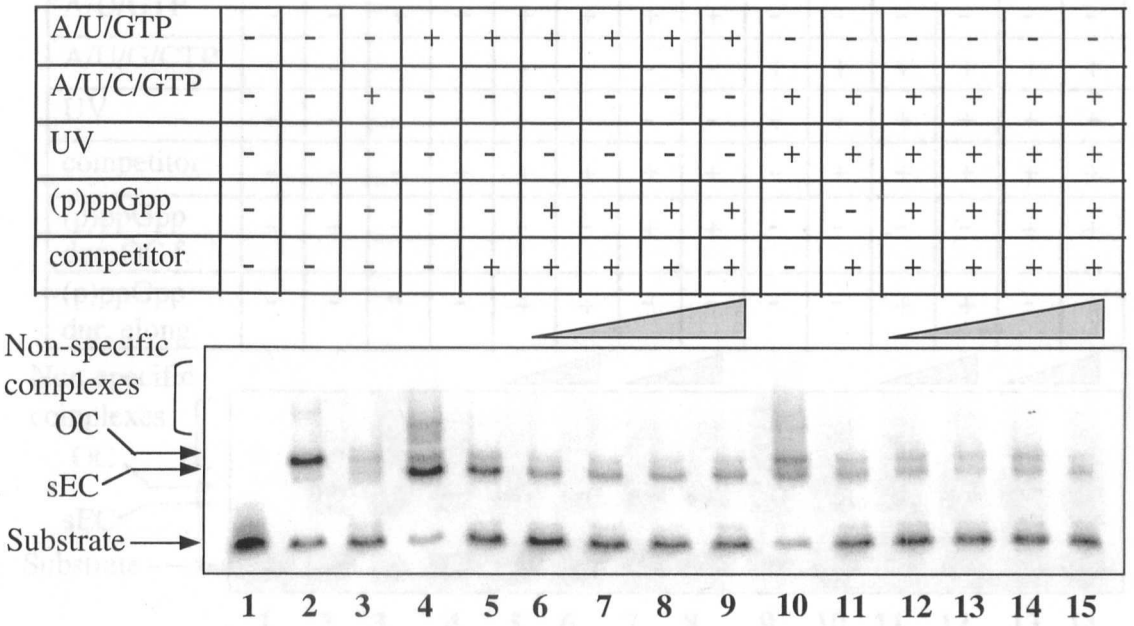

Figure 7.8. Effect of (p)ppGpp on wild type RNAP stalled complexes. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA . RNA polymerase was added as indicated to a final concentration of 10 nM. (p)ppGpp (50 ◿100 ◿200 ◿500 μM final concentration) were added where indicated after the 3 min incubation step along with the nucleotides (final concentration of 2 μM). Unlabelled λ *cro* DNA to 50 - 70 fold excess over the labelled substrate was used as competitor. Competitor also was added as stated after the 3 min incubation step. 2.5 μg/μl heparin was added after the 20 min incubation step. Lane 1: substrate only. Lane 2: RNA polymerase was added for open complex formation. Lane 3: as lane 2, but all four nucleotides were added after the 3 min incubation step to produce run-off transcription conditions. Lane 4: as lane 2, but with three nucleotides added. Lane 5: as lane 4, but with added competitor.Lane 6 - 9: as lane 5, but with (p)ppGpp added. Lanes 10 - 15: as lanes 4 - 9, but UV irradiated template was used and all four nucleotides were added after the 3 min incubation step to produce run-off transcription conditions.

