Endo-exonuclease of *Aspergillus nidulans*

by

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Dedication

This thesis is dedicated to my parents, Kam and Wai Fun Fung-Koa for all the things they have done for me.
Abstract

Endo-exonuclease (EE) has been found in both active and inactive, but trypsin-activatable (TAAE), forms in Aspergillus nidulans. The majority of active EE was found in nuclei, mitochondria, and vacuoles while most of TAAE was found in the cytosol. EE was purified to near homogeneity by successive chromatography on DEAE-Sepharose and ssDNA cellulose. It was found to resemble in enzymatic properties the EE previously purified from Neurospora crassa and implicated in recombination and recombinational DNA repair. A. nidulans EE is an active polypeptide of 28 kDa and is dependent on Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$ for activity with both DNA and RNA. It released small 5’-p-terminated oligonucleotides from DNA. With ssDNA as substrate, the nuclease activity was mixed endo- and exonucleolytic while with dsDNA, the activity was exonucleolytic in character. Superhelical λX174 DNA was nicked by EE to form relaxed circular and then linear dsDNA which was rapidly degraded to shorter fragments. Linearized pBR322 DNA was nicked at specific sites at low concentrations of Mg$^{2+}$ where there was relatively low exonuclease activity, but this nicking required that 5’-p-termini be present on the linear dsDNA. Normal levels of total EE were found in two recombination-deficient mutants of A. nidulans. In addition, the A. nidulans and N. crassa EEs were observed to be immunochemically related. The N. crassa enzyme was also
found to be related to the recC gene product of E. coli.

Using the antibody raised to N. crassa EE, two putative clones of the A. nidulans EE gene were isolated from a genomic library in λgt11. Lysates of E. coli infected with these clones expressed an N. crassa EE cross-reactive polypeptide of approximately 130 kDa.
**Resumé**

L'endo-exonuclease d'Aspergillus nidulans a été isolée sous forme active et inactive, cette dernière pouvant être activée in vitro par la trypsine. L'endo-exonuclease active se retrouve principalement dans les nucléoles, les mitochondries, et les vésicules alors que celle activée par la trypsine est localisée surtout dans le cytosol. L'endo-exonuclease a été purifiée jusqu'à homogénéité par chromatographie sur DEAE-Sepharose et sur cellulose-ssDNA (cellulose contenant de l'ADN simple brin). L'enzyme a des propriétés enzymatiques similaires à l'endo-exonucléase purifiée antérieurement à partir de Neurospora crassa dont l'activité a été impliquée dans la recombinaison et la réparation d'ADN par recombinaison. L'endo-exonuclease d'A. nidulans est constituée d'un seul polypeptide de 28 kDa dont l'activité enzymatique sur l'ADN ou l'ARN, requiert la présence d'ions Mg$^{2+}$, Mn$^{2+}$, ou Zn$^{2+}$. L'enzyme libère de petits fragments d'ADN dont l'extrémité 5' présente des groupements phosphomonoesters. En présence d'ADN monocastré, l'enzyme possède une activité mixte endonucleolytique et exonucleolytique alors qu'en présence d'ADN bicatenaire, elle n'a qu'une activité à caractère exonucleolytique. L'ADN superhélice du phage OX174, a été clivée par l'endo-exonuclease, produisant les formes II et III de l'ADN bicatenaire, la forme III étant rapidement dégradée en petits fragments. L'ADN linearisé du plasmide pBR322 a été coupé à des sites spécifiques en basse
concentration de Mg$^{2+}$ où l'on retrouve une activité exonucléolytique minimale. Toutefois, ces coupures requièrent la présence d'un phosphate à la partie 5'-terminale de l'ADN bicaténaires. Deux mutants d' *A. nidulans* déficients en recombinaison ont des niveaux normaux d'endonucléase. De plus, les endo-exonucléases d' *A. nidulans* et *N. crassa* sont similaires dans leurs propriétés immunochimiques. L'enzyme de *N. crassa* est aussi reliée au produit du gène *recC* d' *E. coli*. En utilisant l'antiserum de lapin contre l'endonucléase de *N. crassa*, deux clones potentiels du gène endo-exonucléase d' *A. nidulans* ont été isolés d'une banque génomique de *gtll*. Des lysats d' *E. coli* infectés avec ces clones expriment un polypeptide de 130 kDa qui réagit avec l'endonucléase de *N. crassa*.
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Preface

This thesis is divided into four chapters: Introduction, Materials and Methods, Results and Discussion. This work, carried out in the laboratory of Dr. M.J. Fraser is entirely my own and has not been published.

The antibody raised to EE purified from a mixture of mitochondria and small vacuoles of *Neurospora crassa* was obtained by Mrs. Helga Cohen. The 24 kDa ÆE-specific inhibitor was purified by Zafer Hatahet. Drs. Terry Chow and Greg May generously supplied respectively the yeast EE clone and the *A. nidulans* genomic library. Total yeast DNA and calf liver tRNA were gifts respectively from Charles Goyer and Dr. Philip Lazurus. Dr. G.R. Smith sent the *E. coli* recBCD deletion mutants.
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<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>apurinic/apyrimidinic</td>
</tr>
<tr>
<td>DEAE</td>
<td>O-diethylaminoethyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetate</td>
</tr>
<tr>
<td>KPB</td>
<td>potassium phosphate buffer</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>SBI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium lauryl sulphate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
</tr>
<tr>
<td>4-NQO</td>
<td>4-nitroquinoline oxide</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris-Acetate - 1 mM EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris-HCl, pH 8.0 - 1 mM EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>10 mM Tris-HCl, pH 7.5 - 1 mM EDTA - 150 mM NaCl</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>ssb</td>
<td>single-stranded binding</td>
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Introduction

This thesis deals with the purification, characterization, and cloning of endo-exonuclease of the fungus, Aspergillus nidulans. The endo-exonuclease of a related fungus, Neurospora crassa is postulated to play a role in recombination and recombinational DNA repair. A brief summary of the known repair pathways followed by a description of isolated fungal nucleases is given.

Repair of damaged DNA

Damage to DNA in cells may occur either spontaneously or be caused by physical or chemical agents. Spontaneous damage includes depurination and deamination of a base that may eventually lead to a transition mutation. It has been estimated that for one mammalian cell, several thousand residues per genome per day are affected in this way. DNA exposed to UV-light and ionizing radiation such as X-rays and \( \gamma \)-rays may contain pyrimidine dimers and other photoadducts as well as single strand and double strand breaks and 5,6- modified pyrimidines. Methylating and other chemical agents cause base changes, monoadducts, and cross-links in DNA. All these alterations are potentially mutagenic and if not removed, most are lethal to the cell.

Various DNA repair mechanisms are involved in the maintenance of the stable structure of DNA. These include
(i) direct repair, (ii) base and nucleotide excision repair in which the damaged base or nucleotide is removed, (iii) recombinational repair where strand switches allow homologous sections of undamaged strands to replace those of the damaged strands, (iv) mismatch repair that removes nucleotides misincorporated during replication or mismatched bases in heteroduplexes generated during recombination and (v) damage inducible but error-prone repair.

Direct repair

An example of direct repair is photoreactivation in Escherichia coli (E. coli) by the enzyme DNA photolyase. This enzyme first recognizes the dimer and then monomerizes pyrimidine dimers to pyrimidines by converting the energy of light absorbed by chromophores in the enzyme to chemical energy in order to break the cyclobutane ring joining the pyrimidines (Sancar et al. 1984). In yeast, two different enzymes with photoreactivating activity have been purified (Sutherland 1981). The gene encoding one of these, PHR1 has been cloned and found to have homology with the bacterial photolyase (Sancar 1985).

A second pathway of direct repair in E. coli involves part of the adaptive response to alkylating agents. A stereoisomer of methylphosphotriester in double stranded (ds) DNA serves as an intracellular signal for induction of the adaptive response. That this response is independent of
the SOS pathway (see later) is demonstrated by the fact that the adaptive response is induced only by alkylation damage (Lindahl et al. 1988). The regulatory protein, Ada, transfers the methyl group from the phosphotriester in DNA to one of its two 'acceptor' cysteine residues (Cys-69) thereby transforming itself into a strong transcriptional activator (Teo et al. 1986). The activated Ada protein binds specifically to the promoters of the ada and alkA genes. Increased expression of at least four genes is known to result from the adaptive response (ada, alkA, alkB, and aidB). The ada gene itself encodes an O6-methyl transferase that repairs O6-methyl guanine by direct transfer of the O6-methyl group to its own Cys-321 to produce a methylated protein which is not regenerated but rather proteolytically cleaved (Demple et al. 1982). The product of the alkA gene is 3-methyladenine DNA-glycosylase that is induced 20-fold by exposure of the cells to low doses of alkylating agents (Karran et al. 1982, Evensen and Seeberg 1982). The alkB gene is in a small operon with ada and its expression is controlled by the ada promoter. The protein product of the alkB gene may be involved in excision repair but its function and the function of the aidB protein which is also under ada control are unknown (Lindahl et al. 1988).

Base and nucleotide excision repair

Base excision repair is accomplished by the actions of
four enzymes, DNA-glycosylase, apurinic/apyrimidinic (AP) endonuclease, DNA polymerase I, and DNA ligase. DNA-glycosylases are small enzymes (25-33 kDa) with narrow substrate specificities (there are at least ten specific types), and no cofactor requirements. They hydrolyze the N-glycosylic bond between the deoxyribose and the modified base to produce an abasic or AP-site. An AP-endonuclease then hydrolyzes the phosphodiester bond either 3' or 5' to the baseless sugar (Sancar and Sancar 1988). DNA polymerase I and ligase repair the excision gap produced. Patients with the human genetic disorder, Xeroderma pigmentosum (XP) are hypersensitive to UV-light and are predisposed to multiple skin tumors. Two types of AP-endonucleases were purified from cultured human fibroblasts of XP patients: AP-endonuclease I and AP-endonuclease II. AP-endonuclease I was found to be deficient in XP complementation group D cells, whereas the levels of AP-endonuclease II were normal (Kuhnlein et al. 1976, Mosbaugh and Linn 1980). It has been proposed that the two enzymes might act in concert to remove a deoxyribose 5'-phosphate from an AP-site.

Cells from XP patients (all complementation groups) have reduced levels of DNA repair synthesis in response to UV-light and to several chemical mutagens. XP cells are defective in nucleotide excision repair. Ten genetic complementation groups have been identified in XP and it has been suggested that each group is deficient in a different
protein. Recently, Wood et al. (1988) found that extracts of XP cell lines were not able to repair damaged circular DNA but were able to repair UV-irradiated DNA containing incisions made by a DNA-glycosylase with an associated AP-endonuclease activity. Therefore, it may be concluded that the defect in these XP cells is in the initial incision step of excision of the damaged nucleotide. When the extracts from two genetic complementation groups (A and C) were combined together, 50% of the repair activity of normal cell extracts was observed.

In nucleotide excision repair, damaged bases such as pyrimidine dimers are removed from DNA as an oligonucleotide. In E. coli, the uvrA, uvrB, and uvrC proteins that are all required for the endonuclease activity were overproduced from plasmids bearing the cloned genes, purified and uvrABC 'exinuclease' activity reconstituted in vitro. This ATP-dependent nuclease makes two simultaneous incisions, one at the 8th phosphodiester bond 5' and the other at the 4th or 5th phosphodiester bond 3' to the mono-or dinucleotide adduct leaving a single-stranded (ss) gap of 12 - 13 nucleotides (Sancar and Rupp 1983). The oligonucleotide is then displaced and the gap filled by DNA polymerase I with the help of helicase II (the uvrD protein) and finally sealed by ligase. Thus, at least six different proteins are required for excision repair in E. coli. In yeast, the proteins of the RAD1, RAD2, RAD3, RAD4, and RAD10
genes were found to be essential for the incision step of excision repair (Reynolds and Friedberg 1981, Wilcox and Prakash 1981). \textit{RAD3} has been found to encode a ssDNA-dependent ATPase (a helicase) and is essential for cell viability (Sung \textit{et al.} 1987). By transfecting UV-sensitive Chinese hamster ovary (CHO) cells with human DNA, a human excision repair gene, \textit{ERCC-1} was cloned and sequenced. The human polypeptide was found to have homology at the amino acid level, at the N-terminal end, with the \textit{RAD10} gene product of yeast and at the C-terminal end with both the \textit{uvrA} and \textit{uvrC} gene products of \textit{E. coli} (van Duin \textit{et al.} 1986, Doolittle \textit{et al.} 1986). However, the role of \textit{ERCC-1} in repair has not been established since it fails to complement any of the XP groups.

Recombinational repair

The above repair pathways act on DNA before the next round of replication takes place. Occasionally, the damage is overlooked and replication of the damaged DNA is attempted. However, many of the alterations in DNA are replication blocks and must be by-passed in the post-replication pathways. One of these pathways, general recombination, involves exchange of DNA sequences between homologous DNA duplexes at essentially any site (Cox and Lehman 1987). The recombination mechanism enables a cell to repair a damaged chromosome in an error-free manner by
borrowing the missing information from a homologous intact chromosome. Basically, two homologous strands are aligned and strands of the same polarity are nicked and switched onto the other duplexes. The Holliday intermediate in which two dsDNA molecules are linked by ss-crossovers is formed by reciprocal strand exchange forming a covalent connection of the duplex molecules. Migration of the cross-over point (branch migration) produces a long heteroduplex region.

Recombination is best understood in the bacterium *E. coli* where three genes were initially recognized to be important for general recombination by isolation of recombination-defective strains, the recA, recB, and recC genes (Cox and Lehman 1987). These strains exhibited increased sensitivity to DNA-damaging agents such as X-rays, UV-light, and alkylating agents and exhibited decreased cell viability.

The recA gene product, a 38 kDa protein referred to as a recombinase is not essential for viability and has many functions (Cox and Lehman 1987). These include (i) a DNA-dependent ATPase, (ii) an ATP-dependent strand switching activity, and (iii) a ssDNA- and ATP-dependent 'protease' activity.

The DNA strand exchange requires a free 3' homologous ss-end (Konforti and Davis 1987) on at least one of the DNA substrates and at least 40-50 base pairs of homology between the two interacting substrates. Complete strand exchange
requires a stoichiometric amount of recA protein and is strongly stimulated by the presence of the ssDNA-binding (ssb) protein of E. coli (Cox and Lehman 1981a). The recA protein first binds to the ssDNA to form filaments (Cox and Lehman 1982). The recA-ssDNA complex pairs with the dsDNA, searches for complementary sequences in the dsDNA, and forms the synaptic complex (a joint molecule) with limited unwinding of the dsDNA. Under the electron microscope, this pairing of linear dsDNA with DNA bearing ss-ends appears like a predicted Holliday structure (DasGupta et al. 1980, DasGupta et al. 1981). Transfer of one homologous strand in the 5' to 3' direction relative to the ssDNA (Cox and Lehman 1981b, West et al. 1981) is followed by renaturation of the complementary strands forming a new duplex and shedding of the recA protein. The new duplex and the newly displaced strand make up a 'D-loop'. While the above reactions occur at a rapid rate, extension of the D-loop with net exchange of DNA strands via branch migration, coupled to ATP hydrolysis occurs at a much slower rate.

RecA-like proteins have also been found in other bacteria such as Bacillus subtilis and Proteus mirabilis. Bacteriophage T4 also encodes the UvsX protein which has a related ATP-dependent DNA strand transferase activity (Formosa and Alberts 1986). An ATP-independent strand transferase with the same polarity as the recA protein has been partially purified from mitotic Saccharomyces
cerevisiae cells (Kolodner et al. 1987). In other eucaryotes, the 70 kDa rec-1 gene product of the fungus Ustilago maydis promotes formation of joint molecules between circular strands and linear duplexes in a reaction similar to that catalyzed by the recA protein but with opposite polarity (see below) (Kmiec and Holloman 1983, Kmiec and Holloman 1984). The rec-1 protein has been shown to have a higher affinity for Z-DNA than for B-DNA (Kmiec and Holloman 1986). Using Z-DNA affinity chromatography, a 74 kDa ATP-dependent strand transferase was partially purified from a human T-lymphoblast cell line (Fishel et al. 1988). A recombinase that carries out a strand transfer reaction between linear dsDNA and homologous circular ssDNA to produce a joint molecule in an ATP-independent fashion has also been partially purified from human B-lymphoid cells (Hsieh et al. 1986). The polarity of branch migration catalyzed by the rec-1 and human proteins is opposite to that catalyzed by the recA protein of E. coli. Another such protein has recently been purified from Drosophila (Eisen and Camerini-Otero 1988).

The recB and recC genes code for two subunits of an ATP-dependent nuclease. Since the discovery of recD, the gene encoding the third subunit of exonuclease V, the nuclease is now referred to as the recBCD enzyme (Amundsen et al. 1986). RecB and recC mutants are sensitive to UV-light and X-rays, possess a residual level (1-2%) of
recombination, lack ATP-dependent exonuclease and ATP-stimulated endonuclease activity, and have low cell viability as a result of the accumulation of lethal DNA lesions (Chaudhury and Smith 1984a).

The activities of the \textit{recBCD} enzyme are all Mg$^{2+}$-dependent; it has (i) ssDNA-specific endonuclease activity that is 7-fold stimulated by ATP (Goldmark and Linn 1972), (ii) ATP-dependent processive exonuclease activity with apparently no polarity with linear but not with covalently closed circular dsDNA (Karu et al. 1973), (iii) DNA-dependent ATPase, and (iv) ATP-dependent unwinding (helicase) activity with ds-DNA (Taylor and Fink 1980).

On linear dsDNA, the \textit{recBCD} enzyme initiates its attack at the ends of the DNA molecule and generates two classes of reaction intermediates: (i) dsDNA with ss tails of several thousand nucleotides at either the 3' or 5' termini and (ii) ss-fragments ranging in size up to 1000 nucleotides (MacKay and Linn 1974). The ss-fragments are then hydrolyzed to small 5'-phosphoryl, 3'-hydroxyl terminated oligonucleotides of 4-5 residues (Goldmark and Linn 1972).

In 4 M salt, the subunits of \textit{recBCD} enzyme dissociate into the $\alpha$ subunit encoded by the \textit{recD} gene, and the $\beta$ subunit composed of the polypeptides encoded by the \textit{recB} and \textit{recC} genes (Lieberman and Oishi 1974, Amundsen et al. 1986). Determined from the cloned genes, the molecular weights of the \textit{recB}, \textit{recC}, and \textit{recD} polypeptides are respectively
133,193 Da, 128,860 Da, and 66,973 Da (Finch et al. 1986a, Finch et al. 1986b, Finch et al. 1986c).

