THE MAJOR INTRACELLULAR ALKALINE DEOXYRIBONUCLEASE ACTIVITIES EXPRESSED IN WILD-TYPE AND REC-LIKE MUTANTS OF NEUROSPORA CRASSA

by

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ABSTRACT

Mutants of Neurospora crassa with the nuh phenotype were tested for mitomycin C and L-histidine sensitivities. It was found that the several mutants with wide spectra of sensitivities to mutagens (uvs-3, uvs-6, nuh-4, and mms(04)), one indication of a Rec phenotype as found for rec mutants of E. coli, were sensitive to mitomycin C and histidine. However, excision-repair deficient mutants which have narrower spectra of sensitivities to mutagens (uvs-2 and upr-1) were not sensitive to these agents.

The expressed and total single-strand deoxyribonuclease (ss-DNase) (after activation with trypsin) activities in extracts of wild-type and mutants log-phase mycelia were determined. The expressed ss-DNase activities of two of the Rec-like mutants were 33% (in uvs-3) to 60% (in nuh-4) of that in wild-type. The total ss-DNase activity of these mutants, however, were the same as wild-type. The expressed double-strand activity was very low in both the wild-type and Rec-like mutants. No differences in total ds-DNase activities were observed.

Over 90% of the expressed ss-DNase in extracts of the wild-type was found to be associated with three nucleases which were isolated by chromatography in turn on DEAE-Sepharose and on Phosphocellulose. All three nucleases showed relatively high activities with ds-DNA as well as with RNA. Two of these nucleases (D1 and D3') had properties very similar to Neurospora endo-exonuclease, an enzyme isolated previously from mycelia. Aged D1 preparations, however, had properties very similar to D2, an enzyme very similar to the Neurospora mitochondrial nuclease. A conversion of D1 to D2 in vitro was shown to occur as D1 aged. The molecular weights of D1, D2, D3' as determined from surcose density gradient centrifugation were

180000, 75000, and 75000 daltons respectively. Two of the Rec-like mutants, uvs-3 and nuh-4, which have been examined, were found to be deficient mainly in the endo-exonuclease while a nuclease deficient but not Rec-like mutant, nuh-3, was found to be deficient in mitochondrial nuclease.

Evidence for the existence of ds-DNase inhibitor(s) is presented, which accounts, at least in part, for the masking of ds-DNase activities in crude extracts. When extracts were treated with trypsin, the ds-DNase activity was found to be 4-fold higher than that of the ss-DNase activity; and in an aged D1 plus D2 fraction (isolated from DEAE-cellulose chromatographic column yielded a protein fraction in the pass-through which specifically inhibits the ds-DNase activity of nucleases from the same column.

RÉSUMÉ

L'action de la mitomycine C et de l'histidine a été mesurer sur des mutants de Neurospora crassa ayant le phénotype Nuh. Nous avons trouvé que plusieurs mutants (uvs-3, uvs-6, nuh-4 et mms(04)) sensibles à un large spectre de mutagènes, le phénotype associé à la sensibilité à un large spectre de mutagènes peut être du type Rec tel qui observé chez le même mutant de E. coli, l'étaient également à la mitomycin C et à l'histidine. Par contre, les mutants uvs-2 et upr-1 deficients au niveau de la réparation dans excision et ayant un spectre de sensibilité aux mutagenes plus restreint par ces deux agents, ne sont pas affectés.

Nous avons déterminé les activités intrinsèques et totale (apres activation à la trypsine) d'extraits de mycélium en phase logarithmique chez des types soit mutants soit original. L'activité intrinsèque de la nucléase de la ss-DNase de deux mutants de type Rec est diminuée de 33% (chez uvs-3) à 60% (chez nuh-4) par rapport au type original. Par contre, l'activité totale de cette enzyme chez ces mutants est comparable à celle du type original. Chez ces mutants, l'activité intrinsèque de la ds-DNase est faible quoiqu' à un niveau seniblable au type original. La ds-DNase totale reste inchangée dans les mutants.

Plus de 90% de la ss-DNase intrinsèque d'extraits du type original est associée à trois nucléases isolées par chromatographie successivement sur DEAE-Sepharose et phosphocellulose. Toutes les trois nucleases sont relativement très actives avec le ds-DNA ou le RNA. Deux de ces nucleases (D1 et D3') ont des propriétés très semblables à l'endo-exonuclease se de Neurospora, enzyme déjà isolée de mycélia. D'autre part, de vieilles préprations de nucléases D1 ressemblent a la D2

(nouvellment preparté) et sont presque simflaires a la nucléase mitochondriale. Nous croyons qu'il y a transformation de D1 en D2 avec le temps. A l'aide de la centrifugation sur gradient de sucrose, en utilisant l'hémoglobine bovine comme marqueur, nous avons trouvé des poids moléculaires de 180000, 75000 et 75000 daltons respectivement pour D1, D2 et D3'. Deux des mutants de type Rec examines soient uvs-3 et nuh-4, sont déficients surtout en endo-exonuclease alorsque le nuh-3, un mutant défférent du type Rec, est a la fois déficient en nuclease et en l'enzyme oriqinant des mitochondrées.

Nous avons demoutre l'existence d'un ou d'inhibiteur(s) de la ds-DNase que serait (ent) responsables du masquage de la ds-DNase dans des extraits non purifiès. Nous trouvons 4 fois plus de ds-DNase que de ss-DNase par un traitement à la trypsine; et dans une vieille fraction de D1 et D2 (isolée pars chromatographie sur DEAE-Sepharose, un repassage sur colonne de DEAE-cellulose révèle dans le volume d' exclusion une fraction qui inhibe spécifiquement l'activité ds-DNase des nucléases obtenues de la même séparation.

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LIST OF ABBREVIATIONS

U.V. or UV	Ultraviolet
ss	single-strand
ds	double-strand
DNase	deoxyribonuclease
RNase	ribonuclease
ss/ds	activity ratio towards single-stranded and double-stranded DNA
KPB	potassium phosphate buffer
Tris	tris (hydroxymethyl)-aminomethane
EDTA	ethylenediamine-tetra-acetate
PMSF	phenylmethyl sulphonyl fluoride
SBI	trypsin soybean inhibitor
DEAE-cellulose	O-(diethylaminoethyl)-cellulose
DEAE-Sepharose	O-(diethylaminoethyl)-Sepharose

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CHAPTER I

1.1 Introduction

1.1.1 Deoxyribonucleases in General

Deoxyribonucleases (DNases) are involved in many important cellular processes. They participate in the repair of damaged DNA, initiation and "proof reading" in DNA synthesis, genetic recombination, packaging of viral genomes, restriction of viral DNA, and many more functions. As a result of these various functions, mutations which affect these enzymes yield cells highly susceptible to a variety of environmental factors. These mutations are often lethal to the cells despite the presence of other unaffected nucleases in the same cells.

DNases have been generally divided into 2 classes based on their modes of action: (i) exonucleases, which requires a 3' or 5' unphosphorylated or phosphorylated terminus and proceed unidirectionally either from 3' to 5' or 5' to 3' by consecutively releasing mononucleotides from the polynucleotide chain. (Most exonucleases, with the exceptions of exonuclease VII of Escherichia coli (14), Bacillus subtilis exonuclease (15), and recBC nuclease of E. coli (16, 17), do not have the ability to excise DNA from both directions); (ii) endonucleases, which do not require a terminus and split internal phosphodiester linkages of the polynucleotides releasing oligonucleotides and, in some cases, mononucleotides as well (The Staphylococal nuclease (18) and the Neurospora crassa endonuclease (3) are two endonucleases which release both oligo-and mononucleotides). However, nucleases which possess both endonuclease and exonuclease activities do exist. This type of nuclease is represented by the RecBC nuclease of E. coli (19, 20) and Neurospora crassa endo-exonuclease (10, 7).

Nucleases can also be classified according to their sugar specificity. They may be specific ribonucleases (RNases) which attack only RNA or

specific deoxyribonucleases (DNases) which attack only DNA or they may be sugar non specific, capable of attacking both DNA and RNA. In the latter case, the term "nuclease" is applied. This classification can be further subdivided according to their preference for ordered or non-ordered structures. Thus, they may be single-strand (ss) specific or double-strand (ds) specific nucleases or they may lack strand specificity. For example, Neurospora endo-exonuclease, one of the enzymes of major concern in this thesis, has been shown (10) to be a nuclease (acting on both DNA and RNA) with two distinct activities associated with a single polypeptide, a sugar non-specific single-strand specific endonuclease activity and an exonuclease activity with DNA only which is not strand specific (acting on ss-DNA and ds-DNA but not on ss-RNA). The nomenclature for such enzymes is still a matter for discussion (91).

1.1.2 Involvement of nucleases in DNA repair process

Cellular repair of damage to macromolecules is known only for DNA. In no other instance is the integrity of a single molecule so vital to the survival of the cell. It is not surprising that the living cell has evolved processes for restoring the integrity of DNA and removing lesions introduced into it by environmental physical and chemical agents, and nucleases are an integral part in this device.