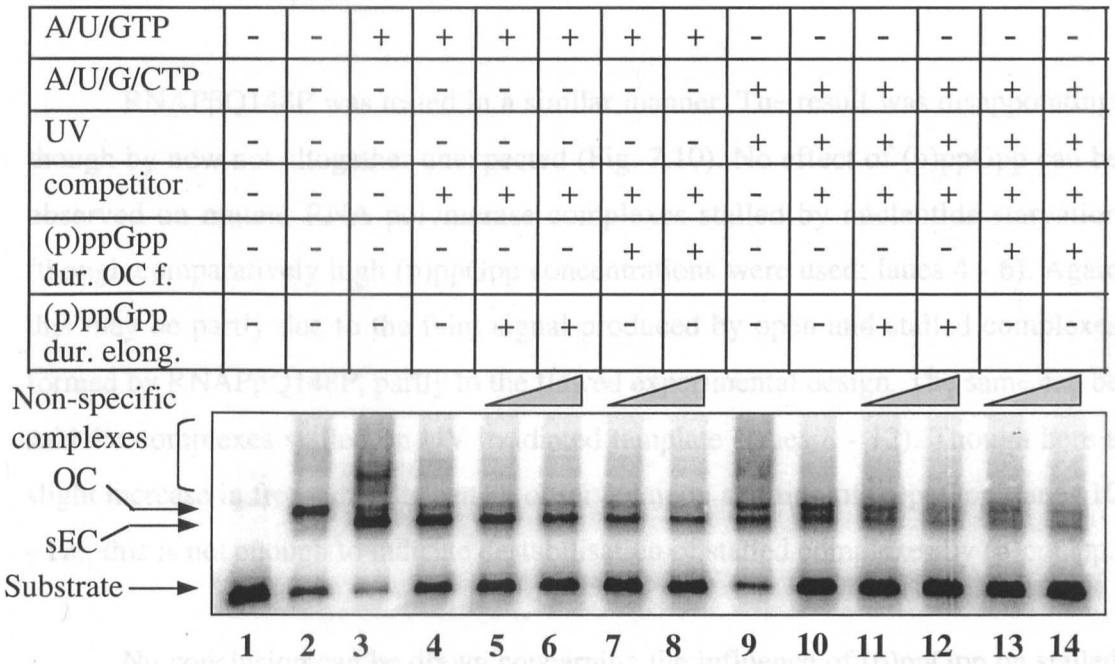

Figure 7.9. Effect of (p)ppGpp, added before or after open complex formation, on the formation of wild type RNAP stalled complexes. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA . RNA polymerase was added to a final concentration of 10 nM. (p)ppGpp (20 ◢ 50 μM final concentration) were added where indicated either at the start (so it was present during both open complex formation and elongation) or after the 3 min incubation step (so it was present during elongation only). Nucleotides (final concentration of 2 μM) were added after the 3 min incubation step as indicated. Unlabelled λ *cro* DNA to 50 - 70 fold excess over the labelled substrate was used as competitor, added where stated after the 3 min incubation step. 2.5 μg/μl heparin was added after the 20 min incubation step. Lane 1: substrate only. Lane 2: RNA polymerase added for open complex formation. Lane 3: as lane 2, but three nucleotides were added. Lane 4: as lane 3, but with added competitor. Lanes 5 and 6: as lane 4, with 20 and 50 μM (p)ppGpp, added after OC formation. Lanes 7 and 8: as lane 4, with 20 and 50 μM (p)ppGpp, added before OC formation. Lanes 9 - 14: as lanes 3 - 8, but UV irradiated substrate was used and all four instead of only three nucleotides were added to produce run-off transcription conditions.

formation or later. As with the previous experiments, clearer results may have been obtained by first allowing formation of the complex in question followed by a further incubation with competitor DNA.

RNAPβQ148P was tested in a similar manner. The result was disappointing, though by now not altogether unexpected (Fig. 7.10). No effect of (p)ppGpp can be observed on mutant RNA polymerase complexes stalled by nucleotide starvation (though comparatively high (p)ppGpp concentrations were used; lanes 4 - 6). Again this may be partly due to the faint signal produced by open and stalled complexes formed by RNAPβQ148P, partly to the flawed experimental design. The same can be said for complexes stalled on UV irradiated template (lanes 8 - 12). Though here a slight increase in free substrate can be observed upon addition of (p)ppGpp (lanes 10 - 12), this is not enough to indicate destabilisation of stalled complexes by (p)ppGpp.

No conclusion can be drawn concerning the influence of (p)ppGpp on stalled complexes formed by mutant RNA polymerase. There are however some indications that (p)ppGpp might indeed destabilise wild type RNA polymerase stalled complexes, both stalled by nucleotide starvation and UV-induced lesions.

7.7. RNAP and RNAP* complex formation at *rrnB P1*

rrnB P1 DNA fragments are widely used in in vitro assays (e.g. Bartlett *et al.*, 1998; Hernandez and Bremer, 1990; Kingston and Chamberlin, 1981). The *rrnB P1* promoter is a strong promoter and it is under negative stringent control. It is usually used as an example for growth-rate regulated genes and rRNA genes that are negatively regulated by (p)ppGpp. While the *λcro* DNA fragment provided a more or less "neutral" substrate, relatively unconnected to stringent response regulation, *rrnB P1* was intended to provide a more natural substrate for *E.coli* RNA polymerase and its modulation by (p)ppGpp. Though several efforts were made, I could not achieve visualisation of complexes with *rrnB P1* and either of the mutant RNA polymerases in these preliminary studies.