The isolated recB polypeptide has been shown to have DNA-dependent ATPase activity but no exonuclease activity whereas the isolated recC polypeptide possessed neither activity. However, when the purified recB and recC proteins were mixed, reconstitution of the nuclease and ATPase activities was achieved (Hickson et al. 1985). In addition, a mutation in the recB gene resulted in defective ds-break repair (Sharma and Smith 1986). Although the recBCD endonuclease will generally not cleave dsDNA, it will nick dsDNA containing the sequence 5'-G-C-T-G-T-G-G-3' (a CHI site) at 4-6 nucleotides 3' to the site. Certain mutations within the recC gene, or a single base pair change within CHI, resulted in a decrease in CHI-specific cleavage (Ponticelli et al. 1985). One class of recBCD mutants, recBC‡ are nuclease-deficient but very recombination-proficient (hyper-rec), resistant to DNA-damaging agents with high cell viability (Chaudhury and Smith 1984b). It was found that an active nuclease is required for CHI activity.

From above observations, the ATPase activity was assigned to the recB polypeptide, with the DNA unwinding activity, CHI recognition and nuclease activity attributed to the proper interaction of all three polypeptides (Smith 1988).
Under conditions similar to those expected in vivo (high ATP and ssb present), there is an increase in dsDNA unwinding relative to exonucleolytic degradation of DNA in vitro (Telander-Muskavitch and Linn 1982). In the presence of 1 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\), and 5 mM ATP, the exonuclease and endonuclease (but not the helicase) activities were completely inhibited (Rosamond et al. 1979). It is possible that the initial action of the recBCD enzyme on dsDNA is unwinding of the DNA strands. The enzyme appears to travel through the dsDNA by unwinding the DNA ahead of itself and rewinding it at a slower rate behind itself to form progressively larger double-looped structures in an ATP-dependent manner (Taylor and Fink 1980). The helicase is most active on dsDNA molecules with nearly flush-ended termini where the 5' and 3' ss-tails are offset by no more than 25 nucleotides (Taylor and Smith 1985). Activity is also seen with ds-circles containing gaps greater than 5 nucleotides in length. The gap would leave enough space to allow the enzyme access to the cleavage site (Telander-Muskavitch and Linn 1982).

A proposed model of general recombination involving the recBCD enzyme is the following (Cox and Lehman 1987, Smith 1988): (i) the recBCD enzyme binds to the flushed ends of the dsDNA and unwinds it with the ATP-driven helicase to produce ss-loops and then double-looped structures. (ii) The enzyme travels along the duplex in the 3' → 5' direction
with respect to the strand cleaved until it encounters a CHI sequence in the correct orientation. (iii) The enzyme then cleaves the strand containing the CHI site a few nucleotides 3' to (behind) the CHI site and continues unwinding the DNA producing 3'-terminated ssDNA that can be assimilated by an adjoining duplex in a strand transfer reaction catalyzed by the \textit{recA} and \textit{ssb} proteins. (iv) The Holliday intermediate is formed and is later resolved into two recombinant DNA duplexes. Whether the \textit{recBCD} enzyme is involved in the resolution of the Holliday intermediate has not been determined. The phage T4 \textit{gene 49} product, endonuclease VII and T7 \textit{gene 3} product, T7 endonuclease I have been shown to cleave cruciform-containing DNA at sites symmetrically located near the base of the Holliday junction. The products formed in each case, were linear dsDNA molecules with hairpin ends containing ss breaks that can be sealed by DNA ligase (Lilley and Kemper 1984, de Massy et al. 1987). In yeast, it has been shown that partially purified extracts contain a 200 kDa nuclease that cleaves Holliday structures \textit{in vitro} (West and Korner 1985, West et al. 1987). Cleavage occurs within homologous duplexes by the concerted nicking of strands of like polarity, and with precise symmetry across the junction (Parsons and West 1988).

Recombination deficiencies of \textit{recB} and \textit{recC} mutants can be suppressed by either of two classes of mutation, \textit{sbcA} or \textit{sbcB} (Smith 1988). In \textit{sbcA} strains, there is a high level
of exonuclease VIII (encoded by the recE gene of the Rac prophage), an ATP-independent 5'→ 3' ds-exonuclease. Thus, this activity can replace the recBCD nuclease in generating 3'OH ss-tailed recombination intermediates. The sbcB− mutant is deficient in exonuclease I, a 3'→ 5' ss-exonuclease capable of destroying the 3'-OH ss-tailed recombination intermediates. The recBCD pathway accounts for 98% of recombinational repair in wild-type E. coli with the remaining 2% accounted for by the recF pathway. In a recBCD− sbcB− background, the recF pathway is induced as part of the SOS response to DNA damage (see later). This pathway requires the recA, recF, recJ, ruv, recN, and recQ gene products the latter of which encodes a 74 kDa ATP-independent endonuclease (Nakayama et al. 1985). The ruv, and recN genes are the only genes known to be under regulation by the recA and lexA genes.

Mismatch repair

Mismatch repair is a minor post-replication repair pathway in E. coli but is of major importance in correction of replication errors and gene conversion in recombination. It is dependent on the mutH, mutL, mutS, and mutU (uvrD) gene products, the mismatch repair enzymes. In vitro, mismatch repair has also been shown to require ATP and the ssb protein. The mutH and mutU genes respectively encode a Mg2+-dependent endonuclease and a DNA helicase (Modrich
Base mismatches may arise as a result of formation of heteroduplex in recombination and as errors in nucleotide incorporation during DNA replication. Normally, during replication parental DNA strands are fully methylated while the newly synthesized daughter strands are not methylated. The mismatch repair complex is able to discriminate between parental and hemimethylated daughter duplexes orienting the mismatch nuclease to make an incision at the mismatches in the unmethylated strand. The misincorporated nucleotides are excised leaving either very long gaps or only small gaps (depending on which mismatch repair system acts) that are filled in by DNA polymerase and sealed by ligase. The repaired duplex is then methylated with S-adenosyl methionine, the substrate of the dam methylase.

**Damage-inducible error-prone (SOS) repair**

In response to damaged DNA consisting mainly of long gaps, the error-prone SOS repair pathway repair is induced in *E. coli*. These long gaps are repaired by a recA-promoted damage by-pass mechanism but the repair is mutagenic. Accompanying this response, there is an increased capacity to reactivate and mutate UV-irradiated bacteriophage, an increased capacity to repair ds-breaks, and an increased ability to carry out long patch excision repair. The induction signal probably involves the appearance of more than normal amounts of ssDNA arising from the halting of
replication forks at damaged sites. Binding of the recA protein to ss-gaps in this post-replication DNA activates the recA protease activity to cleave the lexA repressor and induce expression of the SOS or din (damage inducible) genes (Walker 1985, Little and Mount 1982).

Under normal conditions in the E. coli cell, the repressor encoded by the lexA gene binds to the promoter/operator regions of at least 17 genes that play a role in DNA repair (the din genes), preventing their transcription. In response to DNA damage, the recA protease is activated and cleaves the 23 kDa lexA repressor. Cleavage of the repressor between specific ala and gly residues in the protein greatly weakens the binding of the repressor to the DNA and allows for the increased transcription of the din genes. The uvrA, uvrB, and uvrC genes involved in excision repair, the recA, recN, and ruv genes involved in the recF recombinational repair pathway, and the umuC and umuD genes required for mutagenesis are among the din genes that are highly expressed (Little and Mount 1982).

As the DNA is repaired, the signal for induction of the response is diminished (the ss-gaps are filled), and the recA protease is inactivated allowing lexA molecules to accumulate and to again repress the din genes.

The nucleases of Neurospora crassa

In the fungus, Neurospora crassa, eight nucleases have
been isolated and characterized over a period of several years (see Table I). (i) Linn and Lehman initially isolated a 55 kDa (native molecular mass) ss-specific endonuclease, first from conidia, and then from stationary-phase starved mycelia (Linn and Lehman 1965a, Linn and Lehman 1965b, Linn 1967). This nuclease hydrolyzed ssDNA and RNA to 5'-phosphoryl terminated oligonucleotides and 5'-mononucleotides with a preference for guanosine or deoxyguanosine (Linn and Lehman 1965b). Its activity is dependent on the divalent metal ions, Co²⁺, Mn²⁺, Fe²⁺, and is activated by Mg²⁺. Optimal activity is achieved between pH 7.5 and 8.5. In the presence of 10 mM mercaptoethanol, EDTA, phosphate, or 0.1-2 mM ATP the activity of the enzyme is inhibited (Linn and Lehman 1965, Rabin et al. 1968). The enzyme is very heat stable; the temperature range at which maximal activity is seen is 56-58°C. (ii) A Mg²⁺-dependent endonuclease acting on both DNA and RNA with no strand specificity was found in N. crassa mitochondria (Linn and Lehman 1966). It has a native molecular mass of 75 kDa and was not found to be very heat sensitive. (iii) Another alkaline nuclease released only from mycelia grown in sorbose, DNase C (native molecular weight 65 kDa), has a divalent metal ion requirement for Mg²⁺ and is stimulated by Ca²⁺. It has endonuclease activity with ssDNA and RNA and exonuclease activity with dsDNA (Fraser 1978). (iv) In wild-type log-phase mycelia, more than 90% of the ssDNase
<table>
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<tr>
<th>Nuclease (M.W.)</th>
<th>Localization</th>
<th>Substrates and activity</th>
<th>Divalent Cation(s) Required</th>
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<tr>
<td>(i) endonuclease 55 kDa</td>
<td>intracellular</td>
<td>ss-endonuclease with DNA and RNA; no activity with ds-DNA</td>
<td>Mg²⁺, Co²⁺, Mn²⁺, Fe²⁺</td>
<td>7.5-8.5</td>
<td>protease cleavage product of EE; immunochemically related to EE</td>
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<td>(ii) mitochondrial endonuclease 75 kDa</td>
<td>intracellular</td>
<td>endonuclease with no strand specificity for DNA or RNA</td>
<td>Mg²⁺; inhibited by Ca²⁺</td>
<td>7.5-8.5</td>
<td>immunochemically related to EE</td>
</tr>
<tr>
<td>(iii) DNase C 65 kDa</td>
<td>extracellular</td>
<td>ds-exonuclease with DNA and ss-endonuclease with DNA and RNA</td>
<td>Mg²⁺</td>
<td>7.5-8.5</td>
<td>secreted only in sorbose medium; similar to EE</td>
</tr>
<tr>
<td>(iv) endo-exonuclease (EE) 31 kDa</td>
<td>intracellular</td>
<td>ds-exonuclease with DNA and ss-endonuclease with DNA and RNA</td>
<td>Mg²⁺ (Mn²⁺, Zn²⁺)</td>
<td>7.5-8.5</td>
<td>derived from EE precursor</td>
</tr>
<tr>
<td>(v) exonuclease 72 kDa (periplasmic in conidia?) extracellular</td>
<td>ss-exonuclease with DNA and RNA</td>
<td>Mg²⁺; inhibited by Ca²⁺</td>
<td>7.5-8.5</td>
<td>similar to DNase B</td>
<td></td>
</tr>
<tr>
<td>(vi) DNase B 78 kDa</td>
<td>extracellular</td>
<td>ss-exonuclease with DNA and RNA</td>
<td>Mg²⁺; inhibited by Ca²⁺</td>
<td>7.5-8.5</td>
<td>phosphate repressible; no immunochemical relation to EE; similar to exonuclease</td>
</tr>
<tr>
<td>(vii) N 1 3 12.5 kDa</td>
<td>extracellular</td>
<td>endonuclease with no strand specificity for DNA or RNA</td>
<td>Mg²⁺; inhibited by Ca²⁺</td>
<td>6.0-7.0</td>
<td>phosphate repressible</td>
</tr>
<tr>
<td>(viii) DNase A 65 kDa</td>
<td>extracellular</td>
<td>endonuclease with no strand specificity</td>
<td>Ca²⁺ only</td>
<td>7.5-8.5</td>
<td>phosphate repressible; no immunochemical relation to EE</td>
</tr>
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**Table 1: Neurospora crassa nuclease**

- **Nuclease (M.W.):** Molecular weight of the nuclease.
- **Localization:** Intracellular or extracellular.
- **Substrates and activity:** Indicates the types of substrates and their activities.
- **Divalent Cation(s) Required:** The required divalent cations for the enzyme activity.
- **Optimal pH:** The optimal pH for enzyme activity.
- **Relationships:** Describes the relationship of the nuclease to other enzymes or substances.
activity is expressed by endo-exonuclease (EE), an intracellular nuclease similar to DNase C (Chow and Fraser 1979). It has been shown that this nuclease has ss-specific endonucleolytic activity with DNA and RNA and exonucleolytic activity with both ss and dsDNA and that both activities are associated with a single polypeptide (Fraser et al. 1976). Furthermore, the action of endogenous proteases in vitro abolishes the ds-exonuclease activity converting the enzyme to a ss-endonuclease similar to the one isolated by Linn and Lehman. An inactive but trypsin-activatable form (or precursor) of EE (TAEE) also exists (Kwong and Fraser 1978). It is a hydrophobic polypeptide with a molecular weight of 94 kDa.

(v) An exonuclease specific for ss nucleic acids was later isolated from conidia of N. crassa. This 72 kDa monomeric nuclease attacks ssDNA and RNA from both 5' and 3' termini to produce 5'-mononucleotides. It absolutely requires Mg$^{2+}$ for activity, is slightly activated by 10 mM mercaptoethanol and is not affected by concentrations of ATP up to 2 mM. Like the ss-specific endonuclease above, its pH optimum is 7.5-8.5 but unlike the endonuclease, it is rapidly inactivated at 56°C (Rabin et al. 1972). This enzyme is probably equivalent to the extracellular DNase B described by Fraser (1978) which is phosphate repressible.

(vi) The phosphate-repressible alkaline nuclease, DNase B that is found in culture filtrates resembles the ss-specific
exonuclease described above. It has Mg\(^{2+}\)-dependent exonuclease activity with both ssDNA and RNA and a molecular weight of 78 kDa.

(vii) An extracellular non-specific endonuclease, N\(_3\) whose biosynthesis is repressed by phosphate hydrolyzes ssDNA, dsDNA, and RNA at approximately equal rates. One interesting point about this 12.5 kDa (native molecular mass) active enzyme is that it does not require divalent metal ions for activity. It has a lower optimal pH than the other nucleases described (between 6.0 and 7.0) and is stable up to 55°C (Ishikawa et al. 1969).

(viii) DNase A is a Ca\(^{2+}\)-dependent endonuclease of 65 kDa that is released from log-phase mycelia into culture medium. It has no strand specificity for ss or dsDNA and no activity with RNA. Its activity is not inhibited by ATP but is relatively sensitive to salt (Fraser 1978).

Immunoprecipitation of the mitochondrial endonuclease, the ss-specific endonuclease, and EE isolated from different cellular compartments of \(N.\ crassa\) with the antibody raised to the purified EE followed by SDS-ssDNA activity gel analysis showed that these apparently different intracellular nucleases are related and derived from the EE precursor by limited proteolysis (Fraser et al. 1986). Whether a single gene or more than one gene encodes the different nucleases has not been determined. Possibly, the inactive precursor is differentially cleaved \textit{in vitro} to
give rise either to the ss-endonuclease, the mitochondrial endonuclease, or different forms of EE during extraction and purification (Fraser et al. 1980, Fraser et al. 1986). Antibodies raised to EE cross-reacted with intracellular nucleases of *Aspergillus nidulans* and *Saccharomyces cerevisiae* (baker's yeast), but not with two of the extracellular nucleases of *N. crassa*, DNase A and DNase B.

The activities of *N. crassa* EE are reminiscent of those of the recBCD nuclease of *E. coli* that is deficient in recombination-defective mutants of that organism. Some rec-like mutants of *Neurospora*, **uvs-3**, **uvs-6**, and **nuh-4**, which are sensitive to a wide range of mutagens including UV-light, X-rays, methyl methane sulfonate, nitrosoguanidine and mitomycin C were therefore examined for their EE levels. In addition to having abnormal recombination, they had a lower conidial viability, and a high spontaneous mutation rate. One of them (**uvs-3**) had constitutive levels of excision repair. These mutants also failed to release normal amounts of nuclease activity on DNase test agar relative to the wild-type exhibiting the nuclease halo or **nuh** phenotype (Kafer and Fraser 1979). Examination of culture filtrates of these three mutants and the wild-type revealed that the **uvs-3** mutant released little or no DNase A and little DNase C, the **uvs-6** mutant released a low level of DNase A, and the mutant **nuh-4**, likely an allele of **uvs-3**, also released no DNase A or DNase C and a small amount of DNase B. However,
the nuh-3 mutant which was not sensitive to any of the above-mentioned mutagens released only low levels of DNase B. It may have a general defect in secretion of protein. A slightly UV-light-sensitive mutant, nuc-2, that does not have the nuh phenotype, released normal amounts of the three extracellular nucleases DNase A, B, and C (Fraser 1978), but did not release nuclease N3. It seemed possible from these results that failure to release DNase A and DNase C into the culture filtrates and the sensitivities to a variety of mutagens were characteristic of rec-like mutants. This points to the possible involvement of these nucleases in DNA repair or recombination.

Relative to the wild-type, active EE was found at lower levels in extracts of the rec-like mutants, uvs-3, and nuh-4, but the levels of trypsin-activatable EE (TAAEE or EE precursor) in these mutants were higher than in the wild-type (Chow and Fraser 1979). The DNA-containing organelles, nuclei and mitochondria, of the uvs-3 mutant contained only 10-12% of the active EE found in the same organelles of the wild-type (Fraser and Cohen 1983, Ramotar et al. 1987). In these mutants, there may therefore be a defect in a protease involved in the activation of EE precursor.

Endo-exonuclease of Neurospora crassa

In mycelia, 90% of the active EE was found in the organelles (10% in the nuclei tightly bound to the chromatin
and nuclear matrix, 30% in the mitochondria, and 50% in the vacuoles), while over 90% of the TAEE was localized in the cytosol and in the two DNA-containing organelles, the nuclei, and the mitochondria where it was associated with the inner membrane (Fraser and Cohen 1983, Ramotar et al. 1987). No TAEE was present in vacuoles. In comparison with the wild-type, the UV-light sensitive mutant, uvs-3 had a lower amount of active EE but a higher level of TAEE in the mitochondria (Fraser and Cohen 1983). This is not the case in the nuclei of the mutant where there was a lower level of both active EE and TAEE (Ramotar et al. 1987). In the cytosol of the mutant, a higher level of TAEE was found indicating that perhaps it had leaked from the nuclei. Pretreatment of the cells with the DNA-damaging agent, 4-NQO, resulted in the loss of TAEE and corresponding gain of active EE in the wild-type but not uvs-3 nuclei (Ramotar et al. 1987). It was postulated that the active EE was derived from TAEE in the DNA-containing organelles in response to DNA damage. The uvs-3 mutant did not respond in this way and may be deficient in some process involved in activating TAEE for DNA repair.

