CLONING OF A DNA REPAIR GENE (*uvsF*) FROM ASPERGILLUS

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ABSTRACT

In order to clone the DNA repair gene of *Aspergillus nidulans*, *uvsF*$^-$ *pyrG*$^-$ strains were transformed with a genomic library in a plasmid vector that carried the *pyr-4* gene of Neurospora which complements *pyrG* mutants of Aspergillus. Primary selection was done on media lacking uracil. Growth of rare pyr$^+$ uvs$^+$ transformants was enhanced by overlays with MMS (methyl-methane sulphonate), since *uvsF*$^-$ is sensitive to this agent. Out of the several transformants obtained, four were like wild type. However, one of these grew slower for two days and caught up with the wild type on the third; while the other three were identical with the wild type. These two types also showed a difference in the size of bands in Southerns probed with the vector when the transformant genomic DNA was digested with *Bgl*II. However, both types showed identical bands in similar Southerns when DNA was digested with *Stu*I. The two types also behaved differently in crosses and only those showing the larger *Bgl*II band (the slow grower) gave *uvsF*$^+$ replacement progeny. For rescuing plasmids, transformant DNA was digested with *Bgl*II and self ligated, and used for transformation of *E. coli*. Two types of plasmids, corresponding to the size of bands observed in Southerns of *Bgl*II digested transformant DNA, were obtained; these two had a region in common (<1.0 kb) that was not a simple overlap and gave evidence for rearrangements.

Surprisingly, only the plasmids with the larger insert of Aspergillus DNA (7.2kb, the one rescued from the slow grower) were able to complement *uvsF*$^-$ in the secondary transformation. Northerns of polyA$^+$-enriched mRNA, probed with this plasmid, showed three bands. However, its subclone which spans the shared region hybridized to only one of them (1.0 kb). On screening Aspergillus libraries with the complementing plasmid to obtain the normal *uvsF* sequence, two cDNA and five genomic clones were identified. The two cDNA clones though not identical, cross-hybridized. The one with larger insert, when used to probe Northerns, hybridized to a 1.0 kb mRNA which presumably corresponds to the *uvsF* message. Three out of five genomic clones were identical. The cDNA hybridized to a short segment (2.2 kb) of one of the three types of genomic clones, locating the putative *uvsF* gene sequence.

RÉSUMÉ

Pour cloner le gène de réparation du DNA de *Aspergillus nidulans*, des souches *uvsF*$^-$ *pyrG*$^-$ ont été transformées avec une banque génomique. Cette banque a été construite dans un vecteur portant le gène *pyr-4* de Neurospora qui complémente la mutation *pyrG* d'Aspergillus. La première séléction a été faite sur un melieu sans uracil. L'utilization du MMS (Methyl Methane Sulphonate) a permis une selection secondaire des transformants *pyr*$^+$ *uvs*$^+$ puisque *uvsF*$^-$ est sensible à cet agent. Parmis les quelques transformants obtenus, quatre ressemblaient type sauvage. Mais l'un d'eux poussait plus lentement pendant deux jours, pour rattraper la vitesse de croissance du type sauvage le troisième jour, tandis que les trois autres étaient identiques au type sauvage. Ces deux types montraient aussi une différence avec la technique Southern, quand le DNA génomique des transformants, digiré avec *Bgl*II, a été sondé avec le vecteur. Pourtant, les deux types montraient des bandes identiques, quand le DNA était digéré avec *Stu*I. Les deux types se comportaient différemment dans les croisements et seulement ceux qui montraient la plus grande bande dans les Southerns de *Bgl*II (le transformant à croissance lente), a donné une lignée où *uvsF*$^-$ a été remplacé par *uvsF*$^+$. Pour récupèrer les plasmides, le DNA des transformants a été digéré avec *Bgl*II et ce par la suite été autoligué puis utilisé pour transformer *E. coli*. Deux types de plasmides, correspondant aux bandes observées dans les

Southerns de DNA digéré par le *Bgl*II, ont été obtenus. Ces deux plasmid avaient une région en commun (<1.0 kb), qui n'était pas un chevauchement simple, montrant un réarrangement. Étonnamment, seul le plasmide ayant la plus grande insertion de DNA d'Aspergillus (7.2kb, celui obtenu du transformant à croissance lente) était capable de complémenter *uvsF*$^-$ dans la transformation secondaire. Les Northerns des mRNA polyA$^+$, sondés avec ce plasmide ont montrés trois bandes. Néanmoins, un sousclone de la région commune, n'a révélé qu'une de ces trois bandes (1.0kb). En éxaminant la banque d'Aspergillus avec le plasmide complémentant le *uvsF* pour obtenir sa séquence normale, deux clones de cDNA et cinq clones génomiques ont été identifiés. Les deux clones de cDNA bien que non identique, s'hybridaient. Quand le clone qui contient la plus grande insertion était utilisé, il s'hybridait à un mRNA de 1.0kb qui vraisemblablement correspondait au message de *uvsF.* Trois des cinq clones génomiques étaient identiques. Le cDNA hybridait à un segment (2.2kb) d'un des trois types de clones génomiques, localisant la séquence putative du gène *uvsF.*

-Traduit par Claire Bonfils et Francis Ouellette

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LIST OF ABBREVIATIONS

A	Adenine
Ala	Alanine
C	Cytosine
CHO	Chinese Hamster Ovary
DNA	Deoxyribo-Nucleic Acid
G	Guanine
J/m^2	Joules per square-meter
Lys	Lysine
MMS	Methyl-Methane Sulphonate
MNNG	N-Methyl-N'-Nitro-N-Nitrosoguanidine
MNS	N-Methyl-N-Nitrosourea
mRNA	Messenger RNA
μ	Micron
μm	Micrometer
O^6-mGua	Ortho-6-Methyl Guanine
RNA	Ribo-nucleic Acid
T	Thymine
Tf	Transformant
t-BuO_2H	tert-Butyl Hydroperoxide
UDS	Unscheduled DNA Synthesis
UV	UltraViolet
XP	Xeroderma Pigmentosum

LITERATURE REVIEW

I *Aspergillus nidulans* basics

Life cycle of *A. nidulans* (Clutterbuck 1974):

A. nidulans is a homothallic haploid organism that can reproduce asexually by means of conidiospores or sexually by means of ascospores. Wild type conidiospores are green in colour. Mutations in several genes results in different coloured conidia, facilitating identification of various strains carrying different markers that are visually nonidentifiable. Though Aspergillus is a haploid organism, diploid strains can be obtained as described below: Hyphae of two strains with different nutritional requirements can be forced to fuse in absence of the required nutrients. This results in the formation of a mycelium with two types of nuclei with different nutritional markers i.e. a heterokaryon. At times, two nuclei with different markers fuse to form a diploid nucleus which outgrows the heterokaryon and conidiates better. Diploids are sterile but can be haploidized under special conditions. This property has been exploited to assign newly isolated genetic loci to specific linkage groups. A strain carrying the locus of interest and a strain with markers on all chromosomes are fused to get a diploid. Upon haploidization of this diploid, the locus of interest segregates with the chromosome on which it is located, allowing to assign the locus to that particular chromosome. The locus of interest can then be mapped relative to linked loci of known locations by meiotic analysis.

Genetics and molecular biology of *A. nidulans*:

Aspergillus and Neurospora are two of the best characterized lower eukaryotes, next only to *Saccharomyces cerevisiae*. Foundations of Aspergillus genetics were laid by Pontecorvo and his group (Pontecorvo *et al.* 1953). Aspergillus genetics has been reviewed by Käfer (1977)*.

Though the genetics of *A. nidulans* was well developed, molecular studies have only been possible recently, mainly because of the delayed development of the transformation system as compared to yeast and Neurospora (Ballance *et al.* 1983; Tilburn *et al.* 1983). Before the availability of the transformation system, the usual methods for cloning genes from *A. nidulans* were (1) heterologous probing e. g. cloning of the histone H2A gene using a *Drosophila melanogaster* probe (May and Morris 1987). (2) Complementation of a homologous mutation in another system e.g. the Ornithine Carbamoyltransferase gene was cloned by its ability to complement corresponding mutation in yeast (Berse *et al.* 1983), and the *arom* cluster gene was cloned by its ability to complement the corresponding gene in *E. coli* (Kinghorn and Hawkins, 1982). (3) Selective hybridization as in the case of developmentally regulated genes (Zimmerman *et al.* 1980). With the availability of the transformation system, it is now possible to clone a gene by complementation of an Aspergillus mutation (Ballance and Turner 1986).

Along with the transformation system several recently developed techniques have accelerated molecular studies in

*cited in publication

filamentous fungi in general. It is now possible to electrophoretically separate entire chromosomes of *A. nidulans* (Brody and Carbon 1989), *N. crassa* (Orbach *et al.* 1988), *S. cerevisiae* (Carle and Olson 1985, Clark *et al.* 1986, Schwartz and Cantor 1984) and *Candida albicans* (Magee *et al.* 1988). This has speeded up assigning genetic loci provided a probe is available. Alternately, genetically identified translocations have beenused to assignbands to chromosomes (Brody and Carbon 1989). The same technique could also be used to detect major deletions. Even in genetically poorly understood fungi, the number of complementation groups have been determined by using variations of such high voltage pulse field gel electrophoresis (Magee *et al.* 1988). Functional analysis of various genes can now be achieved by disruption of the gene of interest or replacing it by a mutant copy. This has been successfully achieved in yeast (and has been reviewed by Barnes and Thorner 1985), and in several genes in Aspergillus (Miller *et al.* 1985, Aramayo *et al.* 1989) and in *C. albicans* (Kelly *et al.* 1987).

Several reviews have been written about various aspects of the biology of *A. nidulans* from the molecular point of view (Bennett and Lasure 1985). These mainly refer to development (Timberlake and Hamer 1986, Timberlake 1988), mitosis (Morris *et al.* 1982, Morris 1986, Morris *et al.* 1986) and regulatory genes involved in metabolism (Arst and Scazzochio 1985, Wiame *et al.* 1985).

The recent developments in the molecular studies in

filamentous fungi would be economically important, since quite a few fungi are employed for industrial production of various biochemical products like antibiotics (Bennett 1985).

II DNA REPAIR

The stability of DNA is continuously challenged by various physical and chemical agents which introduce a wide array of changes in DNA. Most mutations are the results of damage caused by these agents, e.g. Heat causes deamination of bases and base loss by glycosylic hydrolysis; UV irradiation produces pyrimidine dimers, 6-4 photoproducts and strand breaks; ionizing radiation results in ring opening, base fragmentation, and single and double strand breaks (Singer and Kusmiérek, 1982). Some examples of chemical agents that damage DNA range from activated oxygen species generated during oxidative metabolism, common metabolites like glucose, inorganic and organic electrophiles including metals, alkylating agents and polycyclic aromatic hydrocarbons (Singer and Kusmiérek 1982).

To ensure the stability of DNA, complex DNA repair mechanisms have evolved that undo the damage caused to DNA. The mechanisms involved in repairing the damage are not simple straight forward biochemical pathways, rather they are more like repair networks or repair systems: more than one way to repair a particular kind of damage, and different types of damages repaired by a particular repair pathway (Von Borstel and Hastings 1977).

TYPES OF REPAIR

Basically all the organisms, in which DNA repair is studied, employ the types of repair mechanisms listed below, with minor variations from organism to organism. The main focus will be on *E. coli* and *S. cerevisiae*, since these are the organisms in which DNA repair is studied in sufficient detail, and in these organisms excision repair will be discussed in more detail, as we think that the gene of interest, *uvsF* of Aspergillus, is an excision repair gene. The reasons why we think it is so, are discussed in publication as well as later on in the literature review (see the section on DNA repair in filamantous fungi).

(1) In **direct DNA repair** the chemical change in DNA is simply reversed. Photolyases that carry out photoreactivation, after exposure of cells to UV, are examples of this class of enzymes. (2) Excision repair: (a) In **base excision repair**, the damaged base is removed by glycosylase, leaving behind an apurinic or apyrimidinic sites; the baseless sugar is then removed by an apurinic or apyrimidinic (AP) endonuclease and DNA polymerase. (2b) In **nucleotide excision repair**, modified bases are removed in the form of oligonucleotides, and the single stranded gaps so generated are then filled in by polymerases. (3) Post replication repair: (a) In **recombinational repair** the gaps that are generated during replication of damaged duplexes are filled in by strand transfer from an intact duplex. (b) In **mutagenic repair** inducible error prone repair system gets activated resulting in increased rate of mutation. (4) In

Mismatch repair the base pair mismatches are repaired by specific repair enzymes.

DIRECT REPAIR

Direct repair involves simple reversal of the covalent modification of DNA. Mainly two types of direct repair mechanisms are known: (1) Photoreactivation is the process in which the enzyme photolyase carries out light dependant reversal of UV induced thymine dimers (Friedberg 1985a). (2) In adaptive response there is a direct removal of methyl groups from the DNA. This is mediated by methyl transferases e.g. O^6-mGua DNA methyl transferase. This mode of response is not described here. It has been reviewed recently by Lindahl *et al.*(1988).

PHOTOREACTIVATION:

DNA photolyases, the enzymes that carry out photoreactivation, catalyze a unique reaction in which the energy in light, in the range of 300-500nm, is utilized to repair pyrimidine dimers in DNA by breaking the cyclobutane ring joining the pyrimidines. *Cis,syn* cyclobutane di-pyrimidines are the only known substrates (Friedberg 1985) and enzyme substrate complex formation is independant of the light requirement and must occur prior to absorbing photoreactivating light (Rupert *et al.* 1958).

Photoreactivation in ***E. coli***:

Enzymatic photoreactivation was first demonstrated in crude cell extracts of *E. coli* (Rupert *et al.* 1958). The *phr* gene, that codes for photolyase in *E. coli*, is not essential for viability.

However, *phr*$^-$ mutants, in addition to being unable to photoreactivate, are also partly deficient in excision repair of pyrimidine dimers (Yamamoto *et al.* 1984) because the enzyme stimulates ABC excinuclease (Sancar *et al.* 1984).

Photoreactivation in ***S. cerevisiae***:

DNA photolyase was first described in extracts of yeast cells in 1960 (Rupert 1960). There are two, apparently distinct, photolyases in yeast cells. One of these is a protein composed of two nonidentical subunits. Neither subunit alone is catalytically active; however, mixing the two restores DNA photolyase activity (Boatwright *et al.* 1975). The second enzyme is not well characterized (Iwatsuki *et al.* 1980).

Resnick (1969) mutagenized UV-sensitive *rad*2 strain defective in excision repair of UV-irradiated DNA and screened for mutants that failed to show improved growth following exposure to UV-irradiation and subsequently to photoreactivating light. Using a DNA transformation assay, extracts of the mutant thus isolated (designated *phr*1) were shown to be defective in DNA photolyase activity (Resnick and Setlow 1972). A gene designated *PHR*2 was identified in yet another mutant deficient (but not defective) in photoreactivation in vivo and in DNA photolyase in vitro.

The *PHR*1 gene has been cloned (Schild *et. al.* 1984, Yasui and Chevallier 1983) by phenotypic complementation of the *phr*1 mutation. When the cloned gene was tailored into an *E. coli* expression vector containing the *tac* promoter and transformed into a mutant of *E. coli* defective in DNA photolyase,

complementation of the Phr$^-$ phenotype was observed (Sancar 1985). Hence, it is likely that the *PHR*1 gene is a structural gene that encodes a yeast DNA photolyase. These complementation studies also suggest that the enzyme encoded by the yeast *PHR*1 gene normally contains a neutral flavin free radical similar to that present in the *E. coli* enzyme (Sancar and Sancar 1984), since *E. coli* is the only possible source of a chromophore for a protein expressed from the cloned yeast gene. In a reciprocal experiment it has also been shown that the cloned *E. coli phr*1 gene complements the phenotype of the yeast *phr*1 mutant (Langeveld *et al.* 1985).

EXCISION REPAIR

BASE EXCISION REPAIR:

In base excision repair, the N-glycosydic bond between the deoxyribose and the unusual or modified base is hydrolysed by a DNA glycosylase (Lindahl 1974). The damaged base is thus removed from the DNA. AP endonucleases hydrolyse (Lindahl and Andersson 1972, Verly and Paquette 1972) the phosphodiester bond adjacent to AP sites generated by DNA glycosylases (or by spontanious or induced depurination or depyrimidination of DNA) (Friedberg 1985). The abasic sugar is then removed from the nick and replaced with the correct nucleotide by the joint action of DNA polymerase and ligase (Lindahl 1979).

DNA glycosylases

There are several DNA glycosylases known and studied in fair

details. Only two most well studied ones will be described here.

(1) Uracil DNA glycosylase:

Uracil in DNA comes from three sources: misincorporation by DNA polymerases, spontanious deamination of Cytosine and deamination induced by Cytosine specific bisulfite, and nitrous acid. Uracil-DNA glycosylase-deficient mutants (*ung*) that contain point mutations, insertions, and deletions have been isolated from *E. coli* (Duncan 1985). Members of the latter two groups have no detectable glycosylase activity, therefore *ung* is not an essential gene. The mutants cannot remove the Ura residues in the DNA produced by Cyt deamination, and therefore have a high rate of G.C to A.T transition (Duncan and Weiss 1982). Uracil DNA glycosylases have also been isolated from yeast and human being (Busby *et al.* 1981, Caradonna and Cheng 1980). Human enzyme has been reported to be physically associated with the 70 kd catalytic subunit of DNA polymerase α, thus leading to the speculation that a multiprotein complex that carries out base excision repair in a concerted fashion may exist (Seal and Sirover 1986).

(2) 3-methyladenine-DNA glycosylase:

3-mAde and other alkylated bases in DNA are produced by treatment of cells or DNA with synthetic alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNS), methyl-methane sulphonate (MMS), dimethyl sulphate and S-adenosyl methionine (Singer and Kusmiéreck

1982). DNA glycosylases that remove 3-mAde have been isolated from various sources. *E. coli* contains two 3-mAde glycosylases, TagI and TagII (Lindahl 1982, Friedberg 1985). TagI is encoded by the *tag* gene. Wildtype cells contain about 200 molecules of TagI, and mutants lacking this enzyme are moderately sensitive to MMS and other alkylating agents (Walker 1985). TagII is encoded by the *alk*A gene. The sequences of TagI and TagII reveal no significant homology. This perhaps reflects, in addition to different evolutionary origins, different mechanisms of action of these proteins as evidenced by the inhibition of TagI but not of TagII by 3-mAde (Lindahl 1982, Walker 1985). The *alkA*$^-$ mutants are sensitive to MMS or MNNG, while *tag*$^-$ *alk*A$^-$ mutants as well as *tag*$^-$ *ada*$^-$ mutants, that are deficient in TagI and unable to induce TagII, are extremely sensitive to killing by alkylating agents. *M. luteus* also has two 3-mAde DNA glycosylase activities and cloned genes complement corresponding *E. coli* mutants (Pierre and Laval 1986).

AP Endonucleases

These enzymes hydrolyze the phosphodiester bond 5' or 3' to an abasic deoxyribose in DNA (Verly and Paquette 1973). AP endonucleases by incising on either side of the abasic sugar, initiate the repair process that eventually results in the replacement of the deoxyribose with a nucleotide. The phosphodiester bonds adjacent to the abasic sugar may be cleaved in four different ways (Sancar and Sancar 1988):

Class I AP endonucleases (3' incision; 3'-OH and 5'-P termini)

All known Class I endonucleases have associated glycosylase activity e.g. Endonuclease III.

Class II AP endonuclease (5' incision; 3'-OH and 5'-P termini) e g. Endonuclease IV.

Class III AP endonuclease (3' incision; 3'-P and 5'-OH termini) e.g. A newly isolated Drosophila AP endonuclease (Spiering and Deutsch 1986).

Class IV enzyme (5' incision; 3'-P and 5'-OH termini) e g The 5'-AP endonuclease associated with redoxyendonucleases.

However, an enzyme from human placenta incises on either side, but never both, of an AP site (Grafstrom *et al.* 1982b). Several reaction mechanisms are possible for cleavage at AP sites and these are discussed by Grafstrom *et al.* (1982a). The following are the best characterized AP endonucleases.

(1)*E. coli* exonuclease III:

This multitalented enzyme is the major AP endonucleases of *E. coli*, accounting for more than 85% of AP endonuclease activity in this bacterium (Lindahl 1982, Weiss and Grossman 1987) It has several different enzymatic activities (Kow and Wallace 1985, Warner *et al.* 1980): (a) Class II AP endonuclease with no associated glycosylase activity. (b) 3'→5' dsDNA specific exonuclease (c) Urea endonuclease. (d) 3' phosphatase activity and perhaps related 3' functions that remove 3' terminal deoxyribose-5'-phosphate to activate the 3' terminus for DNA polymerase I. (e) RNAse H The enzyme hydrolyzes only the RNA part of a hybrid duplex exonucleolytically in the 3'→5' direction. A unifying "common site" model whereby the enzyme "recognizes

the empty space" that exists in all substrates has been proposed to explain these seemingly dissimilar activities (Weiss and Grossman 1987). Exonuclease III mutants (*xth*) are sensitive to killing by MMS (Lindahl 1982), H_2O_2 (Demple *et al.* 1983), and near UV (Sammartano *et al.* 1986). The common feature of all these agents is that they create fragmented bases and/or AP sites. It has been suggested that many mutagens produce AP site as a common intermediate in mutagenesis (Loeb 1985). It is therefore surprising that *xth* mutants (which lack 85% of cellular AP endonuclease activity) have normal spontanious mutation rates. However, when there is an AP site overload such as the one induced by MNNG treatment, *xth*$^-$ cells have mutation rates 5- to 10-fold higher than wildtype cells (Foster and Davis 1987).

(2)*E. coli* endonuclease IV:

This is a Class II AP endonuclease with no other known activity (Lindahl 1982). The gene encoding this enzyme, *nfo*, has been cloned, and overproducing strains as well as deletion and insertion mutants have been isolated (Cunningham *et al.* 1986). The *nfo* mutants have increased sensitivity to alkylating agents MMS and mitomycin C and to the oxidants tert-butyl hydroperoxide (t-BuO_2H) and bleomycin. When strains with various combinations of *nth*, *nfo* and *xth* were constructed it was found that while *nth* had essentially no effect, *nfo* increased the sensitivity of *xth* mutants to MMS, γ-rays, and t-BuO_2H, and enhanced their mutability by MMS. These results are consistant

with redundancy in the function of these two nucleases. Unexpectedly, *nfo* mutants are more sensitive than *xth* mutants to t-BuO_2H and bleomycin (Cunningham *et al.* 1986). It is conceivable that endonuclease IV acts on certain lesions that are not substrates for exonuclease III.

(3)Human AP endonucleases:

Two AP endonuclease activities were partially purified from fibroblasts. It has been reported that AP endonuclease I is absent in xeroderma pigmentosum complementation group D (Linn 1982), whereas an ataxia telengiectasia cell line appears to have an altered form of the second enzyme due to a different posttranslational modification (Kuhnlein *et al.* 1985).

DNA Glycosylase-AP endonucleases

In this case both, DNA glycosylase as well as AP endonuclease, activities are in the same enzyme.

(1) Pyrimidine dimer DNA glycosylase:

So far, this enzyme has been identified only in two sources, *M. luteus* cells and T4 phage-infected *E. coli*. It hydrolyzes the glycosylic bond of the 5' pyrimidine of the dimer and then the phosphodiesterase bond between the two pyrimidines (Lindahl 1982, Friedberg 1985). The T4 phage gene (*denV*) for the enzyme has been cloned and sequenced (Radany *et al.* 1984, Valerie *et al.* 1984). The enzyme apparently carries out two cleavage reactions sequentially as the two reactions are frequently uncoupled (Weiss and Grossman 1987, Liuzzi *et al.* 1987). Phage T4 mutants that are deficient in the enzyme are extremely

sensitive to UV, and thus it seems that T4 Endo V has an important role in phage survival (Lindahl 1982). The *M. luteus* pyrimidine dimer DNA glycosylase (Grafstrom *et al.* 1982a) is similar to the T4 phage enzyme. *M. luteus* mutants deficient in the pyrimidine dimer glycosylase activity apparently have normal UV resistance, because this organism has a nucleotide-excision-repair system as well (Tao *et al.* 1987).

(2) *E. coli* endonuclease III:

This enzyme was originally identified by its endonuclease activity on heavily irradiated DNA. Its substrate include a variety of thymine and cytosine residues damaged by ring saturation, fragmentation or contraction as a result of exposure to ionizing radiation and certain oxidative agents (Katcher and Wallace 1983, Doetsch *et al.* 1986). Endonuclease III hydrolyses the glycosylic bond of the damaged pyrimidine and the phosphodiester bond 3' to the resulting abasic sugar to generate 3'-OH and 5'-P termini (Demple and Linn 1980). The glycosylase and AP endonuclease reactions, unlike pyrimidine dimer glycosylase, are concerted so that enzyme never generates free AP sites during the reaction. Insertion and deletion mutations for the gene, *nth*, encoding the enzyme, are not more sensitive to ionizing radiation or H_2O_2 than wildtype, but they have an increased rate of spontanious mutation (Weiss and Cunningham 1985).

Deoxyribose phosphatase

(Reviewed by Friedberg 1988 and Friedberg 1985)

It is not known if following the incision 5' to the AP site, the abasic sugar can be released by endonuclease III. However, there

exists an enzyme in *E. coli* (Deoxyribose phosphatase) whose sole function appears to be to remove 5' terminal deoxyribose since this function can not be carried out by DNA polymerase I. The enzyme removes deoxyribose from the 5' terminus but not terminal nucleotides and thus may be the missing link in base-excision repair. A similar activity has been found in human cells. The gap created by base-excision-repair enzymes is apparently filled in without nick translation as patches of 2-4 nucleotides are obtained in vivo for both *E. coli* and mammalian cells.