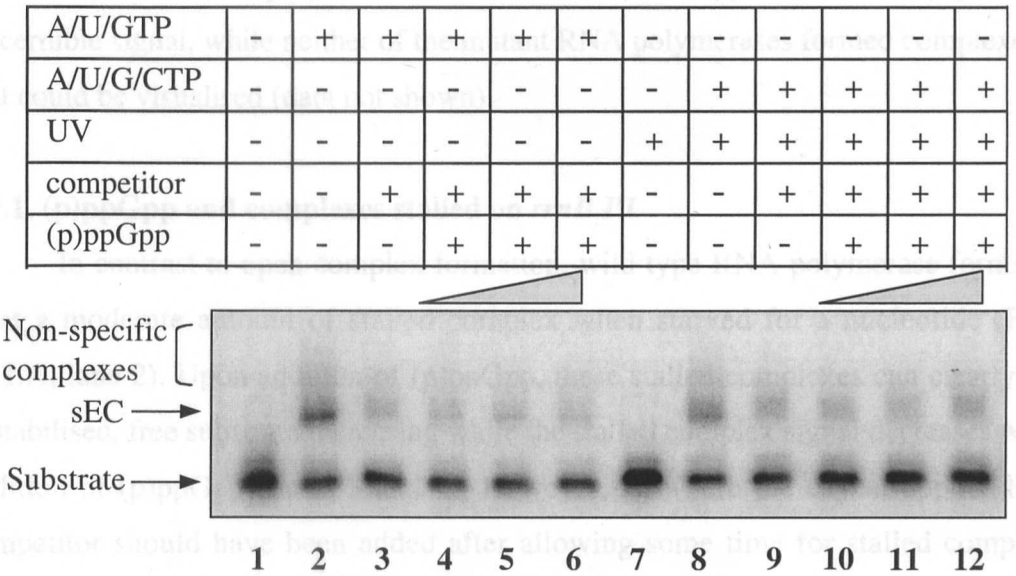

Figure 7.10. Effect of (p)ppGpp on RNAPβQ148P stalled complexes. Reactions (final volume 20 μl) contained 0.5 nM [^{32}P]-labelled λ*cro* DNA . RNA polymerase was added to a final concentration of 10 nM. (p)ppGpp (120 ◿ 300 ◺ 600 μM final concentration) were added where indicated after the 3 min incubation step (so it was present during elongation only), as were nucleotides (final concentration of 2 μM), where indicated. Unlabelled λ *cro* DNA to 50 - 70 fold excess over the labelled substrate was used as competitor, added where stated after the 3 min incubation step. 2.5 μg/μl heparin was added after the 20 min incubation step. Lane 1: substrate only. Lane 2: RNA polymerase was added, as were three nucleotides. Lane 3: as lane 2, but with added competitor. Lanes 4 - 6: as lane 3, with increasing concentrations of (p)ppGpp. Lane 7: UV irradiated substrate only. Lanes 8 - 12: as lanes 2 - 6, but using UV irradiated substrate and all four nucleotides instead of three in order to produce run-off transcription conditions.

RNAP complexes with *rrnB P1* substrate are less stable

It was attempted to form open complexes on *rrnB P1* DNA. It became clear that open complex formation is much less efficient than on *λ cro* DNA. In spite of 20-fold excess of RNA polymerase over DNA, even wild type RNAP hardly gives a discernible signal, while neither of the mutant RNA polymerases formed complexes that could be visualised (data not shown).

7.7.1. (p)ppGpp and complexes stalled on *rrnB P1*

In contrast to open complex formation, wild type RNA polymerase forms at least a moderate amount of stalled complex when starved for a nucleotide (Fig. 7.11.A, lane 2). Upon addition of (p)ppGpp, these stalled complexes can clearly be destabilised, free substrate increasing while the stalled complex signal decreases with addition of (p)ppGpp (lanes 3 and 4). However, also here the caveat applies that competitor should have been added after allowing some time for stalled complex formation, followed by a further incubation step (as in section 7.6). The effect seems significant, but it cannot be attributed to destabilisation of stalled complexes without further tests and seems more likely to be due to destabilisation of open complexes. The latter assumption could not be tested with the *rrnB P1* template as the open complex signal achieved was too weak. The same is true for wild type RNA polymerase complexes stalled on *rrnB P1* by UV-induced lesions. They can be formed, but with less efficiency or less stability than on *λ cro* DNA (Fig. 7.1..B, lane 2). Addition of (p)ppGpp (lanes 3 and 4) has very little effect, which again can be attributed to a decreased half life of open complexes, induced by (p)ppGpp.

Optimal conditions for gel retardation assays with *rrnB P1* substrate have not been achieved yet in these preliminary experiments. Some influence of (p)ppGpp was observed, which could either and probably most likely act at the step of open complex formation, or directly at stalled complexes.

7.8. RNA polymerase and *lac UV5*

lac UV5 is a commonly used control promoter (Barker *et al.*, 2001b; Kajitani and Ishihama, 1984). It is not known to strongly respond to (p)ppGpp and was