The excision-repair pathway for removal of pyrimidine dimers from DNA has been widely studied, especially in E. coli. It is the principal dark-repair mechanism for removal of dimers from one of the DNA strands and subsequent replacement with correctly base-paired nucleotides. The first step in excision-repair is incision, a single endonucleolytic break (or "nick") at or near the lesion, usually on the 5' side of the dimer

(22). In E. coli, this process is carried out by correndonuclease II (23). This enzyme is specific for UV-irradiated DNA in vitro, and produces a single-strand break with a 3'-OH terminus 5' to the pyrimidine dimer. An identical enzyme has also found in Micrococcus luteus (24). In E. coli, mutants in the two genes, uvrA and uvrB, result in the loss of correndonuclease II activity. These two genes have been identified as the structural gene for correndonuclease II (23). T4-endonuclease V, which is induced when T4 infects E. coli, is another UV-damage-specific endonuclease (25, 26, 29). Like correndonuclease II, it nicks at the 5' side of the lesion producing 3'-hydroxyl and 5'-phosphoryl termini. Cells (group A) of patients with the rare human genetic disease, Xeroderma Pigmentosum, which is characterized by extreme sensitivity to sun-light, have been shown to be deficient in the incision step of excision repair (27). The XP cells in culture, when treated with T4-endonuclease, following permeabilization by treatment with Sendai virus, show a marked increase in repair replication (28).

The 3'-OH, 5'-P nick which is generated by correndonuclease II is used by DNA polymerase I to start nick translation and to excise the pyrimidine dimer. The undamaged strand is used as template with the 3'-OH end of the nicked strand as primer for the nick translation nucleotide polymerizing step. The excision of the dimer in the 5'-terminated strand (other side of the nick) is accomplished by action of the 5'→3' exonuclease of the DNA polymerase I, or, alternatively by the action of another enzyme, exonuclease VII (30). In vitro, exonuclease, VII produces oligonucleotides progressively from either the 3'- or the 5'-termini (31) i.e. it is an endo-exonuclease. The nick remaining after DNA polymerase I action is sealed by polynucleotide ligase.

Excision-repair mechanisms also function in repairing damage in DNA caused by alkylating agents. In this case, the excision is of a base rather than of nucleotides (dimers). The modified bases are removed by the action of various specific DNA-glycosylases, with the consequent formation of apurinic or apyrimidinic sites (32). These apurinic or apyrimidinic sites are recognized and cleaved either by endonuclease VI-exonuclease III (33, 34), coded by the Xth gene, or by endonuclease IV (83) which has no associated exonuclease activity. An enzyme like endonuclease VI-exonuclease III is also found in Hemophilus influenzae (35). The gaps resulting from the action of this type of enzyme is filled and sealed by the consecutive actions of DNA polymerase I and polynucleotide ligase as described above.

DNA molecules that have been damaged in both strands even in the same region can be repaired, after replication, through recombination with homologous molecules. The two post-replication DNA helices have been found to contain long gaps opposite pyrimidine dimers (1000-2000 nucleotides long) which are substrates from the recombination enzymes. DNA in which double-strand breaks have been induced by X-rays or DNA with chemically induced inter-strand cross-links also appear to be substrates for the recombination process. The Rec-genes in E. coli control this type of repair. In general, Rec$^-$ mutants are sensitive to a wide spectrum of mutagenic agents, unlike the narrow spectrum of mutagens to which excision-repair mutants are sensitive (36). Exonuclease V of E. coli, the product of the RecB and RecC genes (37) is believed to account for 95-99% of the recombination in that organism. It catalyzes three classes of reactions : ATP-dependent exonucleolytic degradation of single- and double-stranded DNA; ATP-stimulated endonucleolytic digestion

of single-stranded DNA (including closed circular ss-DNA); and DNA-dependent hydrolysis of ATP to form ADP + Pi (17). The involvement of this enzyme in genetic recombination and DNA repair has been implied by a loss of the enzyme activity in RecBC mutants with a concomitant decrease in recombination frequency and increases in mutagenic agent sensitivity (38). The ATPase requires the presence of a polydeoxyribonucleotide, but it need not be coupled to DNA hydrolysis. The reaction proceeds with the nondegradable polymers such as RNA-DNA hybrid molecules and duplex DNA with inter-strand cross-links (19, 40). In the digestion of duplex DNA, the action of exonuclease V is processive; the enzyme is able to act both 3' to 5' and 5' to 3'. During the degradation, the enzyme binds to the termini of both strands of a DNA duplex end and begins to unwind the DNA by tracking down one strand while remaining bound to the terminus of the other. After tracking several hundred nucleotides the long piece of ss-DNA is cleaved and released, leaving a duplex molecule with a long ss-tail. The enzyme can then switch strands and degrade the ss-tail. This leaves a shortened duplex molecule. Winder (41) suggested that ATP hydrolysis is required for DNA unwinding and perhaps for the tracking mechanism. During repair, the enzyme might initiate its action at a preformed gap or large distortion. It could remove or unwind a larger single-stranded piece of DNA, and the resulting large gap or unwound single-stranded DNA "tail" could then initiate a recombination event (strand-switching between DNA duplexes). The single-stranded DNA which generated by the action of exonuclease V is protected by the RecA gene product protein X (43). In presence of protein X, a DNA-binding protein, single-stranded DNA is resistant to both endo- and exonucleolytic activitives of exonuclease V (42). Similar enzymes,

like exonuclease V, have been found in Diplococcus pneumoniae (44), Haemophilus influenzae (45), and Bacillus subtilis (46), but have not been detected in eukaryotes.

A minor recombination pathway independent of the pathway of recombination involving the RecB and RecC genes pathway has been purposed by Horii and Clark (80). This minor pathway is called the RecF pathway after the RecF gene, and also involves the sbcB gene, its product of which is exonuclease I (84). This enzyme degrades single-stranded DNA from 3'-OH termini (85). In $RecB^-$ $RecC^-$ strains it is the inactivation of exonuclease I (Exo I) by sbcB mutations which leads to recombination ability, while the presence of functional Exo I results in recombination deficiency. The independence of the RecBC pathway and the RecF pathway was shown from the fact that RecF mutations did not block recombination by the RecBC pathway, which occurred in $recB^+$ $recC^+$ $sbcB^+$ (or $sbcB^-$) strains, but they did block recombination by the RecF pathway in a $recB^-$ $recC^-$ $sbcB^-$ strain.

"SOS" repair is another dark-repair process which has been shown to be responsible for mutagenesis in E. coli (86). It requires functional recA and lexA genes. The recA gene product, protein X (43), is induced in SOS repair. The lexA gene product is believed to be a repressor of the recA gene. Sedgwick et al (87) purposed that the lexA gene product, together with the recA gene product, forms a complex. This complex of recA-lexA protein is the functional repressor for the recA gene. Gudas and Parder (47) suggested that a product of DNA degradation by exonuclease V may be an activator of protein X, which when " activated", it destroys the lexA product thus de-repressing recA. The destruction of lexA protein may occur because of the proteolytic activity of protein X (70) since it has been shown to hydrolyze the λ repressor protein. The absence of activator will

prevent protein X from exhibiting protease activity and the lexA protein will remain intact. Thus, recA is self-regulated. The view that DNA degradation is involved in the protein X induction has been challenged by Hanawalt (48). He has shown that intracellular degradation of unmodified phage DNA by restriction is not sufficient to induce protein X. A simple DNA degradation is unable to induce protein X. Boyce et al (69) also showed that ultraviolet induction of protein X is independent of the recB mutation. Thus, a more complex system rather than DNA degradation is involved in protein X induction. But in spite of the finding by Hanawalt (48), Boyce et al. (69) showed that a phage λ gene controlling λ-exonuclease can restore the missing function for the induction of protein X in recB⁻ mutants, which is evidence for that DNA degradation is required to derepress the recA gene. A model (49) has been proposed in which all of the SOS functions are derepressed by a common mechanism namely by proteolytic cleavage of the repressor(s) controlling the "SOS" operon(s). DNA degradation, an initial consequence of DNA damage or of a stalled replication fork, may provide a primary danger signal which is amplified through the action of the recA protein (protein X) resulting in the expression of the "SOS" functions, among these functions may be the inhibition of the 3'→5' exonuclease of DNA polymerases I or III, the so-called "proof-reading" functions. If this activity could be suppressed then the long gaps opposite pyrimidine dimers could be filled because now the DNA polymerase could replicate over the dimers in template strands (inserting "random" nucleotides). Thus, although mutagenic, this repair would enhance cell survival.

1.1.3 Neurospora nucleases

A single-strand specific endonuclease acting on DNA and RNA was first isolated and purified from N. crassa conidia, and then from stationary phase starved mycelia, by Linn and Lehman (1, 3, 5). This enzyme has some preference for guanine-containing sequences and degrades the substrates to 5'-mononucleotides and small oligonucleotides terminated in 5'-phosphoryl groups. The enzyme has a high activity against ss-DNA, but only slightly activity with (native) ds-DNA. Using T7 DNA as substrates, the activity toward ss-DNA was found to be approximately 1000-fold of that toward ds-DNA. However, the contaminating ds-DNase activity can be selectively inactivated by reducing agents, such as 2-mercaptoethanol and reduced glutathione. Optimum conditions for ss-DNA hydrolysis occurred between pH 7.5 to pH 8.5, in presence of 10 mM $MgCl_2$ and at salt concentrations between 0.1 and 0.2M. The enzyme had a lower activity at lower salt concentrations, and had a strong temperature dependence. It was active over the range 20°C to 60°C. A concentration of 7×10^{-6}M or higher of ethylenediamine tetracetate (EDTA) inhibited 95% of the ss-DNase activity. This inhibition was not reversed by $MgCl_2$, but a stoichiometric amounts of cobaltous ion (Co^{++}) overcome the inhibition. The ss-DNase activity was maximum when 5×10^{-5}M Co^{++} in presence or 5×10^{-4}M Co^{++} inabsence of Mg^{++} (1). The endonuclease was found to have a molecular weight of 55,000 daltons by sedimentation velocity centrifugation in sucrose density gradients (5). Rabin et al. (12) found that 2×10^{-4}M ATP inhibited the endonuclease activity by 50% in the presence of 10 mM Mg^{++} in a non-competitive fashion. Other nucleoside triphosphates were not effective at this concentration, but inhibited slightly at higher concentrations.