NUCLEOTIDE EXCISION REPAIR:

In nucleotide excision repair damaged bases are removed from DNA as an oligonucleotide and the resulting gap is patched by repair synthesis (Pettijohn and Hanawalt 1964 and references cited therein). Many nucleotide mono- and diadducts are removed by nucleotide excision, and interstrand crosslinks are repaired by a concerted action of nucleotide excision and recombinational repair pathways. Nucleotide excision repair has been documented in prokaryotes and eukaryotes. *E. coli*, yeast, and human cells deficient in this repair pathway have been isolated and have been important in the studies on excision repair (Lindahl 1982, Friedberg 1988, Sancar and Sancar 1988).

E. coli:

Briefly, an ATP dependant nuclease made of three subunits, the ABC excinuclease, hydrolyzes the 8th phosphodiester bond 5' and the 4th and 5th phosphodiester bond 3' to mono- and dinucleotide adducts to remove a 12-13mer from DNA and generate a gap of

the same size that is filled in by DNA polymerase I and sealed by ligase (Sancar and Rupp 1983).

The most striking feature of nucleotide excision repair is its wide range of substrates. The ABC excinuclease excises pyrimidine dimer and 6-4 photoproducts as well as base adducts of many chemicals. It does not remove 3-mAde from DNA nor does it incise DNA that contains base mismatches or one to four nucleotide-long loops or deletions (Thomas *et al.* 1986). It is apparent, therefore, that while most of the adducts removed by the enzyme cause major helical distortions, not all gross helical deformities are recognized. The relatively small size of the enzyme precludes the notion that it contains a different active site for each of the many different adducts that are substrates, thus it seems likely that the adducts that are substrates cause relatively similar helical deformities in DNA and that the enzyme recognizes this altered backbone structure. As the structures of the various adducts are refined, it is possible that a unique DNA backbone configuration specific for interaction with ABC excinuclease will emerge. However, MNNG, which is not known to cause major structural changes in DNA, created substrate for the excinuclease even though the adduct responsible for incision was not identified (Van Houtten and Sancar 1987). Similarly, based on mutation rates observed in *uvr*$^-$ and wildtype cells with a site specifically located O^6-mGua (which does not distort the helix), it has been proposed that the ABC excinuclease or some of its subunits may bind to this adduct but fail to remove it and thus

may interfere with its repair and increase the mutation rate (Chambers *et al.*1985). Clearly, definition of the substrate structure will be an important step towards understanding the action mechanism of the enzyme.

The genes for nucleotide excision repair are *uvrA*, *uvrB*, and *uvrC* (Howard-Flanders 1966); these have been cloned, sequenced, and the subunits have been overproduced in great quantities, and purified to homogeneity and studied in some detail (Husain *et al.* 1986, Arikan *et al.* 1986, Backendorf *et al* 1986, Thomas *et al.* 1985). Mutations in any of the three *uvr* genes make cells extremely sensitive to UV as well to mitomycin C, nitrous acid, psoralen, and many other genotoxic agents.

The UvrA protein:

The UvrA protein is an ATPase and a DNA binding protein, and analysis of the gene sequence reveals some interesting features regarding these functions. The protein contains three Walker "A-type" and "B-type" purine recognition sequences (Walker *et al.* 1982) found in most ATPases. UvrA protein and several other ATPases share extensive homology over about 250-amino acid region (domain) that extends far beyond the Walker sequence (Doolittle *et al.* 1986). The majority of these proteins have only one ATPase domain, whereas UvrA contains three. These three putative ATPase domains of UvrA are interrupted with or seperated from one another by three "Zinc-finger" domains, of which one has extensively diverged, whereas the other two are conserved; indeed UvrA has two Zinc atoms per polypeptide chain.

Thus it has been proposed that UvrA protein originated from the fusion of the Zinc-finger with DNA-binding capacity to an ATPase of energy transducing capacity, and this primordial protein was partially duplicated 3-4 times to generate the present day UvrA protein (Doolittle *et al.* 1986). Association of UvrA with UvrB is ATP dependant, but UvrB does not stimulate the ATPase activity of UvrA. It binds specifically to DNA irrespective of the presence of UvrB (Sancar and Sancar 1988). The binding of UvrA to DNA is increased 10 fold in presence of ATP plus UvrB (Seeberg and Steinum 1982).

The UvrB protein:

The protein has three interesting features. First the protein has a Walker ATP binding sequence even though it is not an ATPase. Second, the 14 amino acid stretch that is 12 amino acids from the C-terminus of UvrB is homologous (13 out of 14) to a region in the middle of UvrC protein. There are other areas of moderate homology between the two proteins such that the two proteins can not be aligned linearly. Third, UvrB is rapidly cleaved in cell free extracts at Lys629-Ala630 by a sequence specific protease that also cleaves Ada, a protein involved in adaptive response, in two places (Arikan *et al.* 1986, Teo 1987). UvrB is a monomeric, globular protein. It does not bind to ssDNA or dsDNA as measured by nitrocellulose filter assay, sucrose density gradient centrifugation (Kacinski and Rupp 1982), or DNAase I footprinting (Van Houtten *et al.* 1987). However, it binds to DNA in presence of UvrA protein.

The UvrC protein:

There are three interesting features of UvrC sequence. One concerns the patches of homology to the UvrB protein. The second, the C-terminal 60 residues of UvrC are homologous to the C-terminal segment of the human repair protein ERCC-1 (Doolittle *et al.* 1986). Third, the amino terminus of the protein is blocked.

The action mechanism of ABC excinuclease:

(1) Formation of the complex: There is no evidence that the ABC excinuclease exists in the absence of the substrate (Seeberg and Steinum 1982). The A subunit is essentially all in dimer form at physiological concentrations, and it makes A_2B_1 complex in the presence of ATP. UvrC does not associate with either subunit or with the A_2B_1 complex in the presence or absence of ATP. Footprint analysis has helped in the elucidation of the assembly process in more detail (Van Houtten *et al.* 1987). UvrA by itself recognizes DNA adducts and binds specifically, making a 33-bp-long DNAse I footprint. ATP is stimulatory to, but not required for this reaction. The UvrA-UvrB complex binds with higher affinity to the damaged DNA and produces a footprint of 19bp, considerably smaller than UvrA alone. This could be due to a conformational change in UvrA to a more compact form that makes mostly specific contacts or due to release of one UvrA from the A_2B_1 complex. The footprints of both, UvrA and UvrA-UvrB complex, are more pronounced on the undamaged strand which shows that the excinuclease makes more intimate contacts with this strand (contrast with photolyase, Van Houtten *et al.*

1987).

(2) ATP hydrolysis: ATP hydrolysis induces conformational changes in UvrA that facilitates its dimerization, and association with the UvrB subunit, and the ATP hydrolysis by the UvrA-UvrB complex induces a conformational change in DNA necessary for cleavage upon binding of the UvrC subunit (Van Houtten *et al.* 1986, Strike and Rupp 1985, Oh and Grossman 1987). However, there seems to be some futile hydrolysis by the UvrA-UvrB or the excinuclease holoenzyme complex when bound at the adduct site. Thus the UvrA-UvrB complex hydrolyzes ATP about twice as fast in the presence of the saturating levels of pyrimidine dimers, and hydrolysis continues at the same rate following addition of UvrC and excision of the dimers (Thomas *et al.* 1985).

(3) ABC excinuclease removes DNA by hydrolyzing phosphodiester bonds on both sides of the modified nucleotides, and it does not become covalently linked to DNA durinig excision (Sancar and Rupp 1983). The incision pattern shows minor variability depending on the type of the adduct: Monoadducts are removed by hydrolysis of the 8th phosphodiester bond 5' and the 5th phosphodiester bond 3' to the damaged nucleotide (Thomas *et al.* 1986, Sancar *et al.* 1985). Thus a fragment of 12 nucleotides is excised, and a gap with 3'-OH and 5'-P is generated. In the removal of intrastrand diadducts there is some staggering on the 3' side (4th or 5th phosphodiester bond 3' to the adduct is cut). In pscralen diadducts the enzyme excises the furan side of the adduct only, generating an oligonucleotide that remains attached

to the other strand (Cimino *et al.* 1985). This unique incision pattern is caused by the structure of the DNA at the crosslink rather than by any affinity of the enzyme for the furan ring. In monoadduct forms both furan side and pyrone side adducts are removed with equal efficiency (Van Houtten *et al.* 1986b).

(4) The subunits making the incisions are not known. The fact that UvrA-UvrB complex makes specific contact at the damaged site, yet does not incise the DNA until UvrC is added, suggests that UvrC may be the nuclease that is targeted to its site of action by the damage recognition subunits, UvrA and UvrB. Recently it has been shown that the excinuclease has only the UvrB and UvrC subunits in the incision complex but neither has an intrinsic nuclease activity on their own (Orren and Sancar 1989). UvrA may thus be involved in delivering UvrB at the damaged site.

(5) The ABC excinuclease makes the two incisions in a concerted fashion (Sancar and Rupp 1983), but depending on the experimental conditions either the 5' or the 3' side of the adduct is cleaved. Since this has been observed only in vitro, it may not have any physiological significance. However, the uncoupling phenomenon has helped in addressing the question of whether there is a rigid temporal order for the two incision events. Since both uncoupled 5' and 3' incisions are observed, it is concluded that one does not have to follow the other (Van Houtten *et al.* 1986a).

Other proteins needed for the activity of ABC excinuclease:

The liquid holding effect is due to UvrD and PolA proteins. In *E. coli* six proteins are enough to carry out excision repair. These

are in addition to UvrA, UvrB and UvrC, UvrD (helicaseII), Pol I, and ligase. Pol I and UvrD are the proteins responsible for the turnover of the enzyme, though the mechanism by which they do so is not well understood (Sancar and Sancar 1988). In vitro, in the absence of UvrD and Pol I proteins, the extent of DNA incision and excision [by ABC excinuclease] of oligonucleotides containing pyrimidine dimers is limited, but not abolished. Addition of UvrD and Pol I proteins increases turnover of the UvrABC complex, thereby facilitating further incision and excision events (Caron *et al.*1985, Husain *et al.*1985, Kumara *et al.*1985). Hence, the overall kinetics of excision repair in vitro is improved in the presence of these two proteins, and this suggests that the role of UvrD and Pol I proteins in nucleotide excision repair is quantitative rather than qualitative (Friedberg 1985b).

***S. cerevisiae*:**

A lot of radiation sensitive yeast mutants were isolated by 1970 (Friedberg 1985, Friedberg 1988). All mutants abnormally sensitive to killing by radiation are designated as *rad*, with identifying locus and allele numbers. Locus numbers less than 50 refer to genes which primarily affect sensitivity to UV radiation or to both UV and ionizing radiation. Locus numbers 50 and above designate genes which primarily affect sensitivity to ionizing radiation. Mutants sensitive to various chemical mutagens were also isolated (e.g. *mms* and *pso* mutants). Approximately 30 mutant loci have been analyzed following exposure of cells to UV or ionizing radiation and have been placed into three epistasic

groups referred to as the *RAD3*, *RAD52*, and *RAD6* groups, after prominent mutant loci in each (Game 1974, Haynes and Kunz 1981). These three groups of genes are thought to reflect three largely nonoverlapping primary cellular responses to ionizing and UV radiation damage to DNA in yeast. Thus, loci in the *RAD3* epistasis group are involved in nucleotide excision repair and those in the *RAD6* epistasis group are required for mutagenesis, while those in the *RAD52* epistasis group are thought to reflect the existence of recombinational responses to DNA damage (Friedberg 1985, Friedberg 1988).

It is important to mention that it has been found that if the epistatic interactions of the mutant loci were reexamined with respect to monofunctional alkylation damage, a different classification of these genes might be expected. Thus, various epistasis groups should not be viewed as repair pathways, but rather as repair systems which can be reassorted for different purposes (Von Borstel and Hastings 1979). This holds true for DNA repiar in any organism. To site three examples: (1) Rad1 protein of yeast is involved in excision repair as well as recombination (Schiestl and Prakash 1988). (2) RecA protein of *E. coli* is involved in recombination repair as well as in regulating SOS repair when challenged with mutagens. RecA is also involved in mutagenic response which gets turned on as a part of SOS circuit (Witkin 1989). (3) RAD 24 gene of yeast participates in excision repair as well as recombination repair (Eckardt-Schupp *et al.* 1987).

Only ***RAD3 group*** which is involved in nucleotide excision repair will be discussed here.

Functional analysis of RAD3 group of genes:

Several lines of evidence suggested that *RAD3* group of genes are involved in nuceotide excision repair.

(1) Direct studies on excision repair of pyrimidine dimers were hampered because *S. cerevisiae* does not possess thymidine kinase activity (Grivell and Jackson 1968); thus, radiolabelled thymine or thymidine is not metabolized to deoxythymidine 5'-triphosphate hence not incorporated into DNA. Unrau and his colleagues (1971) labelled UV-irradiated cells with radioactive uracil, and following the purification of high molecular weight DNA, they chromatographically resolved radiolabelled uracil-uracil, uracil-thymine, and thymine-thymine dimers. Direct quantitation of these photoproducts in RAD and *rad1-1* cells showed that the latter (*RAD3* epistasis group mutants) are defective in the excision of pyrimidine dimers.

(2) Another line of evidence came from the use of photoreactivating enzyme and its specificity for pyrimidine dimers in DNA. It was shown that, when UV-irradiated RAD cells were incubated in the dark, sites that could be reactivated with photolyase during subsequent incubation of their DNA (i.e., pyrimidine dimers) were progressively lost. This dark-dependent loss of photoreactivatable sites was not observed with UV-irradiated *rad2* cells (Resnick and Setlow 1972b).

(3) Later on several workers used the direct measurements of

single-strand breaks in the nuclear DNA of UV-irradiated cells as a function of postirradiation incubation time. This technique involved sedimentation of DNA in alkaline sucrose gradients; thus, RNA is degraded and the use of a general metabolic label such as [^{3}H]uracil is quite satisfactory. However, the drawback is that, at any given moment the number of breaks that can be detected by sedimentation of DNA is very small (Reynolds and Friedberg 1980, Reynolds and Friedberg 1981)

(4) The introduction of strand breaks, with dimer specific endonuclease probes (from *M. luteus* or T4-infected *E. coli*), into DNA isolated and purified following the irradition and incubation of cells, is more sensitive for evaluating defective excision repair than the direct measurement of DNA strand breaks (Prakash 1975). This technique relies on the fact that, if pyrimidine dimers are removed from the genome by excision repair in vivo, sites sensitive to dimer-specific probes are lost and hence are not converted to strand breaks detected by sedimentation velocity.

(5) A number of laboratories have investigated the use of plasmids for evaluating excision repair in yeast cells. Most strains normally contain about 50 to 100 copies of the double-stranded plasmid i.e. the 2μ DNA (Sinclair *et al.* 1967). This plasmid is in many ways a model minichromosome. It is replicated at a specific time in the cell cycle (Zakian *et al.*1979) and is physically associated with histones (Livingstone and Hahne 1979). McCready and Cox (1980) isolated 2 μm plasmid DNA from unirrradiated and UV-irradiated cells incubated for varying

periods of time to allow excision repair. After a dose of 20 J/m^2, ~86% of the plasmid molecules acquired sites sensitive to nicking by the *M. luteus* dimer-specific endonuclease, but following a 3-h preincubation essentially all enzyme-sensitive sites were removed. Consistent with excision repair in nuclear DNA, plasmids isolated from a *rad1* mutants showed no significant loss of dimers (post incubation), even though plasmid-born dimers in this strain were reversible by photoreactivation. *rad2*, *rad3*, and *rad4* mutants showed no detectable repair of plasmid DNA, whereas *rad7* and *rad14* mutants showed residual repair.

Convincing evidence of several genes being involved in excision repair has been obtained by combining several of the experimental strategies discussed above. It has been shown that cells carrying mutations in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10* genes do not carry out any detectable incision of their DNA during postirradiation incubation, whereas those carrying mutations in the *RAD7*, *RAD14*, *RAD16*, *RAD23*, and *MMS19* genes have a significant residual capacity for incising DNA at sites of pyrimidine dimers (McCready *et al.* 1987, Prakash 1977a, Prakash 1977b, Prakash and Prakash 1979, Reynolds and Friedberg 1980, Reynolds and Friedberg 1981, Wilcox and Prakash 1981). Mutants that include deletions of the entire *rad7* and *rad23* genes are only partially defective in excision repair of pyrimidine dimers (Perozzi and Prakash 1986). Hence, the partial defects exhibited by the *rad14*, *rad16*, and *mms19* mutants may not be

fortuitous and that these members of the *RAD3* epistasis group may not be primarily involved in the incision of DNA at sites of pyrimidine dimers [and probably at sites of other forms of bulky base damage], but may have a secondary role in this process, like E. coli UvrD and Pol I proteins (discussed earlier, Friedberg 1988).

Another, not necessarily exclusive, role for these proteins could be their involvement in "opening up" the genome during DNA repair. Such opening up may not be required in actively transcribing genes. There is compelling evidence that in mammalian cells excision repair occurs preferentially in actively transcribing genes (Bohr *et al.* 1986, Bohr *et al.* 1985, Madhani *et al.* 1986).

The observed biochemical differences between the genes of RAD3 group, described above, are consistant with the observed genetic differences between these genes. Mutations in *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10* genes confer markedly increased sensitivity to killing by UV radiation (Cox and Parry 1968), while others [*rad7*, *rad14*, *rad16* (Cox and Parry 1968), *rad23* (cited in Haynes and Kunz 1981), *rad24* (Averbeck *et al.* 1970, Eckardt-Schupp *et al.* 1987), *cdc8* (Prakash *et al.* 1979), and *mms19* (Prakash and Prakash 1979)] are considerably less UV sensitive. The recent cloning of the *RAD4* and *RAD10* genes facilitated the construction of disruption and deletion mutants and led to the demonstration that the *rad4* and *rad10* point mutations, that were less UV-sensitive than *rad1*, *rad2* and *rad3*, are in fact leaky (Fleer *et al.* 1987b, Weiss *et al.* 1987).

Whenever possible, therefore, the phenotypic characterization of radiation-sensitive yeast strains should be carried out with deletion or disruption mutants rather than with point mutants. Similar strategy should be employed wherever possible e. g. *E.coli*, *A. nidulans*.

In the case of the *rad7* and *rad23* mutants, as mentioned before, it has been established that leakiness is not an explanation for their limited UV sensitivity, since deletion mutants have the same phenotype as point mutants (Miller *et al.*1982, Perozzi and Prakash 1986). Comparable evidence for the *rad14*, *rad16*, and *mms19* mutants must await the isolation of alleles with disruptions or deletions (the *RAD24* and *CDC8* genes are essential, hence are inviable if disrupted).

Cloning of *RAD3* group of genes:

Several *RAD* genes have been cloned by complementation of corresponding *rad* mutants. In all cases genetic analysis of the cloned inserts had confirmed that they contain the specific *RAD* genes of interest rather than sequences which nonspecifically complement the phenotype of UV sensitivity. Such genetic analyses are particularly easy in *S. cerevisiae* because transformation of yeast cells with plasmids containing cloned genomic sequences results in integration of the cloned sequences almost exclusively at sites of homology. Hence, it is possible to integrate a cloned wild type gene in very close physical and genetic proximity to a chromosomal mutant allele and establish the identity of the cloned sequence by phenotypic analysis of the integrant derivative. These and other strategies for the genetic

analysis of cloned yeast genes are discussed by Friedberg *et al.*(1988). Analysis of plasmids isolated by phenotypic complementation of selected *rad* mutants has resulted in the unequivocal identification of the *RAD1* (Higgins *et al.* 1983, Yang and Friedberg 1984, Yasui and Chevallier 1983), *RAD2* (Higgins *et al.* 1984, Naumovski and Friedberg 1984), *RAD3* (Higgins *et al.* 1983, Naumovski and Friedberg 1982), and *RAD10* (Prakash *et al.* 1985, Weiss and Friedberg 1985) genes.

Since *RAD4* could not be cloned by complementation of the corresponding mutant, it was cloned by an alternative strategy. The gene is known to be genetically tightly linked to the *SPT2* locus on the right arm of chromosome V. The *SPT2* gene had been cloned (Roeder *et al.* 1985), and an integrating plasmid containing the *spt2-1* mutant allele was shown to complement the UV sensitivity of several *rad4* mutant strains (Fleer *et al.* 1987a). Physical mapping of the *RAD4* gene demonstrated that the entire gene is present in the integrating plasmid containing the *spt2-1* allele (Fleer *et al.* 1987a). However, this as well as all other *RAD4*-containing plasmids propagated in *E. coli* contain inactive alleles (Fleer *et al.* 1987a, Fleer *et al.* 1987b). These alleles arise by selection of mutationally inactivated forms of the gene, presumably because expression of the *RAD4* gene is toxic to *E. coli*.

Cross-species complementation:

The yeast Rad10 and human Ercc1 proteins have been shown to have considerable structural conservation (Van Duin *et al.* 1986).

In an effort to explore a functional relationship between these proteins, the cloned *RAD10* gene was introduced into the UV-sensitive excision-defective CHO cell line UV20 (and into several other cell lines from complementation group 2 of Thompson et al. (1987), the genetic complementation group used to identify the human *ERCC1* gene (Thompson *et al.* 1987). Comparisons of the UV sensitivity of *RAD10*-bearing and control cells reproducibly showed partial complementation of the phenotypes of UV sensitivity. The partial complementation of UV sensitivity may reflect limitations inherent to interspecies complementation. The partial phenotypic complementation is specific for RAD10. Integration and amplication of the *RAD3* gene in UV20 cells has no effect on UV sensitivity, and the *RAD10* gene has no effect on the UV sensitivity of UV-hypersensitive CHO cells from other genetic complementation groups. Thus the structural relationship between human *ERCC1* and yeast *RAD10* is also maintained at the functional level. These results suggests that proteins for nucleotide excision repair may be conserved in many, if not all, eucaryotes and have motivated attempts to use yeast genes to isolate homologous genes from other eukaryotes (McCready *et al.* 1989). Direct probing of human genomic DNA with *RAD10* did not reveal specific hybridization by Southern blot analysis. However, both the *RAD10* and *ERCC1* genes hybridized to common fragments in the Drosophila genome, suggesting that the use of Drosophila probes may facilitate the isolation of human DNA repair genes (cited by Friedberg *et al.* 1987). Probing digests of

Drosophila DNA with the cloned *RAD1* and *RAD3* genes also yielded specific hybridizing fragments (cited by Friedberg *et al.*1987). These will presumably be used to search for homologous sequences in human DNA. A similar approach using affinity-purified polyclonal antibodies purified from antisera to Rad proteins has been used (see above). Antibodies against Rad3 proteins react with two polypeptides in extracts of human HeLa cells (cited in Friedberg 1988). These proteins (Mr, ~150000 and ~95000, respectively) may be distinct species or may be related. Clearly, the next step is to screen human expression libraries in the hope of identifying sequences that prove to be genes related to *RAD3*.

So far there is no evidence for structural or functional homology between yeast and *E. coli* nucleotide excision repair genes or proteins. Antibodies against *E. coli* UvrA, UvrB and UvrC proteins do not cross react specifically with Rad proteins (cited in Friedberg 1988). Similarly, antisera raised againsat the yeast Rad proteins failed to cross react with extracts of untransformed *E. coli* cells or cells transformed with plasmids containing the cloned *uvr* genes. Also, neither transformation of *E. coli uvr* cells with plasmids containing cloned *RAD* genes nor the reciprocal experiment has resulted in phenotypic complementation (Friedberg 1985b). Taking into account these results, it is surprising that human *ERCC1* gene has a region homologous to yeast *RAD10* gene as well as a region homologous to *E. coli uvrA* and *uvrC* genes (van Duin et al. 1988). Several other examples of cross species complementation are discussed

in the "Overview" section of the literature review.

DNA Repair in Humans

Cell strains derived from patients having a hereditary disorder associated with defects in repair of DNA damage such as xeroderma pigmentosum (XP) (Lehmann and Karran 1981) and mutants isolated from established rodent cell lines (Thompson *et al.* 1987, Shiomi *et al.* 1982) provide the tools for genetic and biochemical analysis of DNA repair pathways in mammalian cells (Rubin *et al.* 1983). There do exist mutations, besides XP, in several DNA repair genes in humans "e.g." Bloom's syndrome, ataxia talengiectasia, Fanconi's anemia, and Cockayne's syndrome. All of the known mutations are autosomal recessive and render the individuals harboring them, susceptible to melanomas and other forms of skin cancers, among other symptoms (Kraemer 1983, Friedberg 1985). However, in the present review, main focus will be on the excision repair genes (XP genes and genes cloned by heterologous complementation of mutations) since these are the best studied ones and also, because the gene of interest for my project, *uvsF*, is an excision repair gene.

XP displays a considerable genetic heterogeneity. Genetic complementation experiments have demonstrated the presence of at least ten complementation groups (de Weerd-Kastelein *et al.* 1972, Fischer *et al.* 1985). That XP cells are deficient in DNA repair had been shown by their markedly reduced ability to carry out unscheduled DNA synthesis (UDS) after irradiation with UV. (Cleaver 1968, Reviewed in Friedberg 1985) (In eukaryotic cells

DNA replication occurs only in S phase and not in G2, M or G1 phases. Any DNA synthesis occuring in a phase other than S-phase is referred to as UDS, which reflects presence of DNA repair). Further evidence of XP cells being deficient in DNA repair was the ability of phage T4 endonuclease V, a well characterized DNAglycosylase-AP endonuclease (reviewed earlier), to partially and transiently restore the ability of XP cells to carry out unscheduled DNA synthesis (Tanaka *et al.* 1975, Tanaka *et al.* 1977, Smith and Hanawalt 1978).