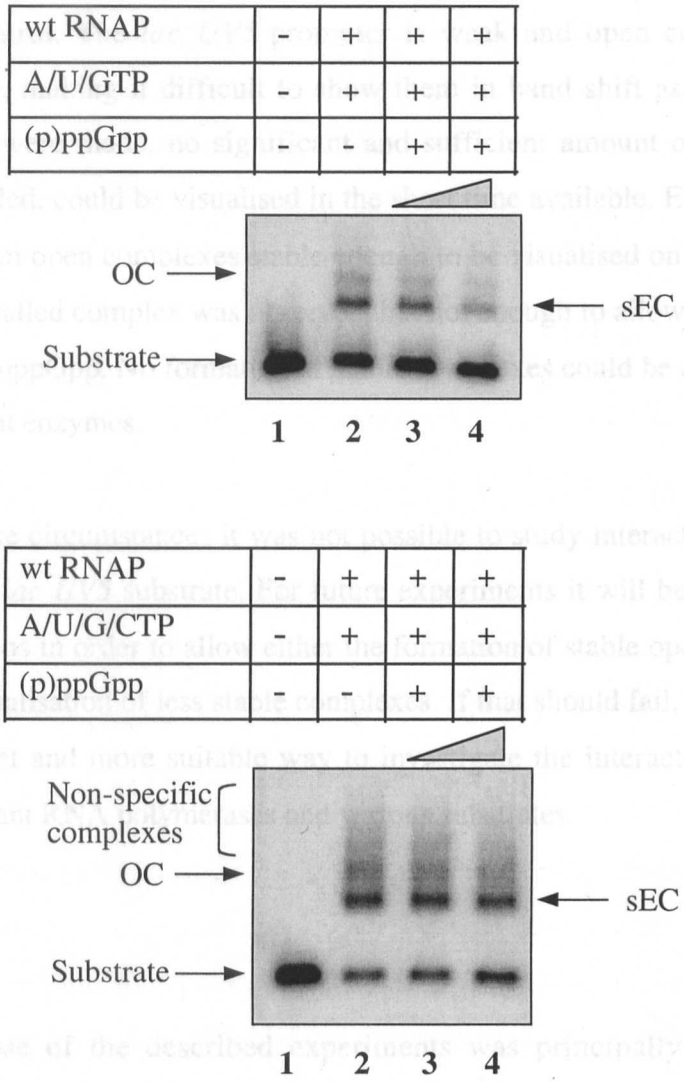

Figure 7.11. Influence of (p)ppGpp on wild type RNAP complexes stalled by nucleotide starvation (A) or UV-induced lesions (B). Reactions (final volume 20 μl) contained 1 nM internally [^{32}P]-labelled *rrnB P1* DNA . RNA polymerase was added to a final concentration of 20 nM. (p)ppGpp (20 ◢ 50 μM final concentration) were added where indicated after the 3 min incubation step (so it was present during elongation only), as were nucleotides (final concentration of 200 μM), where indicated. Unlabelled *rrnB P1* DNA to 50 - 70 fold excess over the labelled substrate was used as competitor, added to all reactions (except substrate onlly) after the 3 min incubation step. 2.5 μg/μl heparin was added after the 20 min incubation step. **A)** Lane 1: substrate only. Lane 2: RNAP was added for open complex formation and three nucleotides and competitor after the 3 min incubation step. Lanes 3 and 4: as lane 2, but with increasing amounts of (p)ppGpp added also after the 3 min incubation step. **B)** Lane 1: UV irradiated substrate only. Lane 2: RNAP was added for open complex formation and all four nucleotides and competitor were added after the 3 min incubation step. Lanes 3 and 4: as lane 2, but with increasing amounts of (p)ppGpp.

intended as a control. The *lac UV5* promoter is weak and open complexes are naturally unstable, making it difficult to show them in band shift assays. Though strenuous efforts were made, no significant and sufficient amount of complexes, either open or stalled, could be visualised in the short time available. Even wild type RNAP did not form open complexes stable enough to be visualised on a gel. A very small amount of stalled complex was observed, but not enough to allow investigation of the effects of (p)ppGpp. No formation of stable complexes could be achieved with either of the mutant enzymes.

Under those circumstances it was not possible to study interactions of RNA polymerases with *lac UV5* substrate. For future experiments it will be necessary to adjust the conditions in order to allow either the formation of stable open and stalled complexes, or visualisation of less stable complexes. If that should fail, I will have to look for a different and more suitable way to investigate the interactions between wild type and mutant RNA polymerases and various substrates.

7.9. Discussion

The purpose of the described experiments was principally to establish methods and test ideas, with the objective to determine whether RNA polymerase - stalled at a UV induced lesion or by amino acid starvation - would dissociate more readily in the presence of (p)ppGpp. If this were the case and (p)ppGpp would indeed decrease the stability of stalled complexes, RNAP* mutants could be expected to also show decreased stability of stalled complexes as they mimic the effect of (p)ppGpp. This could provide an explanation for their increasing the UV resistance of *ruv* and *rec* strains, by simply allowing more efficient repair of lesions, decreasing the incidence of stalled replication forks, and thereby obviating the need for the RecBCD and RuvABC enzyme complexes.

The λ *cro* gene has a very strong promoter and was an ideal choice for a substrate, as it facilitated visualising of open and stalled complexes in band shift assays. Mutant RNA polymerases as well as (p)ppGpp had been shown to decrease the half-life of open complexes in general (Barker *et al.*, 2001b). This fact could be

used as a control and was confirmed by the above experiments. The effect of (p)ppGpp may even have been underestimated. The data also strongly suggests that (p)ppGpp indeed decrease the amounts of stable open complex and mutant enzymes were found to form considerably less open complex under any conditions than wild type enzyme. In spite of this fact, at least the more extensively tested mutant RNA polymerase (RNAPβQ148P) was roughly as proficient at formation of stalled complex as wild type enzyme, as demonstrated by a time course experiment. Due to the addition of heparin at the end of every experiment, only stable complexes were visualised. Due to the nature of heparin (preventing specific interactions but only dissociating unspecific, not weak specific interactions) this does not provide evidence whether or not stalled complexes formed by mutant RNA polymerases are less stable or not. The addition of competitor DNA after stalled complex formation, followed by a further incubation step, would have been necessary to clarify the question further.