Linn and Lehman isolated another endonuclease from N. crassa mitochondria (50). The enzyme had RNase activity as well as ss- and ds-DNase activities in a ratio of about 1:1 (51). The enzyme was not sensitive to mercaptoethanol, and optimum pH was between 6 to 7.5. It was strongly inhibited by EDTA, and had an absolute requirement for divalent metal ions, such as Mg^{++}, Mn^{++}, Co^{++}, but was inhibited by Ca^{++}. Two forms of mitochondria endonuclease have been identified (51), one membrane-bound, which is released by treatment with Triton X-100; the other was found in the soluble fraction. The two forms were recovered in approximately equal amounts. The molecular weight of both forms was some 120,000 daltons as determined by sucrose density gradient centrifugation (51).

An exonuclease specific for single-stranded nucleic acids (DNA and RNA) has been purified from Neurospora crassa conidia (52). The enzyme had a molecular weight of 72,000 and consisted of a single polypeptide chain. It degraded ss-DNA and RNA mainly to 5'-mononucleotides but oligonucleotides were also detected in the digests. No detectable acid-soluble material was released from either double-strand DNA or native (ds) reovirus RNA. The direction of exonucleolytic attack as determined with a hepta-deoxyribonucleotides as substrate was 5'→3'. The purified exonuclease contained some ss-specific endonucleolytic activity as indicated by its ability to destroy rapidly the infectivity of single-stranded circular ϕX174 DNA, but only very slowly convert superhelical S13 replicative form I DNA to the replicative form II DNA. The exonuclease was optimally active with denatured (ss) DNA at pH 8.5 in the presence of 0.01 M Mg^{2+}. However, in absence of Mg^{++} the pattern of degradation of RNA was endonucleolytic in character (52).

The activity was unaffected by 4-8 mM mercaptoethanol and by 1 mM p-hydroxymercuribenzoate, but increases in salt concentration above 0.1 inhibited the activity. ATP concentrations of 0.2-0.4 mM had no affect on the activity. This ss-specific exonuclease has not been detected in mycelia despite attempts to do so (9). This enzyme may be periplasmic since large amounts of activity were released from conidia comply by freezing and thawing (8, 9).

Three different single-strand specific intracellular nuclease activities were isolated by chromatography on phosphocellulose and hydroxyapatite from Neurospora crassa conidia (9). Two of these had the same chromatographic and enzymological properties as the ss-specific endonuclease (1, 3, 5) and ss-specific exonuclease (52) respectively. The third activity appeared to be a mixture or possibly a complex of ss-specific endonuclease and exonuclease activities. The two components of the mixture were resolved by polyacrylamide gel electrophoresis. Although the exonuclease component of the mixture was sensitive to heat inactivation like the purified ss-exonuclease (complete inactivation occurred after 10 to 15 minutes treatment at 58°C), its chromatographic properties were unchanged by the heat treatment. The activity which remained after heat treatment was endonucleolytic in character like that of ss-endonuclease. Sonicates of mycelia subjected to the same purification procedures yielded two chromatographic fractions of ss-endonuclease, one corresponding to the ss-endonuclease and the other to the mixture. However, no exonucleolytic activity was detected in the mixture. It was concluded that two forms of ss-endonuclease existed in log phase mycelia of Neurospora crassa (9).

An endonuclease which was stable to heating up to 74°C and only weakly bound to phosphocellulose, was partially purified from extracts of log phase mycelia of wild-type Neurospora crassa (10) using ss-DNA as substrate. The endonuclease was associated with an equally heat-stable double-strand specific exonuclease. The exonucleolytic activity acted on linear but not circular double-stranded DNA and did not attack double strand RNA. Both endonuclease and exonuclease activities were inhibited by 0.1 to 0.5 mM ATP. The ds-exonuclease activity of this enzyme was inhibited preferentially by a variety of agents and it was also preferentially inactivated by proteases present in partially purified enzyme preparations (endogenous proteases). This inactivation was partially prevented by the presence of a serine protease inhibitor, phenylmethanesulfonyl fluoride (PMSF) and by the presence of bovine serum albumin at 2 mg/ml concentration. Although the endonucleolytic activity was not affected by similar treatment, the chromatographic properties of the enzyme were altered. The residual endonuclease activitiy after these treatments was identical in properties to the ss-specific endonuclease which had been previously described (1, 3, 5). Thus, the ss-strand endonuclease which Linn and Lehman (1, 3, 5) had described appears to be an end-product of processing by proteases, a proteolytic artifact of extraction and purification which arose from this new form of endonuclease with associated ds-exonuclease activity (10), i.e. endo-exonuclease. This endoexonuclease was also found to be associated with a DNA-unwinding activity which was sensitive to trypsin (10). It was suggested that the combined endonuclease and exonuclease activities of this enzyme might constitute a recombination nuclease (10) because

of the similarities of the activities of the two nuclease activities to E. coli exonuclease V (16, 17). Neither enzyme acts on covalently closed circular ds-DNAs at low enzyme concentration and both have an ATP-binding site.

The Neurospora endo-exonuclease described above was found to be a single polypeptide, probably 53,000 dalton in molecular weight as determined from SDS-polyacrylamide gel electrophoresis (7). In crude extracts of mid-log wild-type mycelia, only 25% of total endoexonuclease exists in the active state. The remainder, approximately 75% of total, was found to exist in an inactive form which was activated in vitro either by endogenous PMSF-sensitive protease(s) or by exogenous trypsin. The inactive form of endo-exonuclease has been purified up to 50-fold in 15% yield free of the active enzyme (6, 7). This inactive endo-exonuclease was less acidic and a larger protein than the active nuclease (see below). The inactive form also strongly bound to the hydrophobic matrices, octyl- and phenyl-sepharose, which suggests that the protein may have a relatively large hydrophobic domain (7, 6). The molecular weight which determined by SDS-electrophoresis is approximately 90,000 daltons. It was suggested (6, 7) that this large polypeptide might be an inactive precursor form of the endo-exonuclease. This is consistent with the enzyme being secreted into the culture medium (see below).

Three major alkaline deoxyribonuclease (DNase) activities have been identified in sorbose-containing liquid culture medium in which wild-type Neurospora crassa were grown (11). One of the nuclease, DNase C, had properties very similar to those of the endo-exonuclease (7, 6). Of the other two secreted nucleases, DNase A was found to be a Ca^{++}-dependent endonuclease which had no specificity for ss- or

ds-DNA and no activity with RNA, while DNase B was found to be a Mg^{++}-dependent ss-exonuclease active with both ss-DNA and RNA. The DNase B was thus very similar to the ss-exonuclease purified earlier from Neurospora crassa conidia (52). The native molecular weight of 77,000 determined for DNase B is close to the polypeptide molecular weight of 72,000 determined for the ss-exonuclease.

An orthophosphate repressible extracellular nuclease N_3 which was able to degrade both RNA and DNA was described by Ishikawa et al. (13). Two genes, nuc-1 and nuc-2, were found to regulate this nuclease and other orthophosphate-repressible enzymes. N_3 was found to be a complex consisting of nuclease N_3' and an inhibitor molecule. Nuclease N_3, nuclease N_3' and inhibitor were partially purified and estimated to have the approximate molecular weight of 38,000, 12,500, and 25,000 respectively (57).

1.1.4 Repair-deficient Mutants of Neurospora crassa

Seven DNA repair deficient mutants have been isolated from Neurospora crassa and characterized uvs-1, uvs-2, uvs-3, uvs-4, uvs-5, uvs-6, and upr-1 (21). Two of these mutants, uvs-2 and upr-1, have been shown to be deficient in the excision of pyrimidine dimers in vivo (53). Two other mutants, uvs-3 and uvs-6, resemble recombination mutants of E. coli in that they are sensitive to a wide spectrum of mutagens and have altered mitotic recombination. These two mutants have been shown to be sensitive to ultraviolet light (UV), γ-irradiation, nitrogen mustard, methylmethane sulfonate (MMS) (54), nitrosoguanidine (NG) (55), and L-Histidine (56).

The two rec-like mutants, but not the excision-deficient mutants, have the Nuh phenotype, i.e. they fail to secrete normal levels of DNase activity which is Mg^{++}-dependent and active at pH 8.0 but not at pH 6.0 (55). Two other mutants with a wide spectrum of mutagen sensitivities which have the Nuh phenotype have been isolated: nuh-4 and mms (04) (55). These have been mapped but otherwise have not yet been fully characterized. When grown in sorbose-containing liquid culture medium, the mutants were found to be deficient, relative to the wild-type in the release of alkaline DNases into the medium (11). The uvs-3 mutant released only small amounts of DNase A and DNase C; nuh-4 did not release detectable DNase C and released only a very low level of DNase B; uvs-6 released only a low level of DNase A. A nuh mutant (nuh-3) (55), which was not mutagen sensitive relative to the wild-type released low levels of DNase B (11). Another mutant (nuc-2) (57), which was ultraviolet light-sensitive but does not have the Nuh phenotype, was normal in release of these DNases (11).