In vitro studies with purified *E. coli* repair proteins has been very useful in understanding DNA repair processes in considerable details (discussed earlier). Such an understanding about human DNA repair processes does not exist as purified proteins are not available. As a first step towards characterization of proteins involved in DNA repair in humans, de Jonge *et al.* (1983) and Yamaizumi *et al.* (1986) reported correction of XP group A defect by microinjection of wild type human cell extracts from HeLa cells and placenta. Such crude extracts had an activity that was abundantly present in wild type but not in cells of XP group A, was inactivated by proteinase K but not by RNAse and was specific for XP group A. Furthermore, in order to purify specific factors involved in DNA repair, Yamaizumi et al. (1986) partially purified these proteinaceous factors that are deficient in XP group A cells and determined their molecular size by gel filtration. More recently, Wood and his colleagues (1988) described soluble extracts from human lymphoid cell lines that

perform repair synthesis on covalently closed circular DNA containing pyrimidine dimers or psoralen adducts. In these studies short patches of nucleotides were introduced, by excision repair of damaged DNA, in an ATP dependant reaction. Extracts from XP cell lines failed to act on damaged circular DNA, but were proficient in repair synthesis of UV irradiated DNA containing incision generated by *M. luteus* pyrimidine dimer DNA glycosylase. Repair was found to be defective in all XP cell lines investigated, representing groups A, B, C, D, H, and V. Mixing of group A and C resulted in reconstitution of the DNA repair activity. Taken together all these results suggest that in near future more elegant in vitro experiments with purified human excision repair proteins would be possible, as has been done with *E. coli* proteins.

Cloning of human DNA repair genes:

Cloning of genes involved in excision repair by complementation of human DNA-repair mutants with wild type libraries has, so far, not proved to be successful (Lehmann 1985) [Problems involved in cloning Ataxia talengiectasia genes are discussed by Lohrer *et al.* 1988]. As an approach to characterize these mutants and clone their wild type analogoues, a number of chinese hamster ovary (CHO) cell lines that are sensitive to UV light have been isolated. Genetic complementation revealed that these mutants constitute at least five different complementation groups, which are, like XP, all defective in the incision step of

the excision repair pathway. For obtaining UV resistant transfectants and for cloning of human DNA repair genes, CHO cell lines have proved to be successful (in contrast with XP cell lines) (Rubin *et al.* 1983, McInnes *et al.* 1984, Thompson *et al.* 1985)

Rubin *et al.* (1983) reported the identification of a human DNA repair gene following DNA mediated gene transfer into CHO mutant cells. Westerveld *et al.* (1984), using the strategy developed by Rubin *et al.* cloned the first human DNA repair gene - *ERCC1* by virtue of its ability to complement a UV sensitive CHO cell line. Upon sequencing this gene very interesting features were uncovered. *ERCC1* is homologous to the yeast excision repair gene *RAD10* (van Duin *et al.* 1986, discussed earlier) and its carboxy terminus exhibits homology to parts of the *E. coli* repair proteins *uvrA* and *uvrC* (van Duin *et al.* 1988). Not surprisingly, yeast RAD10 and human ERCC1, both complement excision repair deficient CHO cell lines of the same complementation group (van Duin et al. 1986, Lambert et al. 1988). Thus the structural homology observed between these two genes, is also maintained at the functional level [discussed earlier]. As checked by directed mutagenesis, the highly conserved C-terminus is essential for the function of *ERCC1*, where as the non conserved N-terminus is dispensible (van Duin *et al.* 1988). Recently Tanaka *et al.* (1989) have cloned a mouse DNA repair gene that complements the defect of XP cells of group A.

In spite of progress made, only a few XP genes and none of their products are identified. However, looking at the pace and

direction of research, it should not be long before a comparatively detailed picture of mammalian DNA repair is obtained.

POST REPLICATION REPAIR:

There are two types of post replication repair processes- error free and error prone. Recombinational repair is error free whereas mutagenic response is error prone. In *E. coli*, however, some of the proteins involved in both processes may be coregulated and also some of the proteins may participate in both types of repairs e.g. RecA protein.

RECOMBINATIONAL REPAIR:

E. coli:

The *recA*, *recB*, *recC*, *recD*, *recF* and several other mutations identify recombination repair pathways. The components involved in recombination repair overlap with those involved in recombination, but are not entirely identical with them, since some mutations affect one but not the other activity (reviewed by Dressler and Potter 1982) *E. coli* has two recombination repair pathways. RecA participates in both pathways. One pathway involves RecBCD and the other RecF (reviewed by Walker 1984).

A model which was formulated for *E. coli* two decades ago (Hotchkiss 1974), remains essentially unchanged. A single stranded gap is generated when the polymerase encounters certain nucleotide adducts, and stops replicating and reinitiates

about 1000 bp beyond the adduct. This gap contains a modified nucleotide. This discontinuity or postreplication gap is filled in by the RecA protein, which tranfers the complementary strand from the sister duplex into the gap. With the purification and characterization of the RecA protein (Cox and Lehmann 1987), it has been possible to perform some of the steps of recombinational repair in vitro.

Yeast:

Among the *RAD52* group, the *rad51*, *rad52*, and *rad54* mutants are extremely sensitive to ionizing radiation. These three *rad* mutants are almost completely defective in meiotic and mitotic recombination (Game 1983, Haynes and Kunz 1981, Schild *et al.* 1983). In addition, these mutants have the phenotype of both spontaneous and radiation-induced chromosone instability, leading to monosomy in diploids (Morrison and Hastings 1979, Mortimer *et al.* 1981), and are defective in the repair of double-strand breaks (Ho 1975, Resnick and Martin 1976) and homothallic switching (Malone and Esposito 1980). Finally, these mutants show a sharp reduction of the X-ray resistance normally associated with mating-type heterozygosity (see Game 1983).

The *rad50*, *rad53*, *rad56*, and *rad57* mutants are also classified in the *RAD52* epistasis group, although they are typically less sensitive to ionizing radiation than the *rad51*, *rad52*, and *rad54* mutants. These mutants show other phenotypic differences that distinguish them from the *rad51*, *rad52* and

rad54 (Game 1983). Thus, for example, homozygous diploids are typically more resistant to X-rays than the corresponding haploids. Moreover, these mutants are not defective in the enhanced X-ray resistance associated with mating-type heterozygosity and show rates of X-ray-induced mitotic recombination higher than those of *rad51*, *rad52*, and *rad54* mutants (see Game 1983). The observation of epistatic interactions among all of these mutants, coupled with phenotypic differences between the *rad51*, *rad52*, and *rad54* mutants on the one hand and the *rad50*, *rad53*, *rad55*, *rad56* and *rad57* mutants on the other, has led to the hypothesis that the former group of loci mediate steps common to all repair function mediated by the latter, but that the latter mediate different subclasses of repair events dependent on the former (Game 1983). This situation is analogous to intra-group differences between genes of RAD3 group (discussed earlier).

The observation that the *RAD52* group of mutants are defective in meiosis suggests that the repair of ionizing radiation damage shares biochemical events in common with elements of DNA metabolism in meiosis. There is extensive experimental evidence that normal meiosis has a requirement for recombinational events (reviewed by Game 1983, Schild *et al.* 1983). This is certainly consistent with the phenotypes of the *rad52* mutants summarized above and has led to the implicit assumption that recombination is a key metabolic transaction during the repair of ionizing radiation damage. Recombination events may also be required for postreplicative repair of UV

radiation damage, thus providing a reasonable explanation for the observation that some *RAD52* epistasis group mutants are also slightly sensitive to UV radiation (Kiefer 1987).

MUTAGENIC RESPONSE:

***E. coli*:**

Witkin (1989) has recently reviewed this aspect of DNA repair in *E. coli* from a historical perspective. The following section is a brief overview of the topic.

A number of physiopathological phenomena observed after exposure of *E. coli* to DNA-damaging agents (typically UV) are collectively called SOS response (Walker 1984). A regulon comprising about 20 genes is responsible for this response. In this negatively controlled regulon, the LexA protein, which is the repressor of SOS genes, binds to the SOS box, a nucleotide sequence, overlapping the promoters of the member genes including *lexA* itself and the *recA* gene (reviewed by Little and Mount 1982, and by Walker 1985). DNA damage generates the cofactors (SSB-coated ssDNA and an adenine nucleotide, possibly dATP) necessary to activate RecA to form RecA* that facilitates proteolytic cleavage of LexA protein. The cleavage of LexA turns on the *lexA* and *recA* genes (the regulators themselves), the *uvrA*, *uvrB* and *uvrD* (but not *uvrC*) genes that are responsible for excision repair, *umuDC* genes responsible for mutagenic damage bypass mechanisms, *sulA* and *sulB* that are involved in cell division, and a number of *din* (damage inducible) genes

whose functions are unknown. The SOS response increases cell survival by inducing excision (*uvrA*, *uvrB*, *uvrD*), recombination (*recA*, *recN*, *recQ*, *uvrD*, *ruv*), and mutagenic (*umuDC*, *recA*) repair mechanisms, and by inhibiting cell division to allow more time for repair. Upon recovery of cells from DNA damage, the activating signal (ssDNA) disappears, autodigestion slows down, LexA accumulates, resulting in the low level of constitutive expression of all the SOS genes- characteristic of the uninduced wild type SOS regulon. The repressors of the lambdoid phages are also cleaved by the RecA protein during the SOS response, resulting in phage induction and escape of phage from endangering environment (Walker 1984, Walker 1985, Peterson *et al.* 1988)

The mechanism of UV mutagenesis is just beginning to be uncovered. Bridges *et al.* (cited in Jonczyk *et al.* 1989) have suggested a two step mechanism for UV mutagenesis. The first step (misincorporation) results in the insertion of an incorrect base at a phtoproduct on the template strand. The second step (bypass) requires induced levels of UmuCD proteins and allows replication to continue past the photoproduct.

For the misincorporation step, RecA inhibits the ε proof reading activity of Pol III in vitro (Lu *et al.* 1986) and in vivo (Jonczyk *et al.* 1989). Jonczyk *et al.* have shown that overproduction of the ε subunit of DNA polymerase III, in vivo, counteracts the SOS mutagenic response in *recA441* mutants that express SOS response constitutively. Thus RecA is responsible for the loss of editing capacity of the ε subunit of

DNA polymerase III holoenzyme.

DNA polymerase III (Hagensee *et al.* 1987) and UmuCD (Kato and Shinoura 1977) are also required for mutagenesis. They are probably involved in translesion replication. DNA polymerase II is more strongly amplified during SOS response and is much more efficient than Pol III in bypassing an abasic site in vitro (Bonner *et al.* 1988). An altered form of Pol I (Pol I*), which is error prone in vitro, is present only in SOS induced cells (Lackey *et al.* 1985). Thus all three DNA polymerases seem to be active in SOS mutagenesis.

RecA:

RecA is a crucial multifunctional (structural as well as regulatory) protein that is involved (1) directly in recombination as well as in recombinational repair, (2) in regulation of the SOS circuit, (3) in inhibition of the ε proof reading activity of DNA polymerase III, a reqiurement for the misincorporation step of mutagenesis and (4) in cleavage of UmuD to give UmuD* that is active in and required for mutagenesis (Nohmi *et al.* 1988, Shinagawa *et al.* 1988, Burkhardt *et al.* 1988). [All of these properties of RecA have been discussed earlier.]

Yeast:

As mentioned in an earliar section, *rad6* group of mutants (*RAD5*, *RAD6*, *RAD8*, *RAD9*, *RAD15*, *RAD18*, several *REV* genes, several *UMR* genes, and several *CDC* genes), are hypersensitive to both ionizing and UV radiation (Game 1983,

Haynes and Kunz 1981). Properties of at least some of the mutants in this group is that of defective mutagenesis following exposure to UV radiation. By analogy with the *recA lexA* pathway of inducible error-prone repair in *E. coli*, it has been suggested that the *RAD6* group of genes is involved in error-prone repair in *S. cerevisiae* (Lawrence and Christensen 1976, Lemontt 1973, Prakash and Prakash 1979). This topic has been reviewed by Lawrence (1982).

Like *E. coli*, specific genes of *S. cerevisiae* are expressed in response to DNA-damaging agents (Ruby and Szostak 1985). However, they are not as well characterized as in *E. coli* and will not be discussed here.

MISMATCH REPAIR:

Mismatched nucleotides in the genome can be generated as a result of replicative infidelity during semiconservative DNA synthesis, as a result of spontanious base damage such as deamination of cytosine to generate uracil in DNA, or during recombination (Muster-Nassal and Kolodner 1986).

***E. coli*:**

E. coli has an enzyme, *dam* methylase, that is involved in repairing base pair mismatches. Mutants defective in as well those over producing this enzyme show mutator phenotype. The mutator phenotype of *dam*$^-$ mutants can be understood in terms of a loss in strand bias for repair, while that associated with overproduction can be explained by more rapid methylation of newly synthesized DNA coupled with the reduced efficiency of

correction on symmetrically modified regions. This topic has been reviewed by Modrich (1987) and will not be reviewed here.

Yeast:

Base pair mismatch generated during recombination leads to the formation of heteroduplex DNA molecules which, in eukaryotes, result in non-Mendalian segregation of alleles during meiosis, detectable by conventional tetrad analysis in fungi. Classical models of genetic recombination (Holliday 1964, Hotchkiss 1974, Meselson and Radding 1975) suggest that mismatch correction of a single heteroduplex molecule will cause a deviation from Mendalian 4:4 segregation of tetrads, resulting in gene conversion and hence 3:5 or 5:3 segregations (when meiotic tetrad segregation ratios are presented as octads). If, on the other hand, the heteroduplex is not corrected during meiosis, postmeiotic DNA synthesis at the first mitotic division will result in abnormal 4:4 segregation of alleles (so-called postmeiotic segregation). While other explanations for these abarrant segregations exist (Szostak *et al.* 1983), recent genetic, biochemical, and molecular evidence supports the concept of mismatch correction of heteroduplexes in yeast.

Several genes affecting mismatch repair have been isolated and have been reviewed elsewhere (Friedberg 1985a, Friedberg 1988).

DNA repair in filamentous fungi:

A. nidulans:

Several UV-sensitive mutants increasing mitotic crossing over in *A. nidulans* were known by 1969 (Shanfield and Käfer 1969) By 1975 several more mutations affecting mitotic recombination frequency were reported in haploids and diploids of this fungus (Parag and Parag 1975). In 1986, Käfer and Mayor reported isolation of new UV-induced MMS hypersensitive mutants (*uvs* mutants) and also characterization of these and previously isolated mutants. Out of the known mutants three epistatic pairs were identified by genetic tests. These are (1) *uvsF* and *uvsH* (2) *uvsB* and *uvsD* (3) *uvsC* and *uvsE*.

uvsF shares properties of excision defective mutants of yeast. It has a very high UV induced mutation rate and extremely reduced UV and MMS survival. It is also extremely "hyperrec", although it shows normal levels of meiotic recombination. *uvsH* which is in the same epistatic group as *uvsF*, shares all the above mentioned properties of *uvsF*, but in addition extreme alleles are also sensitive to γ-rays and defective in meiosis. Thus both seem to be involved at least in excision repair.

The *uvsB* and *uvsD* mutations, that belong to the same epistatic group, cause very high frequency of unbalanced, unstable segregants from diploid conidia. These mutants are also hyperrec but exibit only slight increases of UV induced mutation.

uvsC and *uvsE* are *rec*$^-$, cause reduced mitotic crossing over in diploids and increased levels of haploid segregants, are

spontaneous mutators, whereas they also show less UV induced mutation than wild type control.

It is evident that the epistatic pairs of DNA repair genes in *Aspergillus nidulans* do not correspond to those in yeast, therefore studying DNA repair mechanisms in this organism may reveal novel DNA repair pathways or novel interactions between DNA repair genes.

Neurospora crassa:

Inducible nucleotide repair in this organism has been reported by Baker (1983) and its use to study DNA repair has been reviewed by Schroeder (1988).

Overview of approaches used in research on DNA repair:

(1) Isolation and genetic characterization of mutagen sensitive mutants

(2) Cloning of such mutants by homologous (several yeast excision repair genes, reviewed in Friedberg 1988) or heterologous (*ERCC1* gene, van Duin *et al.* 1986) complementation of mutants, by heterologous probing of a library (*ERCC2* gene, Weber *et al.* 1988) or by screening of an expression library with an antibody probe. Recently, Kelley *et al.* (1989) have cloned a Drosophila cDNA that encodes an apurinic endonuclease, using antibody that was originally prepared against a purified human apurinic-apyrimidinic endonuclease.

(3) Molecular characterization of cloned genes. (a) Sequencing a gene may reflect its function if it exihibits homology to a well

characterized gene [e.g. human *ERCC1* gene being homologous to yeast *RAD10* and to *E. coli uvrA* and *uvrC* (van Duin *et al.* 1988)], and may also throw some light on the evolutionary origin of the gene e.g. The *uvrA* gene of *E. coli* [discussed earlier (Doolittle *et al.* 1986)] (b) Site directed mutagenesis may elucidate essential and nonessential regions of the gene and of the protein e.g. *ERCC1* gene (van Duin *et al.* 1988). (c) Gene disruption and replacement wherever possible would, conclusively, show whether the gene is dispensible or not e.g. several genes of the yeast excision repair pathway (reviewed by Friedberg 1988). In certain cases, it may seem that the gene of interest is dispensible due to viability of an isolated mutant, however, the mutant in which the gene is disrupted may be inviable, implying that the original mutant isolated is a leaky one e.g. *rad3* of yeast (Naumovski and Friedberg 1983).

(4) In vitro biochemical and biophysical studies with purified factors, as has been achieved in case of *E. coli* uvrABC excinuclease. Before such studies were carried out, it was thought that the excinuclease makes simultaneous cuts on both sides of the adduct to be removed. Once the subunit genes were cloned, overproduced and in vitro studies carried out it was discovered that the enzyme never cuts on both sides of the adduct simultaneously (reviewed in Friedberg 1985). Once purified, antibodies could be generated against such proteins and used to probe expression library of another organism (Kelley *et al.* 1989; cloning of a Drosophila apurinic-endonuclease specific cDNA,

using human specific antibody).

(5) Once the gene is cloned and the antibody probe available, together these could be used to determine the stage at which the mutant is blocked. If the cloned-gene probe does not hybridize to DNA of the mutant in Southern analysis then it could be interpreted as a deletion mutant; If it does to DNA but not to RNA, then the block could be at the level of transcription. If the cloned gene gives signals in Southerns as well as Northerns but the antibody does not give a signal in Western blots then it could be interpreted as a block at the level of protein synthesis. Positive signals in all the three, Southerns, Northerns and Westerns, may either mean that the mutant produces a nonfunctional protein or mean that the mutant is leaky. As mentioned before, to check whether the mutant is leaky, gene replacement or gene disruption could be carried out.

Besides those mentioned above, there are other ways of doing functional analysis of DNA repair genes, five of which are enumerated in the section on nucleotide excision repair in yeast. Apart from those mentioned, one could also carry out complementation of mutants with a well characterized gene from another system. I have cited several examples in the literature review [complementation of the yeast *phr1* mutant by the corresaponding *E. coli* gene (Langeveld *et al.* 1985) and vice versa (Schild *et al.* 1984, Yasui and Chevallier 1983); complementation of *E. coli* 3-methyladenine-DNA glycosylase mutants by corresponding genes of *M. luteus* (Pierre and Laval

1986).

T4 phage gene *denV* and its product endonuclease V have also been used for cross species complementation studies of excision repair genes. Valerie and his colleagues (1985a) showed that when *denV*$^+$ gene is cloned in *E. coli*, it complements *uvrA*, *uvrB* and *uvrC* mutants as well as, to a lesser extent, *uvrD*. It also increases the UV resistance of *recA*, *recB* and *recC* strains. Also, normal wild type levels of host-cell reactivation of λ phage was observed in *E. coli* strains of following types: *denV*$^+$ *recA*, *denV*$^+$ *uvrA* and *denV*+ *uvrA recA*. The same gene, *denV*, also complements *rad1* and *rad2* mutations of yeast (Valerie *et al.* 1986), *mei-9* and *mus201* mutants of *Drosophila melanogaster* (Banga *et al.* 1989), CHO UV5 cells (Valerie *et al.* 1985b) and also several XP cell lines (Arrand *et al.* 1987, Valerie *et al.* 1987).

[The incision step of the excision repair pathway requires multiple subunits in most organisms studied so far: Yeast requires at least five subunits, humans at least nine and *E. coli* seems to be requiring three. However, in the phage T4 (and *M. luteus*) there seem to be a gene coding for an enzyme, pyrimidine-dimer DNA glycosylase-AP endonuclease, that is a single polypeptide and that can excise pyrimidine dimers from the damaged DNA in a two step process. This is the reason why the T4 enzyme complements more than one excision repair mutations of the same organism, but only partially (reviewed in Friedberg 1985)].

From the literature reviewed so far it has been clear that studies in DNA repair are at a point where, though considerable progress has been made, a lot of questions still remain. The awe and wonder of the research in DNA repair in yeast has been very well captured by Friedberg in a recent review (1988) and the same holds true with such research in other organisms.

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Cloning of the DNA repair gene, *uvsF*, by transformation of *Aspergillus nidulans*.

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Running head: **Cloning of *uvsF* from Aspergillus**

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ABSTRACT

As a first step in the cloning of the DNA repair gene *uvsF* of *A.nidulans*, *uvsF*$^-$ *pyrG*$^-$ strains were transformed with a genomic library. For primary selection, the plasmid vector carried the *pyr-4* gene of Neurospora which complements *pyrG* mutants of Aspergillus. Growth of rare pyr$^+$ uvs$^+$ transformants was enhanced by overlays with MMS (methyl-methane sulphonate), since *uvsF*$^-$ is sensitive to this agent. Four wild type-like transformants showed identical bands in Southerns probed with the vector when DNA was digested with *Stu* I, while *Bgl* II identified two types. These two types of transformants behaved differently in crosses and only those showing the larger *Bgl* II band gave *uvsF*$^+$ replacement progeny. Plasmids rescued by transformation of *E. coli* were also of two types, but had a region in common (1.2 kb). This was not a simple overlap and gave evidence for rearrangements. Only the plasmids with the larger insert of Aspergillus DNA (7.2 kb) were able to complement *uvsF*$^-$ in secondary transformation. Northerns of polyA$^+$-enriched mRNA, probed with this plasmid, showed three bands. However, its subclone which spans the shared region hybridized to only one of them (1.0 kb). On screening Aspergillus libraries with the complementing plasmid to obtain the normal *uvsF* sequence, two cDNA and five genomic clones were identified. One cDNA clone hybridyzed to the 1 kb mRNA which presumably corresponds to the *uvsF* message; it also hybridized to a short segment (2.2 kb) of one of the genomic clones, locating the putative *uvsF* gene sequence.

DNA repair-defective mutants, sensitive to UV or ionizing radiation, were first isolated in *Escherichia coli* (HOWARD-FLANDERS 1968). Such mutants were assigned to several repair pathways on the basis of extensive genetic and biochemical studies (CLARK and VOLKERT 1978; FRIEDBERG 1985). More recently, gene cloning has provided material for molecular studies of these genes, and of the corresponding gene products, their interactions and function (AMUNDSON *et al.* 1986; CUNNINGHAM and WEISS 1985; SANCAR and RUPP 1983). In yeast, radiation-sensitive (*rad*) mutants have also been isolated in large numbers and genetic analysis has identified epistatic and functional groups of genes which correspond fairly well to those found in *E. coli* (HAYNES 1975). Many of these genes have by now been cloned using transformation of *rad* strains of yeast with wildtype genomic sequences which complement (PEROZZI and PRAKASH 1986; FLEER *et al.* 1987; FRIEDBERG 1988) . Potential function of their gene products often can be identified on the basis of amino acid homologies, as deduced from DNA sequences, to well-characterized proteins from *E. coli* and its phages or even mammalian proteins (e.g., ALANI, SUBBIAH and KLECKNER 1989; CHEN and BERNSTEIN 1988; SUNG *et al.* 1987).

In Aspergillus and Neurospora, assignment of *uvs* mutants to repair pathways has been difficult, partly because nonepistatic pairs often are inviable (KAFER and MAYOR 1986; INOUE *et al.* 1981; KAFER 1983), and partly because correspondence to phenotypes of *E. coli* is much less common than in yeast.

Similarly, cloning of genes has lagged behind, especially in *A. nidulans,* because of the comparatively late development of an efficient transformation system (BALLANCE, BUXTON and TURNER 1983; TILBURN *et al.* 1983). The first Aspergillus genes were cloned by their ability to complement homologous genes in *E. coli* (KINGHORN and HAWKINS 1982) or in yeast (BERSE *et al.* 1983). More recently this has been achieved by complementation of mutants using cotransformation of Aspergillus with wildtype library DNA in plasmid vectors that contain a selectable marker (BALLANCE and TURNER 1986).