N. crassa EE has been purified from vacuoles and from mitochondria. A high yield (82%) was obtained with an 1800-fold purification from a mixture of mitochondria and small vacuoles by successive chromatography on DEAE-Sepharose and ssDNA cellulose (Chow and Fraser 1983). Polyclonal
antibodies were raised to the purified protein in rabbits. The ssDNase, dsDNase, and RNase activities were found to co-sediment in sucrose density gradients and to have an apparent native molecular weight of 80 kDa. On silver-stained SDS-polyacrylamide and SDS-ssDNA activity gels, the purified protein had a molecular weight of 28 - 31 kDa indicating that the native protein may exist in the form of an oligomer. EE had Mg$^{2+}$-dependent distributive endonucleolytic activity with ssDNA that was almost fully active at low concentrations of Mg$^{2+}$ (0.1-0.5 mM Mg$^{+2}$) producing 5'-phosphoryl and 3'-hydroxyl terminated fragments. Associated with the endonuclease was a highly processive exonucleolytic activity seen best with linear dsDNA. This released short 5'-p-terminated oligonucleotides (di-, tri-, and tetranucleotides) but when enzyme was present at limiting concentrations, long ss-tailed and ss-gapped linear dsDNAs were produced (Chow and Fraser 1983). If produced in vivo, these products would be potential substrates for recombination. Maximal exonuclease activity was seen in 5-10 mM Mg$^{2+}$.

The activities of EE described above are comparable to some of the activities of the recBCD enzyme of E. coli. However, no DNA-dependent ATPase activity has been found associated with EE, and ATP was not found to have any effect on the nuclease activities of EE. It is possible that a DNA-dependent ATPase subunit may have been proteolytically
degraded during isolation of the organelles and purification of EE. Recently, EE was found to recognize the sequence 5' A-G-C-A-C-T 3' and to make site-specific breaks within or very close to these sites in linear but not closed circular pBR322 dsDNA (Fraser et al. 1989). This sequence is thus analogous to the CHI sequence recognized by the recBCD enzyme of *E. coli*. When the 5'-phosphates were removed from the linear dsDNA, EE did not make these nicks. This demonstrated that EE diffused linearly from the 5'-ends of the DNA with a polarity of tracking and nicking in the 5'→3' direction, that is with a polarity opposite to that of the *E. coli* recBCD nuclease.

In 10 mM Mg$^{2+}$, *N. crassa* EE was found to first nick covalently closed circular superhelical (RFI) X174 DNA in one strand to yield relaxed RFII DNA. The RFII form was then preferentially nicked on the opposite strand to introduce a ds-break at or very close to the first nick to produce linear RFIII DNA (Chow and Fraser 1983). Very little nicking was observed with topoisomerase I-relaxed X174 DNA indicating that the initial break was made in the ss-like region(s) of the supercoiled DNA. The nicks put in RFI DNA by EE were random since linearization of the DNA with a restriction enzyme followed by heat denaturation produced a smear of ss-fragments as seen on agarose gels. The linear DNA was then rapidly degraded by the exonuclease to yield ds-fragments and small oligonucleotides. When low a
concentration of Mg\(^{2+}\) was present, the endonuclease activity predominated and only the RFI and RFII forms of \(\phi X174\) DNA were seen on the agarose gels. It should be noted that the recBCD nuclease of *E. coli* has no activity on covalently closed circular dsDNAs (see above).

Crude extracts of *Neurospora* express a low ss-specific endonuclease activity and no ds-exonuclease activity (Chow and Fraser 1979). This has now been shown to be due to the presence of a highly specific EE inhibitor present in excess amounts in the cytosol (Hatahet and Fraser 1989). The inhibitor is not present in the organelles. The inhibitor has been purified from the cytosol as a heat-stable but trypsin-sensitive polypeptide of 24 kDa (Hatahet and Fraser 1989). It inhibits the ds-exonuclease competitively and completely and inhibits the ss-endonuclease non-competitively but only incompletely (85-95% inhibition). It specifically inhibited EE and the immunochemically related EE of *A. nidulans* (as will be shown), but not any of the commercially available nucleases such as the nuclease of mung bean and the S1 nuclease of *Aspergillus*. Recently, a second heat-shock inducible inhibitor with some properties similar to the above inhibitor was also isolated and partially purified (Ramotar and Fraser 1989). The roles of these inhibitors in the regulation of intracellular EE activity have not been elucidated.
Other nucleases of fungi

In *Saccharomyces cerevisiae*, during the early stage of pre-meiotic DNA synthesis, an increase in a Mg$^{2+}$-dependent alkaline endo-exonuclease which cross-reacts with the antibody to *N. crassa* EE was observed (Chow and Resnick 1983, Resnick et al. 1984). That this nuclease is controlled by the RAD52 gene was demonstrated by a very low amount of this EE in the mutagen-sensitive and recombination-defective rad52 mutant. In addition, during meiosis no increase in this enzyme was detected in the mutant. It had been previously shown that the RAD52 gene was required for meiotic and mitotic recombination and for the repair of ds-breaks in DNA (Prakash et al. 1980, Resnick and Martin 1976). EE from *Saccharomyces* was purified from log phase cells by using an antibody affinity column where the antibody used was raised to the purified *N. crassa* EE. The yeast EE was found to be a 72 kDa monomer. It had distributive ss-specific endonuclease activity and weakly processive exonuclease activity with ds-DNA. Like *N. crassa* EE, it required Mg$^{2+}$ or Mn$^{2+}$ but did not utilize Ca$^{2+}$ as a cofactor for activity. The optimal pH for activity was 7.5. Treatment of different DNAs with the yeast EE yielded the same digestion products as with the *N. crassa* EE. However, no inactive precursor of the yeast EE has been detected (Chow and Resnick 1987). The yeast EE gene has been localized in the nuclei and has now been cloned and found to
encode an essential function (T. Chow, pers. comm.). It is currently being sequenced.

A nuclease with many catalytic properties similar to the *N. crassa* ss-endonuclease (Linn and Lehman 1965) has been purified from *Ustilago maydis*. It has been named nuclease X. It is a 55 kDa single polypeptide that was found in low levels in recombination-deficient mutants (Holloman and Holliday 1973). In contrast to the EEs, it did not appear to require any divalent metal cations for activity although the activity was stimulated many fold by Mg$^{2+}$ and Co$^{2+}$. It had ss-specific endonuclease activity, but no exonuclease activity has been detected (Holloman et al. 1981). Covalently closed circular ssDNA was first cleaved to the relaxed form, then cut to form a linear dsDNA molecule as did the *N. crassa* EE. The products of complete digestion were mononucleotides and small oligonucleotides terminated by 5'-phosphate groups.

Mitochondrial endonucleases showing cross-reaction with the antibody raised to *N. crassa* EE have been purified from *Saccharomyces* and mouse plasmocytoma cells (Dake et al. 1988, Tomkinson and Linn 1986). The 38 kDa yeast mitochondrial endo-exonuclease is localized in the mitochondrial inner membrane like the *N. crassa* EE. It had endonucleolytic activity with ssRNA, ss and dsDNA, and a 5'→3' exonuclease activity with dsDNA. The gene for this nuclease, *NUCl*, has been cloned and sequenced. It was found
to be non-essential to the cell and for mitochondrial function (Zassenhaus et al. 1988, Vincent et al. 1988). The relationship of this endo-exonuclease to the 72 kDa extramitochondrial (nuclear) EE has not been determined, but the two enzymes are encoded by genes located on two different chromosomes of Saccharomyces. The ss-specific endonuclease isolated from mouse plasmacytoma cells is proposed to be a dimer composed of 37 kDa subunits. It required the cations Mg$^{2+}$ or Co$^{2+}$ as cofactors, but did not utilize either Zn$^{2+}$ nor Ca$^{2+}$ for activity.

In Aspergillus oryzae, an intracellular nuclease (nuclease 0) of 46 kDa active on RNA, ssDNA and, to a lesser extent, dsDNA has been purified. Its activity was optimal at pH 8.3 in 0.1 mM Mg$^{2+}$ producing mono-, di-, and trinucleotides with 5'-terminal phosphates. Nuclease 0 was found in partially inactive form in fresh mycelia and was activated during autolysis or by treatment with 3 M urea (Uozumi et al. 1972). Nuclease 0 formed a stable 1:1 nuclease-inhibitor complex with an inhibitor in the soluble fraction derived from fresh mycelia (Uozumi et al. 1976). The inhibitor acted non-competitively on the ssDNase activity and was specific for the nuclease. It was found to have a molecular mass of 23 kDa and was inactivated during the course of autolysis. This resulted in the activation of the nuclease. The purified inhibitor was stable up to 80°C and sensitive to chymotrypsin. This inhibitor thus resembles
very closely that found for Neurospora EE.

Purpose of this thesis

Cloning and sequencing of the EE gene would answer many questions concerning the structure, biosynthesis, and regulation of the EEs in lower eucaryotes. Whether one gene in *N. crassa* encodes the mitochondrial, nuclear and vacuolar enzymes could be determined for example. Availability of the nucleotide sequence of the gene would allow comparison with those of other nucleases postulated to play a role in recombinational repair such as the recBCD nuclease. Disruption of the fungal host gene could also be used to determine if the EE gene of Aspergillus codes for an essential function as is the case for the yeast nuclear EE (T. Chow, pers. comm.).

Aspergillus is preferred over Neurospora for obtaining the cloned EE gene because in Aspergillus truly rec<sup>-</sup> mutants such as *uvsc* and *uvse* are available and there is a much simpler assay system for mitotic recombination than available for Neurospora. The rec<sup>-</sup> mutants are recessive mutants sensitive to UV-light and show decreased spontaneous mitotic intragenic recombination (Fortuin 1971a, Fortuin 1971b, Jansen 1970). They are also spontaneous mutators but show less UV-induced mutation than wild-type controls (Kafer and Mayor 1986). The localization, purification, and characterization of EE in *A. nidulans* is determined here as
a preliminary step to the cloning of the *A. nidulans* EE gene.
Materials and Methods

2.1. Materials  Glass beads were obtained from Sargent Welch. Nitrocellulose filters were obtained from Schleicher and Schuell.

2.1.1. Chemicals: Sucrose, calf thymus DNA, IPTG, PMSF, toluidine blue-O, and Triton X-100 were obtained from Sigma. Pansorbin was obtained from Calbiochem. DEAE-Sepharose, DEAE-Sephadex A25, Sephadex G-100, Sephadex G-50 and CsCl were obtained from Pharmacia. Ethidium bromide, β-mercaptoethanol and TEMED were from Kodak Eastman. Acrylamide, bis-acrylamide, ammonium persulfate and Coomassie blue were from BIO-RAD. Agarose, low melt agarose, and prestained molecular markers were obtained from Bethesda Research Laboratories (BRL). Silver nitrate, EDTA, and glycerol were from BDH Chemicals.

2.1.2. Enzymes: Pancreatic DNaseI, Micrococcal nuclease, bovine spleen phosphodiesterase, snake venom phosphodiesterase and RNase A were obtained from Worthington Diagnostics. Lysozyme, proteinase K, trypsin, and soybean trypsin inhibitor were obtained from Sigma. Klenow and all restriction enzymes were obtained from Pharmacia.

2.1.3. Labelled compounds: $^3$H-thymidine and $-^{32}$P-dATP were obtained from New England Nuclear. $^{125}$I-protein A was obtained from ICN. Aquasol and Econofluor-2 were from New England Nuclear.
2.1.4. Strains: Aspergillus nidulans wild-type (bia1) strain #26, uvsC114 #570, and uvsE182 #572, available from FGSC (Fungal Genetics Stock Center, Dept. of Microbiology, University of Kansas Medical School, Kansas City, KS 66103) were obtained from Dr. E. Kafer, Department of Biology, McGill University.

2.2. Methods

2.2.1. Growth of A. nidulans

Conidia inoculated directly from silica gel stocks were germinated at 37°C on complete medium plus agar (CM-agar) as described by Scott and Kafer (1982) with 1% dextrose as carbon source. Conidia from these cultures were then transferred to flasks containing 10 ml CM-agar and incubated at 37°C for 3 days and then at room temperature for 1 day to obtain larger amounts of conidia. Using these conidia as innocula for liquid cultures, mycelia were grown up in 500 ml CM at 30°C, 20-24 hours shaking gently at 200 r.p.m. in 2 liter flasks in a New Brunswick shaker-incubator.

2.2.2. Cell fractionation

Mycelia were harvested by vacuum filtration through a Buchner funnel. The resulting mat was washed with distilled water, weighed and kept on ice wrapped in foil. All of the following operations were performed at 4°C. The mycelial mat was cut into small pieces and blended in a Waring blendor at
top speed with 2.5 g glass beads in 2.5 ml/s per g mycelia 20% sucrose - 2 mM EDTA, pH 4.5 (extraction buffer) without or with 1 mM phenylmethylsulfonyl fluoride (PMSF). Blending was carried out 3 times for 30 seconds each taking care not to allow the temperature to rise above 15°C. After blending, the glass beads were allowed to settle and the mycelial extract decanted into a graduated cylinder. The glass beads were washed several times with the extraction buffer and the washes pooled with the original extract until the volume of extract was 7.5 ml/g fresh weight of mycelia. The extract was centrifuged at 500 X g, 10 minutes in a Sorvall SS34 rotor to pellet cell debris and remnants of glass beads. Cell fractions were obtained by differential centrifugation. Large vacuoles were pelleted by centrifuging the 500 X g supernatant at 5000 X g for 10 minutes. Further centrifugation of the 5000 X g supernatant at 25000 X g for 40 minutes was used to pellet a mixture of mitochondria and small vacuoles and the supernatant from this step was further centrifuged at 135000 X g to prepare the cytosol fraction (see below). Washed 25000 X g pellets were also stored frozen at -20°C for use in purification of endo-exonuclease. Each pellet fraction was washed once in extraction buffer. The 25000 X g pellet from 45-50 g mycelia was resuspended in 17 ml of extraction buffer. To separate mitochondria and small vacuoles, the washed 25000 X g pellet was loaded on top of four-step gradients made by
layering 3 ml each of 60%, 40%, and 20% sucrose containing 2 mM EDTA pH 6.5 on top of each other. The step gradients were centrifuged at 135000 X g for 90 minutes in a Beckman SW40 rotor. The mitochondria banded at the 40%/60% interface, whereas the small vacuoles sedimented to the bottoms of the tubes. Fractions containing mitochondria were collected, pooled, diluted with 2.7 volumes of sucrose-EDTA and pelleted by centrifugation at 25000 X g for 30 minutes. The 25000 X g supernatant was centrifuged at 135000 X g for 90 minutes to obtain microsomes (pellet) and cytosol (supernatant). All of the organelles obtained were extracted by resuspending in 5 to 10 ml 20 mM Tris-HCl, pH 7.5 - 1 mM EDTA, pH 7.5.

2.2.3. Nuclear extraction

Nuclei were disrupted in the extraction procedure described above. Therefore, another procedure had to be used to recover intact nuclei. In this case, mycelial mats were cut into small pieces and blended with 3 g glass beads in 3 ml/g mycelia of cold 1 M sorbitol- 5% Ficoll 400- 20% glycerol- 5mM MgCl₂- 10 mM CaCl₂ (Buffer A) + 0.25 % Triton X-100 for 30 seconds. Following addition of 3 more volumes Buffer A + Triton, the extract was blended another 30 seconds, after which a further 3 volumes of Buffer A were added and the mixture blended another 30 seconds. After allowing the glass beads to settle, the supernatant was
decanted and centrifuged at 700 X g for 10 minutes in a GSA rotor. The 700 X g supernatant was centrifuged at 9000 X g for 40 minutes in a SS34 rotor and the pellets resuspended in 2.5 volumes of 1 M sucrose- 50 mM Tris-HCl, pH 7.5- 5mM MgCl$_2$- 10 mM CaCl$_2$. The suspension was filtered through glass wool to remove pieces of mycelia and the filtrate re-centrifuged at 9000 X g. This washed pellet contained the nuclei.

2.2.4. Assays

DNA was determined by the method of Giles and Myers (1965) using calf-thymus DNA as standard assuming that a 1.00 mg/ml solution of native DNA has an $A_{260}$ of 20.0. Protein as indicated was determined either by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard or by the method of Bradford (1976) using the BIO-RAD kit, again using BSA as standard protein.

Endo-exonuclease (EE) was assayed by measuring at 37°C, the rate of release of cold 0.1 N perchloric acid-soluble material absorbing at 260 nm from 667 μg/ml ss or dsDNA in the presence of 10 mM MgCl$_2$ and 100 mM Tris-HCl, pH 7.5 or pH 8.0 in a total volume of 0.60 ml according to the method of Fraser (1978). The dsDNA substrate was native calf thymus DNA dissolved in 50 mM Tris-HCl, pH 7.5 by slow stirring in the cold for 2 days and the ssDNA substrate was obtained from the dsDNA by heat denaturation for 15 minutes at 100°C.
followed by quick-cooling on ice. Trypsin-activatable endo-
exonuclease (TAEE) was assayed by the same method after
treatment of the protein with trypsin. For fractions
containing relatively higher concentrations of protein, 400
µg/ml trypsin was used during activation, while for those
containing less protein, 50 or 100 µg/ml trypsin was used
instead. Activation was carried out at 37°C for 30 minutes.
The reaction was stopped by the addition of a two-fold
excess by weight (over trypsin) of soybean trypsin
inhibitor. The difference in nuclease activity measured with
trypsin and that measured without trypsin was taken as a
measure of the amount of TAEE. One unit of activity is
defined as that amount of enzyme which releases 1.0 A₂₆₀
unit of acid-soluble material from ssDNA in 30 minutes under
the conditions of the assay. One unit of dsDNase activity
was defined in the same way when dsDNA replaced ssDNA as
substrate. Fractions collected during the purification of EE
were assayed for nuclease activity using a single time point
assay. The assay mix was only 0.10 ml and was incubated for
30 minutes at 37°C. In other experiments, to examine the
endonucleolytic and exonucleolytic characteristics of the
purified enzyme, the rates of release of products from DNA
with cold 10% TCA or 0.5% UTCA in 10% TCA as precipitants
instead of PCA was followed. TCA does not precipitate
mononucleotides or oligonucleotides containing less than
about 15-17 bases, but precipitates all larger fragments. On
the other hand, UTCA precipitates all products except mononucleotides and very small oligonucleotides.