1.1.5 Protease involvement in cellular processes

It has been known for a long time that proteases participate in the intracellular degradation (catabolism) of proteins. Only recently, however, has it become clear that proteases are involved in the processing of secretory proteins and in the regulation of other physiological functions. Thus, proteases hold much more interest than just as catabolic enzymes.

Most secretory proteins are synthesized on membrane-bound polyribosomes, and pass through the membrane during the translation process. Most of the secretory proteins have been found to have an NH_2-terminal

extension of 15 to 30 amino acids that is rich in hydrophobic residues. Among the proteins which are processed (60) in this manner are pre-proalbumin (64), lysozyme precursor (59) and preproinsulin (65). Blobel and Dobberstein (58) call this hydrophobic N-terminal sequence the "signal sequence" and postulate that its hydrophobic nature allows it to interact with a (hypothetical) membrane receptor, thereby anchoring not only itself but the polyribosomes and mRNA to the endoplasmic reticulum or plasma membrane. The "signal" is normally removed before the polypeptide chain is completely assembled, but its existence has been demonstrated using cell-free protein-synthesizing systems lacking membranes and associated proteases. Translation of fish islet mRNA in a wheat germ cell-free system in the presence of dog pancreas microsomal membranes led to the correct cleavage of the nascent preproinsulin and resulted in the synthesis of authentic fish proinsulin (65).

Many secretory proteases exist in inactive "precursor" states in the zymogen granules. These inactive precursors have higher molecular weights than their corresponding active forms. Thus, secretory proteases such as pepsin, trypsin, chymotrypsin and elastase are synthesized and stored as inactive precursors and undergo proteolytic cleavage when converted to their active forms. Although the pancreatic hormone, insulin, is synthesized in the preproinsulin form, the form which stored in the zymogen granules is proinsulin. During secretion, proinsulin is converted into insulin. Similar processing is found for serum albumin (60), and hen's egg white lysozyme (59).

Other examples of proteolytic modification of enzymes are known. Post synthetic proteolytic transformation of human erythrocyte pyruvate kinase (61) and conversion of one active form of Staphylococcal

nuclease (62) (Nuclease B) into another active form (Nuclease A) have been demonstrated. The conversion of Staphylococcal nuclease B to nuclease A was found to occur extracellularly and can be carried out in vitro with purified Straphylococcal protease (62). This cleavage also removes amino acids from the N-terminal end of the polypeptide as in the case of processing preproinsulin to proinsulin. Some enzyme modifications have also demonstrated in Neurospora crassa. The pyrimidine 3 (pyr-3) gene product, which contains both pyrimidine-specific carbamoyl-phosphate synthase and aspartate carbamoyltransferase, dissociated in vitro due to the action of endogenous PMSF-sensitive protease to an enzyme of lower molecular weight with only aspartate carbamoyltransferase activity (63, 66). Exopeptidases in vitro were unable to bring about this conversion. The "aromatic complex" of Neurospora crassa have also been shown by Gaertner and Cole to be modified in vitro by endogenous PMSF-sensitive protease(s) (67). The aromatic complex is a multienzyme system which catalyzes five consecutive reactions in the central pathway leading to the biosynthesis of the aromatic amino acids. All five enzyme activities are associated with a single polypeptide chain. During extraction and purification, the aromatic complex was found to be very sensitive to the action of endogenous protease(s). Although the activities of the multienzyme system appear to remain intact, the polypeptide was eventually fragmented by protease action into small polypeptides which could be dissociated and shown to contain different activities. It is not known whether these effects on the Neurospora enzymes are mimicked in vivo or have any regulatory role. However, the discovery of an inactive precursor form endo-exonuclease of N. crassa (67) and the fact that the active form of this enzyme is secreted into the culture

medium (11) may indicate a role for protease(s) in, at least, the secretion of this enzyme. The conversion of precursor to active enzyme can be carried out *in vitro* either with endogenous or with exogenous endopeptidases such as trypsin. The action of the endogenous protease(s) is abolished by the serine protease inhibitor, PMSF.

Recently, a role for protease(s) has been suggested for the regulation of "SOS" functions in DNA-repair in *E. coli* (68). The *recA* gene product, protein X, in fact, has been found to possess proteolytic activity. It is able to cleave the λ repressor specifically *in vitro*. The induction of λ in λ-lysogens is one of the functions activated in the "SOS" response, Roberts and Roberts (70) have shown that λ prophage induction is due to a *rec-A* dependent proteolytic cleavage of the λ repressor *in vivo*. Another function which is activated in the "SOS" response in associated with septum formation in cell division of *E. coli* under control of the *lon* gene (89). It has been shown recently that this gene is allelic with the *deg* gene which controls the proteolytic degradation of missense and nonsense proteins in *E. coli* (90).

1.1.6 Purpose of this Thesis

An endoexonuclease with some properties in common with the *E. coli* *recBC* nuclease has been isolated from *Neursopora crassa* (10). Its discovery suggests a possible role DNA-repair and/or recombination in *N. crassa*. An inactive precursor of this *N. crassa* endonuclease has been identified by Kwong and Fraser (6, 7). This precursor can be converted to active endonuclease with either exogenous or endogenous protease(s) (6, 7). In this thesis, the major intracellular alkaline nucleases expressed in wild-type and *Rec*-like mutants of *N. crassa*

are characterized and compared, of which endo-exonuclease is one. In addition, the levels of inactive precursor in wild-type and mutants are compared.

The results are consistent with a role for protease(s) in the regulation of intracellular levels of these enzymes in the wild-type and repair-deficient mutants. Evidence is also presented for the presence in crude fractions derived from mycelia of protease-sensitive ds-DNase inhibitor(s) which may explain the virtually completely masking of ds-DNase activity in mycelia.

CHAPTER II

2.1 Materials

2.1.1 Chemicals

The culture agar for growing Neurospora conidia on agar slants was a product of BBL Division of BioQuest. Neurospora minimal medium, no. 0817-01, used for liquid shake cultures of mycelia and bacto-agar were obtained from Difco Laboratories.

Fish roe type VI DNA, trizma base, bovine serum albumin, bovine pancreatic chymotrypsin, L-histidine, salt free bovine pancreatic trypsin, L-sorbose, d-biotin, soybean trypsin inhibitor were obtained from Sigma Chemical Co. Disodium ethylenediamine-tetraacetate (EDTA), polyethylene glycol 20,000 (Carbowax PEG 20,000), dextrose, D-fructose, potassium phosphate monobasic, potassium phosphate dibasic anhydrous, sodium chloride, sodium citrate, ammonium nitrate, magnesium sulfate, calcium chloride dehydrate, citric acid monohydrate, trichloroacetic acid, uranium acetate, ferrous ammonium sulfate, manganous sulfate monohydrate, boric acid, and sodium molybdate were from Fisher Scientific Company. Zinc sulfate was from Merck and Co. Limited, cupric sulphate from The British Drug Houses Ltd. Perchloric acid 70% from Anachemia Chemicals Ltd. DEAE-cellulose and phosphocellulose from Whatman. DEAE-sepharose from Pharmacia Fine Chemicals. Mitomycin C from Boehringer Mannheim.

2.1.2 Equipment

The controlled environment incubator shaker for the growth of liquid medium cultures was from New Brunswick Scientific Co. Inc. Dry type bacteriological incubator (gravity convection) for incubation of agar plates was made by Blue M Electric Company. 152 Microfuge for

centrifugation in nuclease assays and DU Sepectrophotometer model 2400 for absorbance readings were from Beckman Instruments Inc.

2.1.3 Neurospora strains

WT A (74-OR23-1A), uvs-6 (ALS 35, FGSC #2244), uvs-3 (FGSC #1627) were obtained from Fungal Genetics Stock Center (FGSC) and had been backcrossed to wild-type A for six to eight generations by Dr. Kafer.

uvs-2 (BC II of De Serres stain,ALS 629.3), uvs-4 (BC of De Serres strains), and upr-1 (BC of De Serres strains,ALS 627.9) were obtained from Dr. Schroeder.

nuh-1 (T9.M.2), nuh-3 (FK-003, 35a.7 MF), nuh-4 (FK-004, 45b.3 MF), mei-3 (DNP-469-14), and mms(04) (FK 104, 931.IV. C.4) were obtained from Dr. Kafer.

2.2 Methods

2.2.1 Growth of Mycelia

Conidia were harvested after growing on Neurospora culture agar slants for 5 to 7 days at room temperature. A small loop of conidia was transferred into a sterile Klett tube containing 5 ml of sterile water. The conidial suspension was adjusted to measure 35 Klett units in a Klett-Summerson Photocolorimeter equipped with a blue filter (# 42, transmission range: 400-450 nm) by addition of either more sterile water or more conidia as necessary. A 0.5 ml aliquot of the suspension was innoculated into each 250 ml Erlenmeyer flask containing 100 ml of autoclaved Neurospora minimal medium from Difco Laboratories (27.7 gm per litre of distilled water). Mycelia were grown for 2.5 days in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc.) at 30°C., a shaking rate of 150 r.p.m., under constant illumination from 15 watt fluorescent lamp placed 60 cm. above the window of the incubator chamber. An average yield of 2.5 gm (wet weight) per flask was obtained. Mycelia were collected on a Buchner funnel, washed several times with distilled water, dried by suction, cut into small narrow strips, and stored immediately in a -90°C Revco Ultra-low freezer until use.