In contrast, it could be shown that stalled wild type RNA polymerase complexes are less stable in the presence of (p)ppGpp, whether stalling was achieved by nucleotide starvation or UV-induced lesions. The effect is small, but visible. The fact that RNAPβQ148P gave no indication of decreased stalled complex stability can not be considered conclusive. The experiments performed were far from exhaustive and RNAPβQ148P may not be representative in that respect. A second mutant, RNAPβH1244Q, was also tested, but even less thoroughly, due to time constraints. Open complex formation was very weak in this mutant and experiments involving stalled complexes were therefore severely hampered under the conditions used.

The *rrnB P1* template did not yield as detailed and clear results as one might have expected, because of the low level of open complex formation. This may well at least partly be due to unfavourable reaction conditions, which will be taken into account for future experiments and might increase their quality and value. In spite of that, stalled wild type RNA polymerase complexes were shown to be less stable in the presence of (p)ppGpp on *rrnB P1*.

The data presented here gives a first indication in vitro of why the absence of (p)ppGpp might increases the UV sensitivity of *recB* and *ruv* strains. It is shown here, that the compound not only leads to the formation of less stable open complexes, but also destabilises wild type RNA polymerase complexes stalled by either nucleotide starvation or, more importantly, UV-induced lesions. Both effects may work together to facilitate repair of UV-damage, avoiding collapse of replication forks by stalling of replication and so obviating the need for RuvABC and RecBCD, which would be necessary to repair them. It is not yet clear how mutant RNA polymerases achieve the same effect. Though the data provides no evidence that mutant RNA polymerase stalled complexes are less stable than wild type ones, it corroborates previously presented evidence that mutant RNA polymerases form weaker open complexes. There are two obvious possibilities: First, (p)ppGpp and RNAP* may indeed also destabilise stalled complexes. Only a few experiments have been performed. They were not entirely clear and further investigation is necessary to show whether or not stalled mutant RNA polymerase complexes are less stable than wild type. Second, they may achieve their effect by destabilising open complexes only. This possibility is explored more fully in the final chapter.

Chapter 8.

Discussion

The data presented in this work further strengthens the evidence that connects the stringent response and its effector (p)ppGpp with the repair of stalled or collapsed replication forks. So far evidence had been presented showing that the RuvABC resolvasome acts at arrested replication forks and leads to the formation of double-strand breaks. Seigneur and co-workers (1998) presented a model fitting their experimental results (see chapter 1 for details), also involving the RecBCD enzyme complex as a major player, as it is essential for the repair of double-strand breaks. This idea was further developed by McGlynn and Lloyd (2000) whose data implicate RecG in the repair of collapsed replication fork. RecG is likely to be the major protein in an additional pathway, as outlined in their model (see chapter 1). Their data points to the avoidance of replication fork arrest and collapse by the influence of (p)ppGpp or certain RNA polymerase mutants mimicking (p)ppGpp effects, termed *rpo**. They had identified *rpo** mutants in a *ruv* background, where they increase the survival after UV irradiation to a far greater degree than when transferred into a *recB* background. *ruv* and *recB* cells show a very similar UV sensitivity (see chapter 3) and their effect is not cumulative, supporting the view that they act in the same pathway for the repair of collapsed replication forks. That *rpo** mutations should increase the survival of UV irradiated *ruv* cells more efficiently than that of UV irradiated *recB* cells can be explained by the fact that RecBCD also functions as a major exonuclease and loads RecA more efficiently on 3' DNA ends, while RuvABC has the single task of branch migrating and resolving Holliday junctions. In this work I set out to determine whether *rpo** mutants can also be isolated from a *recB* background and, if yes, whether they differ in any way from *rpo** mutations found in *ruv* strains. I also wanted to further characterise *rpo** mutants and mutations and determine their mode of action.

8.1. The influence of *rpo** mutations on *Escherichia coli* cells in vivo

The data in chapter 3 clearly demonstrates that *rpo** mutations can also be isolated from a *recB* background. Mutations isolated this way also suppress the *ruv* phenotype and can therefore also be regarded as true *rpo** mutations. The mutations investigated by McGlynn and Lloyd (2000) show a higher suppression of *ruv* than of *recB*. After investigation of several other mutations isolated here, it can be concluded that this is a general trend, in agreement with the proposed model. Some mutations however showed no significant difference in suppressing the *ruv* and *recB* phenotypes and there is one (βL571Q) for which the opposite is true. How this higher increase of survival in *recB* cells is achieved is not understood and remains a question to be asked in future studies. The increase of survival after UV irradiation of *ruv* and *recB* cells is the defining characteristic of *rpo** mutations, setting them apart from other stringent mutations. Closer investigation revealed other effects, mostly beneficial.