In Aspergillus, as in Neurospora, complementation of mutation in transformants occurs by stable incorporation of DNA segments, since no replicating plasmid vectors have been developed. For the present work the selection system chosen is based on the Neurospora *pyr-4* gene which complements *pyrG* of Aspergillus (BALLANCE and TURNER 1985). This has the advantage that incorporation is not influenced by the selective gene, which is heterologous, and occurs mainly by homologous recombination directed by the gene sequence of interest. The first gene of our choice, for molecular analysis of DNA repair in *A. nidulans*, was *uvsF* which most likely is an excision repair gene. Since many of the repair genes which have been cloned and sequenced from pro- or eukaryotes are involved in excision (LLOYD and HANAWALT 1981; WEISS and FREIDBERG 1985; VAN DUIN *et al.* 1986), sequence homologies or interspecies complementation tests may well be able to identify its function

at the molecular level. (A preliminary report of this work has been published as an Abstract, KAFER and OZA 1988).

MATERIALS AND METHODS

Aspergillus strains and genetic techniques: Standard *A. nidulans* media and genetic techniques were used (PONTECORVO *et al.* 1953; KAFER 1977; SCOTT and KAFER 1982).

Strains of *uvsF* (KAFER and MAYOR 1986) and *pyrG* (KAFER and MAY 1988) were intercrossed to obtain *uvsF pyrG* double mutants. The genotypes of the two strains used as recipients for transformation of *A. nidulans* were the following:

M3101: *uvsF*201 *pyrG*89 *riboA*1 *yA*2; *wA*3; *pyroA*4

M3115: *uvsF*201 *pyrG*89 *riboA*1 *yA*2; *wA*2; *choA*1; *chaA*1

The genetic map of the left arm of chromosome I and the location of *uvsF* and *pyrG* and several marker loci are shown in Figure 1.

Transformation of Aspergillus: Germinating conidia were used to obtain protoplasts for transformation following exactly the method developed by OSMANI, MAY and MORRIS (1987). Uracil-independant transformants were selected on the following regeneration medium: 1M Sucrose, 0.5% Yeast extract, 20mM Glucose, 5mM $MgSO_4$, supplemented with trace elements and vitamin solution (overlays contained 1% agar, bottom layers 2%). For selection of transformants complemented for *uvsF*, overlays with low concentrations of MMS (0.01-0.015%) in "complete" medium lacking pyrimidines were added after 12-16 hours of incubation. This level of MMS did not preclude growth of *uvsF*

colonies after several days of incubation. All pyr$^+$ transformants were tested by replica transfers onto MMS media lacking uracil.

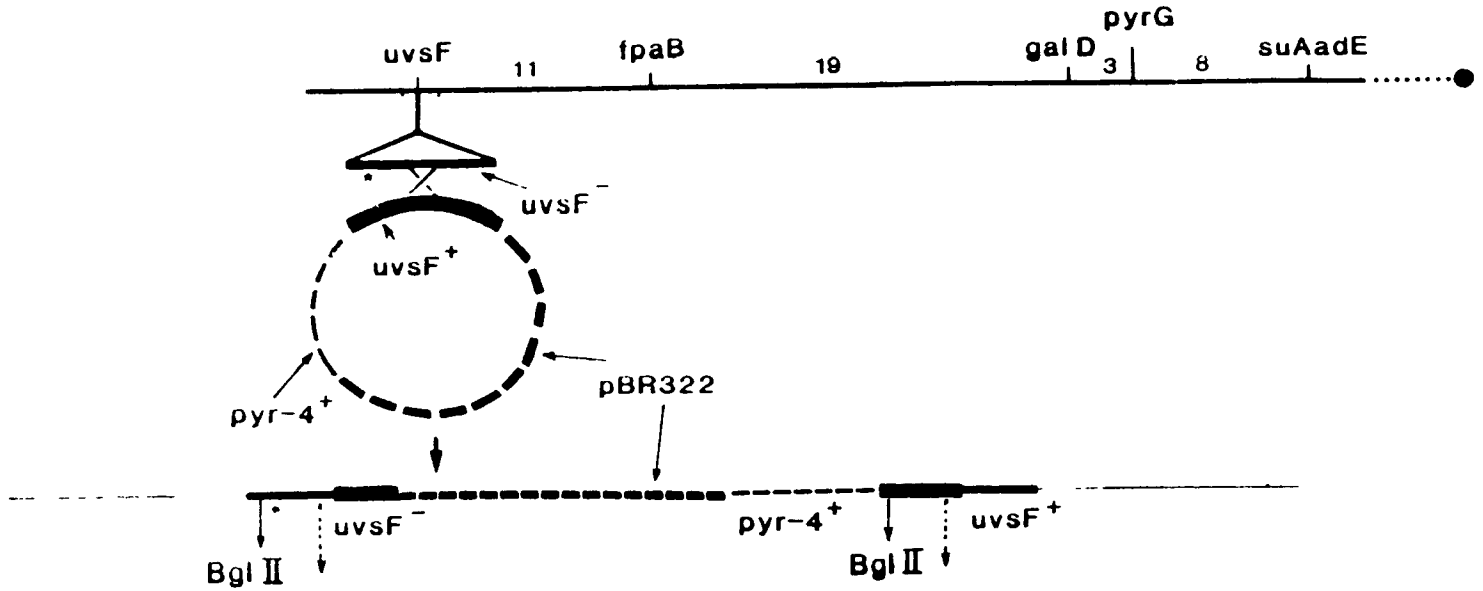
Libraries, vectors and *E. coli* strains: The wildtype genomic library used for primary transformation of *uvsF* strains of *A. nidulans* was prepared in the vector pGM3 by OSMANI, MAY and MORRIS (1987). The host for this library was *Escherichia coli* strain JM83 (VIEIRA and MESSING 1982). This same strain, as well as DH5α (HANAHAN 1983), were used as hosts for all plasmids and for marker rescue experiments. *E. coli* LE392 (BLATTNER *et al.* 1977) was the host for the clones from the Aspergillus genomic library in λ Charon4A (ZIMMERMAN *et al.* 1980; ORR and TIMBERLAKE 1982). The *A. nidulans* wildtype libraries in pGM3 and Charon4A were kind gifts of G. MAY. *E. coli* C600 (HUYNH, YOUNG and DAVIS 1984) was used as the host for cDNA cloned in λgt10. The cDNA library was a generous gift of S. OSMANI. Vectors pGM3 (OSMANI, MAY and MORRIS 1987) and the pUC18-derived pRG3 which contains *pyr-4* of Neurospora (OSMANI *et al.* 1988) were kindly provided by G. MAY and R. WARING (Figure 2a).

Plasmid rescue and molecular techniques: Plasmid rescue from the transformants of *A. nidulans*, preparation of Aspergillus DNA and polyA$^+$-enriched RNA, Northern and Southern analyses, were all done by the methods described by OSMANI, MAY and MORRIS (1987). Nitrocellulose membranes for Northerns and "nytran" membranes for Southerns were washed at high stringency as described by MAY *et al.* (1985). For preparation of

plasmid DNA, the LiCl method was used (VOLLMER and YANOFSKY 1986). For phage DNA isolation, phages were obtained through glycerol gradients. DNA from all sources was purified by CsCl gradient centrifugation (MANIATIS, FRITSCH and SAMBROOK 1982). For smaller samples, high speed runs of 5 or 16 hours were carried out (WEEKS *et al.* 1986). Phosphatase treatment of the vectors, gel electrophoresis and screening of the cDNA and genomic libraries were done as described in MANIATIS, FRITSCH and SAMBROOK (1982). For subcloning, DNA fragments were purified by gel electrophoresis and ligated in low-gelling agarose (STRUHL 1983).

Conditions for restriction digestions, nick translation of DNA with [a^{32}P]dATP or [a^{32}P]dCTP using the BRL kit, and separation of unincorporated dNTPs from the nick-translated probes using BRL columns, were as specified by the supplier. *E. coli* DH5α competent cells were purchased from GIBCO CANADA INC. (GIBCO/BRL, Burlington, Ontario) and transformed according to the protocol supplied.

FIGURE 1. - Meiotic map of chromosome I and insertion of transforming plasmid sequences by homologous recombination. pBR322-based vector with *pyr-4*$^{+}$ from Neurospora. Circle represents centromere. Gene symbols, *A. nidulans*: *uvsF*, UV-sensitive; *fpaB*, resistant to p-fluorophenylalanine; *galD*, unable to grow on galactose as carbon source; *pyrG*, pyrimidine requirement; *suAadE*, suppressor of *adE20*. *Neurospora crassa*: *pyr-4+*, wildtype gene which complements *pyrG*. *BglII*, one (or two?) restriction sites in uvsF. Asterik, mutant site of uvsF201.


uvsF
11
fpaB
19
galD
pyrG
3
8
suAadE
uvsF⁻
uvsF⁺
pyr-4⁺
pBR322
uvsF⁻
pyr-4⁺
uvsF⁺
Bgl II
Bgl II

RESULTS

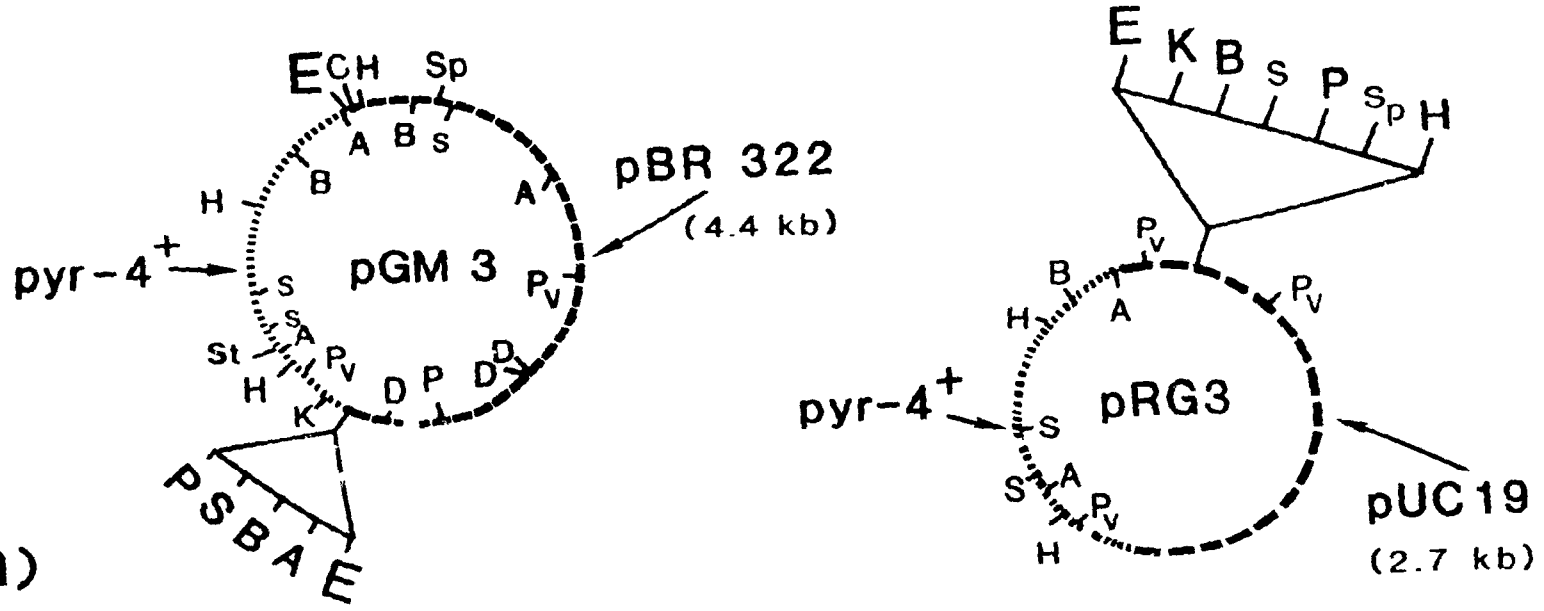
Primary transformation and Southern analysis: Two double mutant strains, *uvsF*$^-$ *pyrG*$^-$ were transformed with an Aspergillus wildtype genomic library in the vector pGM3 that contains the Neurospora *pyr-4*$^+$ gene (Figure 2a; MAY *et al.* 1985). *A. nidulans* transformants were selected first for uracil independance (pyr$^+$) and after regeneration of protoplasts growth of uvs$^+$ types was enhanced by addition of MMS overlays. Among 3000 pyr$^+$ transformants tested, seven with various levels of MMS resistance were obtained, five from one and two from the other strain. All of the pyr$^+$ colonies checked had retained the residual markers of the recipient strains (*riboA*, and *pyroA* or *choA*; see Materials and Methods section). This confirms that none of them were contaminants and indicates that not a single case of transformation for a nutritional marker was obtained.

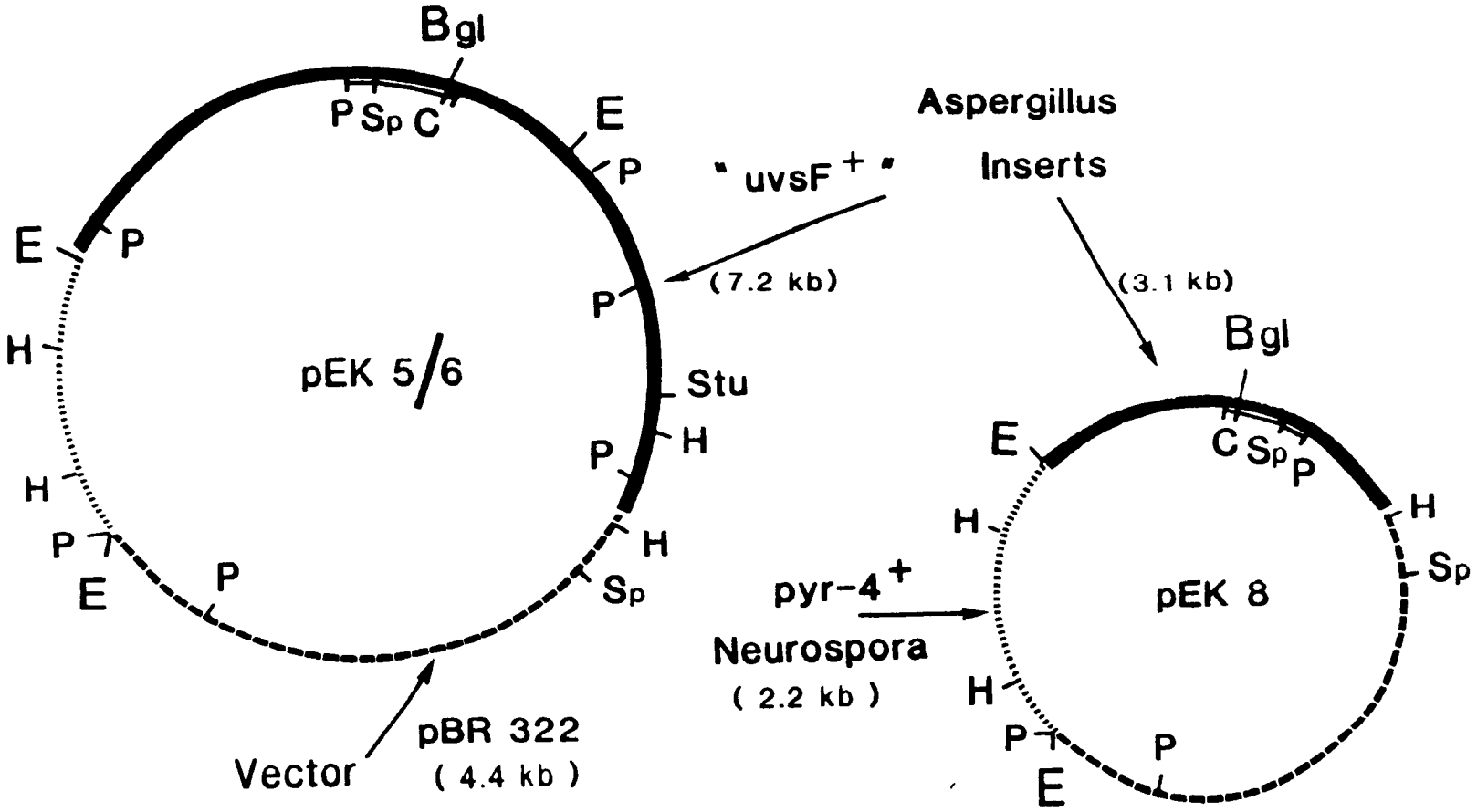
Southern analysis of *Bgl* II-digested genomic DNA of all seven transformants was performed using vector DNA as probes (of pGM3 or pBR322 which have no *Bgl* II sites; Figure 3, a and c) In such Southerns, one transformant which had almost wildtype phenotype (Tf34) showed a larger band (13.8 kb) than 3 other perfectly complemented cases (Tf35, 36 and 37) which all showed similar smaller bands (9.7 kb; Figure 3, a and c). However, no difference was observed between these two types of complemented transformants in genomic Southerns of *Stu*I-digested DNA (Figure 3b).

The remaining three transformants (38, 39 and 40) were only partially complemented and showed multiple bands in Southerns

of *Bgl* II-digested DNA (for Tf40 and 38, see Figure 3, a and c respectively). The latter two were sterile even in heterozygous crosses, whereas the third (Tf39) could be crossed, permitting mapping of the plasmid insertion site(s) to chromosome III. These three transformants were not analysed further.

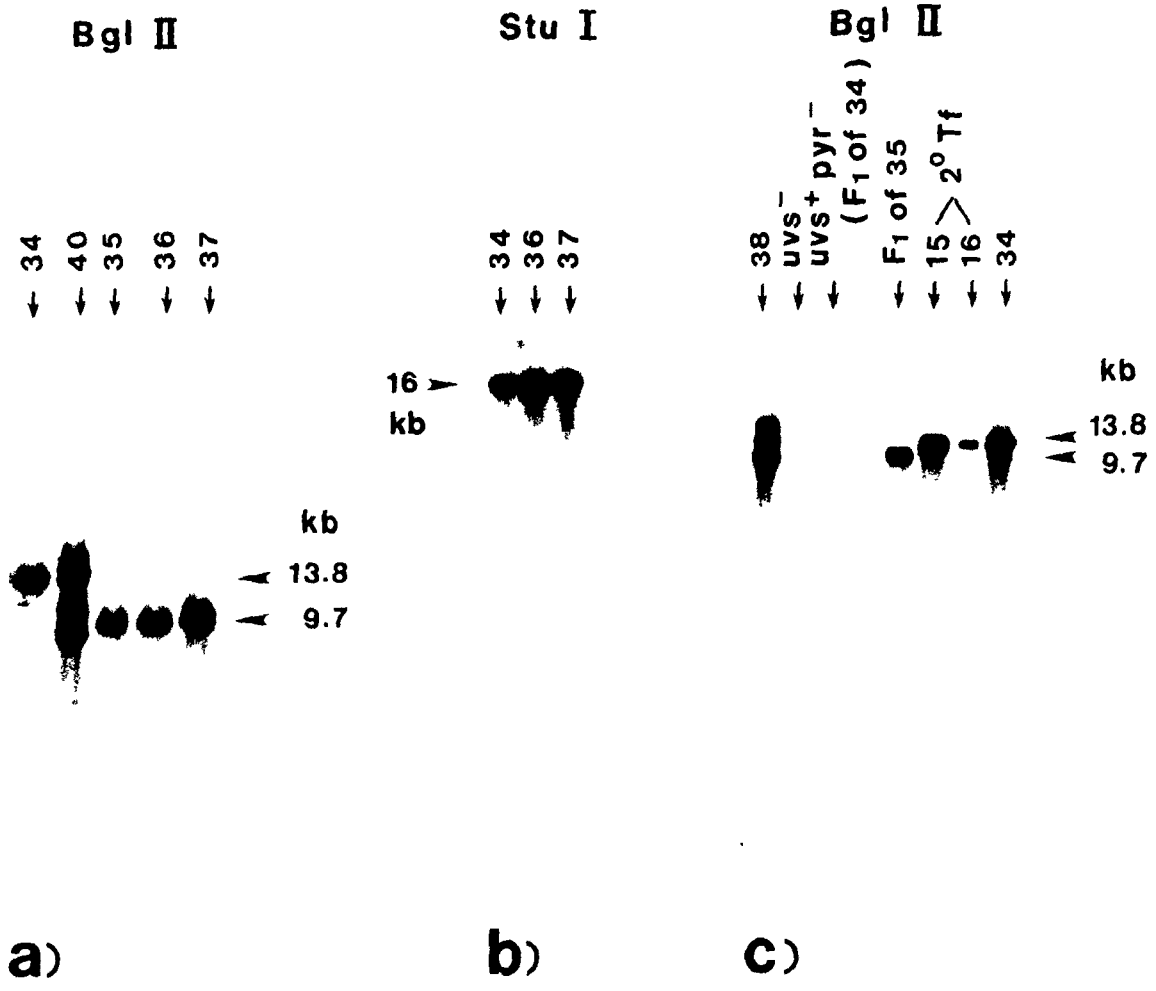
FIGURE 2 - Restriction maps of plasmids. a) Plasmid vectors containing the *pyr-4* gene of Neurospora; pGM3, derived from pBR322, pRG3 from pUC19. b) Rescued plasmids from *Bgl*II-digested DNA of primary uvs$^+$ transformants of Aspergillus; from Tf34, complementing type with larger *A. nidulans* insert (7.2 kb) pEK5/6, pEK8, non-complementing plasmid with shorter (3.1 kb) insert; common segment (<1.0 kb) indicated by double line. Restriction sites: A, *Ava*I ; B, *Bam*HI, C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; P_V, *Pvu*II; S, *Sal*I; Sp, *Sph*I, St, *Stu I*. T, *Sst* II, X, *Xho*I.


E
C
H
Sp
A
B
S
B
A
H
pyr-4+
S
pGM 3
Pv
S
A
St
H
Pv
K
D
P
D
D
pBR 322
(4.4 kb)
P
S
B
A
E
a)
E
K
B
S
P
Sp
H
Pv
B
A
H
Pv
pyr-4+
S
pRG3
S
A
Pv
H
pUC 19
(2.7 kb)


Bgl
P Sp C
E
P
" uvsF + "
Aspergillus
Inserts
(7.2 kb)
(3.1 kb)
E
P
H
pEK 5/6
Stu
P
H
H
P
E
P
H
Sp
Bgl
E
C Sp P
H
H
Sp
pyr-4 +
pEK 8
Neurospora
(2.2 kb)
H
P
E
P
Vector
pBR 322
(4.4 kb)

b)

FIGURE 3. - Southern analysis of transformants, their progeny and controls; genomic DNA digested with *Bgl*II (a, c) or *Stu*I (b), using pGM3 vector as a probe. a) and b): primary transformants (Tf34-40); c) progeny from crosses (F1), secondary transformants (Tf15 and 16) and *uvsF* control


Bgl II
34
40
35
36
37
kb
13.8
9.7
a)
Stu I
34
36
37
16
kb
b)
Bgl II
38
uvs⁻
uvs⁺ pyr⁻
(F1 of 34)
F1 of 35
15 > 2° Tf
16
34
kb
13.8
9.7
c)

Mitotic and meiotic mapping and stabiltiy: The site of insertion of the transforming plasmid was mapped to chromosome I for all four of the well-complementing transformants (Tf34-37), since haploid segregants from heterozygous diploids showed complete linkage of uvs^+ and pyr^+ to markers on that chromosome (5 diploids analysed; 65-95 haploids tested from each). These results confirm and expand the observation, that these transformants, once purified from single conidia, were completely stable in mitosis. Similar results were obtained from one secondary transformant (Tf16, see below) which showed linkage of uvs^+ pyr^+ to chromosome I, while two others which mapped on other chromosomes had become *uvsF* $^-$ and produced low frequencies of *pyr-4*$^+$ loss (4-10 %).

In general, meiotic linkage of the uvs^+ pyr^+ insert in heterozygous crosses of the four well-complementing transformants was consistent with insertion of the complementing sequences by homologous recombination (Figure 1). However, in several respects, results differed for the two types (i.e., Tf34, its Tf progeny and secondary Tf, vs. Tf35-37 and their F_1; Table 1). In all cases normal linkage values were obtained, except for two crosses of the near-wildtype transformant (34) and its F_1 to one well-marked *fpaB* strain, in which meiotic recombination in adjacent intervals was greatly reduced. In contrast, crosses of the three completely complementing transformants (Tf35-37) to this same mapping strain gave normal recombination (section a, Table 1). On the

other hand, Tf34, but not the three others, produced apparently normal replacement progeny in heterozygous as well as homozygous crosses (*uvsF*$^{+}$ *pyrG*$^{-}$ progeny in Table 1) These recombinants had lost the plasmid sequence (third lane in Figure 3c) and never produced *uvsF*$^{-}$ types in crosses to wildtype (several hundred progeny tested). In intercrosses between the two types of transformants, such *uvsF*$^{+}$ *pyrG*$^{-}$ progeny were also produced, but with reduced frequences (section c, Table 1)

TABLE 1. Mapping of inserts and meiotic analysis of stability in primary and secondary transformants (1°, 2° Tf) and their Tf progeny (F_1).