For assays with tRNA as substrate, only 0.5 mM MgCl₂ in excess of the concentration of EDTA was used. Higher Mg²⁺ concentrations inhibited the RNase activity, presumably by stabilizing the secondary structure of the tRNA.

2.2.5. Immunochemical reactions

For immunotitration of TAEE, an aliquot of the cytosol fraction containing 4–5 Units of trypsin-activable ssDNase activity was trypsin activated with 400 μg/ml trypsin at 37°C for 30 minutes. The reaction was stopped by the addition of 800 μg/ml of soybean trypsin inhibitor (a two-fold excess). The TAEE sample or an aliquot containing 4–5 Units of EE, and λIE antibody in different dilutions in phosphate buffered saline (PBS, consisting of 137 mM NaCl-8 mM Na₂HPO₄- 1 mM KH₂PO₄- 3 mM KCl- 3 mM NaN₃) + 2% BSA were mixed in a total volume of 200 μl in a 1.5 ml Eppendorf tube and allowed to sit at room temperature for 5 minutes with gentle rocking. A predetermined excess amount of Pansorbin (commercially available Formalin-fixed Staphylococcal aureus cells) was added and the mixture left on ice for 5 minutes to allow for adsorption of immune complexes and excess IgG. Following centrifugation in a Beckman microfuge for 3 minutes, 100 μl of the supernatant was assayed for remaining nuclease activity. For SDS-ssDNA activity gels (see below),
immunoprecipitates were prepared in the same manner except that a 1:8 dilution of antibody (excess) was used. Following centrifugation, the adsorbed immune complex was washed 3 times with PBS and denatured by boiling in loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 780 mM 2-mercaptoethanol, and 10% glycerol). Following centrifugation for 3 minutes in a microfuge to remove the undissolved Pansorbin, the supernatant was loaded onto the gel.

2.2.6. Purification of EE from a mixture of mitochondria and small vacuoles

EE was purified from the 25000 X g pellet from 50 g mycelia consisting of mitochondria and small vacuoles as described above. The washed pellets (fresh or frozen and thawed), were resuspended in 1 ml/g mycelia of 20 mM Tris-HCl, pH 7.5-1 mM EDTA. This suspension was sonicated on ice four times, each for 1 minute, at 60% power with a Blackstone sonicator, not allowing the temperature to rise above 15°C. The sonicate was then centrifuged at 25000 X g for 30 minutes in a Sorvall SS34 rotor. The supernatant was applied to a 60 ml DEAE-Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.5-1 mM EDTA - 10% glycerol (Buffer A), washed with Buffer A until the A280 was close to 0.0, and then the EE and TAEE eluted with a 0-0.5 M NaCl linear gradient in Buffer A collected in 5 ml fractions. Fractions containing EE activity were pooled, concentrated with PEG
20000, and dialyzed against 2 liters of 20 mM Tris-HCl, pH 7.5 - 5 mM EDTA - 10% glycerol (Buffer B) overnight at 4°C followed by 2 hours in 1 liter of Buffer B. This dialysate is referred to as the DEAE-Sepharose pool, and was applied to a 20 ml ssDNA cellulose column pre-equilibrated with Buffer B, then the column washed with Buffer B until the $A_{280}$ was close to 0.0. EE eluted with 0-1.0 M NaCl linear gradient in Buffer B collected in 3 ml fractions. Fractions containing nuclease activity were pooled, concentrated with PEG 20000 to approximately 5 ml, and dialyzed against 2 changes of 2 liters each of Buffer B overnight at 4°C. This dialysate contained the purified EE.

2.2.7. Gel electrophoresis

Samples were examined by SDS-PAGE at a constant current of 40 - 50 mAmps according to the method of Laemmli (1970). The polypeptides in 5% or 10% 1.5 mm thick slab polyacrylamide gels were visualized either by staining with 0.1% Coomassie blue in 40% methanol- 10% acetic acid for 30 minutes at room temperature, washing several times in 40% methanol- 10% acetic acid and destaining overnight in 10% methanol- 7% acetic acid or by the silver-staining method of Oakley et al. (1980). Activity gels contain, in addition to the polyacrylamide, 10 ug/ml each of ss-DNA and BSA. Denatured nuclease samples were electrophoresed and allowed to renature by washing out SDS according to Lacks (1980).
with 40 mM Tris-HCl, pH 7.5 - 2 mM EDTA - 0.1% Triton X-100 overnight at room temperature. The next day, the nuclease was activated by incubating the gel with 200 mls of 10 mM MgCl₂ and 10 mM CaCl₂ in the above buffer - Triton X-100. Bands of DNA digestion were localized by staining the undigested DNA in the gel with 2 μg/ml ethidium bromide. Pancreatic DNase I (31 kDa), and Micrococcal nuclease (16.7 kDa) were run as controls for the renaturation of nucleases.

2.2.8. Western Blots

Immediately after electrophoresis, SDS-PAGE gels were rinsed briefly in the blotting buffer (25 mM Tris base - 192 mM glycine - 20 % methanol). Proteins were electrophoretically transferred to nitrocellulose as described by Towbin et al. (1979) at a constant current of 0.7 Amps for 1.5 hours in the above buffer. To check for complete transfer of proteins, the gel was then stained with Coomassie blue. The blot was quickly rinsed in Tris buffered saline (TBS, 10 mM Tris-HCl, pH7.5 - 1 mM EDTA - 150 mM NaCl) and then transferred to a heat-sealable food bag containing 10 ml of 5% skim milk - 0.02% Na azide in TBS (blocking buffer). The bag was sealed and shaken at room temperature for 30 minutes in order to saturate the free binding sites on the filter. Antiserum raised to purified N.

crassa EE was added to a final dilution of 1/500 and the bag returned to the shaker and left there overnight. The filter
was removed from the bag and washed 3 times in TBS for 10 minutes each. The blot was then put into another heat-sealable food bag containing 1/7500 dilution of alkaline phosphatase conjugated anti-rabbit goat IgG. The bag was sealed, shaken at room temperature for 1.5 hours, and then the blot was removed from the bag. The blot was washed 3 times in TBS for 10 minutes each, air dried and then developed with the alkaline phosphatase substrates 5-bromo-4-chloro-3-indolyl-phosphate (165 ug/ml) and Nitro Blue tetrazolium (330 ug/ml) in 0.1 M Tris-HCl, pH 9.5 - 0.1 M NaCl-0.005 M MgCl₂ as described by Promega for the ProtoBlot system.

2.2.9. Large scale preparation of plasmid pBR322 DNA

From a -70°C frozen stock of E. coli HB101 harbouring the pBR322 plasmid, an overnight culture of cells in LB medium containing 50 ug/ml ampicillin was grown at 37°C. This was used to inoculate two flasks containing 500 ml LB medium containing 50 ug/ml ampicillin and the cells grown in a shaker incubator overnight at 37°C. The cells were pelleted by a low speed centrifugation and resuspended in 25 mls of 50 mM glucose - 10 mM EDTA - 25 mM Tris-HCl, pH 8.0-5 mg/ml lysozyme. After 15 minutes at room temperature, 50 ml of freshly prepared 1% SDS - 0.2N NaOH was added and the mixture allow to sit on ice for 15 minutes. Then 38 ml of 3M K-acetate was added, and the mixture vortexed and returned
to the ice bath for another 15 minutes. A low speed centrifugation in a table top IEC centrifuge at 2500 r.p.m. for 10 minutes was used to pellet the cell debris. The supernatant was extracted with an equal volume of phenol:chloroform (1:1) and two vol of 100 % ethanol added to the aqueous phase. DNA was allowed to precipitate at -20°C for 20 minutes, then it was pelleted by centrifugation at 10000 r.p.m. for 10 minutes, washed with 70% ethanol, dried and resuspended in 3.8 ml of 10 mM Tris-HCl, pH 8.0-1 mM EDTA (TE buffer) with 4.2 g of cesium chloride and 200 ul of 10 mg/ml ethidium bromide. Plasmid DNA was banded by overnight centrifugation at 60000 r.p.m. in a VTi80 rotor at room temperature. The covalently closed circular DNA band, visualized under UV, was collected and diluted 3 times with distilled water. DNA was precipitated 2 times with 0.1 vol 3M Na-acetate, 2 vol of 100% ethanol at -20°C, washed with 70% ethanol, dried and resuspended in TE buffer. The yield was 90 ug/500 mls of cell culture.

2.2.10. Preparation of E.coli $^3$H-DNA

E. coli HB101 cells grow well in LB medium which is commonly used and therefore readily available. An overnight culture of E. coli HB101 cells was used to inoculate 500 ml LB medium and this was warmed to 37°C over 30 minutes. Deoxyadenosine (dA) in sterile distilled water was added to 20 ug/ml followed immediately by addition of 2 μCi/ml $^3$H-
thymidine (specific activity 20 Ci/mmol). Incubation at 37°C was continued for 3 hours at which time there was a second addition of dA and ³H-thymidine, followed by further incubation at 37°C for 3 hours. The cells were then harvested and kept frozen at -20°C. The pellets were resuspended in 10 mls TE buffer containing 2 mg/ml lysozyme and kept at room temperature for 5 minutes. To increase the EDTA concentration, 400 µl of 0.5 M EDTA, pH 8.0 was added prior to addition of SDS to a final concentration of 1%. The suspension was kept on ice for 10 minutes to allow for lysis of the cells and was then extracted twice with an equal volume of phenol. Two volumes of 100% ethanol were added to the final aqueous phase and the DNA allowed to precipitate at -20°C for 30 minutes. The DNA was pelleted, washed, dried and resuspended in TE buffer as described above. The specific activity of the DNA obtained was 2.9 X 10⁴ c.p.m./µg DNA.

2.2.11. Action of EE on superhelical OX174 DNA and on linearized pBR322 DNA

Samples of 125 ng OX174 RFI DNA (50 µg/ml) and 1 µg EcoRI-linearized pBR322 DNA (dephosphorylated or not) were each treated EE in 100 mM Tris-HCl, pH 7.5 with 5 or 10 mM MgCl₂ in a total volume of 25 µl for 5 minutes at 37°C. The reactions were stopped by addition of 2 µl of 0.5 M EDTA, pH 8.0 and the mixtures put on ice. After cooling, 2.5 µl of 10% SDS was added to each and the mixtures either heated to
65°C for 5 minutes or denatured in boiling water for 5 minutes and then quick-cooled on ice. After addition of 0.2 vol of DNA loading buffer (30% glycerol - 0.25% bromophenol blue), the samples were electrophoresed at a constant current of 60 mAmps on 1% agarose gels containing 40 mM Tris - 20 mM acetate - 1 mM EDTA, pH 7.7 (TAE buffer). The gels were stained with 0.5 µg/ml ethidium bromide for 20 minutes at room temperature and then photographed over UV-light.

2.2.12. Digestion products of EE

Digestion products released by EE from ss and dsDNA were examined by filtration through Sephadex G-100 as described by Birnboim (1966). Reaction mixtures (0.65 ml containing 4 Units EE ssDNase or 8 Units EE dsDNase) were made up according to the routine assay as described earlier. Aliquots of 200 ul were removed at 0, 15, and 30 minutes and added to 20 ul cold 0.2 M EDTA, pH 6.5 to stop the reaction. Each digest was loaded onto a 0.6 by 38 cm Sephadex G-100 column that was equilibrated and then washed with 0.10 M Tris-HCl, pH 7.5 - 10 mM EDTA. Fractions of 0.50 ml were collected and the absorbance at 260 nm (A260) determined for each one.

Small oligonucleotide products of digestion of dsDNA were resolved by chromatography at room temperature on DEAE-Sephadex A25 in 20 mM Tris-HCl, pH 8.0 containing 7 M Urea (Buffer A) as described by Kumagai et al. (1980). 20 Units
of EE was added to 2 ml of the routine assay mix and incubated at 37°C for 40 minutes to obtain complete hydrolysis. The mixture was then heated to 100°C for 2 minutes to denature the enzyme, diluted 5 times with buffer A. As mononucleotide marker, 0.5 μCi of ³H-dTMP was added and the mixture loaded onto an equilibrated 1.1 X 30 cm DEAE-Sephadex A25 column. The column was washed with 15 ml of Buffer A, eluted with 500 ml linear 0 - 0.4 M NaCl gradient in buffer A and collected in 2 ml fractions.

To determine what type of endonucleolytic breaks were made by A. nidulans EE, labelled ssDNA was used as the substrate. In 1 ml containing 100 mM Tris-HCl, pH 7.5 - 10 mM MgCl₂, denatured E.coli ³H-DNA was incubated with or without 0.5 U of EE for 2 minutes at 37°C (10% hydrolysis). The enzyme was then denatured by boiling and the mixtures each divided into two equal portions. To determine the nature of the 5' termini of the products, bovine spleen phosphodiesterase was added to one portion of the digest and snake venom phosphodiesterase was added to the other portion at 37°C. Incubations were carried out at 37°C, and 100 μl samples withdrawn at 0, 10, and 20 minutes, added to 60 μl of carrier DNA and then 160 μl of 10% TCA was added to each sample. The tubes were kept on ice for 10 minutes to allow for complete precipitation of undigested DNA. Following centrifugation for 1.5 minutes, 200 μl of the supernatant was added to 10 mls Aquasol and counted in a Beckman
2.2.13. Extraction of DNA from Aspergillus and Neurospora

Aspergillus and Neurospora DNAs were each prepared from 5-6 grams of mycelia frozen at -70°C. Each frozen mat was quickly ground to fine powder in a mortar and pestle kept at -70°C. The powdered material was then resuspended in 5 ml 10 mM Tris-HCl, pH 7.5 - 100 mM EDTA per gram of mycelia. Sarkosyl and RNaseA were added to a final concentrations of 2% and 0.4 mg/ml respectively and the mixture incubated at 65°C for 30 minutes. Proteinase K was then added to a final concentration of 200 µg/ml and the mixture further incubated at 37°C for at least 3 hours. Following centrifugation at 2250 r.p.m. in a SS34 rotor for 5 minutes to pellet cell debris, the supernatant was extracted twice with phenol, twice with phenol:chloroform:isoamyl alcohol (24:24:1) and twice with chloroform:isoamyl alcohol (24:1). To the final aqueous phase was added an equal volume of 20% PEG 8000-2.5 M NaCl to allow for precipitation of DNA on ice over a period of at least 1 hour. The DNA was pelleted by centrifugation at 11200 r.p.m. in a SS34 rotor for 15 minutes. The pellet was dissolved in 4.5 mls TE buffer and extracted once with chloroform to get rid of traces of PEG. To the aqueous phase was added 0.1 vol of 3 M Na-acetate, pH 5.2 and 2.5 vol of 100% ethanol. The DNA was allowed to precipitate at -70°C for 15 minutes and was pelleted,
washed, and resuspended in 1 ml of TE buffer as described previously. Typical yields were 3 mg and 6 mg of DNA respectively from 6 g mats of *Aspergillus* and *Neurospora* mycelia.

2.2.14. **Large scale preparation of plasmid Pgc10 DNA**

From a yeast genomic library in a yeast episomal plasmid constructed from pBR322, T. Chow isolated a yeast EE clone (Pgc10) by screening with a partial yeast EE clone in gtl1 (T. Chow, pers. comm.).

*E. coli* HB101 cells harbouring the Pgc10 plasmid (the yeast EE clone obtained from T. Chow, see Figure 15A) were inoculated into 500 mls of LB medium containing 50 ug/ml of ampicillin and grown at 37°C overnight to stationary phase by shaking at 200 r.p.m. in a New Brunswick shaker-incubator for 16 hours. Cells were pelleted by low-speed centrifugation, washed with TE buffer, and resuspended in 10 ml fresh TE buffer and frozen at -70°C for 30 minutes. The cells were then thawed at room temperature and lysozyme added to a final concentration of 1 mg/ml. After incubation at room temperature for 20 minutes, 8 ml of 0.4% deoxycholate and 8 ml of BRIJ-58 were added, mixed, and left on ice for 5 minutes. NaCl (5M solution) was added to a final concentration of 50 mM, and the mixture centrifuged at 30000 r.p.m. for 60 minutes in a Ti60 rotor at 4°C. Solid NaCl was added to the supernatant to a final concentration
of 1 M and this solution extracted with an equal volume of phenol. DNA and RNA were precipitated from the aqueous phase with 0.1 vol 3M Na-acetate, pH 5.2 and 3 vol 100% ethanol at -70°C for 30 minutes. Centrifugation at 10000 X g for 25 minutes pelleted the nucleic acid which was then dried under N₂ and resuspended in 7 ml TE buffer containing 800µl of 10 mg/ml ethidium bromide. Solid cesium chloride (CsCl) was added so that the final concentration was 0.928 g/ml solution which has an approximate density of 1.55 g/ml. This mixture was centrifuged at 35000 X g for 48 hours at 10°C in a Ti50 rotor to separate the DNA from the RNA. The supercoiled DNA band was visualized with a UV-lamp, collected and diluted with 3 vol of distilled water. DNA was precipitated twice with 0.1 volume 3 M Na-acetate, pH 5.2 and 3 vol 100% ethanol at -70°C for 20 minutes and then pelleted, washed, dried and resuspended in TE as described previously.

2.2.15. Restriction of DNA and agarose gel electrophoresis

Where indicated, cleavage of DNAs with restriction endonucleases was performed as described by the standard procedures recommended by the manufacturer.

Agarose gels (0.7% to 1.0% in TAE buffer, 40 mM Tris-Acetate - 1 mM EDTA) were electrophoresed to separate products of restriction enzyme digestion at a constant current of 60 mAmps for 2 to 3 hours in TAE buffer. In some
cases, 0.5 μg/ml ethidium bromide was added to the gel and in others, the gel was stained after electrophoresis with a 0.5 μg/ml solution of ethidium bromide.

2.2.16. Extraction of DNA from low melt agarose gels

Hexadecyltrimethylammonium bromide (Kodak) with a ammonium cation (QN⁺) was dissolved in a mixture of 100 ml each n-butanol and distilled water to make a 0.5% solution. The solution was vigourously shaken and the two phases allowed to separate overnight. The QN⁺-butanol and QN⁺-aqueous phases were stored separately at room temperature.