2.2.2 Enzyme Assays

Single strand deoxyribonuclease (ss-DNase) and double strand deoxyribonuclease (ds-DNase) activities were measured by following the rates of release of acid-soluble material (A_{260}) from heat-denatured and native DNA respectively as described by Fraser et al.

(10). Heat-denatured DNA was prepared by heating native DNA in boiling water for 15 to 20 minutes and then cooling rapidly on ice. An appropriate amount of nuclease was incubated at 37°C in 0.1 M Tris-HCl buffer pH 8.0, containing 10 mM $MgCl_2$ and 0.67 mg/ml heat-denatured or native DNA. Aliquots of 100 µl were transferred to cold microfuge tubes containing 100 µl of a 'carrier' solution (2 mg/ml native DNA solution in 0.05 M Tris-HCl buffer pH 7.5 to ensure complete precipitation of DNA) at regular time intervals. To each of the microfuge tubes, 150 µl of cold 1 N perchloric acid (PCA (in the routine assay)) or 10% trichloroacetic acid (TCA) or 0.5% uranyl-acetate (UTCA) in 10% TCA (for the qualitative determination of the endo- or exonucleolytic character of the nuclease) was added immediately to precipitate the undegraded DNA. The suspensions were mixed thoroughly by vortexing, held on ice for approximately 15 minuites, and then centrifuged for 1.5 minutes in a Beckman Microfuge. Aliquots (200 µl) of the supernatant were then transferred into tubes containing 800 µl of distilled water. The absorption at 260 nm was determined with a Beckman DU Sepectrophotometer. The " 0 minute " A_{260} reading was used as the blank. The linear portion of the rate curve was used to calculate the activity. One unit of nuclease activity was defined as the amount of enzyme which released 1.0 A_{260} unit of acid-soluble material in 30 minutes under the conditions of the assay. For single time " point " assays, incubations were carried out (under the same conditions as above) in microfuge tubes containing 100 µl each of reaction mixture. In each case, the reaction was stopped after the appropriate time by the rapid successive additions of 100 µl of DNA

"carrier" solution and 150 µl of 1 N PCA. After standing on ice and centrifuging as described above, 200 µl of the supernatant was diluted into 800 µl of water and the A_{260} of the solution determined.

Ribonuclease activity was assayed by the same procedure using Brewer's yeast tRNA as substrate in place of DNA except that no $MgCl_2$ was added to reaction mixture.

For measurements of total DNase activities, trypsin activation of the nuclease was carried out by incubating the samples with either 100 µg/ml trypsin (crude extracts) or 10-20 µg/ml trypsin (partially purified fractions) at room temperature for 30 minutes. The reaction was stopped by addition of three times the concentration of soybean trypsin inhibitor. After standing at room temperature for 30 minutes, the sample were assayed for nuclease activity as above.

The activity before trypsin activation was taken as a measure of "expressed" nuclease activity, while activity after trypsin activation was taken as a measure of the "total" nuclease activity (active plus inactive nuclease). The difference between these two activities was used as a measure of the inactive endo-exonuclease precursor present (see Results and Discussion). The level of activation by trypsin was calculated by dividing the total nuclease activity by the expressed nuclease activity.

Treatment of nuclease preparations with EDTA was carried out by preincubating enzyme with EDTA (15 mM in the preincubating medium) for 5 minutes at room temperature, and then diluting into a metal ion free reaction mixture for assay of activity. The final EDTA concentration in the reaction mixture was 5 mM. Restoration of nuclease activity of EDTA-treated enzyme by divalent metal ions was determined by diluting into a reaction mixture which contained either 10 mM $MgCl_2$

or 10 mM $CaCl_2$ (final concentration). Nuclease activity of untreated enzyme assayed in a metal ion free reaction mixture was used as the control for the above inhibition and restoration assays. This was compared with routine assay to give the dependency of the nuclease activity on Mg^{++}.

Inhibition of nuclease activity by p-hydroxymercuribenzoate (PHMB) was determined by preincubating equal volumes of saturated PHMB solutions (about 1 mM) and enzyme for 30 minutes at room temperature. Inhibition of nuclease activity by ATP was determined by adding ATP (0.5 mM final concentration) to the routine assay mixture containing an excess of Mg^{++} (10 mM, final Mg^{++} concentration).

2.2.3 Protein Determinations

Protein concentrations were determined by the method of Lowry et al. (2). Bovine serum albumin was used as a standard. For crude extracts only, one volume of sample was first precipitated by one volume of cold 10% trichloroacetic acid (TCA), the precipitate was washed twice with ether and redissolved in 5 volumes of 0.1 N KOH, as recommended by Linn and Lehman (3). This procedure removed TCA-soluble material which interferred with the Lowry determination.

2.2.4 Estimation of Molecular Weight by Sucrose Density Gradient

Sedimentation coefficients were determined according to the method of Martin and Ames (4) by centrifugation in 5 to 20% linear sucrose gradients. Sucrose solutions were made up in Buffer A (0.02 potassium phosphate buffer, pH 6.5) and stored frozen until used. Gradients (4.8 ml) were preformed with a gradient maker in polyallomer tubes

and used immediately. Samples containing at least 4 units of nuclease activity in 200 µl were layered on top of the gradients. A Beckman SW-50,1 rotor was used for centrifugations at 37,000 r.p.m. in a Beckman L2-65B centrifuge for 24 hours. Fractions of 200 µl each were collected from the bottoms of the tubes. Bovine haemoglobin ($S_{20,W}$: 4.3S) was used as a molecular weight marker (0.5 mg/tube). The haemoglobin concentration was determined in the gradient fractions by following the absorbance at 440 nm (A_{440}) of samples diluted 10-fold in distilled water. Nuclease activity in each fraction was determined by "point" assay as described above.

2.2.5 Extraction of Mycelia

Frozen mycelia were ground to a fine powder in a mortar and pestle at dry-ice temperature. A 20% w/v suspension in ice-cold 0.02 M potassium phosphate buffer, pH 6.5 (buffer A) was sonicated over ice with a Blackstone (Model 552) sonicator (1 cm probe) for 6 minutes at full power. The temperature of the suspension was kept below 20°C during sonication. Sonicates were centrifuged for 10 minutes at 4100Xg in a refrigerated RC2-B Sorvall centrifuge. The supernatant ("crude extract") was decanted and held in an ice-bath for assays or chromatography.

2.2.6 Fractionation of Extracts

2.2.6.1 Chromatography on DEAE-Sepharose Column

Extracts of 5 gm (wet weight) mycelia in Buffer A were chromatographed on 100 ml (packed volume) DEAE-Sepharose columns (which had been previously equilibrated with Buffer A) at a flow rate of

approximately 30 ml/hr. The column was washed with Buffer A until the A_{280} was below 0.05 and then 300 ml of a linear 0 to 0.5 M NaCl gradient in buffer A was used to elute the column. Fractions of 2.5 ml were collected. The salt gradient was monitored with a conductivity meter. Expressed and total ss-DNase activities were assayed in each fraction for the wild-type strain but only the former activity for mutant strains. Fractions with nuclease activity were pooled as indicated in results and dialysed overnight in buffer B (4 mM KPB pH 6.5) to remove salt. Fresh DEAE-Sepharose was used for the chromatography of crude extracts each time. The regeneration of these columns after such usage proved to be inefficient.

2.2.6.2 Chromatography on Phospho-Cellulose Column

Pooled and dialysed active fractions (conductivity checked) from DEAE-Sepharose were applied to 15 ml (packed volume) phospho-cellulose columns, which had been pre-equilibrated with Buffer B. The equilibrations was checked by conductivity and pH measurements, both of which proved necessary because of the spontaneous hydrolysis of phosphate from the absorbent. The phosphocellulose columns were washed with Buffer B until an A_{280} reached base line. The columns were then eluted with 140 ml linear 4 mM to 0.3 M gradients of potassium phosphate buffer, pH 6.5. Expressed ss-DNase activity was assayed in all cases and RNase activity was measured in some cases in each fraction derived from the column. Fractions with nuclease activity were pooled as indicated in Results and dialysed overnight against Buffer A. In cases where insufficient activity was recovered in the dialyslate, it was necessary to concentrate the pooled fractions by applying poly-

ethyleneglycol (Carbowax 2000) to the outside of the dialysis bag. After concentration, the fraction was then dialyzed against buffer A to remove polyethyleneglycol (PEG).

2.2.6.3 Chromatography on DEAE-Cellulose Columns

Pooled fractions equilibrated with buffer B which had been obtained from either DEAE-Sepharose or from phosphocellulose were applied to 20 ml (packed volume) DEAE-cellulose column, which had previously equilibrated with buffer B. After washing with buffer B until the A_{280} reached base line, the columns were stripped immediately with 5 volumes of 0.13 M potassium buffer, pH 6.5. Fractions with nuclease activity were pooled, and dialyzed against buffer A overnight as in the case of phosphocellulose fractions above.

2.2.7 Solid Media for Growth of Neurospora crassa

The preparation of solid media was according to methods described by Davis and De Serres (92). Sugars were added to the media as the carbon source. However, sorbose was found to have a metabolic effect on the cell wall in Neurospora such that the cells would grow into colonial form. The addition of 1 gm% sorbose thus restricted each conidia to grow locally into a colony and prevented the growth from speading out over the plates, which would obscure the scoring of results.

Other additions were also found to be essential to the growth of Neurospora. These were biotin and a trace element solution (for preparation see below).