Strains to which transformants were crossed	Progeny of interest (phenotype)			Frequency of relevant recombinants from crosses of 2 types of transformants					
				Wild type-like Tf (35-37)			Near-wild type Tf (34) its F_1 and 2° Tf		
	uvs	pyr	gal	%	No. of crosses	No. tested	%	No. of crosses	No. tested
a) Mapping of uvs^+ insert									
uvsF fpaB galD	-	(±)	+	24	(2)	371	4	(2)	242
ursF +pyrG galD	+	(±)	-	30[a]	(3)	252	26 ± 2	(5)	605
b) Tests for stability (Tf x haploid strains)									
$uvsF^+$ $pyrG^+$	-	-		20 ± 4	(4)	425	14	(1)	95
uvs^+ pyrG	-	-		25 ± 2	(3)	314	14	(1)	172
	-	+		0			3		
ursF pyrG	+	-		0	(3)	151	8[b]	(3)	230
	-	+		0			0[b]		
	+	+		>50[c]			>50[c]		

uvsF pyrG$^+$	+	−	0	(4)	531	20 ± 6	(4)	484
	−	+	36 ± 2[c]			37 ± 2[c]		
	+	+	33 ± 3			30 ± 4[c]		
	−	−	30 ± 3			13 ± 5		
c) Tests for stability	(Tf x Tf)							
Selfed or homo-	+	−	0	(5)	553	14	(1)	101
zygous crosses	−	+	0			0		
	−	−	33 ± 2			0		
1° Tf (F, of 34)	+	−	—	—	—	7		
x 2° Tf	−	+				2	(1)	200
	−	−				36		
Crosses between	+	−				4		
the two Tf types	−	+		(2)	419	3		
	−	−				33		

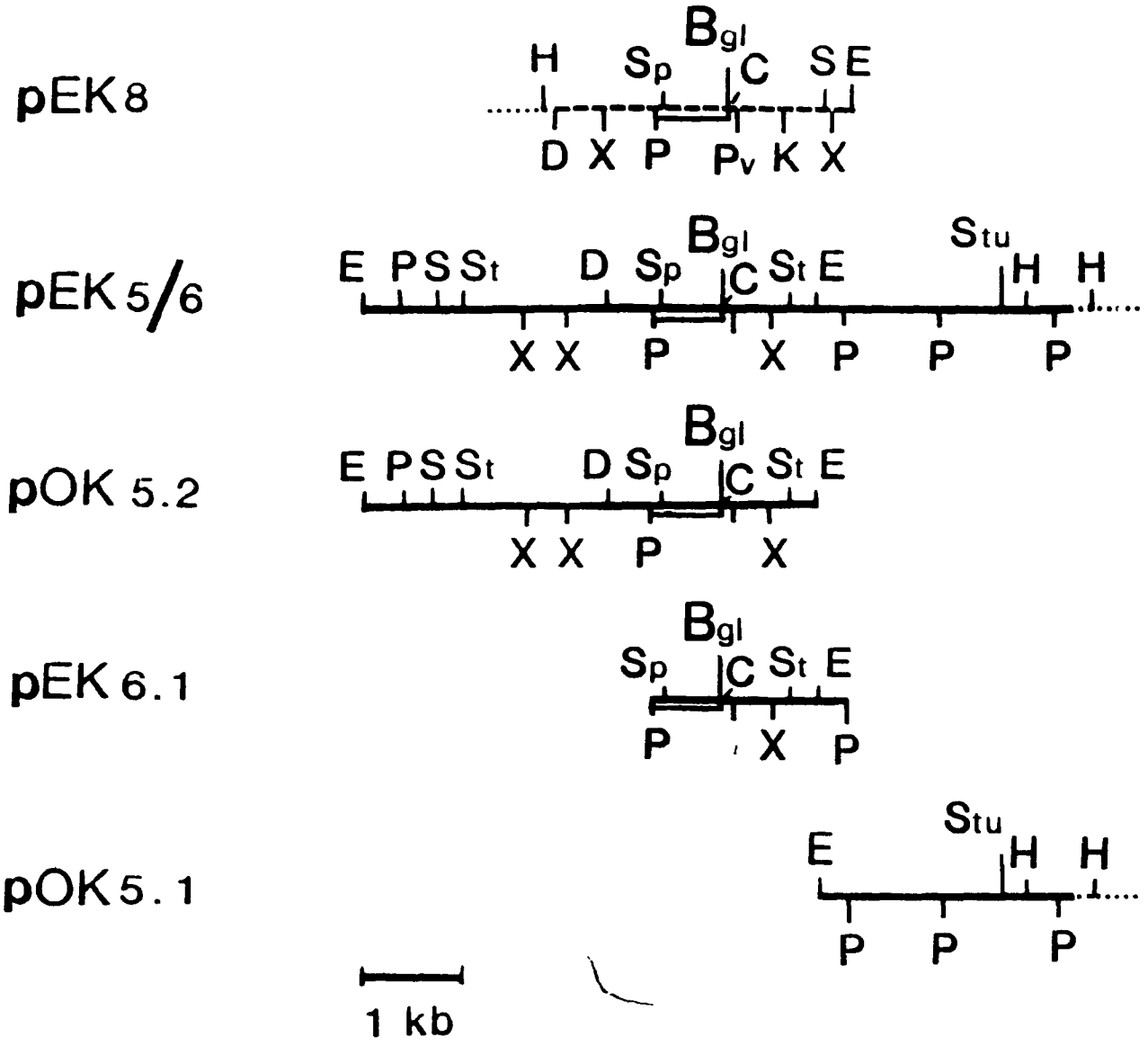
[a] Estimate assumes close linkage of galD to pyrG, as found in standard crosses (Figure 1).

[b] Results shown for 1° Tf34; 2° Tf16 produced very different and variable results in two crosses.

[c] On media containing standard amounts of pyrimidines, pyr$^+$ colonies have a selective advantage.

Marker rescue experiments: Genomic DNA from all of the four complementing transformants was digested with *Bgl* II, since neither the plasmid vector nor *pyr-4*$^+$ contain *Bgl*II sites (NEWBURY, GLAZEBROOK and RADFORD 1986) . When ligated and used to select amp^r transformants of *E. coli*, 5-10 colonies were obtained in each case. Plasmids isolated from the resulting *E. coli* strains were checked by restriction analysis using diagnostic enzymes. Two types of plasmids were identified and, as expected, a single *Bgl* II site was found in all these plasmids which also contained the 2.2 kb *Eco*RI fragment of *pyr-4*$^+$. Any plasmids rescued from the three wildtype-like uvs$^+$ pyr$^+$ transformants had identical restriction patterns and the expected total size of 9.7 kb (shown for pEK8 from Tf35 in Figures 2b and 4). Similarly, those isolated from Tf34 in two independant rescue experiments (pEK5 and pEK6), showed the expected total size of 13.8 kb and shared restriction patterns (pEK5/6; Figures 2b and 4). Detailed comparison of restriction maps between the two types of plasmids revealed a short common region of less than 1.0 kb. However, this internal segment has opposite orientation relative to the vector and is not a simple overlap region (Figure 4).

FIGURE 4. - Restriction maps of inserts in rescued plasmids; pEK8 with short insert from Tf35 (top line); pEK5/6 with long insert from Tf34 (seond line) and subclones of the latter (for enzyme abbreviations see Fig. 2, except B = *Bgl*II in this figure).


pEK8
H
Sp
Bgl
C
S
E
D
X
P
Pv
K
X
pEK5/6
E
P
S
St
D
Sp
Bgl
C
St
E
Stu
H
H
X
X
P
X
P
P
P
pOK5.2
E
P
S
St
D
Sp
Bgl
C
St
E
X
X
P
X
pEK6.1
Sp
Bgl
C
St
E
P
X
P
pOK5.1
E
Stu
H
H
P
P
P
1 kb

Secondary transformation: Unexpectedly, only the plasmids with the larger insert (pEK5/6) were regularly able to complement *uvsF*$^-$ in secondary transformation and produced phenotypes as well as genetic behaviour identical to the primary tramsformant (34). Such secondary transformants all showed the same band as the primary Tf34 in Southerns of *Bgl*II-digested DNA (Tf15 and 16, Figure 3c).This was the case not only for the uvs$^+$ pyr$^+$ types (like Tf 16) , but also for *uvsF*$^-$ pyr$^+$ types (like Tf15 with *pyr-4*$^+$ inserted in chromosome V). In contrast, none of the plasmids with the smaller insert were able to complement uvsF mutants (e.g., pEK8 produced not a single MMS-resistant case among >400 pyr$^+$ transformants tested).

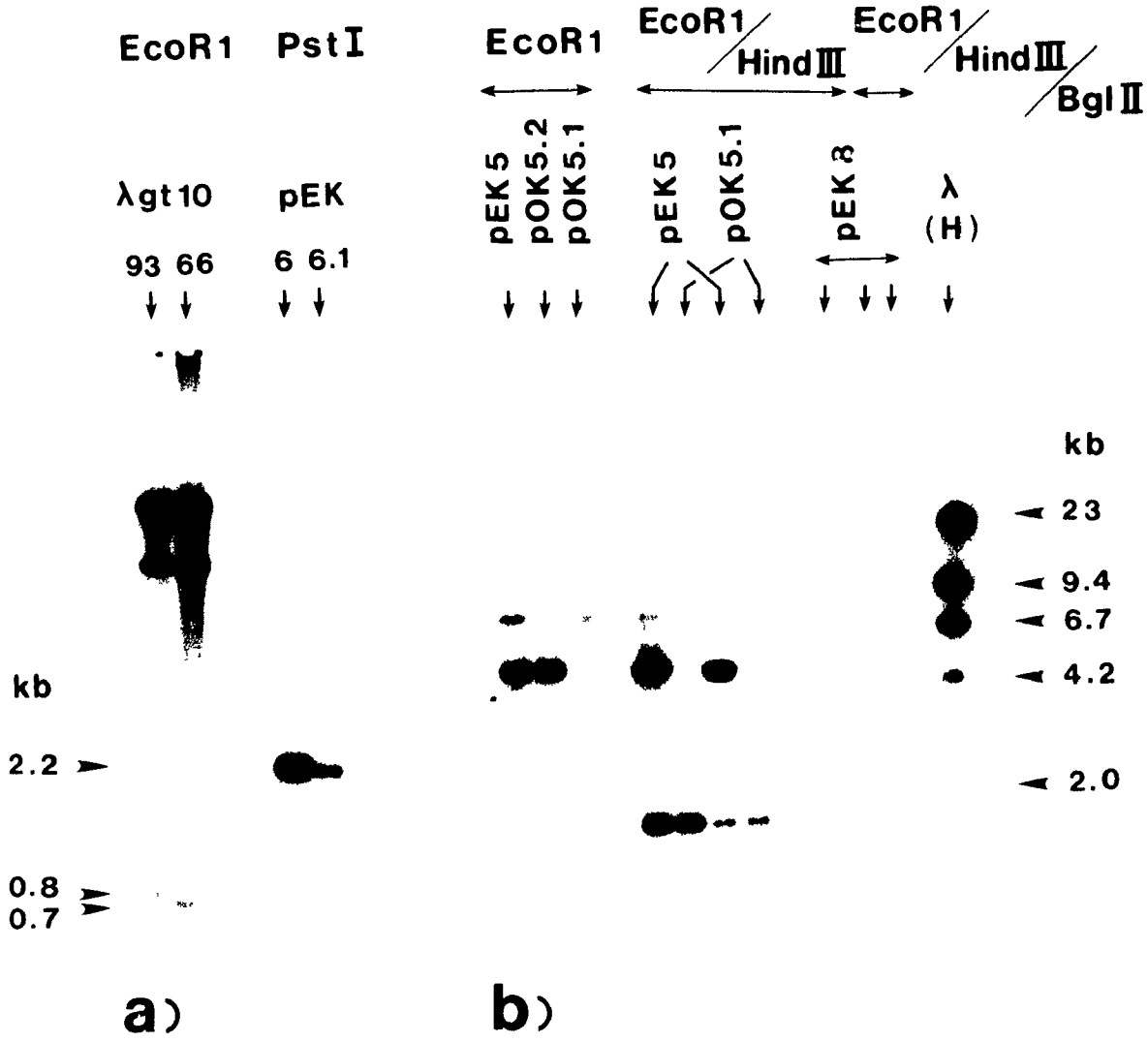
To identify the smallest complementing segment of the plasmid with the larger insert (pEK5/6), various restriction fragments were isolated and subcloned in pRG3 (Figure 2a) and used for transformation. None of these complemented *uvsF* in secondary transformation, even though two of them (pOK5.2 and pEK6.1, Figure 4) contained the common (overlap) sequence (<1.0kb) and adjacent regions on one or the other side (150-890 pyr$^+$ transformants tested on MMS media for each subclone).

Screening of the cDNA Library: Since none of the subclones complemented the *uvsF* mutation, the original complementing plasmid itself (pEK5 with the larger insert) was used as the probe for primary screening of the two available Aspergillus libraries, namely the cDNA library in the λgt10 vector (of OSMANI, MAY and MORRIS 1987) and the wildtype genomic library

in the Charon4A vector (of ZIMMERMANN *et al.* 1980).

From the cDNA library, two clones were obtained (λgt10.93 and 10.66) which contained similar size inserts, as checked on agarose gels (about 1200 bp; results not shown). Both inserts were cut once by *Eco*RI, producing insert bands of approximately the following sizes: λgt10.93, 800 bp and 400 bp; gt10.66, 700 bp and 500 bp. Since the inserts of λgt10.93 and 10.66 cross-hybridized under stringent conditions (Figure 5a), only λgt10.93 was used for detailed cross-hybridization analysis of the rescued plasmids and their subclones from primary Aspergillus transformants (Figure 5, a and b). The following results were obtained: The cDNA insert hybridized to, (1) both of the *Eco*RI fragments of the complementing pEK5 insert (pOK5.1 and pOK5.2; Figure 5b); (2) the insert of the subclone pEK6.1 and the corresponding *Pst*I overlap fragment of pEK5/6 (Figure 5a); (3) part of the insert of pEK8, namely and significantly, the fragment that contains the region common to the two plasmids ("overlap"; Figures 4 and 5b).

FIGURE 5. - Southern blots probed with the cDNA clone λgt10.93 hybridizing to a) cDNA clone 10.66 and itself, as well as to rescued plasmid pEK6/5 and its *Pst*I-subclone, pEK6.1; b) λgt10.93 hybridizes to both *Eco*RI subclones pEK5/6, pOK5.2 and 5.1, and to pEK8 (lesser extent of hybridization)


EcoR1
PstI
λgt10
pEK
93 66
6 6.1
kb
2.2
0.8
0.7
a)
EcoR1
EcoR1
HindIII
EcoR1
HindIII
BglII
pEK5
pOK5.2
pOK5.1
pEK5
pOK5.1
pEK3
λ
(H)
kb
23
9.4
6.7
4.2
2.0
b)

Analysis of the genomic clones: Using pEK5 as probe, five genomic clones representing three different types were obtained from the Charon4A library. Three of the five clones, namely Charon4A.1, 4A.119 and 4A.200 gave indistinguishable restriction patterns in digests by *Eco*RI or *Bgl*II, as well as in double digests by *Eco*RI and *Bgl*II, while 4A.46 and 4A.137 produced unique patterns (Figure 6). Thus the first three were considered identical. Only one of each type (4A.1, 4A.46 and 4A.137) was used for the search of *uvsF* genomic sequences.

To locate the putative *uvsF*$^+$ sequences in Charon4A clones, the *Eco*RI fragments of the cDNA insert of λgt10.93 were subcloned into the *Eco*RI site of the plasmid vector pRG3. Two types of subclones were obtained, with inserts of either the large or the small *Eco*RI fragment. Used as probes against various digests of the three types of Charon4A clones, both gave the same results, hybridizing only to Charon4A.137 (results not shown).

In parallel with the above tests, the rescued plasmids and some of their subclones were used as probes in Southerns of the three types of Charon4A genomic clones. While the original complementing plasmid (pEK5/6) hybridized to all three types of clones (during screening), the *Pst* I subclone from this plasmid which spans the "overlap" region (pEK6.1), hybridized only to Charon4A.137. In contrast, when the noncomplementing rescued plasmid with the shorter insert (pEK8) was used as a probe, it

hybridized not only strongly to 4A.137, but also to 4A.46 and not at all to 4A.1 (not shown). Therefore, only the 4A.137 clone was likely to contain the genomic sequence of *uvsF*.

To map the *uvsF* sequences within the large insert of Charon4A.137 by Southern analysis, this clone was probed with the cDNA and pEK6.1 subclones. With most enzymes (including *Bgl*II, and *Eco*RI, not shown) the above-mentioned probes hybridized to more than one band. However, single bands were obtained in the double digest of *Stu*I and *Sal* I, and in single digests with *Dra*I or *Pst* I (7.5 kb, 4.0 kb or 2.2 kb; Figure 7b, lanes 9, 1 and 10, respectively) . From the combined results of a large number of single, double and triple digests and their blots, it was possible to deduce that the *uvsF* gene squence was most likely located within a 4.0 kb region at the 3' end of the Charon4A.137 insert (cDNA probes hybridized to fragments on both sides of the three restriction sites indicated in that region; Figure 8).

FIGURE 6. - Agarose gel of restricted DNA from 5 Charon4A clones which hybridize to the insert of the complementing plasmid pEK5/6, revealing a total of 3 types.

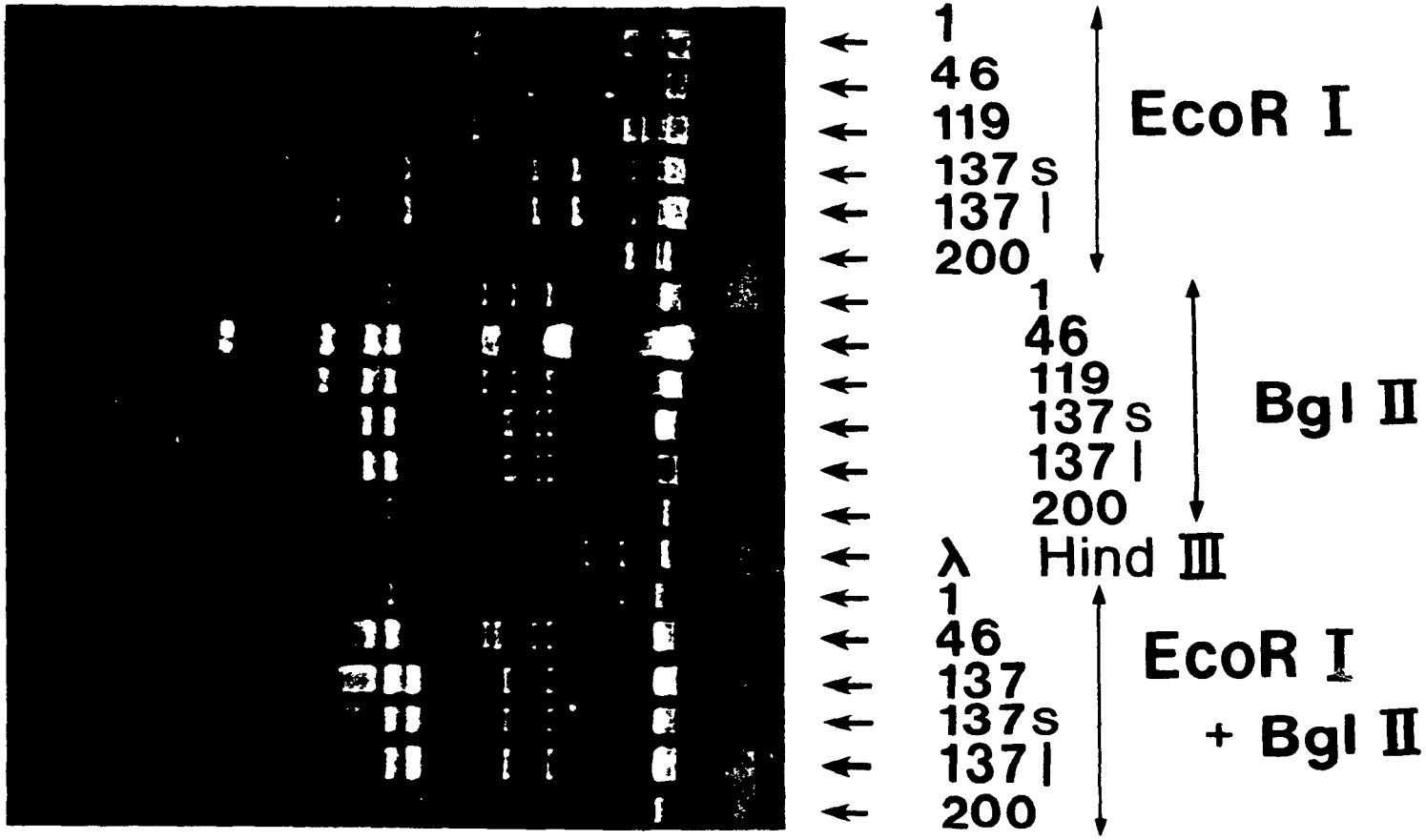
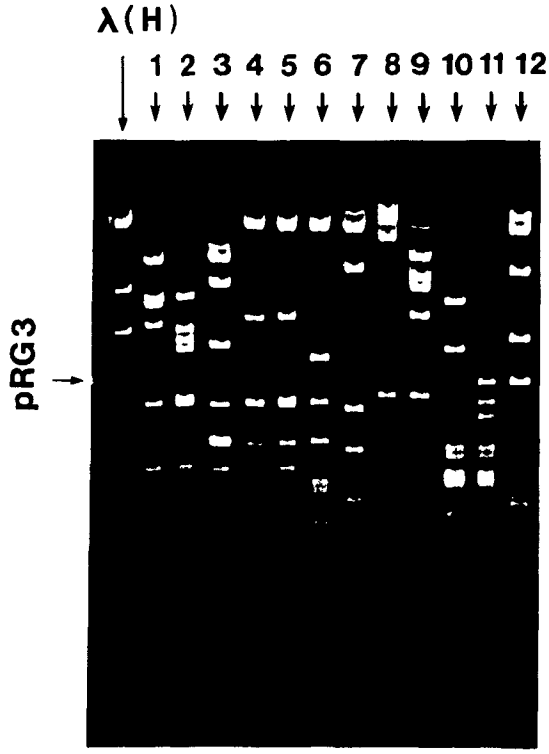
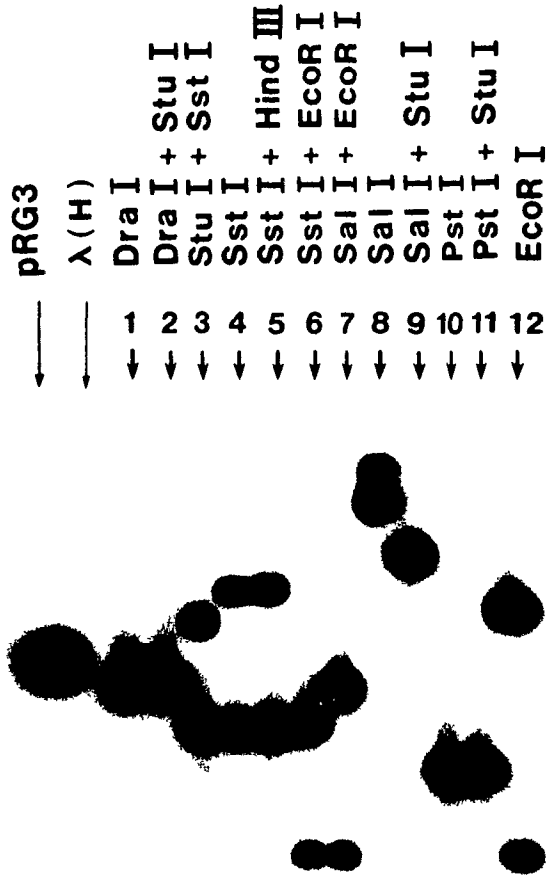

1
46
119
137 s
137 l
200
EcoR I
1
46
119
137 s
137 l
200
Bgl II
λ Hind III
1
46
137
137s
137l
200
EcoR I
+ Bgl II

FIGURE 7. - a) Various digests of the Charon4A.137 clone, b) probed with pRG3-39L, i.e., the larger *Eco*R I fragment of the cDNA insert from 10.93, subcloned in pRG3 (probing with pEK6.1 or pEK6 gave the same results).