Extraction of DNA from low melt agarose gels was performed according to Langridge et al. (1980). After electrophoresis, the DNA band was visualized over a long-wave UV-lamp, cut out of the gel and placed into a 1.5 ml Eppendorf tube. The gel piece was melted by placing the tube in a 65°C water bath for 5 minutes and the volume measured. Equal volumes of QN⁺-butanol and QN⁺-aqueous (both prewarmed at 37°C) were added, the mixture vortexed and then centrifuged in the microfuge. The upper butanol phase was removed to a new tube and the QN⁺-aqueous-agarose reheated to 65°C for 2 minutes and reextracted with QN⁺-butanol. Salt (0.25 vol 0.2 M NaCl) was added to the combined butanol extracts and the mixture vortexed and centrifuged. The lower aqueous phase containing DNA was transferred to a new tube and extracted with an equal volume of chloroform to remove
the QN⁺. Following extraction, 0.1 vol 3 M Na-acetate, pH 5.2 and 2 vol 100% ethanol were added to the aqueous phase. The DNA was allowed to precipitate at -70°C for 30 minutes and then was pelleted by centrifuging for 15 minutes. The pellet was washed with 70% ethanol, dried, and left in pellet form until needed.

2.2.17. Labelling of DNAs used as probes in Southern blot analysis and screening of libraries

Labelling of DNA fragments was accomplished by using the oligo-labelling kit obtained from Pharmacia as described by Feinberg and Vogelstein (1983, 1984). Briefly, the following ingredients were combined in a total volume of 50 ul: 50 ng of denatured DNA, a mixture of hexanucleotides (oligos), dATP, dGTP, dTTP, 50 uCi ³²P-dCTP (specific activity 3000 Ci/mmol), 120 ng/ml BSA, and 2 Units of E. coli polymerase I Klenow fragment. This mixture was allowed to incubate at room temperature for 5 h after which an additional 1 Unit of Klenow fragment was added followed by further incubation at room temperature for 3 hours. To stop the reaction, the mixture was heated to 65°C for 10 minutes. The mixture was then chromatographed on a 1 ml Sephadex-G50 spin column to remove unincorporated nucleotides.

2.2.18. Southern blot analysis

Immediately after electrophoresis, the agarose gel was
photographed over a UV-lamp, and the gel transferred to a plastic container with a solution of 0.5 M NaOH - 1.5 M NaCl. The gel was gently shaken in this solution for 45 minutes to denature the DNA. To neutralize the gel, the above solution was replaced with a 0.5 M Tris-HCl, pH7.5 - 1.5 M NaCl solution and shaken for a further 45 min. The electrophoretically separated DNAs in the gel were then transferred to nitrocellulose in 10X SSC overnight by capillary action as described by Southern (1975). The nitrocellulose was soaked in 6X SSC for 5 minutes, air dried and baked for 2 hours at 80°C under vacuum between two sheets of Whatman 3MM paper. The baked filter was soaked for 2 minutes in 6X SSC and transferred to a sealable plastic bag containing 15 ml of 6X SSC - 0.5% SDS - 5X Denhardt’s solution (1X Denhardt’s is 0.02% Ficoll - 0.02% polyvinylpyrrolidone - 0.02% BSA). The bag was sealed after squeezing out most of the air bubbles and incubated at 42°C for 3 hours (prehybridization). The bag was opened and denatured 32P-labelled DNA (Specific activity 9.0 x 10^8 c.p.m./ ug DNA) added to a concentration of 2.20 x 10^5 c.p.m./ ml and the bag resealed and returned to the incubator for 40 hours. The filter was removed from the bag and washed 2 times with 2X SSC - 0.1% SDS for 15 minutes at room temperature followed by 2 washes at 42°C in 1X SSC - 0.1% SDS for 15 minutes each. After air drying the filter at room temperature, it was wrapped in saran wrap and exposed
to Kodak XAR5 X-ray film for 2 days at -70°C with a Dupont 'Cronex lightning plus FA intensifying' screen.

2.2.19. Screening a λgt1l library of Aspergillus genomic DNA

The initial round of screening involved using a high density of plaque forming units (pfu), 5 × 10³ pfu per plate. As the purification of a phage clone progressed, lower density platings were used (100 - 200 pfu per plate).

The λgt1l phage vector is 44 kilobases long (Figure 15B) and includes the lacZ operon, CI857 (the temperature-sensitive repressor which is inactive at 42°C), and S100 (the amber mutation which renders the phage lysis-defective in hosts which lack the amber suppressor, SupF). Foreign DNA is inserted into the unique EcoRI restriction site located 53 basepairs upstream from the β-galactosidase translation termination codon. This allows for the expression of fusion proteins with β-galactosidase. The Aspergillus nidulans genomic DNA λgt1l library was obtained from G. May, Rutgers University. The size of the pieces inserted range from 4-7 kilobases.

Cells of E.coli strain Y1090, a lon mutant that produces large amounts of the lac operon repressor (to prevent lacZ-directed expression of fusion proteins early in plaque formation), were grown at 37°C overnight in LB medium supplemented with 0.2% maltose and 50 μg/ml ampicillin. Foreign or abnormal proteins in E. coli are usually degraded
by the lon protease. The lacZ-directed expression was induced by inactivating the repressor with isopropyl β-D-thiogalactoside (IPTG). The cells were pelleted and resuspended in 0.4 vol 10 mM MgSO_4. An aliquot of 200 μl of these cells was mixed with the appropriate dilution of the gt11 library in 50 mM Tris-HCl, pH 7.5 - 10 mM MgSO_4 - 100 mM NaCl - 0.01% gelatin (SM buffer) and incubated at 37°C for 30 minutes to allow for adsorption of the phage. A suspension of the infected cells was made by adding 4 ml top agar (0.75% agar in LB containing 10 mM MgSO_4), vortexed and poured onto the surface of 1.5% LB-agar plates. The plates were allowed to stand for 15 minutes at room temperature to let the layer of agar solidify. In the case of screening with an antibody, the plates were then inverted and incubated at 42°C until tiny plaques were visible, usually 3 - 4 hours. Induction of lacZ fusion protein synthesis was accomplished by overlaying each plate with a dry nitrocellulose circle previously soaked in 10 mM IPTG. The plates were then incubated overnight at 37°C and the next day, allowed to cool and the nitrocellulose lifted after the orientation of the filter on the plate had been marked. The filters were rinsed briefly in TBS to remove traces of agar and then placed (10 back to back) in a sealable food bag containing 10 mls 5% skim milk - 0.02% Na azide in TBS (blocking buffer). The rest of the procedure followed that described above for the Western blot except that a final
dilution of 1/100 of EE antiserum was used, and the immune complex was detected by reaction with 0.1 \text{uCi} \ 125\text{I}-protein A/ml blocking buffer. The washed and dried filters were exposed to Kodak XAR5 X-ray film at -70^\circ C for 2 days.

In other experiments, toluidine blue-O plates were used in screening the gt11 expression library as described by Lindler and Macrina (1987). To prepare these plates, toluidine blue-O was added to DNase test agar to a final concentration of 0.01\%, maltose to 0.1\%, and MgSO$_4$ to 10 mM. The top agar contained 10 mM IPTG in addition to the above, but in DNase test agar of half the concentration. E. coli Y1090 cells were infected with the phage as described above and the plates incubated at 42^\circ C for 5-6 hours. Small amounts of solutions of Micrococcal nuclease, pancreatic DNase I, and restriction endonuclease EcoRI were spotted onto a separate plate as controls.

In the case where a DNA fragment was used as probe to screen the library, the inverted agar plates containing the plaques were incubated at 37^\circ C overnight. In the morning, the plates were put in the cold to harden the agar. The plates were then overlayed with nitrocellulose circles, the plaques lifted after 4 min and the DNA denatured with alkali by floating the circles in a 0.5 M NaOH - 1.5 M NaCl solution for 5 min. The circles were then transferred to a 0.5 M Tris-HCl, pH 7.5 - 1.5 M NaCl solution for 10 minutes in order to neutralize the DNA. The filters were rinsed in
6X SSC to remove any adhering agar. The pre- and hybridization as well as the washing conditions were the same as for the Southern blots described earlier.

In both cases, the plates were stored at 4°C while the filters were being processed. The plaques on the agar plate giving a positive signal were removed and incubated in SM buffer for at least 1 hour. The phage were replated in subsequent rounds of screening.

2.2.20. LacZ - fusion protein production

Induction of fusion protein production was essentially as described by Huynh et al. (1985). E.coli Y1089 cells that are similar to E. coli Y1090 cells, but contain a mutation which enhances the frequency of phage lysogeny were grown at 37°C in LB medium supplemented with 0.2 % maltose and 50 µg/ml ampicillin overnight. The cells were pelleted and resuspended in an equal volume of 10 mM MgSO₄. These cells were infected with the phage at a multiplicity of infection of 5 at 37°C for 30 minutes, then 10 ml of LB medium containing 50 µg/ml ampicillin was added and the tubes incubated at 30°C with shaking until the A₆₅₀ reached 0.5. The temperature was then rapidly increased to 42°C and after 30 minutes, IPTG was added to a final concentration of 10 mM and the cells further incubated at 37°C for 1 hour. Cells were harvested at room temperature, resuspended in loading buffer and denatured by boiling for 5 minutes. After
vigorous vortexing, the suspensions were frozen at -20°C until ready for electrophoresis and immunoblotting.

2.2.21. Large scale recombinant phage preparation and DNA extraction

E. coli Y1088 cells that are defective for host-controlled restriction and modification enzyme activities were grown as described for strain Y1089. The cells (0.8 ml culture) were infected with 10⁵ pfu of phage, 10 ml of LB medium containing 10 mM MgSO₄ was added and incubation continued at 37°C overnight to allow for complete lysis (this is the phage inoculum). A large scale preparation of phage was made by inoculating 500 ml LB medium containing 10 mM MgSO₄ with a 1:100 dilution of an overnight culture of Y1088 and then incubating at 37°C until the A₆₅₀ reached 1.0. At this point, the phage inoculum was added to the culture and incubation continued at 37°C until there was complete or near complete lysis. Chloroform was then added to 2% to ensure complete lysis. This was followed by the addition of RNase A and DNase to final concentrations each of 1 μg/ml and incubation continued for another 30 min at 37°C. After addition of NaCl to 1 M, the culture was allowed to sit on ice for 1 hour. Centrifugation at 8250 r.p.m. in a GSA rotor for 10 min at 4°C brought down cell debris and chromosomal DNA but left the phage in suspension. Phage were precipitated by the addition of solid PEG 8000 to 10% and
incubation in the cold for 2 hours. Centrifugation at 8250 r.p.m. for 10 min, pelleted the precipitated phage. The phage were resuspended in 10 ml SM buffer and extracted 2 or 3 times with an equal volume of chloroform until a clear interface was obtained. Solid CsCl was added to the aqueous phase to a final concentration of 0.5 g/ml and this solution was loaded on top of a 4 ml CsCl step gradient consisting of layers of 1.45, 1.50, and 1.70 g/cm³ CsCl. The tubes were centrifuged at 22000 r.p.m. in a SW40 rotor in a Beckman ultracentrifuge for 4 hours. The bluish band containing the phage was detectable by eye at the 1.45/1.50 g/cm³ interface. The band was collected and dialyzed against two changes of 1 liter each of 10 mM NaCl - 50 mM Tris-HCl, pH 8.0 - 10 mM MgCl₂ for a total of 2 h to remove the CsCl. The dialysate was transferred to a 50 ml test tube, and 0.5 M EDTA, proteinase K, and SDS added respectively to final concentrations of 20 mM, 50 ug/ml, and 0.5%. Incubation was carried out at 65°C for 1 hour to allow for bursting of phage heads and inactivation of nucleases and then 0.1 vol of 3M Na-acetate added and the solution extracted twice with an equal volume of phenol : chloroform (1:1). The DNA was precipitated from the aqueous phase by adding 2.5 vol of 100 % ethanol and holding at -20°C for 30 minutes. The DNA was pelleted, washed, dried, and resuspended in 100 µl TE buffer as described previously.
2.2.22. Immunochemical study of relationship between recBCD nuclease of E. coli and endo-exonuclease of N. crassa

The E. coli strains used were obtained from G. Smith (Table 7) and were grown up overnight at 37°C with gentle shaking in 5 ml standard LB medium (V186) or 5 ml LB medium containing 50 μg/ml ampicillin (plasmid-harbouring strains). Cells were pelleted and resuspended by vortexing briefly in 200 ul 10 mM Tris-HCl pH 8.0 - 1 mM EDTA (TE buffer) containing 1 mg/ml lysozyme. After standing for 10 min at room temperature, SDS was added to each lysate to a final concentration of 0.6%. After a further 10 min., 70 ul of 4X loading buffer was added and each mixture heated to 100°C for 5 min, vortexed vigourously and then aliquots of each lysate loaded in duplicate onto 5% polyacrylamide gels. Gel electrophoresis and Western blot analysis were performed as described earlier.
3.1. Compartmentation of Aspergillus nidulans EE

In A. nidulans the majority of active EE was found in mitochondria, microsomes, and vacuoles, while most of the inactive form (TAEE) was found in the cytosol fraction (Table 2). Quantitatively, 57% of the active EE was found in the organelles (nuclei, mitochondria, vacuoles) while close to 80% of the inactive EE was found in the cytosol. The ratio of the level of active enzyme found in the large vacuoles to that found in the small vacuoles which co-sedimented with mitochondria averaged at about 3 (data not shown). Only 1% and 9% respectively of the total TAEE has been detected in A. nidulans mitochondria and nuclei, and there was 2.6% of the inactive EE found in the vacuoles. However, in the latter case, this small amount probably arises from contamination from other fractions, especially the cytosol. The ratio of total TAEE to total active enzyme in all isolated cell fractions was only 1.3, whereas in crude sonicates made in 20 mM KPB (potassium phosphate buffer) that ratio was found to be 63 (not shown in Table 2). One can infer from this that TAEE was much more stable in KPB than in the Tris buffer used during fractionation and extraction of the organelles. It should also be noted that there was great variability in the enzyme levels recovered from experiment to experiment. This result may indicate
Table 2: Intracellular distribution of A. nidulans endo-exonuclease.

Levels of endo-exonuclease are reported in Units/10 g fresh weight of mycelia. Nuclei were prepared separately from the other cell fractions as described in Materials and Methods. The averages for five experiments are reported. The total active enzyme extracted varied from 1640-2300 Units/10 g while the total inactive enzyme varied from 1630-3860 Units/10 g.
<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Active (Units/10g)</th>
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<tbody>
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<td>320</td>
<td>270</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>350</td>
<td>75</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>590</td>
<td>35</td>
</tr>
<tr>
<td>Microsomes</td>
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<td>290</td>
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<tr>
<td>Cytosol</td>
<td>390</td>
<td>2260</td>
</tr>
<tr>
<td>Total</td>
<td>2220</td>
<td>2930</td>
</tr>
</tbody>
</table>
different extents of uncontrolled proteolysis during different extractions and fractionations (see below).

Immunotitrination of the cytosol fraction (135000 X g supernatant) with the antibody raised to N. crassa EE showed that 70 % of the ssDNase was immunoprecipitable while 30 % was not (Figure 1). This indicates that there were at least two forms of trypsin-activable EE, one form cross-reactive with the antibody and the other form not cross-reactive with antibody. When the A. nidulans cytosol fraction was trypsin-activated and electrophoresed on a SDS-ssDNA activity gel, bands indicating polypeptides with nuclease activity of nearly 90 kDa and 28 kDa appeared (Figure 2, lane 3) whereas without trypsin activation, no activity was seen (not shown in Figure 2). This points to a possibility that there may be a trypsin-activatable EE precursor of greater than 90 kDa. Immunoprecipitation of this activated fraction followed by SDS-ssDNA activity gel analysis revealed a 'smear' of activity with polypeptides ranging in size from 50 kDa to 31 kDa (Figure 2, lane 5). Activity gel analysis of the 25000 X g supernatant ('crude cytosol') gave the same results as above (Figure 2, lanes 4 and 6). These results indicate clearly that proteolytic fragments of EE (the immunoprecipitable nuclease) retain their activity.

3.2. Purification of A. nidulans EE

The A. nidulans EE was purified from the sonicate of a
Figure 1: Immunotitration of inactive endo-exonuclease in the cytosol fraction of A. nidulans.
Aliquots of cytosol containing 4 Units of trypsin-activatable nuclease were incubated with increasing dilutions of antibody raised to the N. crassa endo-exonuclease for 5 minutes at room temperature, and the immunocomplexes adsorbed on Pansorbin and removed by sedimentation. The remaining activity in the supernatant was assayed using the standard nuclease assay as described in Materials and Methods.
Figure 2: SDS-ssDNA activity gel analysis of inactive endo-exonuclease of A. nidulans. After electrophoresis, the SDS was washed out of the gel, the gel activated in the presence of 10 mM Mg$^{2+}$ and 10 mM Ca$^{2+}$, stained with ethidium bromide and photographed over UV-lamp. The 25000 X g supernatant and 135000 X g supernatant or cytosol fraction were treated with 400 μg/ml trypsin at 37°C for 30 minutes. The reaction was stopped by addition of 800 μg/ml of soybean trypsin inhibitor. Immunoprecipitates were obtained by incubating with 1/8 dilution of antibody against the N. crassa enzyme for 5 minutes at room temperature, adding Pansorbin and incubating on ice for 5 minutes to adsorb the immune complex. The pellet was washed with phosphate buffered saline (PBS), denatured and loaded onto a 10% acrylamide gel. Only lanes 3 to 6 are relevant.

Lane 3: 2.5 Units of trypsin-activated enzyme from the cytosol fraction; lane 4: 2.5 Units of trypsin-activated enzyme from 25000 X g supernatant, lane 5: Immunoprecipitate of lane 3; lane 6: Immunoprecipitate of lane 4; lane 7: 20 μg of prestained molecular weight protein standards (BRL); lane 8: enzyme standards, pancreatic DNase I and Micrococcal nuclease (31 kDa and 16.6 kDa, respectively). Molecular weights of marker proteins are indicated in kDa on the right.
mixture of mitochondria and small vacuoles (25000 X g pellet) by successive chromatography, first on DEAE-Sepharose and then on ssDNA cellulose. Most of the proteins of the sonicate of the 25000 X g pellet did not bind to DEAE-Sepharose whereas the majority of the Mg$^{2+}$-dependent nuclease activity bound to the column and was eluted between 0.04M and 0.26 M NaCl (Figure 3, Panel A). On ssDNA cellulose, most of the nuclease activity was eluted in two peaks in the gradient: a small weaker-binding fraction and a larger tighter-binding fraction (purified EE, see later). The latter EE fraction was eluted between 0.33 M and 0.50 M NaCl (Figure 3, Panel B) with no detectable protein as monitored by the absorbance of fractions at 280nm. In other preparations, TAAE was also assayed and found to be in the pass-through fractions when chromatographed on ssDNA cellulose. The precise elution profile on ssDNA cellulose depended on the batch of ssDNA cellulose used; with some other batches, EE was found to elute with higher salt concentrations (0.5 M to 0.77 M). In Panels A and B, it can be seen that there were some losses of nuclease activity in the pass through fractions of both columns. Whether these activities were EE or some other Mg$^{2+}$-dependent nuclease has not been determined.