Cell viability is increased even in the absence of UV induced damage. While *recB* and (p)ppGpp^0 strains show viabilities of 65% and 40% respectively, this is decreased drastically in *recB* (p)ppGpp^0 double mutants (< 8%). Introduction of a *rpo** mutation leads to restoration of the strains to viabilities of usually 65 - 80%. These data confirm that double-strand breaks arise in many cells even without externally induced damage, and are resolved by a process that involves RecBCD. The absence of (p)ppGpp aggravates the condition and *rpo** mutations suppress it, often more than merely compensating for the absence of (p)ppGpp and raising viability above that of a *recB* only strain.

The phenotype is also greatly changed. *recB* cells show only slight signs of filamentation. (p)ppGpp^0 cells appear to be in a worse condition with more filamentous cells. This phenotype is enhanced in *recB* (p)ppGpp^0 double mutants, where the cells are very large and filament profusely. Introduction of an *rpo** mutation into such a strain more or less cures it. Filamentation is very much reduced or even completely absent. Another remarkable characteristic is the diminished cell

size of most *rpo** mutant strains. The small cell size is explained by the *rpo** mutations conferring a stringent phenotype. The cells behave as if under stringent control in any condition. (p)ppGpp has been found to suppress filamentation in *ftsZ* (Harry, 2001) mutant cells (Powell and Court, 1998). That (p)ppGpp^0 cells form filaments has already been observed by Xiao and co-workers (1991). Suppression by stringent mutations, of which *rpo** mutations are a subclass, is therefore not surprising. Small cell size and reduced filamentation are therefore likely to be common to all stringent mutants and not a specific characteristic of the *rpo** phenotype. The latter must have additional effects that help promote survival of UV-irradiated cells.

The growth rate of *rpo** mutants is not improved. *rpo** mutants in liquid culture and under optimal conditions grow just as slowly as the very sick *recB* (p)ppGpp^0 strain. This is not surprising, considering that RNAP* mimics the effect of (p)ppGpp, which downregulates most household genes that are necessary for exponential growth. RNAP* keeps the cells healthy and viable in the absence of RecBCD and (p)ppGpp, it allows them to grow on minimal medium, but it also confers slow growth, just as wild type RNAP in the presence of (p)ppGpp would. Whether this reduced growth rate is important in recovery from UV-irradiation is not clear at this stage.

8.2. The location and nature of *rpo** mutations - implications

Twenty-seven mutations were identified by sequencing. With the exception of one three amino acid deletion, all of them were point mutations leading to single amino acid changes. Twenty-one were found in *rpoB* and only six in *rpoC*. This distribution does probably not reflect the real circumstances, as preferentially rifampicin resistant *rpo** strains were chosen for further investigation (see chapter 3). According to Cashel and co-workers (1996) stringent mutations are equally distributed between *rpoB* and *rpoC* and rare mutations conferring stringency can be found in *rpoD*, encoding σ^{70}, *E.coli*'s main sigma factor. The distribution does not

necessarily have to be the same for *rpo** mutations, it is however likely that more *rpoC** mutations can be found and possibly also *rpoD** mutations.

The mutations mostly concern conserved amino acids and lie in or very near the initially defined conserved regions of the proteins (Jokerst *et al.*, 1989; Sweetser *et al.*, 1987). The location of the mutations on linear maps of RpoB and RpoC is not very illuminating, as they are spread more or less over the whole length, forming a few clusters on RpoB. Mapping of the concerned residues on the three dimensional map of *Thermus aquaticus* RNA polymerase (Zhang *et al.*, 1999) however reveals an interesting pattern. Most of the mutations lie on the inside surface of the DNA, ideally placed to make contact with the DNA substrate or the RNA product respectively. A large number of the *rpoB** residues form a pocket adjacent to and partly overlapping with the so-called Rif-pocket, explaining the connection between rifampicin resistance and the *rpo** phenotype. When rifampicin binds to the RNA polymerase, it physically blocks the path of the elongating RNA (Campbell *et al.*, 2001), making the rifampicin binding pocket also a good target for *rpo** mutations that modulate the activity of RNA polymerase.

The effect of an *rpo** mutation is in all likelihood the result of both its location and the nature of the amino acid change. In the time available it could not be attempted to clarify this issue, which probably demands detailed biochemical studies. It seems significant however, that of the twenty-seven identified mutations, six are changes from or to proline (βQ148P, βP153L, βH447P, βL533P, βH551P and βT563P), causing structural changes in the region concerned. Some changes substitute hydrophobic amino acids (e.g. β'K215E), some concern changes in size of the side chains (e.g. βR151S), whereas other changes seem very insignificant (e.g. βL448I). It can be said that *rpo** mutations are either in a position to make direct DNA or RNA contact or are likely to induce structural changes influencing substrate or RNA interactions.

8.3. Induction of the stringent response by UV light

Near UV-light has been shown to stimulate the production of (p)ppGpp (Kramer et al., 1988). It has never been tested whether or not UV light has the same effect, though there are some indications that this might be the case (Nystrom *et al.*, 1992). As this question is of considerable importance to the matter in hand, considering that elevated levels of (p)ppGpp increase the survival of UV irradiated ruv cells, thin layer chromatography was used in order to determine the levels of (p)ppGpp under various conditions in different strains (Cashel, 1969).