λ(H)
1 2 3 4 5 6 7 8 9 10 11 12
pRG3

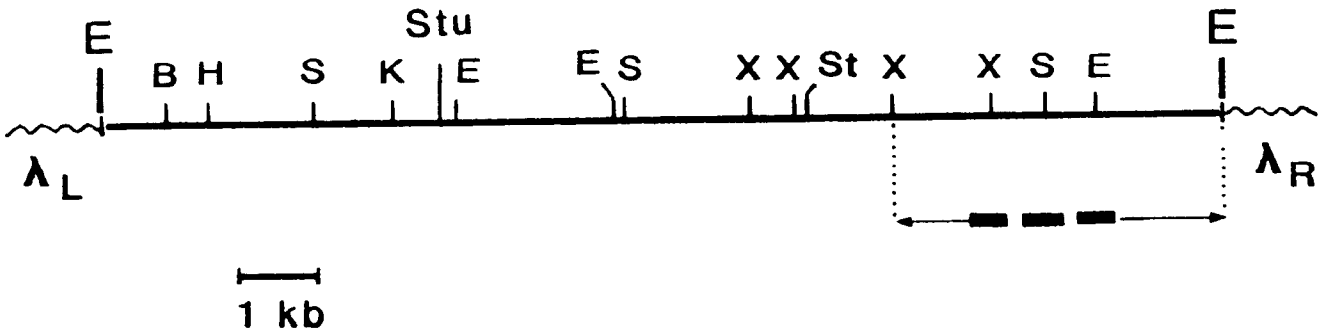
a)


pRG3
λ(H)
Dra I
Dra I + Stu I
Stu I + Sst I
Sst I
Sst I + Hind III
Sst I + EcoR I
Sal I + EcoR I
Sal I
Sal I + Stu I
Pst I
Pst I + Stu I
EcoR I
1 2 3 4 5 6 7 8 9 10 11 12

b)

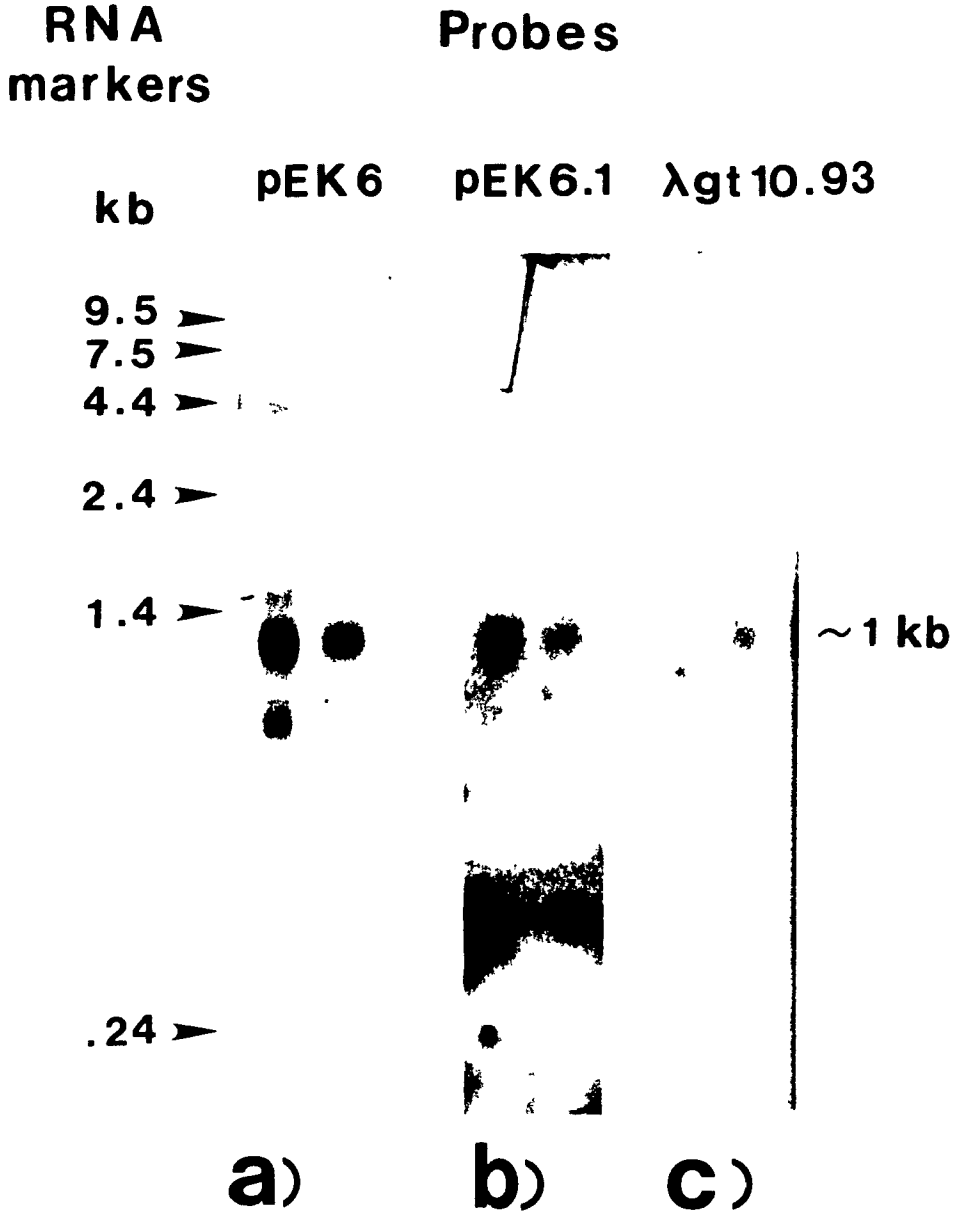
FIGURE 8. - Restriction map of *A. nidulans* insert in Charon4A.137. Regions of maximum (⟵ ⟶) and minimum (---) possible extent of hybridization to cDNA insert of λgt10.93 or subclone pEK6.1 indicated between arrows (4 kb out of a total size of 14.5 kb); λL and λR, left and right arm of vector Charon4A (B, *BamH*I; E, *EcoR*I; H, *Hind*III; K, *Kpn*I; S, *Sst*I; Stu, *Stu*I; X, *Xba*I. For the map of the vector arms, see MANIATIS, FRITSCH and SAMBROOK 1982).

genomic 4A·137



Northern Analysis: In Northern analysis of polyA$^+$-enriched wildtype RNA using pEK5/6 as a probe, three bands were seen (1.4 kb, 1.0 kb and 0.8 kb), but by far the brightest band was that of approximately 1.0 kb (Figure 9a). When for such Northern either pEK6.1 or the subclones of the cDNA were used as probes, only the 1.0 kb band was obtained which, therefore, may represent *uvsF* mRNA (Figure 9, b and c).

FIGURE 9. - Northern analysis of poly A^+-enriched RNA from *uvsF*$^+$ strain (different amounts loaded in duplicate lanes). RNA markers on original gel provide for approximate estimates of mRNA size (HAUGE 1988).


RNA markers
Probes
kb
pEK6
pEK6.1
λgt10.93
9.5
7.5
4.4
2.4
1.4
.24
~1 kb
a)
b)
c)

DISCUSSION

The results presented here demonstrate some of the similarities and differences between Aspergillus transformation systems and those of yeast and Neurospora, when genes are cloned by complementation of mutants with genomic library segments in plasmid vectors. As in yeast, stable single-copy incorporation of complementing DNA and linked vector sequences occured repeatedly at the locus of interest, presumably by homologous recombination. Furthermore, in one type of complemented transformants, meiotic recombination in heterozygous crosses regularly produced progeny which had lost the vector and *pyr-4*$^+$; these recombinants all had also lost the duplication for *uvsF* sequences and were of two types, either *uvs*$^-$ and indistinguishable from *uvsF*201, or *uvs*$^+$ and indistinguishable from wildtype (8-15% of each type; Table 1). On the other hand, several unexpected features observed for the primary transformants and the plasmids rescued from these, indicate that the situation in Aspergillus is more complex Not only can incorporation occur also at non-random heterologous sites (DIALLINAS and SCAZZOCCHIO 1989) but in addition, rearrangements are not uncommon. The usual procedure, adopted here, is therefore to rescue complementing sequences from selected transformants and to use these as probes to screen genomic (BALLANCE and TURNER 1986) or cDNA libraries (OSMANI *et al.* 1988).

Aspergillus also seems to differ from Neurospora where an unusual mechanism for inactivation of duplicate gene sequences has recently been discovered (SELKER and GARRETT 1988; CAMBARERI *et al.* 1989). Apparently, during the mitotic divisions which precede meiosis in the ascogeneous hyphae, one or both copies of duplicate segments undergo high frequencies of G-C to A-T mutation (as well as methylation). In our analysis of the many crosses between transformants and normal-sequence strains, the corresponding types would presumably become *uvs*$^-$, while remaining pyr$^+$ (retaining the Neurospora *pyr-4* sequences which are heterologous to *pyrG*). Such types were either very rare or absent in crosses in which they can be identified (3 types of crosses each, in sections b and c of Table 1).

In the current case, rearrangements have obviously occurred during the incorporation of at least one of the segments that complement the *uvsF* mutant, since the two recovered sequences are not colinear; while they contain a short common region which hybridizes to the same cDNA segment, the adjacent sequences have very different restriction maps (rearrangements during the production of the library are less likely, but cannot be ruled out). Our hypothesis is, that the larger plasmid that complements in secondary transformants contains the rearranged sequence, for the following reasons: a) the corresponding transformant (34) is not perfectly complemented (in contrast to the other three uvs$^+$ Tf) and only produces normal uvs$^+$ phenotype (presumably monosomic for *uvsF* $^+$) after meiotic recombination

and loss of vector sequences; b) in interaction with a specific marker strain, it shows drastically reduced recombination, not found for the other three transformants. If this hypothesis is correct, two properties of the three transformants with normal uvs$^+$ phenotype need explanation: a) crosses of the latter Tf types, while producing fairly high frequencies of *uvsF*$^-$ *pyrG*$^-$ types when vector sequences are eliminated, never showed any *uvsF*$^+$ *pyrG*$^-$ progeny (i.e., cases of incorporation of *uvsF*$^+$ and excision of *uvsF*$^-$); b) rescued plasmids from the *Bgl* II-restricted DNA of these strains do not complement. While no satisfactory explanation of the first of these findings has been found, both of them may be a consequence of the relative position of the mutant site of *uvsF*201 and the extent or orientation of the inserted gene sequence, or possibly the polarity and preferred start sites for recombination. The second observation, however, may well be the result of two, rather than one, *Bgl* II sites in the normal sequence of *uvsF*, assuming that one such site has been lost by rearrangement in the incompletely complemented transformant (34).

To test the proposed hypothesis, plasmids could be rescued from the wildtype-like transformants using either *Stu*I-digested DNA or partial digests with *Bgl*II. Such analysis might indeed support our hypothesis and demonstrate that the large 16 kb *Stu*I sequence from wildtype-like transformants (35-37) can complement *uvsF* in secondary transformation. However, there is no guarantee that such clones would be without

rearragements, even though a few cases have been reported in which the normal gene sequence has been obtained from rescued complementing plasmids (e.g., for *pyrG* of Aspergillus by OAKLEY *et al.* 1987). It will, therefore, be more useful to identify the smallest fragment from the genomic Charon4A.137 clone which complements *uvsF* ⁻ in transformation. Such a segment is likely to contain the complete gene sequence of *uvsF* and, if our hypothesis is correct, should be one of the fragments which hybridized to the isolated cDNA clones, as well as to the subclone containing the common region of the two rescued plasmids (i.e., the 2.3 kb *Pst* I insert of pEK6.1). The smallest fragment that cross-hybridized to the cDNA insert is a 2.2 kb single band, identified in *Pst* I-digests of the 4A.137 clone. This segment may well be the same sequence as the insert of pEK6.1. Since the latter does not complement *uvsF*, the next larger genomic fragment identified (*Dra*I digest, 4.0 kb), may be the most useful one for direct complementation tests in Aspergillus transformation.

An alternate possibility is to use the complete cDNA insert of λgt10.93 which may be able to complement *uvsF*. However, the finding that the two cross-hybridizing cDNA clones show somewhat different positions of an *Eco*RI cut in their inserts, suggests that at least one of them is not complete, even though they presumably overlap to a large extent. The size of a complete cDNA is however not expected to be much larger than the total of 1.2 kb estimated for the insert of λgt10.93, since

the hybridizing mRNA showed a size of approximately 1 kb.

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RESULTS (Not shown in publication)

My personal contribution toward the work presented in the publication is listed below:

1) **Figures:** 2c (Southern analysis of transformants and their progeny); 3b and 4 (detailed restriction analysis of the rescued plasmids); 5,6,8 and 9 (all the work on cDNA and Charon 4A genomic clones); 7c (probing of the Northern with λgt10.93). Figure 1 was planned by Dr. Käfer and me.

2) Four of the 25 crosses listed in Table I.

3) Experimental results not shown in Figures and Tables.

(a) Making the subclones pOK5.2 and pOK5.1.

(b) Secondary transformation experiments with rescued plasmids pEK5 (larger insert) and pEK8 (shorter insert) as well as with the pEK5-derived subclones pOK5.2 and pOK5.1.

(c) All the work involving cDNA library and Charon 4A genomic library.

(d) Purification of polyA$^+$-enriched mRNA was done by Dr. Käfer. Stripping, the Northern blots that she had probed with pEK5/6 and pEK6.1 (Figure 7,a and b) and reprobing with λgt10.93 was done by me (Figure 7c).

The rest of the work was done by Dr. Käfer.

For some of the work described in the publication, data has not been shown. Any such results and their discussion are presented in the next section. Since these results are already described as well as discussed in publication, they will not be discussed in depth.

1) Mitotic mapping and stability:

In 3 of the 4 diploids that were analysed (tester strains with: Tf3135, its F_1 and the secondary transformant of the plasmid with the larger insert-pEK5/6), *uvs*$^+$ and *pyr*$^+$ showed complete linkage with markers on chromosome I, whereas in a *uvsF*$^-$ *pyr*$^+$ secondary transformant of pEK5, *pyr*$^+$ segregated with the markers on chromosome V. In *Bgl*II-digested genomic DNA probed with the vector (pGM3), such *uvsF*$^-$ *pyr*$^+$ progeny showed the same size band (13.8Kb) as the original primary transformant Tf34 (Figure2c), suggesting that the entire plasmid along with *uvsF*$^+$ and *pyr*$^+$ has integrated on chromosome V and that *uvsF*$^+$ is not expressed at this site. Alternately, *uvsF* sequence is not present in this secondary transformant. In such a case, a possible reason (though less likely) for this secondary transformant giving the same size band as its primary transformant (34), could be a chance occurance of flanking *Bgl*II sites at a distance that would give that particular size of band. The results of diploid analyses are summarized in Table II. I also made and analysed another standard diploid that was made to obtain strains with new combination of markers. These strains were then used for other diploid analysis (see Table III; strains 3171 and 3175, that were progeny of the standard diploid MD2800).

Table II: Genetic Analysis of Primary and Secondary Transformants and Progeny thereof: Mitotic Stability and Mapping

Tf# (in recipient#)	Tester strain #	Linkage Group	Exceptions / Total	Loss of uvsF+ &/or pyr+ #/relevant tested
a) 1° Tf or its F_1				
3134 (in 3101)	3171	I	0/70	0/40
3135 (in 3115)	3099	I	0/95	0/66
3213 F_1 of 3135	3172	I	0/179	0/114
3136 (in 3115)	3224	I	0/63	0/57
3137 (in 3115)	3272	I	0/81	0/76
3139 (in 3101)	3272	III	3/70	--
b) 2° Tf or its F_1				
3216 (in 3101)	3174	I	0/58	0/37
3215 (in 3101)	3171	V	10/87 (11%)	0/35
3362 (F_1 of 3215)	3171	V	0/81	0/78
c) Incorporation uvs+ pyrG-				
3176 (in 3101) F_1 of 3134	2487	*uvs* F^+ I pyrG	0/79	0/30

Table III: Genotypes of strains used and their stock numbers

a) as recipients

b) as testers in diploid analysis
c) in crosses (Meiotic analysis and mapping)

Strain#	Genotype
a) 3101	uvsF201 pyrG89 riboA1 yA2; wA2; pyroA4
3115	uvsF201 pyrG89 riboA1 yA2; wA2; choA1; chaA1
3114	uvsF201 pyrG89 yA2; wA2; choA1; chaA1
b) 2487	sulA1 anA1 biA1; wA2 cnxE16; galA1; methG1; nicA2; sbA3; choA1; chaA1
3099	pyrG89; AcrA1; pabaB22
3171	pyrG89; AcrA1; ActA1; pabaB22; nicA2; sB3; malA1; chaA1
3172	pyrG89; AcrA1; ActA1; pabaB22; nicA2; sB3; malA1; riboB2; chaA1
3174	pyrG89; AcrA1; ActA1; nicA2; sB3; malA1; chaA1
3224	pyrG89; AcrA1; ActA1; pyroA4; nicA2; sB3; riboB2; chaA1
3272	pyrG89; AcrA1; ActA1; pabaB22; nicA2; sB3; malA1; fwA2
c) 1996	galD5 suA1adE20 anA1 biA1; chaA1
2343	uvsF201 fpaB37 galD5 suA1adE20 riboA1 yA2 adE20
3099	pyrG89; AcrA1; pabaB22
3135	uvsF201 (uvsF$^+$pyr4$^+$) pyrG89 riboA1 yA2; wA2; choA1; chaA1
3202	uvsF201 (uvsF$^+$pyr4$^+$) pyrG89 yA2; wA2 cnxE16; chaA1
3203	uvsF201 (uvsF$^+$pyr4$^+$) pyrG89 yA2; wA2 cnxE16; choA1; chaA1
3213	uvsF201 (uvsF$^+$pyr4$^+$) pyrG89 yA2; wA2 cnxE16; choA1; chaA1

2) Meiotic mapping and stability:

As already mentioned in the publication, in the complemented transformants, *uvsF*$^+$ and *pyr*$^+$ were tightly linked to each other. They gave recombination frequency, with the markers on the left arm of chromosome I, as expected of the plasmids integrated by homologous recombination. In such a case the integrated Aspergillus sequence would be duplicated as shown in Figure I, thus providing a site for intrachromosomal recombination. Depending on the site of recombination one would expect either *uvsF*$^+$ or *uvsF*$^-$ progeny with the loss of the sequence between the points of recombination. Surprisingly, no replacement progeny (*uvsF*$^+$ *pyrG*$^-$) was observed either in heterozygous or in homozygous crosses involving the wildtype-like transformant (35), while there was a high level of plasmid instability giving about 30-40% *uvsF*$^-$ *pyrG*$^-$ progeny. In the heterozygous cross *uvsF*$^-$ *pyrG*$^-$ progeny could either mean a crossover or plasmid loss. An excess of *uvsF*$^-$ *pyrG*$^-$ progeny was obtained than would be expected just by crossing over Thus plasmid instability was observed in the heterozygous cross as well, but could only be approximated (unlike the homozygous crosses it could not be quantified exactly). In order to quantify such instability in the heterozygous cross of Tf35, a strain with a marker tightly linked to *pyrG* was necessary. A standard cross was performed to obtain a strain with *galD* and *pyrG*, since these markers are only three map units apart (Figure1). In such a case one could follow these two fairly closely. Plasmid

instability of about 30% was observed in such cases. The *pyrG galD* progeny that was obtained in such a cross was also used, by Dr. Käfer, as a mapping strain for determining the order of *pyrG* and *galD* on the left arm of chromosome I (Kafer and May 1986, see the reference list in publication).

It is intriguing that the normal replacement progeny (*uvsF*$^+$ *pyrG*$^-$) is not observed in any crosses with the wildtype-like transformants. In absence of any molecular evidence one can just speculate that due to the topology of the DNA sequence at the site of integration, and its possible ways of interaction with the enzymes involved, recombination may be favoured only in one direction giving rise to only one of the two possible types of progeny.

I performed a total of 5 crosses (4 out of 25 crosses listed in Table I and a standard cross). Three out of these were homozygous transformant crosses involving F1 of Tf35, the fourth one was a heterozygous cross of Tf35 with a *uvsF*$^-$ *pyrG*$^+$ strain and the fifth one (the standard cross) was between *pyrG* and *galD* strains (see above).

3) Restriction analysis of the rescued plasmids:

Several lines of evidence had shown that the two types of transformants from which the plasmids were rescued were different, suggesting that rearrangements had occured atleast in one of the two cases. (a) The primary transformants from which these were rescued, are phenotypically different. (b) They behave

quite differently in crosses (see the publication). (c) In southern analysis of the *Bgl*II digested DNA, they gave different sized bands. To confirm the occurance of such rearrangements, we decided to perform detailed restriction analysis of the plasmids rescued from the well complemented primary transformants. Such analysis showed that the two plasmids had quite different restriction maps and also revealed a 1.2Kb region in common, between the two plasmids, that was not a simple "overlap" (Table IV).

Putting together these results convinces us that there has been rearrangements in the genome of, atleast, one of the two transformants necessitating screening of the wild type libraries, to obtain the wildtype sequence of *uvsF* gene, rather than relying on the rescued plasmids for further studies (see section 5). However, it would be necessary to sequence the rescued inserts as well as mRNA and comparing their sequences to confirm our hypothesis.

Table IV: Results of restriction analysis of the vectors (pGM3) and the rescued plasmids (pEK8 and pEK5) [a]

# of sites in / Restriction Enzymes	pGM3	Inserts of	
		pEK8	pEK5
Ava I	4	1	6
Acc I	6	3	4
Bam HI	3	0	0
Bcl I	0	0	0
Bgl II	0	1	1
Cla I	1 [b]	1 [b]	1 [b]
Eco RI	2	0	1
Hind III	3	0	1
Kpn I	1	1	0
Pst I	2	1	5
Pvu II	2	1	0
Sal I	4	1	1
Sma I	2	0	1
Sph I	1	1	1
Sst I	1	0	2
Sst II	2	0	1
Stu I	0	0	0
Xba I	0	2	4
Xho I	1	2	3

a) For the maps of these plasmids refer to Figures 3 and 4 on page--- and page -----.

b) Cla I site in the vector pGM3 is lost due to the way the library was constructed (Osmani et.al. 1987), hence the Cla I sites in the rescued plasmids are distinct from the one in the vector.

4) Secondary transformation:

In secondary transformation experiments it was only the plasmid rescued from the near wildtype-like Tf34, (the one with the larger insert, pEK5/6) that was able to complement *uvsF*. The plasmid rescued from the wildtype-like Tf35 and Tf36 (the one with the smaller insert, pEK8) did not complement *uvsF* in several different transformation experiments, neither did any of the pEK5/6 derived subclones. The results of these experiments are summarized in the publication and details are shown in Table V. *Eco*RI and *Pst*I sites must be within the *uvsF* complementing sequence because by cutting the rescued plasmid pEK5/6 with these enzymes, the *uvsF* complementing ability of pEK5/6 is lost. In the Southerns of *Pst*I digested pEK5/6, probed with the cDNA, only one band lights up However, when the corresponding fragment from pEK5/6 is subcloned, it looses its *uvsF* complementing ability. The reason why this fragment does not complement *uvsF* may be because there are two very closely linked *Pst*I sites in the rescued plasmid so that when it is cut with this enzyme it may loose a small fragment (that may be essential for the complementation of *uvsF*) that may not be detected on Southerns or on the gels. It should be noted that even in the Southerns of *Pst*I digested genomic clone 4A.137 only one band lights up which is of the same size as the band that lights up in the case of pEK5/6 (Figures 6 and 8). The answer to the question, whether the gene has a *Pst*I site, can be obtained by sequencing the gene of interest.

Table V: Tests of pyr$^+$ selected transformants for complementation of uvsF.

Plasmid	Total pyr+ Tfs. tested on MMS	Growth on MMS +	±	-
pEK5	236 [a]	168 (71%)	29 (12%)	39 (17%)
pEK8	285	-	-	285
pEK6.1	555	-	-	555
pOK5.2	890	-	-	890
pOK5.1 (+pGM3/pRG3)	93	-	-	93

a) Of the total Tfs. tested ~82% were pyr$^+$, all of which were tested on MMS.

5) Screening of the wild type libraries and analysis of the clones:

The details of the screening of the wild type libraries and Southern analysis are given in Table VI.

The cDNA subclones as well as the subclone of the plasmid with the larger insert with 1.2 Kb "overlap" (pEK6.1), hybridize only to the DNA of the genomic clone Charon4A.137 and not to that of 4A.41 and 4A.46 (results for the larger cDNA subclone used as probe are shown in Figure 10). Thus it is 4A.137 that has the wild type sequence of the gene of interest. In order to locate *uvsF*$^{+}$ sequence on a single small restriction fragment of 4A 137 insert, various single and double digests were probed with cDNA subclones and with pEK6.1 (results for the larger cDNA subclone are shown in Figure 11). Most digests gave more than one band suggesting a cut within the gene. On the other hand *Bam*HI, *Kpn*I, *Hin*dIII and *Stu*I, produced a single hybridizing fragment each of high molecular weight (Figure 11b). These bands, however, are too large to locate the gene Double digests using combinations of the latter two enzymes did not result in a significant reduction in size of the bands (Figure 12b; lanes 2,4,6,8, and10). The only enzymes that gave bands of smaller size were PstI (2.2Kb), DraI (4.0Kb) and StuI+SalI double digests (7.5Kb). The results of these are shown in the publication (Figure 7; lanes 10, 1 and 9).

Restriction and Southern analysis also helped in deducing the restrction map of 4A.137 insert (Figure 9). Two restriction bands of particular digests, that lighted up using the cDNA probe, could easily be assigned neighbouring positions, and verified by using

various single and double digests. For example, in such Southerns two *Eco*RI bands light up 1.6kb and 6.2kb which could thus be placed side by side (refer to Figure 8, fragments on either side of the *Eco*RI site at the 3' end). Moreover, restriction map of the λ Charon4A arms was available from Maniatis *et al.* (1982, see the reference list in publication), which also simplified the task further.

Table VI: Details of screening of the *A. nidulans* wildtype libraries using pEK5 as probe and hybridization response of the isolated clones to plasmid and cDNA inserts.

Library	# of pfu screened	pfu/plate screening		Clones obtained (size of the insert)	Hybridization of the obtained clones to the insert of					
					pEK		pOK		λ gt.10	
		1^0	2^0		8	6.1	5.1	5.2	.66	.93
cDNA in λ gt.10	~5000	~500	~25	λ gt10.66 (1.2 kb)	NC[a]	+	NC	NC	+[b]	+
				λ gt10.93 (1.2 kb)	+	+	+	+	+	+[b]
Genomic in λ Charon 4A	~5000	~500	~25	4A.1 (2.9 kb)	-	-	NC	NC	-	-
				4A.46 (12.5 kb)	+	-	NC	NC	-	-
				4A.137 (14.5 kb)	+	+	NC	NC	+	+

a. NC = not checked

b. Self-hybridization

FIGURE 10. a) Various single digests of three Charon 4A clones, b) probed with pRG3-93L, i.e., the larger *Eco*R1 fragment of the CDNA insert from λg10.93, subcloned in pRG3 (probing with the other cDNA subclone and pEK6.1 gave the same results). c) The same probed with pEK8, i.e., the noncomplementing rescued plasmid.