The A. nidulans EE was purified 11200-fold with 16% yield from the sonicate of a mixture of mitochondria and small vacuoles (Table 3). The most effective step in
Figure 3, Panel A: Chromatography of the sonicate of the 25000 X g pellet fraction on DEAE-Sepharose. Panel B: Chromatography of the DEAE-Sepharose pool on ssDNA cellulose. Proteins were estimated in each fraction from $A_{280}$ (○ ○); ssDNase was estimated using the single time point (30 minutes) assay for PCA-soluble material released from ss-DNA $A_{260}$ (△ △); conductivity was measured with a Radiometer conductivity meter (● ●); < > indicates the pooled fractions described in the text.
protein (O, A280); ssDNase (△, A260)
Table 3: The purification of *A. nidulans* endo-exonuclease from the 25000 X g pellet fraction, a mixture of mitochondria and small vacuoles.

The purification described here was from 50 g mycelia. See Materials and Methods for purification procedure. Activity expressed is that with ssDNA.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (Units)</th>
<th>Protein Specific Activity (Units/mg)</th>
<th>Yield (%)</th>
<th>Purification (- fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial Extract (16500)</td>
<td>3070</td>
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<tr>
<td>25,000 X g pellet fraction (16500)</td>
<td>47</td>
<td>350</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>DEAE-Sepharose gradient fraction</td>
<td>7900</td>
<td>4.8</td>
<td>1650</td>
<td>48</td>
</tr>
<tr>
<td>ssDNA cellulose gradient fraction</td>
<td>2600</td>
<td>0.043</td>
<td>60500</td>
<td>16</td>
</tr>
</tbody>
</table>


purification was chromatography on the affinity column, ssDNA cellulose. The specific activity of the pure enzyme is calculated to be 60500 units/mg but this may be an underestimate due to the difficulty in accurately assessing the low amount of recovered protein. On a silver-stained SDS-PAGE gel, no visible bands were detected in the lane containing 0.38 ug protein (36 units EE) (Figure 4A, lane 7). It has been observed for the N. crassa EE that the polypeptide did not stain well with either silver or with Coomassie blue (Chow and Fraser 1983). By comparing lanes 5 and 6 of Figure 4A, one can see that there was considerable elimination of proteins on DEAE-Sepharose chromatography. However, after chromatography on DEAE-Sepharose, 52% of the nuclease activity had been lost.

On SDS-ssDNA activity gel, an active polypeptide of 28 kDa appeared in the lane containing the purified EE (Figure 4B, lane 8). One can also see that proteolysis was taking place as the purification progressed. In the crude extract (Figure 4B, lane 1), a single polypeptide of 38 kDa was present, but in the sonicate of the mixture of mitochondria and small vacuoles, a 33 kDa as well as a 38 kDa band was visible (Figure 4B, lane 5). After chromatography on DEAE-Sepharose, only one active polypeptide of 28 kDa was seen (Figure 4B, lane 6). It is important to note that all the active polypeptides present in the crude extract, the sonicate and the purified EE were immunoprecipitated by the
Figure 4A: SDS-polyacrylamide gel electrophoresis of *A. nidulans* endo-exonuclease. After electrophoresis, the gel was silver stained. Only lanes 5 to 9 are relevant. 
Lane 5: 13 ug of sonicate of 25000 X g pellet; lane 6: 5.2 ug of DEAE-Sepharose pool; lane 7: 0.38 ug purified endo-exonuclease; lane 8: 0.54 ug ss-DNA cellulose weaker binding pool; lane 9: 1 ug of molecular weight protein standards: 200K, 97K, 67K, 43K, 30K, and 18K.

Figure 4B: SDS-ssDNA activity gel analysis of *A. nidulans* endo-exonuclease. After electrophoresis, the SDS was washed out of the gel, the gel activated in the presence of 10 mM Mg$^{2+}$ and 10 mM Ca$^{2+}$, stained with ethidium bromide, and photographed over UV-lamp. Only lanes 1, and 5 to 10 are relevant. 
Lane 1: 4 Units of crude extract; lane 5: 4 Units of the sonicate of 25000 X g pellet; lane 6: 4 Units of DEAE-Sepharose pool; lane 7: 4 Units of ss-DNA cellulose weaker binding pool; lane 8: 4 Units of purified endo-exonuclease; lane 9: 10 ug of prestained molecular weight protein standards (BRL); lane 10: enzyme standards, pancreatic DNase and micrococcal nuclease (31 K and 16.6 K, respectively).
antibody against *N. crassa* EE (Figure 5, Panel A, lanes 2, 4 and 10). This implies that the 38 kDa, 33 kDa, and 28 kDa polypeptides were all immunochemically related and that the 33 kDa and 28 kDa peptides are likely derived from the 38 kDa peptide through proteolysis during the purification process. Figure 5, Panel A, lanes a and b show a SDS-ssDNA activity gel analysis of an extract of *A. nidulans* mycelia made in the presence of the protease inhibitor, PMSF. A large active polypeptide of 90 kDa was present and was also found to be immunocross-reactive with the antibody to the *N. crassa* enzyme. The weaker binding fraction from ssDNA cellulose also contained an active polypeptide of 28 kDa, but this polypeptide had less activity and showed less cross-reaction with the *N. crassa* EE antibody than purified EE (stronger-binding fraction, Figure 5, compare lanes 7 and 8 with lanes 9 and 10). The purified *A. nidulans* EE was the same size as EE purified from a mixture of the same organelles of *N. crassa* and was also immunoprecipitated by antibodies raised to the *N. crassa* enzyme (Figure 5, Panel B). Also, it can be seen that, although equal amounts of each enzyme were loaded onto the gel (4 Units), the *N. crassa* EE appeared to renature more efficiently (Figure 5, Panel B lanes 1 and 2). The reason for this difference is unknown.
Figure 5: SDS-ssDNA activity gel analysis of immunoprecipitates of *A. nidulans* endo-exonuclease-containing fractions. Immunoprecipitates were obtained by incubating the sample with 1/8 dilution of antibody against *N. crassa* endo-exonuclease at room temperature for 5 minutes. Pansorbin was added and the mixture incubated on ice to adsorb the immune complex. The precipitate was resuspended, denatured and applied to the 10% polyacrylamide gel as described in Materials and Methods. Panel A, lane a: Immunoprecipitate of 10 Units of crude extract + PMSF; lane b: 4 Units of crude extract + PMSF; lane c: 20 ug of prestained molecular weight protein standards; lane l: 4 U of crude extract - PMSF; lane 2: Immunoprecipitate of 10 Units of crude extract - PMSF; lane 3: 4 Units of sonicate of 25000 X g pellet; lane 4: Immunoprecipitate of 10 Units of sonicate of 25000 X g pellet; lane 5: 4 Units of DEAE-Sepharose pool; lane 6: Immunoprecipitate of 10 Units of DEAE-Sepharose pool; lane 7: 4 Units of ss-DNA cellulose weaker binding pool; lane 8: Immunoprecipitate of 10 Units of ss-DNA cellulose weaker binding pool; lane 9: 4 Units of purified endo-exonuclease; lane 10: Immunoprecipitate of 10 Units of purified endo-exonuclease; lane 11: antibody and Pansorbin control; lane 12: 20 ug of prestained molecular weight protein standards. Panel B, Lane 1: 4 Units of purified *N. crassa* endo-exonuclease; lane 2: as Panel A, lane 9; lane 3: as Panel A, lane 10.
3.3. Properties of A. nidulans endo-exonuclease

The purified enzyme has activity with ssDNA, dsDNA, and RNA (Table 4). The highest activity seen was with the ssDNA, while the activities with dsDNA and RNA were each about one-fifth of that seen with ssDNA. The ratio of ssDNase to dsDNase activities varied somewhat ranging from 2.0 to 9.0 from preparation to preparation and depended on the pH. In Tris buffer, the pH optimal for ssDNase activity was 7.5 and that for dsDNase was 8.0 (Figure 6).

For both ssDNase and dsDNase activities, there was an absolute requirement for the cation Mg\(^{2+}\) as cofactor (Table 5). In addition, the cations Mn\(^{2+}\) and Zn\(^{2+}\) also served as cofactors for ssDNase activity (this was not determined for the dsDNase activity). Maximal ssDNase activity was seen with 5 mM Mg\(^{2+}\) and 2 mM Mn\(^{2+}\) while only very low amounts of Zn\(^{2+}\), in the 10\(^{-6}\) to 10\(^{-5}\) M range, were required for activity. Higher than optimal concentrations of Mn\(^{2+}\) and Zn\(^{2+}\) resulted in inhibition of the ssDNase activity. Ca\(^{2+}\) did not act as cofactor for either activity, but in the presence of 10 mM Mg\(^{2+}\), Ca\(^{2+}\) (even at 2 mM) inhibited the ssDNase activity slightly. The effect of Ca\(^{2+}\) on the dsDNase activity of EE was different; in the presence of 10 mM Mg\(^{2+}\), 2 mM and 10 mM Ca\(^{2+}\) stimulated the dsDNase activity respectively by 11 and 13 %.

The results above showed that A. nidulans EE has activities and cofactor requirements similar to the N.
Table 4: Substrate specificity and activity of A. nidulans endo-exonuclease.

The activities were determined according to the routine assay as described in the Materials and Methods. Rates of release of cold 0.1 N perchloric acid-soluble material absorbing at 260 nm from 667 μg/ml ss or dsDNA in the presence of 10 mM MgCl₂ and 100 mM Tris-HCl, pH 7.5 or 8.0 in a total volume of 0.60 ml were followed. With tRNA as substrate, only 0.5 mM MgCl₂ in excess of the EDTA concentration was used.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA</td>
<td>900</td>
</tr>
<tr>
<td>dsDNA</td>
<td>180</td>
</tr>
<tr>
<td>tRNA</td>
<td>190</td>
</tr>
</tbody>
</table>
Figure 6: pH-dependence of *A. nidulans* endo-exonuclease. Activity was determined using the routine nuclease assay in 0.1 M Tris-HCl buffer of the appropriate pH (6.8, 7.5, 8.0, or 9.5), 0.01 M MgCl$_2$, and 0.677 mg/ml ssDNA (O--O) or dsDNA (Δ Δ).
Table 5: Divalent metal cation dependence of ssDNase and dsDNase activities of *A. nidulans* endo-exonuclease. Assays were performed in 0.1 M Tris-HCl pH 8.0 as described in Materials and Methods except that the appropriate amount of the cation was substituted in place of 10 mM Mg$^{2+}$. Activities are reported as percentages of those found with 10 mM Mg$^{2+}$. 
<table>
<thead>
<tr>
<th>Divalent Metal Cation(s)</th>
<th>Concentration (mM)</th>
<th>ssDNase activity %</th>
<th>dsDNase activity %</th>
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<tr>
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<td>Mg^{2+}</td>
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<td>--</td>
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<tr>
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</table>
crassa enzyme. They also show strong immunological cross-reaction. A 1:1 volume ratio of A. nidulans EE to antiserum raised to the N. crassa EE (excess antibody) resulted in complete inhibition of the nuclease activities of A. nidulans EE (data not shown). To determine quantitatively, how similar the two enzymes were, an immunotitration of the ssDNase and dsDNase activities with the antibody to N. crassa EE was performed. From the titration curve (Figure 7), the antiserum dilution at which 50% of the enzyme activity was inhibited was 1:512 ($2^9$). The original antigen, that is the N. crassa EE, also had the same titration profile with the antibody. Therefore, the A. nidulans EE is as good an antigen as the N. crassa enzyme. It may be concluded that there is very likely high structural homology between these two enzymes.

A 24 kDa inhibitor specific for N. crassa EE has been purified in our laboratory. This inhibitor competitively inhibited the dsDNase of N. crassa completely and non-competitively inhibited the ssDNase but only incompletely (70% -95%). A titration curve showing inhibition of the ss and dsDNase activities of A. nidulans EE with this inhibitor indicated that there was complete inhibition of 3 Units of both activities with 10.6 Units of inhibitor (Figure 8). One unit of inhibitor is defined as that which inhibits 1 Unit of the ssDNase of N. crassa EE by 100%. In addition, it can be seen in Figure 8 that, at the lower inhibitor
Figure 7: Immunotitration of purified *A. nidulans* endo-exonuclease with antibody against *N. crassa* endo-exonuclease.

Aliquots containing 5 Units of ssDNase (○ ○) or dsDNase (△ △) were incubated with increasing dilutions of antibody for 5 minutes at room temperature and the immunocomplexes adsorbed on Pansorbin and removed by sedimentation. The remaining activity in the supernatant was assayed using the standard nuclease assay as described in Material and Methods.
Figure 8: Titration of purified *A. nidulans* endo-exonuclease with inhibitor of *N. crassa* endo-exonuclease. Aliquots containing 3 Units of ssDNase (●●●) or dsDNase (▲▲▲) were assayed in the presence of increasing amounts of inhibitor using the standard nuclease assay as described in Material and Methods. 1 Unit of inhibitor is defined as that amount that will inhibit 1 Unit of ssDNase of *N. crassa* endo-exonuclease by 100%.
concentrations, the ssDNase activity was better inhibited than that of dsDNase. It should be noted that 5 times more protein was required to show the same units of dsDNase activity as ssDNase. This confirms the previous finding where the A. nidulans EE was the only other nuclease of more than a dozen tested (Hatahet and Fraser 1989) which was blocked by this N. crassa inhibitor.

3.4. Analysis of digestion products of A. nidulans EE

To elucidate the endonucleolytic and exonucleolytic modes of degradation of DNA by the enzyme, nuclease assays using TCA and UTCA as precipitants instead of PCA were performed. TCA leaves large and small oligonucleotides and mononucleotides in solution while UTCA leaves only small oligonucleotides and mononucleotides in solution. Therefore, a true exonucleolytic activity would show very similar rates of release of acid-soluble material and a true endonucleolytic activity would show a much faster rate of release of acid-soluble material in TCA than in UTCA. Figure 9 shows the relevant rate curves for A. nidulans EE. For ssDNase activity, the ratio of the slope of the rate curve seen with TCA to that seen with UTCA is 3.22, while for the dsDNase activity, that same ratio is 1.70. Therefore, it is concluded that the enzyme has mainly endonucleolytic activity with ssDNA and mainly exonucleolytic activity with dsDNA (see below).
Figure 9: Rates of release of acid-soluble material absorbing at $A_{260}$ from ssDNA and dsDNA using TCA and UTCA as precipitants.

The release of acid-soluble material in the presence of *A. nidulans* endo-exonuclease from ssDNA using TCA (●—●) and UTCA (○○) as precipitants and from dsDNA using TCA (▲▲) and UTCA (▲▲) as precipitants was followed over 45 minutes using the standard nuclease assay as described in Materials and Methods.
When digests of ssDNA with \textit{A. nidulans} EE were analyzed by gel filtration on Sephadex G-100, one can see that at the earlier time, the products ranged in size from that of undigested DNA to small oligonucleotides (Figure 10, Panel A). At the later time, more small oligonucleotides appeared and large fragments of ssDNA disappeared but there was still a wide range in sizes of the products. The gel filtration profiles of digestion products of dsDNA are very different (Figure 10, Panel B). At 15 minutes, the majority of the products were small oligonucleotides with little undigested DNA remaining and by 30 minutes when complete hydrolysis was achieved, the only products detected were small oligonucleotides eluting slightly ahead of the marker mononucleotide.

The profile of the dsDNA digest is consistent with an exonucleolytic mode of degradation while that of the ssDNA digest demonstrates a mixed endo- and exonucleolytic mode of degradation. An exclusively endonucleolytic type of hydrolysis would be represented by polynucleotide products of large sizes at the earlier times that get progressively smaller as the digestion proceeded.

To show that the small products of digestion of dsDNA with \textit{A. nidulans} EE were indeed oligonucleotides, chromatography of the products on DEAE-Sephadex A-25 was performed. The main products were seen to be di-, tri-, and tetra-nucleotides with no mononucleotides released since no
Figure 10: Gel filtration of digests of ssDNA and dsDNA with A. nidulans endo-exonuclease through Sephadex G-100. Reaction mixes, in a total volume of 0.65 ml, contain 4 Units of ssDNase or 8 Units of dsDNase in 0.1 M Tris-HCl, pH 8.0 and 0.01 M MgCl$_2$ were incubated at 37°C. 200 µl aliquots were removed at 0, 15, 30 min and added to 20 µl of cold 0.2 M EDTA to stop the reaction. Each digest was loaded onto a 0.6 X 38 cm Sephadex G-100 column equilibrated with 0.10 M Tris-HCl, pH 7.5 - 0.01 M EDTA, and the column washed with the above buffer. The absorbance at 260 nm of each fraction was determined. The arrow on each panel indicates the position of elution of the marker mononucleotide, dAMP. Panel A: ssDNA; Panel B: dsDNA. Symbols, ▲-▲: 0 minute control; ○ ○: 15 minute digest; Δ-Δ: 30 minute digest.
A₂₆₀ absorbing material was eluted with the Δ₃H-dTMP mononucleotide marker (Figure 11). The first peak of Δ₃H c.p.m. contained labelled thymidine which did not bind to the column.

The nature of the termini produced by EE in ssDNA was determined by treating heat-denatured *E. coli* Δ₃H-DNA with *A. nidulans* EE to achieve approximately 10% hydrolysis and then subjecting these products to hydrolysis by either snake venom phosphodiesterase (SVP) or bovine spleen phosphodiesterase (SP). Both of these enzymes are 5'→3' exonucleases; SVP requires a 5'-phosphate terminus to exert its activity, while SP requires a 5'-hydroxyl terminus. The EE-treated DNA was a good substrate for SVP but not for SP as determined by the rate of release of acid-soluble Δ₃H-c.p.m. (data not shown). As a control, *Micrococcal* nuclease-treated DNA proved to be a good substrate for SP while the untreated DNA was not. It may be concluded that the *A. nidulans* EE, like its *N. crassa* counterpart cleaved DNA to small oligonucleotides containing 5'-phosphorylated and 3'-hydroxyl termini.

3.5. Action of *A. nidulans* EE on superhelical and linear DNAs

The results presented above show that the *A. nidulans* EE has enzymological properties in common with the *N. crassa* EE and that the products of its hydrolytic action on DNA
Conductivity in mmoles (\(\uparrow\))

Fraction Number

\[ A_{260} \quad (\bullet) \]

\[ ^3H-dTMP \ (10^{-4} \text{ cpm}) \quad (\circ) \]
substrates are similar to those of the enzyme in *N. crassa*. To extend the comparison, the actions of the *A. nidulans* EE on well characterized viral and plasmid DNAs were studied.