Conidia from strains grown for 5 to 7 days on agar slants were used for innoculation of plates. Small amounts of conidia were transferred with a sterile steel needle from slants to the plates.

2.2.7.1 Biotin

10 mg% biotin solution was prepared by dissolving 5 mg of biotin

in 50 ml of distilled water. The solution was divided into 5 ml aliquots and stored frozen until use.

2.2.7.2 Trace Element Solution

5.00 gm of Citric acid $\cdot 1\ H_2O$, 5.00 gm $ZnSO_4 \cdot 7\ H_2O$, 1.00 gm $Fe(NH_4)_2 SO_4 \cdot 6\ H_2O$ (iron in ferrous state), 0.25 gm of $CuSO_4 \cdot 5\ H_2O$, 0.05 gm $MnSO_4 \cdot 1\ H_2O$, 0.05 gm H_3BO_3 (anyhydrous Boric acid), and 0.05 gm $Na_2 MoO_4 \cdot 2\ H_2O$ were added in the order indicated into 90 mls of distilled water. After all the material was completely dissolved, the solution was adjusted to 100 ml by further addition of distilled water. Chloroform (1 ml) was then added to prevent growth of bacteria and the solution was stored at 0-4 C.

2.2.7.3 Vogel's Medium N

The Vogel's N medium was prepared as a stock 50 times the concentration required. The following materials were added to 750 ml distilled water in the following order. 127 gm of Na_3Citrate$\cdot 2\ H_2O$, 250 gm KH_2PO_4, 100 gm NH_4NO_3, 10 gm $MgSO_4 \cdot 7\ H_2O$, 5 gm $CaCl_2 \cdot 2\ H_2O$ (predissolved in 20 ml of distilled water), 5 mls of 10 mg% biotin (prepared as above), and 5 ml trace element solution (prepared as above). After all material was dissolved, the solution was adjusted to 1 ℓ. with distilled water and the solution was stored at room temperature.

2.2.7.4 Tests for U.V. Sensitivity

The U.V. sensitivity of each strain was tested. It served as a preliminary method to check for mutants, and to separate the repair deficient mutants from non repair deficient mutants. The solid media

was prepared with Vogels'-N (20 mls of 50X concentrated solution) and 20 gm of Difco agar were added to 780 mls of distilled water in a 2 ℓ. Elenmeyer flask, and stoppered with sterile cotton; to another 500 ml Erlenmeyer flask, which contained 10 gm of sorbose, 0.5 gm glucose and 0.5 gm fructose in 200 ml of distilled water. The two flasks were autoclaved and cooled to about 45 C, and then mixed (total volume: 1 ℓ), poured into the autoclaved plates immediately (approximately 25 ml per plate). The plates were stored for overnight before innoculation. The innoculated plates then exposed to U.V. irradiation (U.V. intensity of 2 $\mu w/cm^2$ x 100 at a distance of about 30 cm) for time interval chosen (0,1,2,3 minutes). The U.V.-irradiated plates were incubated at 34 °C in dark, so that photoreactivation was held minimum. Sensitivity of stains was recorded as inhibition of growth relative to wild-type after 2 and 3 days growth at 34 °C in dark.

2.2.7.5 Tests for Mitomycin C Sensitivity

The Rec mutants in Eschericia coli were found to be sensitive to a wide spectra of mutagens (36). One of these mutagens which rec mutants were sensitive to was mitomycin C (36). Thus, positive results for mitomycin C sensitivity would enhance the possibility but not prove that the mutants were rec -like. Mutants which were sensitive to mitomycin C but not rec-like have been found in Micrococcus radiodurans (93). Solid mitomycin C contained media was prepared same as the solid media used for U.V. sensitivity, except that after autoclaving and cooling to about 45 °C, appropriate amounts of a 1 mg/ml stock solution of mitomycin C was added to the sugar solution before mixing. The plates were stored in dark 4 to 5 hr. before innoculation. Test plates contained 0, 5, 10 and 20 $\mu g/ml$ of mitomycin C. Mitomycin C was sensitive to

light, thus it was essential to preform all steps involving handling of mitomycin C in dim light.

2.2.7.6 Tests for Histidine Sensitivity

The preparation of histidine containing plates was essentially the same as that for U.V.-sensitivity plates. Appropriate amounts of L-histidine (0, 250, and 500 µg/ml final concentration) were added to and autoclaved with the sugar solution. Since histidine was not light sensitive, working in dim light was not necessary.

CHAPTER III

3.1 Results

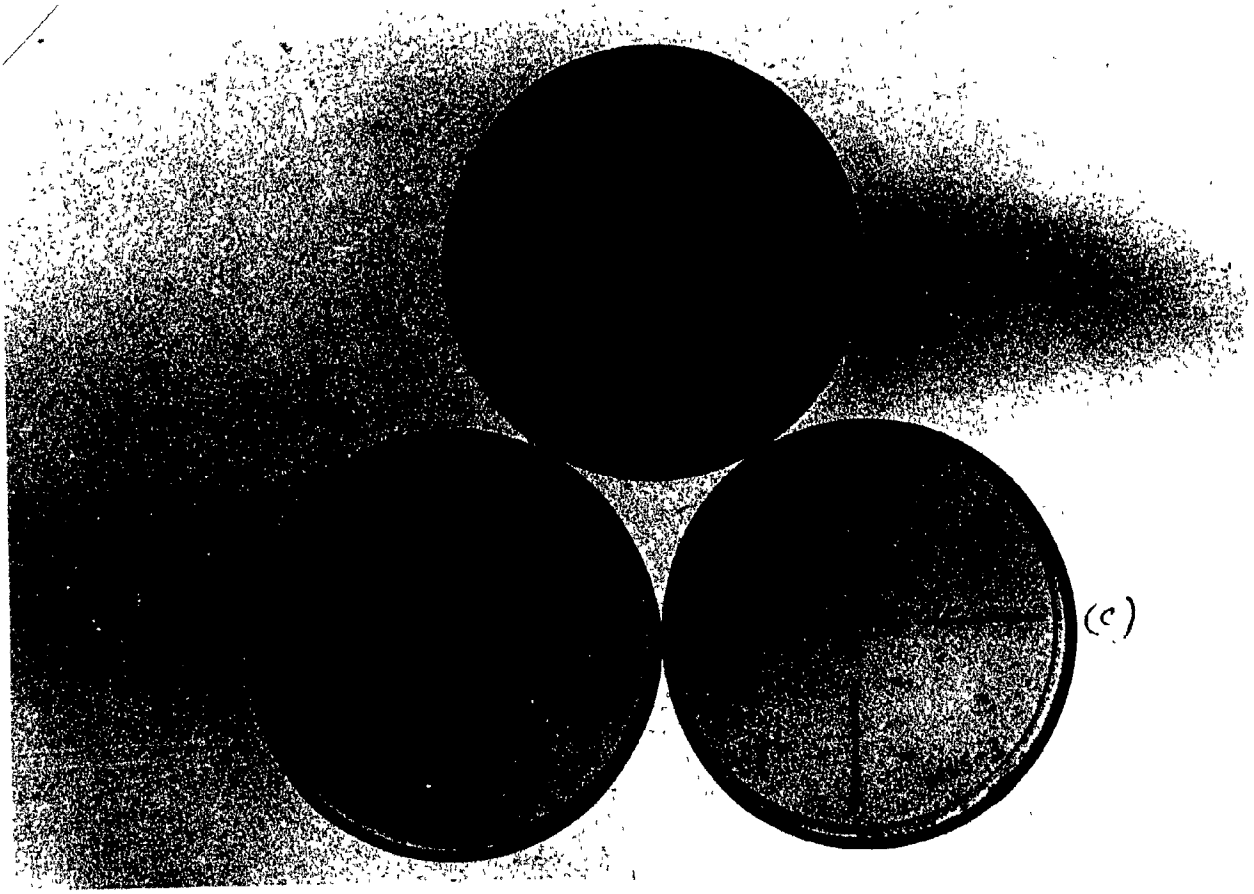
3.1.1 L-Histidine and Mitomycin C Sensitivities

It has been found that the rec⁻ mutants in E. coli were sensitive to a wide spectra of mutagens (36). The excision repair mutants of E. coli, on the other hand, were sensitive to a narrow spectra of mutagens. One of the mutagens which rec⁻ mutants of E. coli was sensitive to is mitomycin C. Thus, DNA repair mutants in Neurospora which show sensitivity towards mitomycin C would strengthen the possibility that they are rec-like mutants.