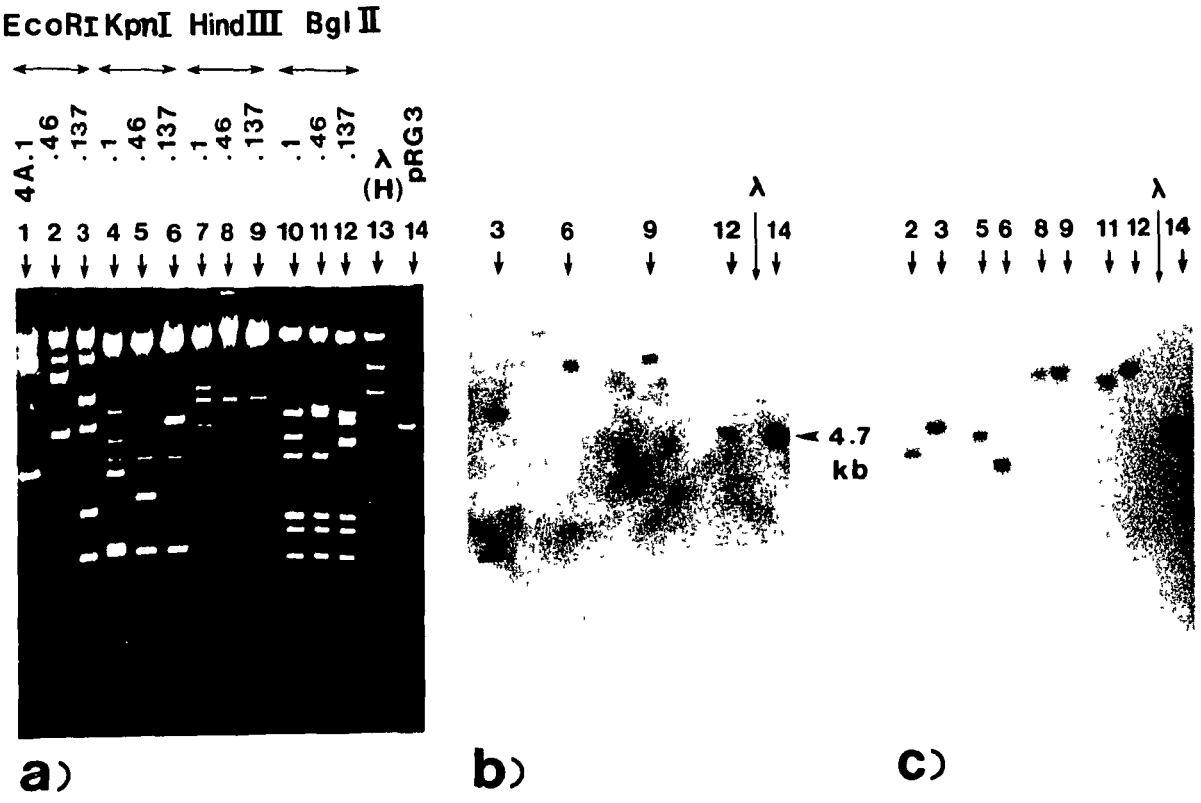
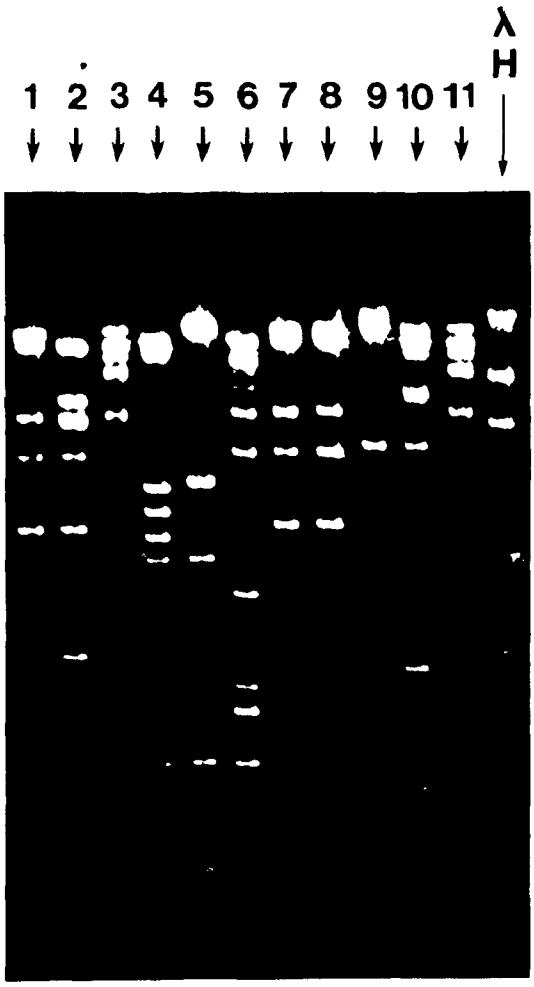
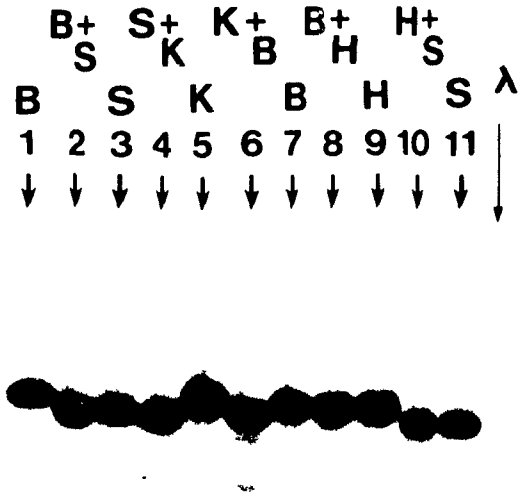

EcoRI KpnI HindIII BglII
4A.1 .46 .137 .1 .46 .137 .1 .46 .137 .1 .46 .137 λ (H) pRG3
1 2 3 4 5 6 7 8 9 10 11 12 13 14
a)
3 6 9 12 14 λ
4.7 kb
b)
2 3 5 6 8 9 11 12 14 λ
c)

FIGURE 11. a) Various single and double digests of Charon 4A-137 clone, b) probed with pRG3.93L, i.e., the larger EcoR1 framment of the cDNA insert from λgt10.93, subcloned in pRG3 (probing with the other cDNA subclones and pEK6.1 gave the same results). B, *BamH*I; H, *Hind*III; K, *Kpn*I; S, *Stu*I.

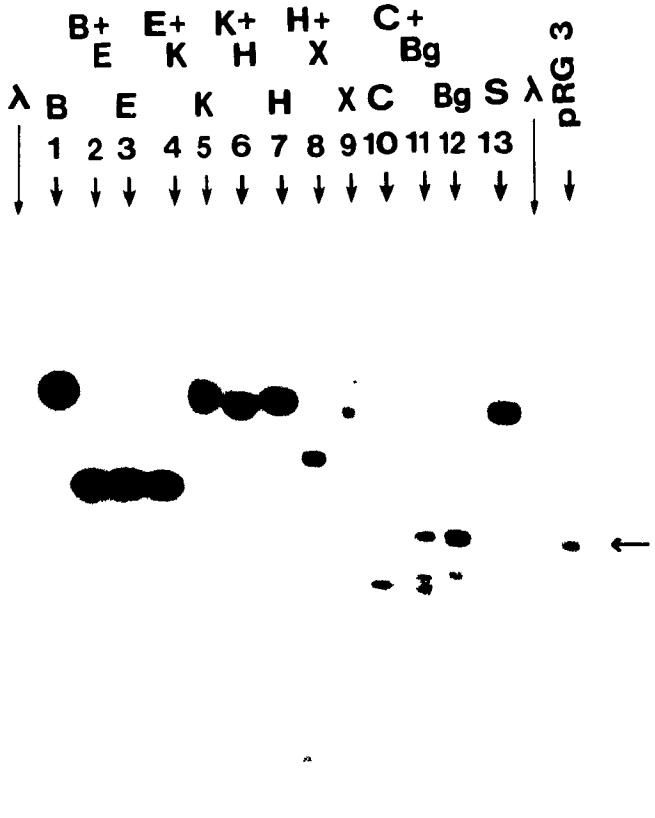


a)

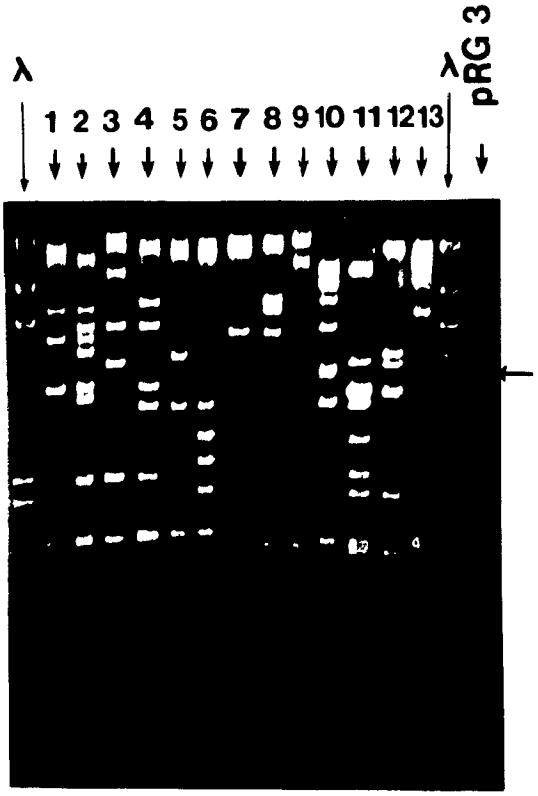


b)

FIGURE 12. b) Various single and double digests of Charon4A.137 (different from Fig. 11), a) probed with pRG3.93L, i.e., the larger EcoR1 fragment of the cDNA insert from λgt10.93, subcloned in pRG3. (Probing with the other cDNA subclones and pEK6.1 gave the same results.) B, *BamH*I; Bg, *Bgl*II; C, *Cla*I; E, *EcoR*I; H, *Hind*III; K, *Kpn*I; S, *Stu*I; X, *Xba*I; λ, *Hind*III digested λ markers. (a and b are not to the same scale; arrows on the right of both figures correspond to the 4.7 kb size of pRG3.)


B+ E+ K+ H+ C+
E K H X Bg
λ B E K H X C Bg S λ pRG 3
1 2 3 4 5 6 7 8 9 10 11 12 13

a)


λ λ pRG 3
1 2 3 4 5 6 7 8 9 10 11 12 13

b)

FUTURE DIRECTIONS:

On the basis of literature reviewed and ideas already discussed at the end of the literature review, I shall propose experiments that can be carried out to extend the project that I have been working on.

As a first step towards understanding DNA repair in Aspergillus we have cloned the gene *uvsF*. This can be confirmed by obtaining a subclone from the wild type genomic or cDNA clone that can complement the mutation of interest. The wild type gene and the cDNA could be sequenced as the next step, as this would also show whether there has been any rearrangements in one of the two rescued plasmids, if the latter are also sequenced along with. As discussed earlier, sequencing may provide some clue as to its function if it has any homology to a well characterized gene (as in human *ERCC1* and yeast *RAD10* genes; van Duin *et al.* 1986, van Duin *et al.* 1988, see the overview section of the literature review). To analyse the function of the cloned gene one could carry out gene disruption and gene replacement experiments which are now possible with Aspergillus (Miller *et al.* 1985, Aramayo *et al.* 1989). Site-directed mutagenesis could be carried out to localize the essential and nonessential functions of an indispensible gene, and to localize regions of any peptide sequence that may affect its turnover. Further, functional analysis could be done (1) by checking whether the phage T4 gene *denV*$^{+}$ can complement *uvsF* and (2) by one or more of the five ways listed in literature review in the section on yeast

nucleotide excision repair system.

The cloned gene could be used to overproduce the gene product and to carry out in vitro studies as in the case of purified *E. coli* ABC excinuclease (see the literature review). Southern, Northern and Western analyses could be carried out to determine the stage at which the gene function is blocked (discussed in the overview section of the literature review).

APPENDIX

I. ASPERGILLUS PROTOPLASTING and TRANSFORMATION

(**Osmani** *et al*, **1987**, J. Cell Biol. 104: 1495)

A) Growing up of **young conidia**:
Using suspension (low "Tween 80") from recent plate
or from previous experiment,
inoculate > 5 x 10^6/plate into overlay (MAGUU, ~30-40 ml),
and pour on top of **4-6 plates** (YAGU; see p.3, A).

Incubate: approx. 28h at 37° C,
or 32h at 32°,
[or some ts, >2 days at 25°].

Also make 2 YAG or CM plates to check for revertants or contamination

B) Harvesting of **young conidia**: use 10-15 ml of 0 2% Tween 80,
and glass spreader to rub off conidia,.pouring fluid from one plate to next.
Suck up suspension from last plate (use attachment on 10 ml pipette),
and transfer to sterile plastic centrifuge tube (15 ml)

Spin to pellet conidia, **2k, 3'** max [decant to discard Tween 80]
Wash 2x with sterile H_2O (~10 ml), spin 2k, 3'. Vortex to resuspend,
but leave black part of pellet behind (= conidiophores).
[Tween is very bad for protoplasts.]

Count: Suspend final pellet in 2-4 ml H_2O = "**original**" suspension
(or 1 ml H_2O, if few conidia)
Dilute 1/100: 50 µl into 5 ml H_2O, and if good suspension,
make 10^{-3} (0.5 ml into 4.5 ml).
Make **hemacytometer** count of dilution
and calculate concentration of original suspension
Needed, 10^9 conidia (more if poor strain) to inoculate 50 ml liquid culture,
(wanted ~2 x 10^7/ml; poor growth if >3 x 10^7/ml).

C) Liquid culture: Use 10^9 conidia for 50 ml media
in 125 ml **siliconized flask**
Incubate either: **a)** 3 1/2 - 4 (max) hrs on **first day**, (put at 4° overnight)
and continue until germination on **2nd day** (1 hr or more)
Total: **5 hrs** (or more for some strains), use YAGU media (see p.3, B),

or **b)** for **1-day expt**, start early and use YAGUU media;
incubating **4 1/2 - 5 hrs** (or more), until germination

Use **32°** with **moderate shaking**· New Brunswick shaker, ~150.
To assess germination, **check under high-power microscope**,
use, when spores are gourd-shaped or have short germ tubes.
Longer germ tubes also work well and protoplasting will be faster

D) Protoplasting:

Transfer germinated conidia to 50 ml sterile centrifuge tubes and spin at 1.8 - 2k, 3' max. (low-speed Table-top [or IEC] centrifuge); RT is OK, but use low break setting

Decant or suck off supernatant (discard, autoclave).

Resuspend soft pellet in 20 ml of **solution II** (p.4).

Add 20 ml of **Protoplasting Mix***, for40 ml total (make just before needed).

Transfer to siliconized, sterile 125 ml flask

Incubate at 32°, with gentle shaking (~100 New B. shaker) for >1 hour, until "all nuclei" are found in protoplasts (with vacuole).

Check with **phase contrast** high-power microscope.

Break up clumps by vigorous pipetting (after 1 - 1 1/2 hrs).

***Protoplasting Mix**: Use 20 ml of **sol. I** (p.4), and dissolve .
0 8 g **BSA**, (final conc. 20 mg/ml)
Add 0.5 ml 1 M $MgSO_4$
(stir in beaker, then keep cold);
last minute, add 120 mg **Novozyme** (final conc. 3 mg/ml)
and stir to dissolve.
Filter sterilize into 50 ml sterile tube. **Keep cold, in ice.**

E) Harvesting of protoplasts & wash:

Centrifuge (**4°C**) 1.7 - 2k for 2-3' (low break) [discard supernatant] and suspend pellet in **solution III** (15-20 ml); to wash, repeat 2›. **Keep on ice**

Resuspend final **pellet** (± well drained) in 1 ml of **sol. V** (+ **$CaCl_2$**) to obtain **competent protoplasts**, [these can be kept overnight in ice, in cold unit].

F) Transformation:

In microfuge tubes mix and hold **on ice 20'**: 100 µl **protoplasts**
\+ 2-4 µg of **DNA** (e.g , 2µl of 2µg/µl plasmid DNA)
\+ 50 µl **solution IV (PEG)**, mix well by pipetting.

Then add 1 ml/tube of **solution IV** and mix by tipping and tapping.

Hold at **RT, 20'**.

Also make and melt overlay agar, 4-5 ml/tube.

Plate in regeneration agar-overlays **for transformation**:
from each tube add increasing amounts (10, 100, 500 µl, and rest) to **selective** overlay agar and pour on selection plates
From "0" DNA tube make 2 plates (~500 µl each).

G) Protoplast Survival: Dilute from "0" DNA into **sol. III**, down to 10^{-5}.
Add 100 µl from each dilution to **control** regeneration overlay tubes and pour onto non-selective plates (= Sucrose YAGU; 1 tube/plate).

Dilutions: 50 µl into 5 ml = 10^{-2}
and 0.5 ml into 4.5 for 10^{-3};
repeat to get 10^{-4} and 10^{-5}.

Count after 2 days (37°), and calculate total number of **viable** protoplasts per strain.

ASPERGILLUS TRANSFORMATION MEDIA

A) Plates to grow up conidia (see p.1)

Bottom layer: YAGU (150-300ml/flask)	per liter	Overlay: MAGUU (30-50ml/flask)	per 500 ml
0.5% YE	5 .0 g	2% Malt Extract	10.0 g
2% Dextrose=Glucose	20.0 g	2% Glucose	10.0 g
5 mM $MgSO_4$	1.25 g	0.1% Peptone	0.5 g
10 mM Uridine	2.40 g	5mM Uridine	0.6 g
		10mM Uracil	0.6 g
Trace Elements (TE)	1 ml*	TE	0.5 ml*
Vitamin Solution	1 ml*	Vitamin Sol	0.5 ml*
1.5% Agar**	15.0 g	2% Agar**	10.0 g

* From stock solution for standard.MM medium.

** Weigh out agar into each flask, add solution and autoclave, or use half of H_2O to steam agar, mix "solutions", dispense and autoclave.

B) Liquid Media for growth: Use YAGU (as above, but no agar)

Make max. 500 ml and dispense 50 ml per siliconized 125 ml flask.

Or: if 1-day experiments, for faster growth use YAG + Uridine 5mM + Uracil 10mM

C) Selection and Regeneration Plates

Selection:		per liter	
1 M Suc YAG	1 M Sucrose	342 g	
	0.5% YE	5.0 g	
	20 mM Glucose	3.6 g	
	5 mM $MgSO_4$	1.25g	
	TE + Vitamins	1 ml each.	For **plates** use **2%** Agar (weigh into flasks)

Regeneration:

1 M Suc YAGU: Add 10 mM Uridine to M Suc YAG, using M stock solution.

D) Selection and Regeneration Overlays

Use same media as for plates (C), but for **overlays add 1%** Agar Weigh into flasks and keep at 4°.

Also: oven sterilize tubes + metal caps (100 x 13 mm).

To plate protoplasts:

Autoclave **overlay** media before transformation (mix well) dispense while hot, 3 5 - 4 ml/tube (more if room cold), and keep at 47° C (blocks or water bath) until needed.

ASPERGILLUS TRANSFORMATION SOLUTIONS

Solution I — **Needed per strain:**

		for 500 ml	
0.8M	Ammonium Sulphate*	52.8 g	(start with
0.1M	Citric Acid (anhydrous	9.6 g)	400 ml H_2O)
	or monohydrate	10.5 g	

pH to 6.0 with KOH pellets (about 100), make up to 500 ml, dispense as 5 x 100 ml and autoclave.

Solution II — **II:** 20 ml

		for 500 ml	
1%	Yeast Extract	5 g	(start with
2%	Sucrose	10 g	400 ml H_2O)

Make up to 500 ml and autoclave (in 50 ml & 100 ml lots).

Solution III — **III:** >80 ml

		for 1 liter		or:
0.4M	Ammonium Sulphate*	52.8 g		Use 2%
1%	Sucrose	10.0 g	(start with	sucrose,
50 mM	Citric Acid (anhydrous	9.6 g)	800 ml)	autoclaved.
	or monohydrate	10.5 g		To get III,

pH 6.0 with KOH pellets (>100). mix 1:1 with solution I.

Make up to 1 liter, . dispense 5 x 200 ml, and autoclave.

*used ultra-pure, but best ordinary grade is also OK.

Solution IV — **IV:** 5 ml

		for 100 ml	
25 %	PEG 6000 (8000 now)	25.0 g	(start with
0.1 M	Calcium Chloride $\cdot 2H_2O$	1.47 g	<70 ml H_2O)
0.6 M	Potassium Chloride	4.47 g	
10 mM	Tris-HCl, pH 7.5	1 ml of 1 M	

Make up to 100 ml, heat, filter hot to sterilize [use suction and disposable filter unit]. Dispense as 50 ml aliquots, into sterile plastic disposable centrifuge tubes; **store frozen (-20°).**

Solution V — **V:** 1 ml

Mol W			for 100 ml	
74 6	0 6 M	Potassium Chloride	4.47 g	(start with
147.0	250 mM	Calcium Chloride $\cdot 2H_2O$	0.73 g	80 ml H_2O)
192 2	10 mM	MES (Sigma) buffer	0.19 g	

pH to 6.0 with KOH (not much needed, use 1 N).

Make up to 100 ml and filter sterilize; Dispense in <15 ml aliquots into sterile tubes; **store at -20°.**

II PLASMID DNA "Mini" PREP (Rapid Lysis; LiCl)

[Vollmer and Yanofsky, 1986, PNAS 82: 4869, with minor modifications).

Protocol

1) **Culture:** Inoculate 10 ml LB +amp (50 or 100 µg/ml) with single colony. Incubate O/N, at 37°.

2) **Harvest*:** Centrifuge cultures at **4000 rpm,** 4°C, **5 min**
(40 ml plastic centrifuge tubes); pour off supernatant.
* save 1 ml of original culture before centrifuging.

3) **Resuspend** in **100** µl of ice cold **Solution** I (Tris-EDTA-Glucose).

4) **Transfer** to microfuge-tube (1 1/2 ml); hold at Room Temp **5 min.**

5) **Lysis:** Add **200** µl of fresh **Sol. II** (NaOH, SDS)
mix by inversion, hold on ice **5 min.**

6) **Neutralize:** Add **150** µl of **Sol. III**, invert, vortex **10"**, hold on ice **5 min.**

7) **Sediment:** Microfuge (room temperature is OK) **5 min.**

8) **Transfer** supernatant to fresh tube: avoid transferring traces of pellet; (if doubtful, spin again).

9) **Ethanol Precipitation:** Add 2 volumes of 95% **EtOH** (at RT),
(~ 2 x 400 µl); vortex; hold at RT **2 min.**
Then microfuge at RT for **5 min.**

10) **Dry Pellet** briefly in "speed vac" and resuspend in 200 µl of dd H_2O

11) **Precipitate Non-DNAs** Add 200 µl of **5M LiCl.**
Hold at **-20**°C for **5-10 min.**
Microfuge at **4**°C **10 min.**

12) **Ethanol Precipitation:** Transfer **supernatant** to fresh tube
Add 1/20 Vol of **4M NaCl** (~ 20 µl)
and add 2 Vol (~ 800 µl) of ice cold **95% EtOH.**
Hold at **-70°C** (dry-ice, ethanol bath) for **20 min,**
then microfuge (in the cold) for **10 min.**

13) **Wash Pellet**, rinse with 70% EtOH; dry pellet (in speed vac) **15 min.**

14) **Resuspend** in 100-200 µl sterile TE (or H_2O) at RT;
For digestion, use 1-2 µl; keep at 4°,
when analysed, store at **-20°**

SOLUTIONS for Plasmid DNA Mini-prep (LiCl)

Solution I

		Stock solution	for 100 ml use:
25 mM	Tris-HCl, pH8.0	(1/40 of 1M)	2.5 ml
10 mM	EDTA	(1/50 of 0.5 M)	2 ml
50 mM	Glucose	(1/20 of 1M)	5 ml
			[+ 90.5 ml H_2O];

autoclave or filter sterilize.

Solution II (make fresh; keep at room temperature):

0.2 N	NaOH	(1/10 of 2N)	200 µl	for 2 ml;
1%	SDS	(1/10 of 10%)	200 µl	(need
			[+1.6 ml H_2O]	200 µl/tube)

no need to sterilize

Solution III (=3 M K-acetate, pH4.8)

For 6.7 ml use: 3.6 ml of 5M K-acetate
2.4 ml H_2O
0.69 ml Glacial Acetic Acid (pH will be correct);
keep in cold unit, no need to sterilize.

III. PHAGE LIBRARY SCREENING

An outline of phage library screening is given below, followed by detailed protocols.

OUTLINE:

Primary screening:

A. Checking of library titre.

a) Preparation of "plating" bacteria.

b) Plating of serial dilutions of bacteriophage λ to determine the titre.

c) Amplify the library (if necessary) and determination of the titre of the amplified library.

d) Storing of phage libraries.

B. Screening the library.

a) Plating of bacteriophage for transfers.

b) Transfers onto membrane or nitrocellulose filters.

c) Hybridization with a radio-labelled probe.

d) Washing of filters, exposure of X-ray films and developing of the films

e) Picking of positive clones.

Secondary Screening:

A. Checking of titre of the primary clones picked.

B. Screening of the primary clones picked

(determine the titre and store in DMSO)

PROTOCOLS (modified from Maniatis *et. al.*, 1982).

A. Checking of Library Titre.

a) Preparation of "plating" bacteria.