Superhelical or RF DNA of the bacteriophage ϕX174 was treated with 2 to 16 Units/ml of the *A. nidulans* EE at 37°C in the presence of 0.1 M Tris-HCl pH 7.5 and 0.01 M MgCl₂ for 5 minutes. EE first nicked one strand of the superhelical DNA to yield the relaxed RFII form, demonstrating a true endonucleolytic action on this covalently closed circular DNA. This is seen as a shift in mobility of the DNA in Figure 12, lane 2 to a slower form. The other strand was then nicked close to the site of nicking of the first strand yielding a linear DNA molecule, RFIII (Figure 12, lane 3). This linear DNA was quickly degraded by EE to yield smaller fragments, seen as fast-running 'smears' in Figure 12, lanes 3-6. With the highest concentration of enzyme (lane 6), it appears that most of the DNA has been hydrolyzed to small oligonucleotides.

Treatment of linearized pBR322 DNA with *A. nidulans* EE in 0.1 M Tris-HCl pH 7.5 containing 5 mM MgCl₂ at 37°C briefly followed by electrophoresis of the products on agarose gels resulted in the appearance of a short 'ladder' of DNA along with the unit-length DNA (data not shown). This signifies that the enzyme recognizes and nicks at specific sites in the DNA. That the enzyme made endonucleolytic nicks in the linear dsDNA was apparent when the above treated
Figure 12: Actions of *A. nidulans* endo-exonuclease on ϕX174 RFI DNA.

Aliqouts of 125 ng of RFI DNA (50 ug/ml) were treated with increasing amounts of enzyme in 0.1 M Tris-HCl, pH 7.5 and 0.01 M MgCl₂ at 37°C for 5 minutes. The reactions were stopped by addition of excess EDTA and SDS and the mixes electrophoresed on 1% agarose gel. The gel was stained with 0.5 ug/ml ethidium bromide and photographed over UV-lamp. Going from top to bottom, the arrows point to the RF II, RFIII, and RF I forms respectively.

Lane 1: untreated ϕX174 RFI DNA; lanes 2 - 6: ϕX174 RFI DNA treated with 1.8, 5.4, 9.0, 12.6, and 16.2 Units/ml of endo-exonuclease.
plasmid DNA was heat-denatured and quick-cooled prior to electrophoresis. This treatment yielded a smear of low molecular weight DNA. When the linearized DNA was EE-treated for a longer period, there was extensive hydrolysis of the DNA (Figure 13, lane 5). In Figure 13, lane 7 it is seen that no hydrolysis of DNA occurred when the linearized plasmid dsDNA had been pre-treated with calf intestinal phosphatase before incubation with EE. Therefore, for EE to act on this linearized DNA, it required that 5'-phosphate termini be present. When the above DNA is heat-denatured and electrophoresed, unit length and a smear of slightly less than unit-length DNA is present (Figure 13, lane 8). Here, the denatured DNA appears to have renatured and the smear results from non-specific nicking and low exonuclease activity. Thus, both internal nicking and ds-exonuclease activity of EE depend on the presence of 5'-p-termini on the linear duplexes.

3.6. Attempts at cloning the A. nidulans EE gene(s)

Recombination-defective mutants of N. crassa and Saccharomyces cerevisiae, uvs-3 and rad52 respectively, have lower intracellular levels of active EE. The levels of EE in two recombination-deficient mutants of A. nidulans, uvcc and uvse were examined to determine if this was also the case in this species. If the levels of EE were indeed lower in one or both of these mutants, cloning of the A.
Figure 13: Actions of *A. nidulans* endo-exonuclease on linearized pBR322 DNA.

Superhelical pBR322 DNA was linearized with restriction enzyme EcoRI and linearized pBR322 DNA was dephosphorlyated with 2 X 1 Unit of calf intestinal phosphatase for 15 minutes at 37°C. 1 ug of DNA was treated with 22 Units/ml of enzyme in 0.1 M Tris-HCl pH 7.5 and 0.005 M MgCl₂ at 37°C for 5 minutes. The reaction was stopped by addition of excess EDTA, some samples were denatured and quick cooled, and the samples electrophoresed on 1% agarose gel. The gel was stained with 0.5 ug/ml ethidium bromide, and photographed over a UV-lamp.

Lane 1: 450 ng / DNA-EcoRI standards; lane 2: 625 ng untreated pBR322 RFI DNA; lane 3: 735 ng linearized pBR322 DNA; lane 4: same as lane 3 but denatured; lane 5: treated linearized pBR322 DNA; lane 6: same as lane 5 but denatured; lane 7: treated dephosphorylated linearized pBR322 DNA; lane 8: same as lane 7 but denatured.
*nidulans* EE gene might simply involve the complementation of the mutation.

It should be noted that in the presence of 1 mM PMSF, a larger amount of intracellular active EE was recovered from wild-type *A. nidulans* (compare Tables 2 and 6). The levels of inactive EE were also higher in the mitochondria and vacuoles.

Table 6 shows the intracellular distribution of EE found when extracts and cell fractionations were carried out in the presence of the protease inhibitor, PMSF for the wild-type and the two mutants, *uvsc* and *uvse*. In terms of the total units (active as well as inactive) of EE in the cell, there was essentially no difference between the wild-type and the two recombination-deficient mutants. *Uvse* had more active EE but less inactive EE than the other two strains. This might be accounted for by growth conditions and handling of the cells.

Therefore, the choice of cloning the *A. nidulans* EE gene by complementing either of the *rec* mutations was eliminated and other approaches were taken. Other approaches include using the yeast EE clone (made available by T. Chow) or the polyclonal antibody raised to *N. crassa* EE to probe an *A. nidulans* genomic DNA expression library with the goal of isolating a segment or segments of the *A. nidulans* EE gene.

The yeast EE clone (Pgc 10, Figure 15A) was isolated
Table 6 : Comparison of endo-exonuclease levels in the wild-type, and two recombination deficient mutants of *A. nidulans*, *uvsc* and *uvse*.

Extracts were made and cell fractionations carried out in 1 mM PMSF as described in Materials and Methods. The levels in nuclei were not determined in these experiments. Levels are reported as ssDNase activity in Units/10 g mycelia.
<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>A. nidulans strain</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>wild-type</td>
<td>uvsC</td>
<td>uvsE</td>
</tr>
<tr>
<td>Active enzyme</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vacuoles</td>
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<td>870</td>
<td>1770</td>
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<tr>
<td>Mitochondria</td>
<td>1420</td>
<td>690</td>
<td>1220</td>
</tr>
<tr>
<td>Microsomes and Cytosol</td>
<td>1440</td>
<td>2010</td>
<td>1470</td>
</tr>
<tr>
<td>Active Total</td>
<td>3530</td>
<td>3570</td>
<td>4460</td>
</tr>
<tr>
<td>Inactive enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuoles</td>
<td>210</td>
<td>240</td>
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</tr>
<tr>
<td>Mitochondria</td>
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<td>0</td>
</tr>
<tr>
<td>Microsomes and Cytosol</td>
<td>1250</td>
<td>1250</td>
<td>920</td>
</tr>
<tr>
<td>Inactive Total</td>
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<td>1490</td>
<td>950</td>
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<tr>
<td>Totals</td>
<td>5570</td>
<td>5060</td>
<td>5410</td>
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</table>
from a yeast genomic library in a yeast episomal plasmid constructed from pBR322 by probing with a partial yeast EE clone in \( \lambda \)gtll. This partial yeast EE gene was obtained by screening a yeast genomic library in \( \lambda \)gtll with the antibody raised to purified \( \textit{N. crassa} \) EE (Chow, pers. comm.). Disruption of the BglII site in the yeast insert of Pgc 10 followed by its transformation into yeast cells replacing the host gene abolished nuclease activity. The polyclonal antibody to \( \textit{N. crassa} \) EE is of high titer, is highly specific for the antigen, and completely inhibited the \( \textit{A. nidulans} \) active EE. Furthermore, it immunoprecipitated the purified active enzyme and the active precursor forms of 33 kDa, 38 kDa, and 90 kDa.

To determine the degree of homology that exists between the yeast EE and the fungal EEs at the DNA level, Southern blot analysis was performed. Total genomic DNAs from \( \textit{A. nidulans} \) and \( \textit{N. crassa} \) were restricted with the BamHI, BglII, and EcoRI endonucleases and electrophoresed on 0.8% agarose gel. DNA was transferred to nitrocellulose filter and probed with the denatured 3.4 kb \(^{32}\text{P}\)-labelled SalI-BamHI fragment of Pgc 10. This 3.4 kb fragment consists of most (75%) of the yeast insert DNA and 275 nucleotides of the pBR322 plasmid. The hybridization conditions were 6X SSC - 0.5% SDS - 5X Denhardts at 42\( ^\circ \)C for 40 hours. Washes were in 2X SSC - 0.1% SDS at room temperature and 1X SSC - 0.1% SDS at 42\( ^\circ \)C for a total of 30 minutes each. Under these
conditions (relatively weak stringency), the yeast EE probe hybridized well to *N. crassa* DNA but to a much lesser extent to *A. nidulans* DNA. In each digest, one band of *N. crassa* DNA shared homology with the yeast DNA (Figure 14, lanes 6, 7, and 8). This implies that there is only one copy of the EE gene in the *N. crassa* genome. There was less homology between the yeast EE probe and *A. nidulans* DNA (Figure 14, lanes 1, 2, and 3) since no distinct band of *A. nidulans* DNA hybridized to the yeast probe. Total yeast DNA was used as a hybridization standard (Figure 14, lane 4).

Screening more than $10^6$ recombinants of the *A. nidulans* genomic DNA *gtll* library with the same DNA fragment failed to produce any positive signals. This result correlated with above finding; since the yeast EE clone did not hybridize to *A. nidulans* DNA on a Southern blot, one would not expect any positive signals from screening an *A. nidulans* genomic library using it as probe.

An expression vector is capable of producing a polypeptide encoded by the DNA insert fragment. A DNase in *S. sanguis* necessary for repair of DNA damaged by UV-light was cloned in a *gtll* expression library using toluidine blue-O (Lindler and Macrina 1987). Toluidine blue-O, a metachromatic dye, when complexed with DNA stains blue but when complexed with nucleotides, stains pink. A clone bearing a DNase gene could in theory be isolated from the *A. nidulans* expression library on toluidine blue-O plates by
Figure 14: Southern blot hybridization of genomic DNAs of *A. nidulans* and *N. crassa* versus a 3.4 kb fragment of the yeast endo-exonuclease clone.

Genomic DNA was obtained as described in Materials and Methods, restricted with endonuclease, and electrophoresed on 0.8% agarose gel. Following electrophoresis, the DNA in the gel was denatured in 1.5 M NaCl in 0.5 M NaOH and neutralized in 1.5 M NaCl in 0.5 M Tris-HCl, pH 7.5. After transfer of DNA to nitrocellulose according to Southern, the blot was probed with a $^{32}$P-labelled fragment of the yeast endo-exonuclease clone. Hybridization was at 42°C for 40 hours in 6 X SSC - 0.5% SDS - 5 X Denhardt followed by washes at room temperature in 2 X SSC - 0.1% SDS and at 42°C in 1 X SSC - 0.1% SDS.

Lanes 1 - 3: 99 ug of *A. nidulans* DNA restricted with BamHI, BglII, and EcoRI respectively; lane 4: EcoRI-restricted yeast DNA; lane 5: DNA-EcoRI standards; lanes 6 - 8: 128 ug of *N. crassa* DNA restricted with BamHI, BglII, and EcoRI respectively.
Figure 15 A: Restriction map of the yeast clone in a pBR322-yeast hybrid plasmid (Pgc10).

On the plasmid map, an EcoRI site is at 0 bp, the unique BamHI site at 375 bps, and the unique SalI site at 650 bps. The yeast library was originally constructed by cloning into the unique BamHI of the yeast episomal plasmid (10700 bps). The BamHI sites were lost. The yeast DNA insert of Pgc10 is 4.5 kb and contains one restriction site for each of BamHI, BglII, and HindIII.

Figure 15B: Restriction map of the \gtll vector.

The letters used to denote the restriction enzyme sites are:
P: PvuI; K: KpnI; E: EcoRI. The arrow shows the direction of transcription of the lacZ operon. The A. nidulans genomic library was cloned into the EcoRI site (dotted vertical line).
looking for pink plaques on a blue background. However, of more than $10^6$ recombinants screened, none produced pink plaques. It may be concluded that the expressed fusion proteins did not have any associated nuclease activity.

The polyclonal antibody was used to attempt to isolate a segment or segments of the A. nidulans EE gene by screening an expression library of A. nidulans genomic DNA in λgt11. From $10^6$ recombinants screened, two positive plaques were isolated which upon purification, gave only positive clones (clones RT1 and RT2). DNA was extracted from each of the purified phage in order to rescue the A. nidulans DNA inserts (Figure 15B). However, restriction of both recombinant DNAs with EcoRI did not release any insert DNA. To confirm that there was indeed a piece of DNA inserted into the vector, restriction patterns of the DNA isolated from the clones were determined and compared with those of the non-recombinant vector. An agarose gel of such a comparison is shown in Figure 16; the restriction patterns of the clones were identical to each other but were different from that of the vector. It is likely that the two clones are identical and that the EcoRI cloning sites were lost in each case. From the restriction pattern, it was determined that the insert DNA is small in size (100 nucleotides) and that in this insert, there is a PvuI restriction site. Putative A. nidulans insert DNAs were extracted from low melt agarose gels, labelled with $^{32}$P and
Figure 16: Agarose gel analysis of the DNAs extracted from the phage clones restricted with endonuclease PvuI. DNA was extracted from the purified phage clones as described in Materials and Methods. The DNA was then restricted with 12 Units of PvuI overnight at 37°C. 
Lane 1: DNA-EcoRI standards; lane 2: \gtl1 vector DNA restricted with PvuI; lanes 3 and 4: DNA from \gtl1 phage clones 1 and 2 restricted with PvuI.
used as probe on Southern blot analysis of total A. nidulans DNA. However, none of the extracted DNA hybridized to the genomic DNA indicating that the DNAs isolated were not A. nidulans DNA.

Western blot analysis of the β-galactosidase fusion proteins expressed by the two clones in E. coli demonstrated that there was cross-reaction between the hybrid polypeptide and the antibody to N. crassa (Figure 17, lane 1) as well as the monoclonal antibody to β-galactosidase (not shown in Figure 17). The size of the expressed protein was determined to be 120 kDa by running an aliquot of it (Figure 17, lane 4) side by side with β-galactosidase (molecular weight of 116 kDa) on a 5% SDS-polyacrylamide gel. The hybrid protein migrated a little slower than the β-galactosidase. This is consistent with the insert DNA being a small piece.

3.7. Immunochemical reaction of antibody to N. crassa EE with the recBCD nuclease of E. coli

N. crassa EE, Saccharomyces cerevisiae EE, and E. coli recBCD nuclease all share some common enzymic properties and are deficient in recombination-defective mutants of the respective species (Chow and Fraser 1979, Chow and Resnick 1983, Chaudhury and Smith 1984). Since the yeast EE is immunocross-reactive with antibodies raised to the N. crassa EE (Chow and Resnick 1987), it was of interest to determine if there is any immunochemical relationship between the N.
Figure 17: Western blot analysis and expression of a β-galactosidase-fusion protein from the phage clone. Induction of the β-galactosidase fusion protein was as described in Material and Methods. The cells were lysed and proteins denatured in loading buffer prior to electrophoresis in duplicate on 5% SDS-polyacrylamide gel. Half the gel was stained with Coomassie blue and for the other half, the proteins were transferred to nitrocellulose and probed with a 1/500 dilution of antibody to *N. crassa* EE overnight. After washing the blot in TBS, antirabbit goat IgG conjugated to alkaline phosphatase was added and incubated for 1.5 hours. Cross-reactive polypeptides were visualized by developing with the alkaline phosphatase substrates, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT).

Lanes 1 and 2 are from the Western blot and lanes 3 and 4 are from the Coomassie blue stained gel. Lanes 1 and 4: lysate containing fusion protein; lanes 2 and 3: 10 µg of prestained molecular weight protein markers (BRL). Molecular weights of marker proteins are indicated in kDa on the right. The arrowhead indicates the position of the β-galactosidase fusion protein.
crassa EE and the recBCD nuclease (exonuclease V). Mutants of recBCD were made available to us and are summarized in Table 7. The V186 strain contains a deletion of the entire genomic region between thyA and argA which includes all the recB, recC, and recD genes. The other strains are derived from the V186 strain and harbour multicopy plasmids carrying either all the recBCD genes or deletions of one or more of these genes. Briefly, V682 lacks entirely the recB and recD genes, V182 has all of the recBCD genes and this has a 'wild-type' genotype, while V653 also has all of the recBCD genes except that it contains the recB21 allele which is an insertion that reduces the expression of the recD gene product.

The above-mentioned strains were streaked onto LB-agar plates, irradiated with UV-light in the dark, immediately covered with aluminium foil, and incubated at \(37^\circ C\) overnight. Table 7 summarizes the relative UV sensitivities of these strains. These results confirm the lack or presence of the recB, recC, and/or recD genes.

Figure 18 shows a Western blot of lysates of four of the above strains probed with antiserum raised to N. crassa EE. Two immunoreactive polypeptides of 175 kDa and 130 kDa are present in the lysates of each of the strains bearing plasmid (lanes 2, 3, and 4) while only the 175 kDa polypeptide is present in the lysate of the deletion mutant. The Western blots were performed three times, each with
Table 7: Bacterial strains used to study immunochromatographic relationship between *N. crassa* EE and the recBCD enzyme

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Genotype</th>
<th>Relative UV Sensitivity</th>
<th>Source and Reference</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V186</td>
<td>Δ(thyA-argA)232</td>
<td>++</td>
<td>G.R. Smith</td>
</tr>
<tr>
<td>V182</td>
<td>Δ(thyA-argA)232(pDWS2)</td>
<td>+</td>
<td>G.R. Smith</td>
</tr>
<tr>
<td>V653</td>
<td>Δ(thyA-argA)232(pSA21)</td>
<td>++</td>
<td>&quot;</td>
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<tr>
<td>V682</td>
<td>Δ(thyA-argA)232(pAFT2)</td>
<td>+++</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pDWS2</td>
<td>thyA⁺-argA⁺ (recBCD⁺)</td>
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<td>pSA21</td>
<td>thyA⁺-argA⁺, but recB21</td>
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</tr>
<tr>
<td>pAFT2</td>
<td>thyA⁺-recC⁺, (Δ(recB-argA))</td>
<td></td>
<td></td>
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</table>
Figure 18: Western blot of lysates of recBCD deletion mutants probed with antibody against *N. crassa* endo-exonuclease.