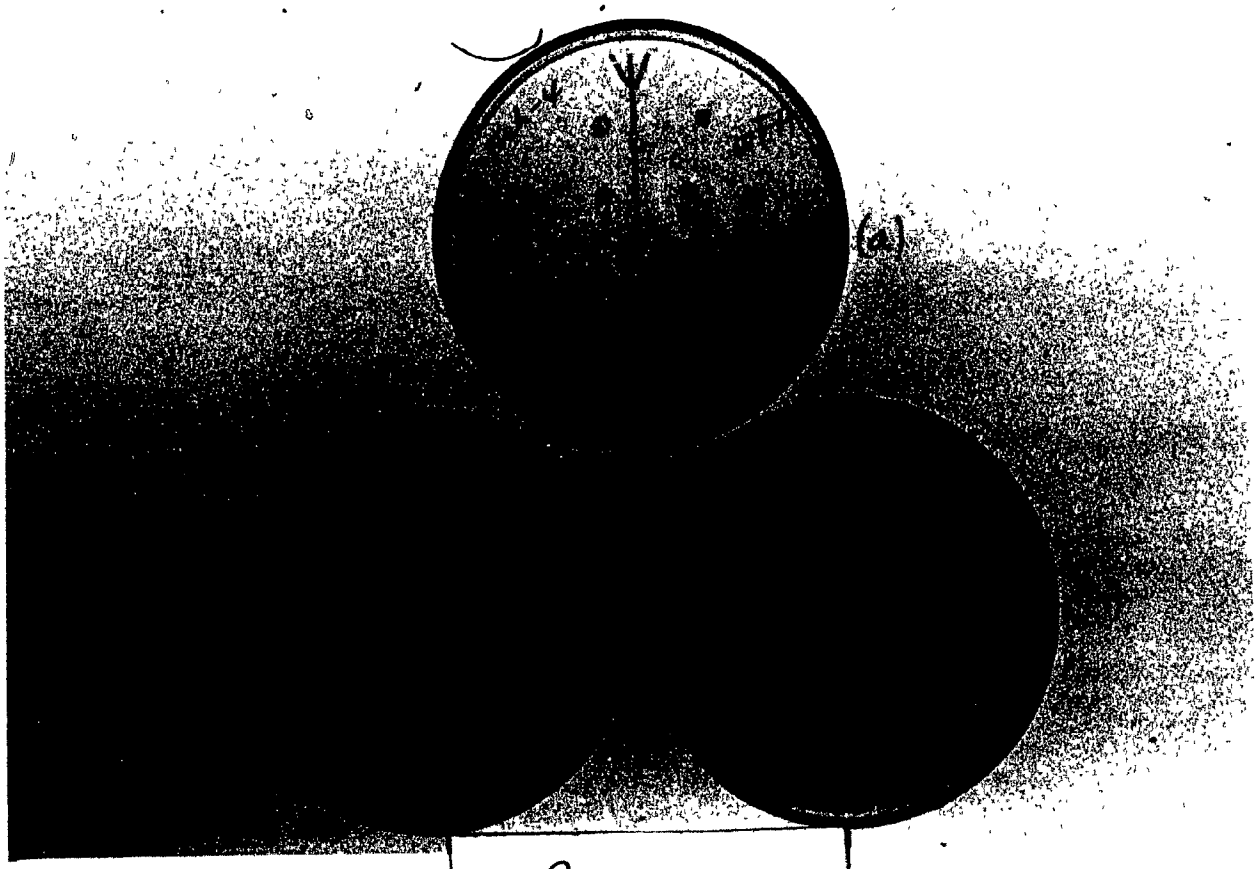
Wild-type and several mutagen sensitive mutants (uvs-3, uvs-6, mms(04), mei-3, nuh-4, nuh-3, upr-1, uvs-2 and uvs-4) were tested for sensitivity to histidine and mitomycin C. The growth of each strain was determined by the size of its colonies at the end of 2 and 3 days at 34°C when plated on solid media containing either histidine or mitomycin C.

At 0 µg/ml of either reagent (controls), all mutants grew as well as the wild-type (Fig 1, 2 and Table 1, 2). The presence of L-histidine (250 µg/ml or 500 µg/ml) or mitomycin C (10 µg/ml or 20 µg/ml) had remarkable effects on the growth of the some of the mutants: uvs-3, uvs-6, mms-(04), and mei-3 showed a strong inhibition of growth (Fig 1,2). No effect on growth was seen with the wild-type, nuh-3, and the two excision repair mutants: upr-1 and uvs-2 (Table 1,2), and only partial inhibition of growth was observed for nuh-4 and uvs-4 on histidine medium. When tested on mitomycin C medium, nuh-4 exhibited strong inhibition of growth. Table 3 summarizes the sensitivity of each strain towards L-histidine and mitomycin C. The mutants are

Fig 1: L-histidine inhibition of the growth of wild-type and mutants. The concentrations of L-histidine are: (a) 0 μg/ml, (b) 250 μg/ml, and (c) 500 μg/ml. The photos were taken after the strains had grown for 2 days at 34°C after the innoculation.



(c)



(a)

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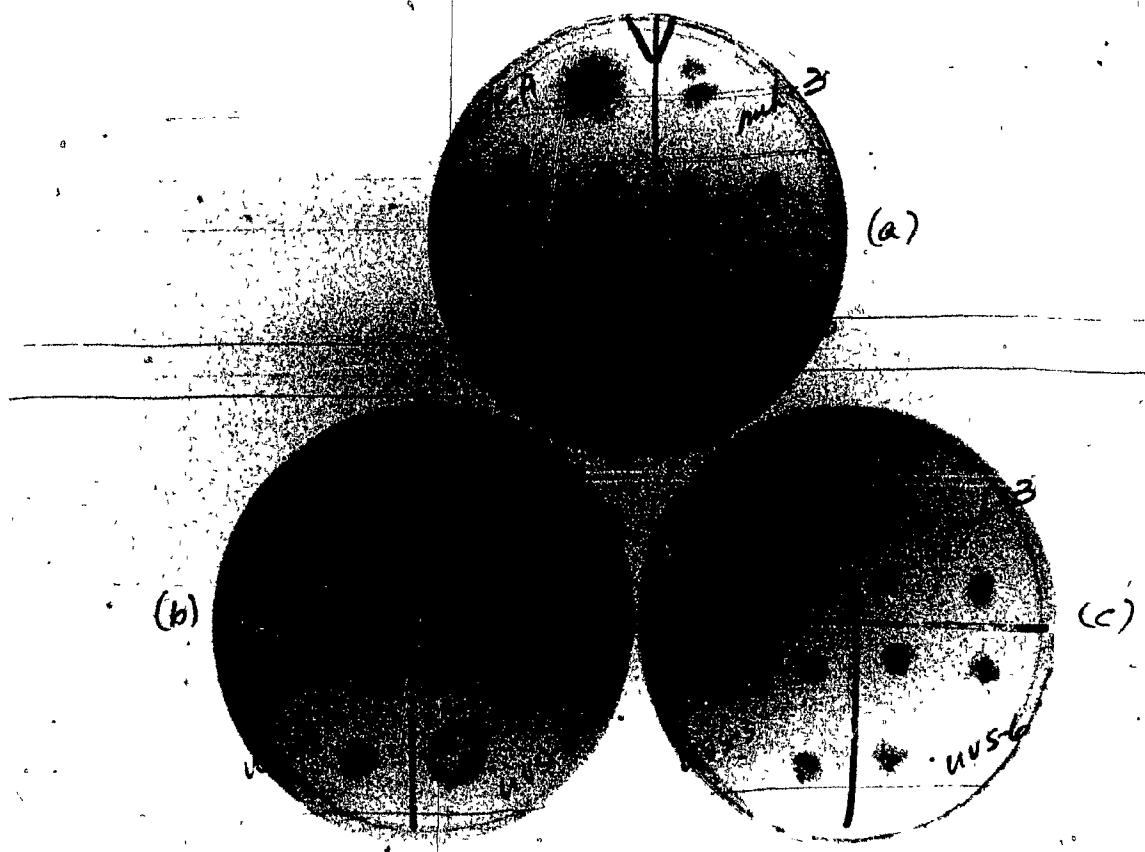
Fig 2: Mitomycin C inhibition of the growth of wild-type and mutants. The concentrations of mitomycin C are: (a) 0 μg/ml, (b) 10 μg/ml, and (c) 20 μg/ml. The photos were taken after the strains had grown for 3 days at 34°C after the innoculation.