A working assay could be established, clearly showing induction of (p)ppGpp synthesis after amino acid starvation. Exposure to UV light however negatively influenced the performance of the method, so that no answer could be obtained. Other methods will have to be used in order to determine (p)ppGpp levels. High pressure liquid chromatography may represent another way for directly measuring (p)ppGpp.

8.4. Influence of (p)ppGpp and behaviour of RNAP* in vitro

(p)ppGpp is known to regulate transcription by modulating RNA polymerase, similar to a sigma factor, upregulating some genes, downregulating others. In contrast to sigma factors it does not recognise specific sequences, but rather depends on the intrinsic properties of the promoter-RNA polymerase interaction, as the data of Barker and co-workers (2001a) suggest. They found that (p)ppGpp exerts the same effect on all promoters, namely the decreasing of open complex half lives, which has different consequences, according to the promoter in question. Apart from their effect on promoters, the compounds have also been implicated in NusA dependent termination (Kingston and Chamberlin, 1981) and transcriptional pausing (Kingston *et al.*, 1981). The available evidence strongly suggests that the increase in survival of UV irradiated *ruv* and *recB* cells is also due to effects of (p)ppGpp or RNA polymerase mutations on transcription, though probably not mainly due to differential gene expression (McGlynn and Lloyd, 2000). A first step towards

investigating the mechanism of this suppression of UV sensitivity was made in this work. Different scenarios could account for the observed phenomenon. Differential gene expression could act in concert with direct effects of (p)ppGpp or RNAP* that lower the incidence of replication fork blocks. It is also possible that the latter mechanism acts alone. The most likely explanation for how modulation of RNA polymerase can decrease the incidence of stalled replication forks would be its speedy removal from lesions to allow repair (Fig. 8.1). How can (p)ppGpp or *rpo** mutations achieve that? Again there are different possibilities. (p)ppGpp or RNAP* might facilitate the removal of RNA polymerase from lesions by Mfd. Modulated RNA polymerase could simply have a generally lower affinity to DNA, allowing it to dissociate more frequently and more easily. The demonstrated effect of (p)ppGpp and stringent RNA polymerases on promoters (see above) may also account for the phenomenon.

In order to investigate the mechanism of the increase in UV survival caused by *rpo** mutants and (p)ppGpp, wild type and two different mutant RNA polymerases were purified for in vitro studies. Three different DNA substrates were chosen for band shift studies: *λcro*, containing the strong *λcro* promoter and part of the gene sequence. *rrnB*, containing the *rrnBP1* promoter, which is under negative stringent control, and some of the following sequence. *lacUV5*, containing the *lacUV5* promoter and some of the following gene sequence, to act as a control for the *rrnBP1* promoter. The aim of the in vitro experiments was to investigate the influence of (p)ppGpp and *rpo** mutations on the behaviour of RNA polymerase stalled by either nucleotide starvation or UV-induced lesions. These initial experiments confirmed that (p)ppGpp decreases the amount of open complex formed and that RNAP*s, just like stringent RNA polymerases, form weaker open complexes. Stalled complexes seem to be formed with equal efficiency by both wild type and at least one mutant polymerase under the conditions used. I found however that (p)ppGpp decreases the amount of stalled wild type RNA polymerase. The possibility that this is just a consequence of the weaker open complex formation in the presence of (p)ppGpp was excluded by addition of the compound after open complex formation. Addition before open complex formation further decreases the amount of stalled complex.