The lysates were electrophoresed on 5% 1.5 mm thick polyacrylamide gel at constant current and the separated polypeptides transferred to nitrocellulose at constant current of 0.7 amps for 1.5 hours in 25 mM Tris-base-192 mM glycine and 20% methanol. The blot was blocked in 5% skim milk - 0.02% Na-azide in 10 mM Tris-HCl pH 7.5-1 mM EDTA - 150 mM NaCl (TBS) and incubated with 1/500 dilution of antibody in the blocking buffer overnight. Following washes in TBS, the blot was incubated in anti-rabbit goat IgG conjugated to alkaline phosphatase for 2 hours. After washing in TBS, the blot was developed with the alkaline phosphatase substrates, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT).

Lane 1: strain V186; lane 2: strain V682; lane 3: strain V653; lane 4: V182 (See Table 7 for their genotypes).

Molecular weights of marker proteins are indicated in kDa on the right. The arrowhead indicates the position of the immunoreactive 130 kDa polypeptide.
different lysates and each with the same result. The three plasmid-bearing strains tested which all yield the 130 kDa cross-reactive polypeptide have in common an intact recC gene whose protein product has a known molecular weight of 130 kDa. Unfortunately, the recC<sup>-</sup> strain (V683) sent to us was no longer viable and it was not possible to further confirm the above result by testing this strain.

When lysates of the 'wild-type' strain, V182 were assayed for exonuclease activity using E. coli <sup>3</sup>H-dsDNA in the presence and absence of 250 μM ATP, an ATP-stimulated activity was found. This activity was not inhibited by incubation of the lysate with a 1:4 dilution of antibody against <i>N. crassa</i> EE prior to the nuclease assay (data not shown).

The yeast mitochondrial enzyme also showed cross-reaction with the antibody to <i>N. crassa</i> EE and the gene for this enzyme, <i>NUC1</i>, has been cloned and sequenced (Dake et al. 1988, Vincent et al. 1988). Its predicted amino acid sequence was therefore aligned with the predicted sequence of the recC polypeptide (Finch et al. 1986). Extensive homology was found M.J. Fraser in this laboratory by aligning 306 of the 329 amino acids of the yeast mitochondrial nuclease sequence with the carboxy-terminal one-quarter of the amino acid sequence of the recC polypeptide (data not shown). No significant homology was found between the amino acid sequence of the yeast
mitochondrial nuclease and those of the recB and recD polypeptides. Thus, it seems likely that the nuclease active site(s) of the recBCD enzyme will be found localized entirely in the C-terminal region of the recC polypeptide.
Discussion

In terms of the total amount of active and inactive EE (Units/10 g mycelia), there was 20% of each of these forms of EE in A. nidulans as in N. crassa but the intracellular distributions were similar (Fraser and Cohen 1983). In both organisms, the majority of active EE was found in mitochondria and vacuoles, while most of the inactive form (TAEE) was found in the cytosol fraction. In A. nidulans, a relatively large amount of active EE was found in the microsomes fraction. Unlike N. crassa, no TAEE was detected in A. nidulans mitochondria. In yeast, a 72 kDa nuclease that is inhibited by the antibody raised to N. crassa EE has been isolated, but no precursor has been detected. Similarly, no precursor was detected for the 38 kDa yeast mitochondrial nuclease, nor could one be predicted from the amino acid sequence of the cloned yeast mitochondrial nuclease gene (Dake et al. 1988, Vincent et al. 1988). The low amount of TAEE found in A. nidulans mitochondria and nuclei may result from proteolytic activation during extraction and handling since this readily occurred in extracts of N. crassa mitochondria. Addition of the protease inhibitor, PMSF, during extraction of organelles resulted in the recovery of higher amounts of TAEE from the mitochondria (Table 6).

In A. nidulans, 70% of the trysin-activatable ssDNase
in the cytosol fraction (as compared to 60% of the N. crassa TAEE) was immunoprecipitated by the antibody to N. crassa EE. In N. crassa, two forms of TAEE were present in the cytosol that differed in their immunochemical reactivity (Fraser et al. 1986). These forms and the different forms found in A. nidulans cytosol may represent precursors of the nuclear and mitochondrial EE. It is possible that the two precursor forms are structurally different so that the antibody raised to mitochondrial and vacuolar EE was capable of recognizing only the mitochondrial EE precursor. Trypsin treatment of the cytosol fraction resulted in immunoprecipitable active polypeptides of 90 kDa and 28 kDa on SDS-ssDNA activity gels. This result indicated that the precursor was larger than 90 kDa and that the protease-resistant nuclease core was 28 kDa, the same size as the purified EE.

The chromatographic profiles of A. nidulans EE were very similar to those seen previously with N. crassa EE (Chow and Fraser 1983). Neurospora EE was eluted with 0.07 to 0.20 M NaCl from DEAE-Sepharose and with 0.50 to 0.80 M NaCl from ssDNA cellulose, as compared respectively to elutions with 0.04 to 0.26 M and 0.33 to 0.50 M for the Aspergillus EE (Figure 3). In addition, a weaker-binding peak of EE activity was also eluted from the ssDNA cellulose column, but in this case, it contained many contaminating proteins as seen on silver-stained SDS gels (data not
shown). In both organisms, the weaker-binding pool may contain EE with minor modifications that affect its binding to ssDNA, its activity, and its immunochromical reactivity with the antibody raised to EE. Chromatography of the sonicate of the mixture of small vacuoles and mitochondria on DEAE-Sepharose resulted in loss of 52% of the nuclease activity. This is a crude fraction containing many contaminating proteins including proteases. The loss in nuclease activity may be attributed to the actions of these proteases. Another factor that may play a role in this loss is the affinity of the *A. nidulans* EE for DEAE-Sepharose. A fraction of the Mg$^{2+}$-dependent nuclease activity passed through the DEAE-Sepharose column. It may be that the *A. nidulans* EE differs structurally from the *N. crassa* EE and therefore has a lower affinity for this column.

The *A. nidulans* EE has been purified to near homogeneity from a mixture of mitochondria and small vacuoles by successive chromatography on DEAE-Sepharose and ssDNA cellulose. Its specific activity is calculated to be 60500 Units/mg protein although it was difficult to determine with accuracy the low amount of protein in the purified preparation. In comparison with the *N. crassa* enzyme, there was a much lower yield of the *A. nidulans* EE. A possible explanation is that the extraction protocols used were developed for *N. crassa* and not *A. nidulans*. It has been observed that for the same weight mycelia, twice as
much DNA was extracted from *N. crassa* than from *A. nidulans*. This may be attributed to the greater difficulty of disrupting the outer cell wall of *A. nidulans*.

In the presence of the protease inhibitor, PMSF, an immunoprecipitable 90 kDa active polypeptide was seen by SDS-ssDNA activity gel analysis of the crude extract. On the other hand, active polypeptides of 38 kDa and 33 kDa were present in the sonicate of the 25000 X g pellet. In *N. crassa*, SDS-ssDNA activity gel analysis revealed active polypeptides of 88 kDa and 76 kDa in the crude extract and of 66 kDa and 43 kDa in sonicates of the 25000 X g pellet (Fraser et al. 1986). It is possible that proteolysis is occurring at a much faster rate in *A. nidulans*. Polypeptides of 90 kDa, 76 kDa, 43 kDa, 38 kDa, and 33 kDa were also intermediates in the processing of TAEE to EE in *A. nidulans*. The smaller proteins of 38 kDa and 33 kDa have fewer protease cleavage sites available by virtue of their size. As a result, they are less susceptible to cleavage than the 76 kDa and 43 kDa proteins. Thus, in both organisms, rapid proteolysis occurred during extraction and purification of EE with the products of these proteolytic actions retaining their activity. By running the *A. nidulans* and *N. crassa* EEs side by side on a SDS-ssDNA activity gel, it was demonstrated that both preparations of purified enzyme contained active polypeptides of 28 kDa (Figure 5, Panel B).

Besides having identical polypeptide molecular weights,
the A. nidulans EE and N. crassa EE share many other properties in common. Both enzymes act on ssDNA, dsDNA, and RNA in a pH optimum range between 7.5 and 8.0. The divalent metal cations Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$ were utilized in both cases as cofactors for ssDNase activity (Table 5). In the case of the A. nidulans EE, Mn$^{2+}$ was a better cofactor than Mg$^{2+}$ for ssDNase activity, while the reverse was true for the N. crassa EE (Chow and Fraser 1983). Maximal ssDNase activity was detected only in a narrow concentration range of Zn$^{2+}$ (10$^{-6}$ to 10$^{-5}$ M) in both cases. Higher than optimal concentrations of Zn$^{2+}$ and Mn$^{2+}$ inhibited the ssDNase of both enzymes. The Ca$^{2+}$ cation did not serve as cofactor for the ssDNase or dsDNase activities of either enzyme but in the presence of Mg$^{2+}$, Ca$^{2+}$ stimulated the dsDNase of both fungal enzymes (Table 5).

The immunotitration curves for the two EEs with increasing dilutions of antibody raised to purified N. crassa EE were virtually identical. In addition, 1 unit of the N. crassa EE-specific inhibitor inhibited 1 unit (ssDNase activity) each of N. crassa EE and A. nidulans EE. Together with all of the above results, these two observations imply that the two enzymes are very similar in structure.

The products of digestion of ss and dsDNA released by the A. nidulans and N. crassa EEs were identical. Both give 5'-phosphoryl, 3'-hydroxyl terminated di-, tri-, and
tetranucleotides. With ssDNA as substrate, both EE s showed a mixed endo- and exonucleolytic mode of degradation, while dsDNA was degraded exonucleolytically by EE. The actions of the two EE s on superhelical OX174 DNA and linear pBR322 DNA were also very similar. With superhelical DNA, both enzymes made a nick initially in one strand yielding the relaxed form. A second nick was then introduced into the other strand at a site close to the first nick generating a linear molecule. This linear molecule was then rapidly degraded to shorter fragments. It has been demonstrated that N. crassa EE in the presence of low Mg\(^2+\) made site-specific nicks in linear pBR322 DNA and that this site-specific nicking required a 5' -phosphate at the terminus (Fraser and et al. 1989). It has been shown here that A. nidulans EE made ss-breaks (nicks) in linear pBR322 DNA which also required 5' -p-termini on the dsDNA. However, the rates of hydrolysis of pBR322 DNA by the two enzymes differed; the A. nidulans EE hydrolyzed ss and dsDNA quicker than that of N. crassa. In summary, the EE purified from A. nidulans had the same activities, very similar properties, and produced the same products as the well-characterized N. crassa EE. The two enzymes also showed immunochemical identity.

Several strategies for cloning the A. nidulans EE gene were available. The simplest was complementation of a mutation that resulted in a recombination deficiency. However, in two rec\(^-\) strains of A. nidulans, normal
intracellular levels of EE were found. Since EE has been implicated to play an important role in recombination and recombinational repair, it is possible that EE is required for cell survival. In yeast for example, disruption of the nuclear EE gene was found to be lethal (T. Chow, pers. comm.) and therefore recovery of null mutants was not possible. Therefore, nuclear EE plays an essential role in the yeast cell. However, recBC- mutants of Escherichia coli show only a partial reduction in viability (Smith 1988). A. nidulans mutants lacking EE might not be viable although mutants with lower levels of active EE, like the uvs-3 mutant of N. crassa, might be found.

The 3-phosphoglycerate kinase (PKG) gene from A. nidulans was cloned by using the corresponding cloned yeast gene as hybridization probe (Clements and Roberts 1985). This gene differed from the yeast gene in having a 57 basepair intron. By analogy, the isolation of the yeast nuclear EE gene made it possible to clone the related A. nidulans gene in a similar manner. Southern blot analysis of total A. nidulans genomic DNA with the yeast nuclear EE clone showed that a copy of the A. nidulans EE gene existed but that there was only weak homology between these organisms with respect to this gene, although homology with Neurospora DNA was much stronger (Figure 14). One factor that might contribute to this weak hybrization could be the presence of introns in the A. nidulans EE gene. In contrast,
most if not all genes isolated so far from \textit{S. cerevisiae} have not been found to contain introns. One way to overcome the problem caused by introns is to perform Northern blot analysis of \textit{A. nidulans} total or messenger RNA with the yeast EE clone as probe. In addition, the results should indicate the size and number of transcripts made.

It was not surprising to find no positive clones during the screening of the \textit{A. nidulans} genomic DNA library in the \textit{\lambda gt}11 vector given the results of the Southern blot. It was shown (see Results) that in terms of total protein, EE was present in small amounts in mycelia of \textit{A. nidulans}. It is possible that the entire \textit{A. nidulans} genome is not represented in the \textit{\lambda gt}11 library.

The attempt to clone the \textit{A. nidulans} EE gene by looking for DNase activity expressed by \textit{\lambda gt}11 clones that show up as pink plaques on toluidine blue-O stained plates was unsuccessful. This approach not only required a transcript with in-frame codons to allow for synthesis of the proper nuclease polypeptide but also that an intact active site in the protein be produced. It was highly improbable that expression of an active nuclease polypeptide by the \textit{\lambda gt}11 clones occurred.

That the active and inactive forms of EE in \textit{A. nidulans} were recognized and immunoprecipitated by the antibody raised to \textit{N. crassa} EE made it feasible to screen the above \textit{\lambda gt}11 library with the antibody as probe. However, the
cross-reactive clones isolated did not contain the EcoRI sites into which the A. nidulans DNA was originally cloned. These sites may have been lost during the construction of the library or during phage recombination. The presence of an inserted piece of DNA and cross-reaction of the expressed β-galactosidase fusion protein with the antibody to N. crassa EE has been demonstrated (see Results). The size of the inserted DNA was estimated to be 100 nucleotides, enough to code for 33 amino acids. Since antibodies recognize antigenic sites in the form of tertiary rather than primary structure, the above 33 amino acids was sufficient to generate structures recognized by the antibody. However, the rescue of the small piece of insert DNA was not successful.

Sequencing of the recombinant and non-recombinant vectors from a site within the lacZ gene in both directions would give the nucleotide sequence of the inserted piece of DNA. The A. nidulans sequence so obtained may then be used as a probe to screen other A. nidulans DNA libraries with the goal of obtaining a full-length clone. Protein sequencing of a portion of the purified A. nidulans EE would allow for the synthesis of an oligonucleotide that may also be used as a probe. Due to the existence of introns in the A. nidulans genome, a cDNA library is preferred for gene cloning.

An alternative to cloning the A. nidulans EE gene is to obtain a clone of the N. crassa EE gene by screening an N.
crassa genomic library. Southern blot analysis has demonstrated that there is more homology between the yeast and the N. crassa EE genes. The A. nidulans gene should have greater homology with that of N. crassa than that of yeast. The N. crassa EE gene may then be used to screen an A. nidulans DNA library to isolate the corresponding EE gene.

Isolation and sequencing of the A. nidulans EE gene would answer questions concerning its biosynthesis, role, and regulation in the cell. Whether one or more genes in A. nidulans code for the mitochondrial, nuclear and vacuolar enzyme may be determined by Northern blot analysis of messenger RNA with the isolated EE clone. It is likely that one gene encodes all three proteins since only one band cross-hybridized to the yeast EE clone on Southern blot analysis. The yeast cytoplasmic and mitochondrial histidine tRNA synthetases are encoded by one gene, the HST1 gene (Natsoulis et al. 1986). Two transcripts of different lengths are generated by the use of two different initiator codons. It may be that, in A. nidulans, three different messenger RNAs are transcribed from one EE gene.

In DNA-containing organelles, nuclei and mitochondria EE may play a role in recombination and recombinational repair. A. nidulans EE was able to act on superhelical DNA to produce ss- and ds-breaks (Figure 12). Ds-DNA with ss-tails and long ss-gaps may also be generated. These products are potential substrates for recombination. In addition, A.
nidulans EE possess properties similar to and therefore belongs to a distinct class of nucleases that have been implicated in recombination and recombinational repair, such as the EEs of N. crassa and S. cerevisiae. Lower levels of the E. coli recBCD enzyme, the 72 kDa yeast nuclear EE, N. crassa EE, and Ustilago nuclease α were found in recombination-defective mutants of their respective organisms.

A component of the recBCD enzyme, the recC polypeptide, the yeast nuclear EE, and the 38 kDa yeast mitochondrial EE have all been shown to be related immunochemically to the N. crassa EE. No immunochemical cross-reaction was seen with other fungal and plant nucleases with sugar non-specific but ss-specific endonuclease activity such as the S1 nuclease of A. oryzae, the P1 nuclease of P. citrinum, and the mung bean nuclease. Here, it was demonstrated that the A. nidulans EE cross-reacted with the antibody to N. crassa EE. It may be concluded that all these polypeptides share some homology. In fact, high homology has been found between the yeast mitochondrial nuclease and the carboxy-terminal end of the recC polypeptide. It has not been determined if nuclease 1 of Ustilago is related to EE of N. crassa. Nuclease 1 is similar in catalytic properties to the ss-endonuclease isolated by Linn and Lehman; no exonuclease activity has been detected. It is possible that the portion of the polypeptide with the exonuclease activity was
proteolytically cleaved as is the case in *N. crassa*. Active EE is also present in the vacuoles where it may be involved in the turnover of DNA and RNA.

Finally, success in cloning and sequencing the *A. nidulans* EE gene would shed some light on a number of problems involving EE structure and function. For example, the *A. nidulans* EE gene could be mutagenized and transformed into wild-type cells to replace the host gene with the mutagenized copy (Ballance et al. 1983). In this case, survival of cells would demonstrate whether or not EE is an essential enzyme. In addition, the sequence of the cloned *A. nidulans* EE gene might help resolve the debate of whether TAEE is an inhibitor-active EE complex or a common precursor from which both proteins are derived. In *A. oryzae*, a 22 kDa inhibitor of nuclease O strongly resembling the *N. crassa* 24 kDa EE-specific inhibitor has been purified. The inhibitor forms a 1:1 complex with nuclease O, which is likely identical to EE as discussed earlier. At present, immunochemical studies with antibodies raised to the *N. crassa* inhibitor (Z. Hatahet, D. Ramotar, and M.J. Fraser, unpublished), favor a common origin for the EE and its inhibitor. Thus, site-directed mutagenesis studies on the isolated *A. nidulans* EE gene could not only answer questions concerning the structure and function of EE but also questions concerning its regulation.
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