1. **Inoculate** a single **bacterial colony** into 50 ml of LB containing **10 mM $MgSO_4$**, supplemented with **0.2% maltose**, into a 250 ml flask and grow at 37° overnight.
2. **Centrifuge** the cells at **4000 g** for **10** minutes at room temperature.
3. Discard the supernatant and **resuspend** the **cell pellet** in cold sterile 0.01 M $MgSO_4$ (0.4 x the volume of the original culture).
4. **Store** at **4°** . The bacterial suspension may be used for up to three weeks. However, the highest plating effeciencies are obtained with fresh cells (0-2 days old).

b) Plating of serial dilutions of phage λ to determine the titre.

1. Prepare 10-fold serial **dilutions** of **bacteriophage stocks** in **SM**. **Dispense 0.1ml** of each **dilution** to be assayed into each of two test tubes (13-mm x 100-mm).
2. **Add 0.1 ml** of plating **bacteria** to each tube. **Mix** by shaking. **Incubate** at 37° for 20 minutes for **bacteriophage adsorbtion.**
3 **Add 3.0 ml** of **medium** (47°) containing melted **0.7% agar** to the first tube, vortex gently, and immediately **pour** onto a **labeled plate** containing

30-35ml of hardened bottom agar medium. Try to avoid air bubbles. Swirl the plate gently to ensure an **even distribution** of bacteria and top agar. **Repeat** with each of the tubes.

4. Close the **plates** and let them **stand** for **10 to 15 minutes** at room temperature to allow the top agar to harden. Invert the plates and incubate at 37°. **Plaques** begin to appear after about 8 hours and should be **counted** or **picked after 12-16 hours** of incubation.

NOTE:
Although there can be considerable variability, most plate stocks or liquid culture lysates of bacteriophage λ contain **10^9 - 10^{11} / ml.** phages.

c) Amplification of the libraryand preparing stocks of bacteriophage λ from single plaques.

Two techniques are commonly used:

(1) **Plate lysates**, in which phages are propagated in bacteria grown in soft agar, and (2) **Small-scale liquid cultures**, in which the phages are grown in bacteria in liquid medium.

Although the yields from the two procedures are approximately equal, the first has the advantage that one can determine, merely by looking at the degree of confluence of the plaques, whether or not the bacteriophage has grown successfully; hence the first-technique was used in our laboratory.

To achieve **maximum yield**, the number of bacteriophages plated should be adjusted so that the outer edges of the expanding **plaques just touch** after approximately **12 hours** of incubation. An **inoculum of 10^5** plaque-forming

units (**pfu**) is usually sufficient to produce confluent lysis on an 85mm plate (567 cm^2). By the end of the period of growth, there should be no patches of uninfected bacteria.

Steps of method 1:

1. **Mix 10^5 pfu** of bacteriophage (or 1/20 of a resuspended plaque) with **0.1ml** of plating **bacteria**. **Incubate** at 37° for **20 minutes**.

2. **Add 3.0 ml** of melted **top agar** at 47°, mix, and pour onto a labeled 85mm plate containing 30ml of hardened bottom agar, LB + 10 mM $MgSO_4$, supplimented with 0.2% maltose. Freshly poured plates give the best results, but older plates, 1-4 days old, give satisfactory yields.

3. Invert and **incubate** the plate for **8-12 hours**, until lysis is confluent.

4. Turn the plate over, **add 5ml of SM**, and **store** the plate at **4°** for several hours with intermittent, gentle shaking.

5. With a pasteur pipette, **harvest** as much as possible of the **SM.** Add **1 ml** of **fresh** SM and store the plate for **15 minutes** in tilted position to allow all the fluid to drain into one area. **Again remove the SM** and **combine** it with the first harvest. Discard the plate.

6. **Add 0.1 ml** of **chloroform** to the pooled SM, **vortex** briefly, and **centrifuge** at **4000g** for **10 minutes** at **4°**.

7. Recover the **supernatant** and **add chloroform** to **0.3%**. The **titer** of bacteriophage (approximately **10^{10} / ml**) usually remains unchanged as long as the stock is **stored** at **4°**.

d). Storing phage libraries and purified plaques

Master stocks of important λ bacteriophages should also be **stored** at **-70°**.

Add dimethylsulfoxide (**DMSO**) to the bacteriophage stock to a **final concentration** of **7% v/v**. Mix gently. Plunge the container into **dry ice-ethanol bath**. When the liquid has frozen, transfer the container to a freezer at -70°C for long-term storage.

To **recover** the bacteriophage, **scrape** the frozen **surface** of the liquid with a sterile, 18-gauge needle. **Streak** the needle over the surface of a **plate** containing **indicator bacteria** in order to obtain bacteriophage λ plaques.

B. Screening Bacteriophage λ Plaques by Hybridization.

To **screen** a library of **Aspergillus** DNA (genome complexity, 2×10^7 bp), a total of at least **2000** recombinant **plaques in Charon 4A** must be examined. In the following example, the volumes given are suitable for screening approximately 500 plaques in a 90mm-diameter petri dish.

a) Plating bacteriophage for transfers.

1. **Mix** aliquots of a **packaging mixture** or bacteriophage λ **stock** containing up to 500 bacteriophage particles in a volume of **50 ml** or less with **0.1 ml** of plating **bacteria**. **Incubate** at 37° for **20 minutes**.

2. Add **3.5ml** of molten (50°) **top agar** (1%) and **pour** onto a 90 mm agar plate. The **plates** must be **dry**, otherwise the layer of top agar peels off with the filter. Usually, 2 days-old plates that have been dried for several additional hours at 37° with the lids slightly open work well.

3. **Incubate** at 37° until the **plaques** reach a diameter of approximately **1.5 mm** and are just beginning to make contact with one another **(10-12 hrs).** The plate should **not** show **confluent** lysis.

4. **Chill** the plates at **4°** for at least **1 hour** to allow the top agar to harden.

b) Transfers onto membrane or nitrocellulose filters.

1. **Number** dry, nonsterile **nitrocellulose filters** (from S & S) with a soft pencil or a ball-point pen.

2. At room temperature, **place** a dry **nitrocellulose** circle neatly **onto** the surface of the top **agar** so that it comes into **direct contact with** the **plaques**. Be careful not to trap air bubbles. The filter should be handled with gloved hands; finger oils prevent wetting of the filter and affect transfer of DNA. Mark the filter in three or more asymmetric locations by stabbing through it and into the agar beneath with an 18-gauge needle.

Once in contact with the top agar the filter wets very rapidly and **transfer of** bacterio**phage DNA occurs** quickly. Therefore, do not move the filter once contact with the plate is made The easiest way of placing the filter on the plate is to hold it by its edges, bending it slightly so that the middle of the filter makes contact with the center of the plate. Let wetting action pull the rest of the filter onto the plate.

Make certain that the **keying marks** are **asymmetrically placed** and that both the filter and the plate are marked.

3. After **30-60 seconds**, use blunt-ended forceps to **peel off** the **first filter** and **immerse** it, DNA side up, in a shallow tray of a **denaturing solution** (1.5 M NaCl, 0.5 M NaOH) for **30-60 seconds**. **Transfer** the filter

into **neutralizing solution** (1.5 M NaCl, 0.5 M Tris-HCl [pH 8.0]) for **5 min. Rinse** the filter in **6x SSC** and place it **on** Whatman **3MM** paper **to dry**.

4. Place a **second**, dry **filter onto** the **same plate** and mark it with ink at the same locations. After 1-2 minutes, peel the filter off the plate. Denature, neutralize and rinse the filter, as in step 3 above

Generally, the first filter is left in contact with the plaques for 30-60 seconds and **subsequent filters are left on about 30 seconds longer** or until the filter is completely wet We did not make more than 2 replicas.

If any top agar peels off the plate with the filter, remove it by gently agitating the filter in the denaturing solution.

5. After all the **filters** are **dry**, **wrap** them **between** sheets of Whatman **3MM paper. Fix** the **DNA to the filter** by **baking for 2** hours at **80°** in a vacuum oven. Overbaking can cause the filters to become brittle.

c) Hybridization with a radio-labelled probe.

1. **Hybridize** the filters to a **^{32}P-labeled probe**. [See the publication for protocols of prehybridization, nick-translation, hybridization and washing conditions.]

NOTE: (1) Any **filters not used immediately** in hybridization reactions should be **wrapped** loosely **in** aluminum **foil** and **stored** under **vacuum** at room temperature

(2) We screened a total of about 5000 plaques / library

d) Washing of the filters, exposure of X-ray films and developing of films.

e) Picking of plaques.

1. Place **1.0 ml of SM** in a 13-mm x 100-mm polypropylene tube. Add a **drop** of **chloroform.**

2. Using a pasteur pipette **stab** through the **chosen plaque** into the hard agar beneath. Pull gently so that the plaque, together with the underlying agar, is **drawn into** the pipette. For the primary screen (not well isolated plaques) use the **broad end of a pasteur pipette**. For well isolated plaques use the narrow end

3. **Wash** out the fragments of agar **into 1.0 ml of SM** containing a **drop of chloroform. Let stand** at room temperature for **1-2 hours** to allow the bacteriophage particles to diffuse out of the agar. An average plaque yields **10^6 - 10^7 infectious phage** particles, which can be **stored** indefinitely at **4° in SM/chloroform** without loss of viability.

NOTE

Because bacteriophage λ can diffuse considerable distances through the top agar layer, choose well-seperated plaques*. For the same reason, it is advisable to pick plaques shortly after the bacterial lawn has grown up and the bacteriophage plaques have first appeared.

* for secondary screens.

IV. PHAGE DNA PROTOCOL (LARGE SCALE)

(Maniatis *et al.*, 1982; with minor modifications.)

Locate or prepare appropriate bacterial host (fairly fresh plate) and phage of good titre (pref. > 10^8, 10^9 - 10^{11}/ml).

A) Bacterial and Phage Culture

Day 1 (pm): For **bacterial inoculum** [10^{10} needed for each 500 ml culture]:

1) **Inoculate** 1 colony into 100 ml NZCYM and incubate with vigorous shaking at **37°**, **O/N.**

Day 2 (am):

2a) **Measure OD_{600}** [visual, use plastic cuvettes]; OD of **1 - 1.2** is stationary phase and can be used to inoculate, keep cells at **4°** to start O/N culture later.

or

2b) For log-phase inoculum: reinoculate, using 1-5 ml into 100 ml NZCYM; incubate (with hard shaking) at **37°**, for **<8 h,** until **OD_{600}** of fresh culture is **0.8**

3) For either type of **inoculum,** withdraw aliquots of 10^{10} bacteria [**1 OD unit = 8 x 10^8** cells /ml]. Transfer into sterile plastic centrifuge tube [flip top, >40 ml].

Centrifuge cells (preferably at **RT**) . use 4000g for **10 min.** Discard supernatant. [~ **5.3 k**, check chart]

4) **Resuspend cells** adding 3 ml **SM** (see p. 4)

5) **Add phage**, mix rapidly, gently. Use for **λgt 10**: 5 x 10^7 - 8 x 10^8 / 10^{10} cells; for **Charon 4A**. 5 x 10^8 - 10^{10} / 10^{10} cells, estimating inoculum from titer.

6) **Incubate** (with intermittent shaking) at **37°, for 20 min.**

7) For **large culture**, add each 3 ml of culture-mix to 500 ml of NYZM (prewarmed in 2 liter flask) and incubate (hard shaking, >200 strokes), **37°** **O/N,** (at least 9 - 12 hrs, **max.** 16 - 18 h).

Bacterial lysis by phage

Day 3 (am):

8) **Check**: when cleared (no more Schlieren patterns in homogeneously dense culture) or when bacterial debris evident.

[If in doubt, test 1 ml ineach of 2 tubes at 37° for 10 min,adding 2-3 drops of chloroform to one tube; it will show lysis,.if it is almost ready].

9) Add 10 ml **chloroform** / 500 ml and incubate with hard shaking at **37°** for **30 min.**

10) Cool in water (fill basin ahead of time) to **RT**; **~10 min.**

B) Phage Purification:

1) **Add DNase,** and **RNase,** to get 1 µg/ml (or up to 10 mg/ml); [i.e., for 500 ml culture, 0.1-0.5 ml of 10 mg/ml stock]. Let stand at room temperature,for **30 min.**

2) **Add NaCl** to get 1 M [i e 29.2g / 500 ml] ; dissolve by swirling, put **on Ice** for **1 hr.**

3) **Centrifuge** to remove debris; use 11 000 g at **4° 10 min,** (if 250 ml bottles, ~ **8.7 k**). Combine supernatants in clean flasks.

4) **Add** solid **PEG 8000** to get 10 % [50 g to 500 ml]. Stir (at RT) until completely dissolved.

5) **Cool** in ice water, at least **1 hr.**

6) **Spin** (as above) at **4°,** 11 000 g (**~8.7 k**) **10 min;** carefully drain all PEG.

7) **Suspend pellets** in 3-5 ml **TM** buffer (in corex tubes). Use up to 5 ml TM / 500 ml culture, if glycerol gradient will be used; may be kept at **4° O/N** at this stage.

8) **Extract** 1x with **chloroform,** adding an equal volume; mix well (no vortexing), and spin in cold, **~8 k, 10 min.**

[Sign up for ultra-centrifuge.]

9) Make **glycerol step gradient** in ultra-centrifuge tubes:

a) add at the bottom **3 ml** of **40%** glycerol (in TM) , and
b) carefully overlay with **4 ml** of **5 %** glycerol in TM,
c) add layer of phage in TM (3-5 ml) and
d) fill up with TM (up to neck, no air bubbles).

10) Carefully balance pairs of tubes (weigh, W5/12) .
and spin in **ultra-centrifuge at 4°** (low break) **35 k** for **60 min.**

11) Pipette off and squeeze out glycerol,
cut tube to **dissolve** phage pellet in **TM**
(0.5 ml / 500 ml culture; total up to 1.5 ml for small CsCl tubes)

C) DNA Purification from Phage:

1) **Add** to phage in TM (0.5 - 1.5 ml) :

		from **10 mg/ml stock**
a) **DNase** to get at least 5µg/ml.	{ or up to }	1 - 2 µl/ml
and **RNase** to get at least 1 µg/ml.	{5x more }	0.5 - 1 µl/ml.

Incubate in water bath, **37°** for **30 min,** then add:

b) **EDTA** (0.5 M stock), to get **20 mM**, use 1/25; i e , 40 µl per ml.

c) **Proteinase K** (1 mg/ml stock), to get **50 mg/ml** use1/20;
i.e., 50 µl per ml.

d) **S D S** (20% stock), to get **0.5%** use 1/40, i.e. 25 µl per ml.
Incubate in water bath, **65°** for **1 hr.**

2) <u>Optional</u>: Transfer to corex tube, to **extract 1 x** with **chloroform** (as B 8, before glycerol gradient).

3) For **CsCl gradient** (3 ml tubes, table top model):
Measure volume of clear supernatant; make up to **2.25 ml.**

Add **CsCl,** using the ratio of 1 ml : 1.08 g; i.e., **2.43 g** CsCl.
Put on platform shaker to mix and **dissolve.**
Add **EtBr,** 100-150 µl of stock (10 or 5 mg/ml in H_2O).
Weigh to **balance** and fill up with CsCl-TM mix , or TM.

4) **Heat seal** 3 ml ultra-centrifuge tubes (in lab of Dr. Brown).

5) **Centrifuge** in table top ultra-centrifuge (W5/3) . 15°, **75 k, 4 hrs** or **O/N.**
(Head and adapter caps are stored in W5/9.)

MEDIA AND SOLUTIONS FOR PHAGE DNA PREPARATIONS

Liquid NZCYM, medium for bacteria (Maniatis *et al.*, 1982, p. 68):

	per liter	
NaCl	5 g	Adjust pH to 7.5 with NaOH,
Casamino acids	11 g	(use 1-1.5 ml of 3N per liter).
Bacto-yeast extracts	5 g	
$MgSO_4 \cdot 7H_2O$	2 g	

Autoclave: 500 ml in 2 liter flasks, and 100 ml in 1/2 liter flasks.

SM for phage storage and dilution (Maniatis *et al.*, 1982, p. 70):

	per liter:
NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2.0 g
1 M Tris·Cl (pH 7.5)	50 ml
2% gelatin	5 ml

Sterilize by autoclaving, and store in 50 ml lots.

TM buffer (= 50 mM Tris, 10 mM $MgSO_4$]:

	per liter:
1 M Tris·Cl (pH 7.75)	50 ml
1 M $MgSO_4 \cdot 7H_2O$	10 ml

Sterilize by autoclaving; store volumes of different sizes.

V ASPERGILLUS "MINI" DNA PREPARATION.

(May *et al.*, 1985, J. Cell. Biol. 101:712)

Solutions:

A. 10 mM Tris-HCl, 100 mM EDTA, **2% Sarkosyl**, pH 8.0.
B. **RNase A**, 10 mg/ml in 50 mM NaOAc, pH 4.5,
heated in a boiling water bath for 5 minutes.
C. **20 % PEG 6000** (or 8000), 2.5 M NaCl.

Grow liquid culture overnight, in 25-100 ml media, using
for **inoculum** > 10^6 conidia / ml, to get **>0.2 g wet weight** of mycelium;
e.g , use 25 ml YAG (20 mM Glucose) in 125 ml flasks.

Harvest mycelia on Miracloth (using Buchner funnel and vacuum water tap),
rinse with cold water, press dry (almost completely) and
weigh out **0.2 g**rams; place into microfuge tube (2 samples each).
Freeze all the material on dry ice, and **lyophilyze** (**> 3 hrs**, or O/N).
Keep **extra material** for later use, frozen at **- 80°**

Grind the freeze-dried material to a fine powder in the microfuge tubes,
using battery-operated motor and disposable tips (beware of spatter),
or, use ice-cold mortar and pestle, adding ground dry ice and a pinch of sand

Add **1 ml** of **solution A** to ground mycelia in microfuge tubes
Vortex briefly (or invert tubes) to **mix** well, place at **65°** for **10 min.**

Add **5 µl** of **solution B** and incubate at room temperature for **5 min.**

Split into **2** portions (2 tubes from each) and extract
2x with **phenol** (very toxic, handle with care),
2x with **phenol: chloroform: Isoamyl alcohol;**
2x with **chloroform: Isoamyl alcohol**
In each case, add equal vollume, mix well and spin for 5 min.;
transfer top aqueous phase to fresh tube (avoid touching interphase)

Add: 0.5 ml of **solution C** and hold the tubes **on Ice** for **2 hrs or more.**
Sediment the prepicipate in the microfuge (pref. **at 4°**) for **10 min.**

Resuspend the pellet in **200 µl TE**, add 20 µl of 3 M **NaOAc**,
combine the contents of the duplicate tubes and
add **1 ml** cold **ethanol** (95%) to **precipitate** the DNA
Sediment the DNA in the microfuge for **5 min**
Wash with 70% ethanol (i.e., rinse and respin),**dry** in speed vac and
dissolve in ~100µl **sterile TE** at RT (but **store** in cold; for long term at **-20°**).

Check conc. on a mini gel (uncut). Cut 10µl of this DNA in 30µl for a gel or Southerns

VI. SARKOSYL HYBRIDIZATION

(after Maniatis *et al.*, 1982; as modified by G. May, Nov. 1985)

A. Prehybridization: Incubate with gentle agitation at **65° C** for **1 hour**.

Buffer:	**Make from stock**	For **50** ml use:
5 x SSC	20 x SSC	12.5 ml
1 % Sarcosyl	20 % Sarcosyl	2.5 ml

Use sealable bag (sides sealed), cut off amount needed, seal one end.
Put nitrocellulose (or nytran) filter in bottom corner, add ~**12 ml buffer,**
and seal second end,cut off left upper corner
Roll bag over edge of table, get rid of most of the air through open corner,
and make sure filter is well wetted (use paper towel to distibute buffer all over).

Seal corner and check that air tight, "hang" in 65° water bath, for 1 hour;
attach weight (clamp) at the bottom, remaining air will cause bag to stand vertically;
or place in shallow plastic box filled with 65° water, and put into water bath for agitation.

B. Hybridization.

To **add** the **boiled** (and quick-cooled) **radioactive probe** to the bag,
cut open one corner, tip the bag at an angle and pipette probe into fluid,
being careful not to get too close to the filter.

Incubate in water bath at **65°** for **24 - 48 hours.**

C. Wash (after hybridization):

To open plastic bag, cut on 2 adjacent sides and carefully lift filter (using 2 forceps)
into sandwich box containing 100-300 ml wash buffer (dump radioactive fluid
into appropriate discard container)
Wash 2 x , using **3 x SSC**
0.5 % Sarkosyl, at 65° for **30 min.**
Float box in waterbath with gentle shaking; change buffer after first wash.

Wash 2 x , using **0.1 x SSC** **at 65°** for **30 min**
(change buffer, not box).

D. Dry at room temperature (~ 15 min),
mark wells with radioactve pen (wrap in saran) and
put into folder with **X-ray film** ; expose in ultra-deep freeze,
for **2 - 4 hours** if probe is **hot,** or longer, before **developing**.

VII. ALKALINE LYSIS PLASMID DNA PREP & CsCl GRADIENT

(Maniatis, *et al.*, 1982; as modified by G May, Nov 1985).

	Solutions:	in 100 ml	**or**	use stock:	Make stock 100 ml
A:	50 mM Glucose	0.91 g		5 ml of 1 M;	18.16 g
	25 mM Tris base	0 30 g		2.5 ml of 1 M;	12.1 g (pH 8.0)
	10 mM EDTA(4Na)	0.37 g		2 ml of 0.5 M;	18.5 g

Adjust pH to 8.0, using conc. HCl; autoclave or filter sterilize; **keep cold.**

Lysozyme: Use 5 mg/ml final concentration. Make stock solution: 50 mg / ml **sol. A.**

	Make fresh:	in 100 ml	**or**	use stock:	100 ml :
B:	1% **SDS**	1.0 g		5 ml of 20%;	20 g
	0.2 M **NaOH**	0.8 g		10 ml of 2 M;	8 g

no need to sterilize; **keep at RT.**

C: 5 M **KOAc, pH 4.8.**

Use: 18 g KOH pellets **or** 60.0 ml Ka-Acetate of 5 M (49 g/100 ml)
+ 29 ml Glacial Acetic Acid +11.5 ml Glacial A cetic Acid;
add H_2O to fill up to 100 ml; **keep cold.**

For inoculum:

Streak bacterial + plasmid culture out on fresh LB + amp plates; grow 0/N.
Use single colony for small liquid culture (~5 ml) in test tube (LB +amp);
incubate at least **6 hrs,** until turbid.

Main culture

Use 0.5 - 2 ml to inoculate a 2 liter flask with 200 ml LB plus antibiotic;
grow at 37° (shaking >200 rpm) for >18 hrs (less for cosmids or library DNA).

Harvesting

Sediment bacteria in 250 ml (or 500 ml) bottles, **5 k** for **5 min** at **4°.**
Make sure good washers in bottles give tight seal; use fixed angle head.

Lysis

Decant supernatant (and autoclave in flasks); from 200 ml culture.
resuspend bacteria in **3.6 ml** of **solution A;**
transfer to high speed centrifuge tubes (50 ml).
Add **0.4 ml** of **lysozyme** (10 x stock) [for 400 ml use double amounts]
and **incubate** at room temperature (RT) for **5 min.**

Add **8 ml** of **solution B** and place on ice for **10 min.**
Add **6 ml** of **solution C** and keep on ice for **10 min.**

Sediment the precipitate in high speed centrifuge,
at 4° 15 k 15 min,
or, if fixed angle head, **12 k 20 min.**

DNA precipitation

Decant supernatant into measuring cylinder; add **0.6 vol.** of **isopropanol;** hold at RT for **15 min.**

Sediment precipitate (in corex tubes; warm rotor, RT) at **9 k** for **10 min.**
Wash with (20 ml) 70% ethanol (spin again) and dry (use fixed angle head).

CsCl-Gradient

Dissolve DNA in **8 ml** sterile **TE** (10 mM Tris, 1 mM EDTA, pH 8.0).

Add **8.67 g CsCl** (grind caked CsCl with pestle and mortar);
dissolve by gently tipping up and down.
Fill into screw-top **ultracentrifuge** tube with pasteur pipette (check washer).

Add **EtBr** (stock:10 mg/ml in H_2O or TE): 250 - 400 ml/tube; **keep dark.**

Balance carefully (use fine balance); if necessary fill up with mineral oil.

Centrifuge, using Ti-70.1 roter (fixed angle), at **38k**, for **48 hrs**
[see instructions, for use of ultra centrifuge]. (at least **40** hrs).

To collect DNA: Work in red light.
Open top (small screw,using special screw driver);
fix outer screw in stand (place beaker with bleach underneath).
Pierce with **needle** of 5 ml **syringe** from below,
taking 1 - 1 1/2 ml of red band(s).
Take lower band, if *E. coli* DNA is present, but
large plasmids may also give 2 bands (nicked vs. supercoiled).
Take upper band first and
transfer to medium-size test tubes.

Extract EtBr with **NaCl-saturated butanol** (top layer); add equal volume.
Shake or use pipette to mix.
Collect pink top phase with pasteur pipette
and discard (after inactivation with bleach);
repeat several times (twice more when bottom phase looks clear).

To precipitate DNA, add, first equal vol. of H_2O,
then 95% **ethanol**, adding 2 x current volume (= 4 x vol. of DNA in CsCl).
If no precipitate visible, transfer to low temperature, min. **2h**, **-20°.**

Spin in corex tube, **9 k** for **10 min.**
Decant supernatants.
Wash pellet with 70% ethanol (rinse only) and spin **9 k** for **5 min,**
and **dry** in lyophilizer.

Dissolve in sterile **TE,** 200 ml -1 ml, depending on amount of precipitate.