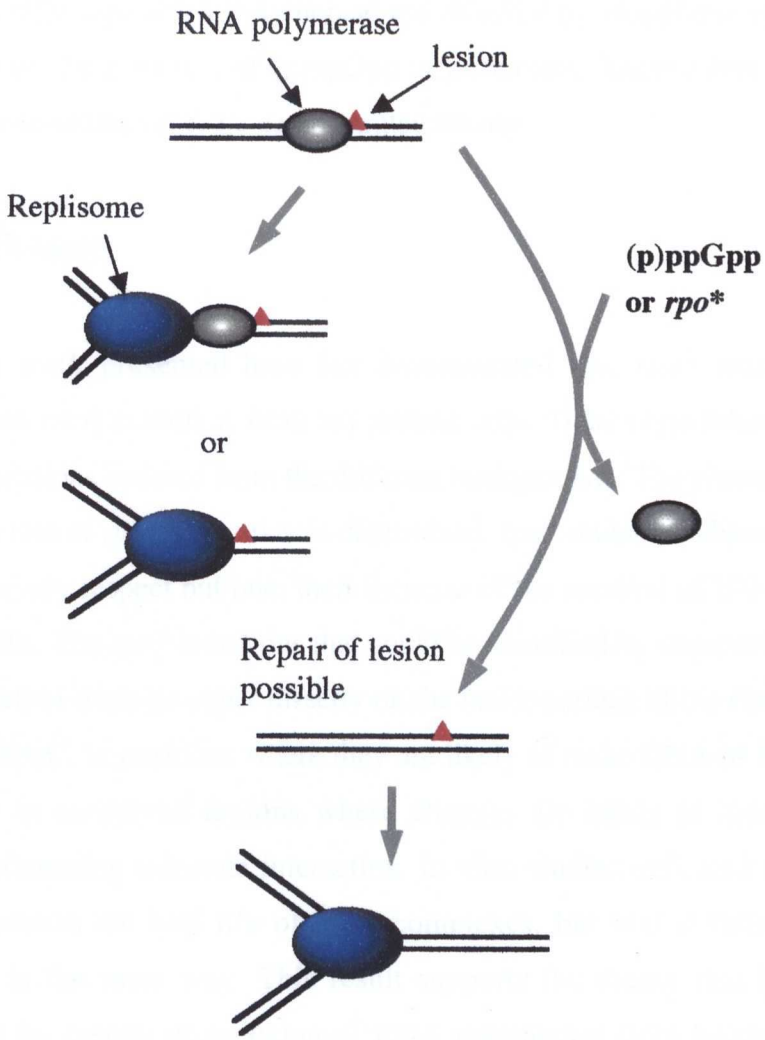

Figure 8.1. A model summarising how RNA polymerase could increase the frequency of replication fork bocks and how (p)ppGpp or *rpo** mutations could relieve this effect. RNA polymerase stalled at a lesion could delay its repair or itself form a block for replication. The presence of (p)ppGpp or an *rpo** mutation could facilitate dissociation of RNA polymerase, removing a possible obstacle and facilitating access for repair systems.

The biochemical evidence obtained in this work gives some indication that (p)ppGpp could increase the survival of UV irradiated *ruv* and *recB* cells by destabilising RNA polymerase stalled at lesions. Both open and stalled complexes formed by wild type RNA polymerase are affected by (p)ppGpp. *rpo** mutations clearly mimic the presence of (p)ppGpp at promoters. Their effect on stalling of RNA polymerase has yet to be demonstrated clearly.

8.5. Conclusions

The work presented here has demonstrated that *rpo** mutations can be isolated from *recB* as well as from *ruv* mutant cells. There is no inherent difference between mutations isolated from the different backgrounds. The phenotype, viability and growth rate of *rpo** mutants was determined. *rpo** mutants behave like stringent mutants in every respect but one: their increase of the survival of UV-irradiated *ruv* and *recB* cells. The *rpo** mutations that could be identified by sequencing lie in *rpoB* and *rpoC*. All of them lie either directly on the inside surface of the clamp formed by RpoB and RpoC, in positions where they are likely to make DNA or RNA contacts, or they lie in conserved regions where changes are likely to induce structural changes, influencing substrate interaction. In vitro studies indicated that (p)ppGpp not only reduces the half life of open complexes, but that it influences stalled complexes in the same way. This result supports the theory that (p)ppGpp and RNAP* act by speedy dissociation of RNA polymerase from lesions to facilitate repair, thereby reducing the incidence of stalled replication forks. The results presented go a long way towards establishing the methods and conditions that might reveal how *rpo** affects RNA polymerase in a way that helps UV-irradiated cells to survive.

8.6. Future work

It would be of interest to ascertain whether *rpo** mutations can also be found in *rpoD* (encoding σ^{70}), as stringent mutations are known to exist in that gene and *rpo** mutations represent a subgroup. One stringent mutation that is not *rpo** has

been mapped on the sequence and lies in a very different location from any *rpo** mutation. In order to show the significance of this more stringent mutations will have to be sequenced.

Determining whether UV light induces the production of (p)ppGpp will be necessary to demonstrate the relevance the compounds for the compensation of the detrimental effects of UV light. Indirect assays for (p)ppGpp induction may need to be developed based on transcription of the (p)ppGpp-regulated *rrn* operons. The use of mass spectrometry could be an option.

The biochemical studies on wild type and mutant RNA polymerase presented here are far from complete. While they have provided some interesting results, much remains to be done. This includes more exhaustive studies with the two mutant RNA polymerases isolated so far and the isolation of one or two more mutant polymerases. Further optimising of the reaction conditions for some of the substrates will be necessary and other DNA substrates may have to be considered. Though it seems unlikely that stalled mutant RNA polymerase complexes are less stable (as the addition of heparin allowed only stable complexes to be visualised), an experiment where competitor is added after stalled complex formation is still missing. Introduction of specific damage into the DNA substrate (instead of or in addition to UV-induced damage) could prove useful. Different methods may have to be considered in order to obtain conclusive results. They could include use of a BIACORE (biomolecular interactions can be studied using surface plasmon resonance). It will also be necessary to test directly whether breakage of replication forks is affected by (p)ppGpp and *rpo** mutations, and if so whether such breakage occurs at sites of intense transcriptional activity, such as at *rrn* operons. *rrn* operons have been linked with genetic instability, consistent with DNA breakage being common at these sites.

It would also be interesting to perform experiments involving micro arrays in order to determine possible differences in gene expression between wild type RNAP under the influence of (p)ppGpp and RNAP containing an *rpo** mutation.

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