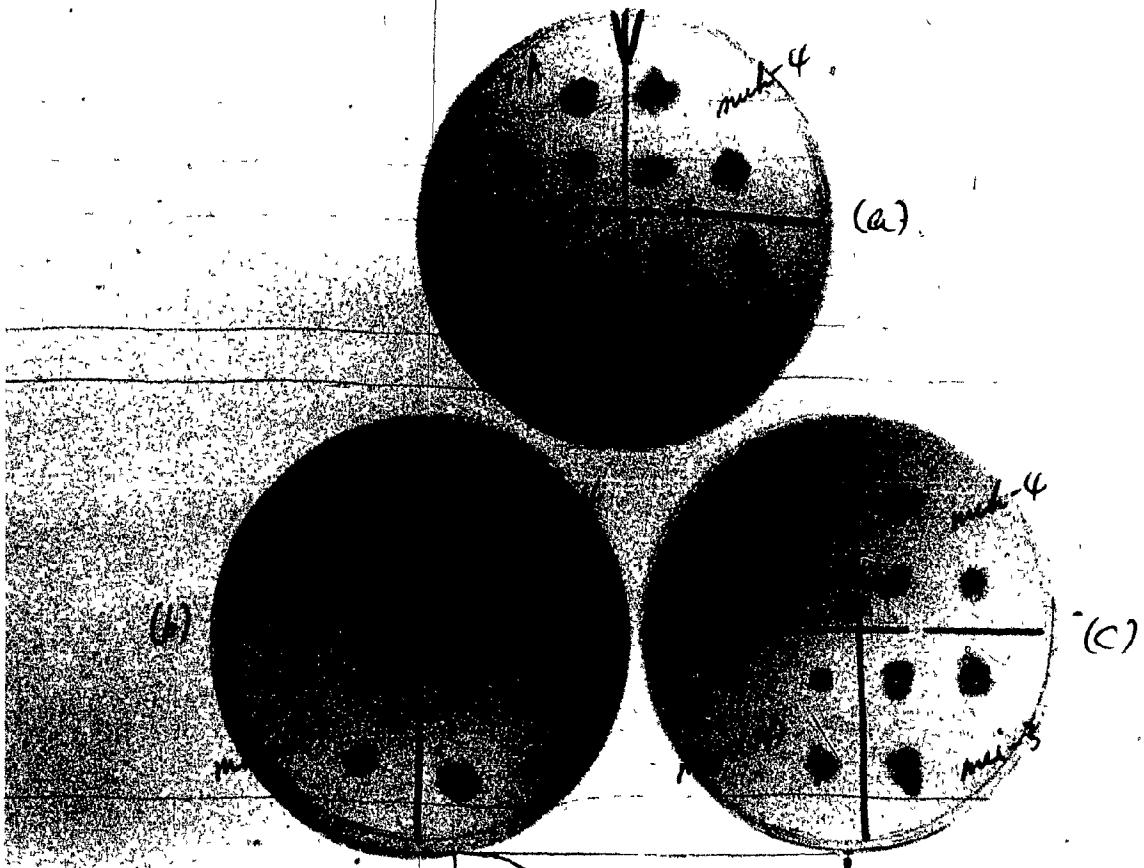


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Table 1: The growth of wild-type A in the absence of L-Histidine is used as the control. The growth of a strain is determined by its colony size at the end of the growth period (2 days at 34°C). A score of +++ is given to the control. Colonies with sizes comparable to the control are scored +++ . Other scores, ++ , + , and - represent diminishing colony sizes. A - score represents no growth.

Table 1

Growth of wt-A and mutants in presence of L-histidine

Strains	Concentration of L-histidine (mg/ml)		
	0	0.25	0.50
wt-A	+++	+++	+++
uvs-3	+++	+	+
uvs-6	+++	-	-
mms(04)	+++	+	+
mei-3	+++	+	+
nuh-4	+++	++	++
nuh-3	+++	+++	+++
upr-1	+++	+++	+++
uvs-2	+++	+++	+++
uvs-4	+++	++	++

Table 2: The growth of wild-type A in the absence of mitomycin C is used as the control. The growth of a strain is determined by its colony size at the end of the growth period (3 days at 34°C). A score of +++ is given to the control. Colonies with sizes comparable to the control are scored +++ . Other scores of ++ , and + represent diminishing colony sizes. A + score represents the smallest size.

Table 2

Growth of wt-A and mutants in presence of Mitomycin C

Strains	Concentration of Mitomycin C (μg/ml)		
	0.0	10	20
wt-A	+++	+++	+++
uvs-3	+++	++	+
uvs-6	+++	++	+
mms(04)	+++	++	+
mei-3	+++	++	+
nuh-4	+++	++	+
nuh-3	+++	+++	+++
upr-1	+++	+++	+++
uvs-2	+++	+++	+++
uvs-4	+++	+++	+++

Table 3: Summary of the sensitivity of each strain towards L-Histidine and mitomycin C. The concentrations of L-Histidine and mitomycin C are 500 and 20 μg/ml respectively. Sensitivities are scored according to the inhibition of growth by the two agents. S = sensitive, R = resitance (resistance is only meant to imply a resistance to the killing effects of the agent that is comparable to, and not greater than, the wild-type).

Table 3

Sensitivity of wt-A and mutants to L-histidine and mitomycin C (Summary)

Strains	Agents	
	L-histidine	mitomycin C
wt-A	R	R
uvs-3	S	S
uvs-6	S	S
mms(04)	S	S
mei-3	S	S
nuh-4	S	S
uvs-4	S	R
upr-1	R	R
uvs-2	R	R
nuh-3	R	R

divided into three classes: those sensitive (S) to both agents, those sensitive to only one of two agents and those not sensitive (R) to either agent relative to the wild-type. The symbol R = resistance is only meant to imply a resistance to the killing effects of the agent that is comparable to (and not greater than) the wild-type.

3.1.2 Nuclease Activity in Crude Extracts

The intracellular levels of expressed and total ss-DNase activities of the wild-type and several mutants in mid-log and end-log growth in sucrose medium are compared in Table 4. Each specific activity value reported is the average of at least three and in some cases up to eight separate determinations (on different cultures of mycelia) and none of these differed from the average by more than 10 - 15%. Only one mutant (nuh-1) had total ss-DNase activity consistently lower than the wild-type, but several mutants had expressed levels of ss-DNase activity which were significantly lower than the wild-type in both mid-log and end-log phase growth viz. uvs-3, nuh-4, nuh-3 and nuh-1. The ss-DNase levels in these mutants were respectively 37%, 61%, 39% and 28-65% of wild-type. The expressed ds-DNase activities in all cases were very low like that of the wild-type, and in every case the ds-DNase was activated on treatment with trypsin (Table 5).

3.1.3 Fractionation of the ss-DNase Activity of Extracts

When care was taken to avoid overloading of columns, all the ss-DNase activity of crude extracts of 2.5-day log phase mycelia was found to absorb at pH 6.5 both on DEAE-cellulose and on DEAE-sepharose. The ss-DNase activity eluted from DEAE-cellulose with 0.13 M potassium

Table 4: Total ss-DNase activities were obtained by pre-treatment with 100 µg/ml of trypsin for 30 min at room temperature. SBI (3 times the amounts of trypsin) was added to stop further action of trypsin. Control experiments have shown that the trypsin treatment used fully activated the nuclease activity.

Each specific activity value reported is the average of at least three and in some cases up to eight separate determinations; none of these differed from the average by more than 10-15%.

Table 4

Specific ss-DNase activities of extracts of wild-type and mutant mycelia grown in sucrose medium

Strains	Mid-log			End-log		
	-T	+T	Trypsin activation *	-T	+T	Trypsin activation*
wt-A	18	58	3.2	20	83	4.2
uvs-3	6	52	8.7	8	71	8.9
nuh-4	11	62	5.6	12	67	5.6
uvs-6	12	50	4.2	16	73	4.6
nuh-3	7	47	6.7	8	83	10.4
nuh-1	5	35	7.0	13	57	4.4

* Trypsin activation= +T/-T

Table 5: Total ds-DNase activities are obtained by pre-treatment with 100 µg/ml of trypsin for 30 min at room temperature. SBI (3 times the amount of trypsin) is added to stop further action of trypsin. Control experiments have shown that the trypsin treatment used fully activated the nuclease activity.

Table 5

Specific ds-DNase activities of extracts of wild-type and mutants mycelia grown in sucrose medium

Strains	Mid-log			End-log		
	-T	+T	Trypsin activation*	-T	+T	Trypsin activation*
wt-A	0.6	9	15	0.6	11	18
uvs-6	0.4	6	15	0.8	12	15
nuh-1	0.6	16	27	1.0	18	18
nuh-4	0.6	9	15	0.9	29	32
nuh-3	0.3	8	26	0.5	6	12
uvs-3	0.3	9	30	1.5	10	6.7

* Trypsin activation= +T/-T

phosphate buffer, pH 6.5 (6), however, differed from that eluted from DEAE-Sepharose in having a relatively low ratio of ss-DNase/ds-DNase activity (ss/ds of approximately 6 to 8 (cf refs 6,7)) as compared to the high ratio (ss/ds = 20 to 50) found for most of the fractions eluted from DEAE-Sepharose and for extracts.

The DNase activity of the DEAE-cellulose "strip fraction" was quite stable, the ss/ds ratio remaining at about 7 for nearly 2 weeks at 0-4°C. This fraction also contained all of the trypsin-activatable DNase activity (mainly, if not entirely, endo-exonuclease precursor (refs 6, 7 & 8)). The activations of ss-DNase and ds-DNase activities after treatment with trypsin were identical and in range 3.5 - 4.0 fold for different preparations derived from 2.5 to 3.0 day cultures of mycelia. This is in contrast to the differential activations of ss-DNase and ds-DNase activities (4-fold versus 20-fold) seen in fresh extracts treated with trypsin (Table 4 & 5).

3.1.3.1 Fractionation on DEAE-Sepharose Columns

Fractions eluted from DEAE-cellulose with buffer gradients proved much less stable and gave poorly reproducible elution profiles. As a result, chromatography of extracts on DEAE-Sepharose was adopted as a first step in the fractionation of the major intracellular DNases of mycelia. This has resulted in reproducible protein and ss-DNase profiles (Fig 3 & 4). The chromatographic profile for a crude extract of a 2.5 day culture of wild-type mycelia is shown in Fig. 3 . It can be seen that two protein "peaks" were resolved in fractions 1 - 120 containing unadsorbed protein. However, in some preparations of wild-type, a small third protein peak existed in fractions 80 - 90.

Fig. 3: Chromatography on DEAE-Sepharose of a crude extract of wild-type mycelia grown for 2.5 days in liquid culture. The extract of 5 g (wet weight) of mycelia is made in buffer A and applied to a 100 ml column (packed volume) of DEAE-Sepharose equilibrated with buffer A. After washing with more buffer A, a linear gradient of 0.0-0.5 M NaCl in buffer A is applied. Fractions of 2.5 ml are collected at a flow rate of about 1.0 ml/min. Protein in each fractions is estimated by determining the A_{280} (open circles). The ss-DNase activity (expressed) is estimated by A_{260} (closed circles). Total ss-DNase activity (dashed line shown for gradient fractions only) is estimated by A_{260}. Conductivity is determined by measurements with a conductivity meter (open triangles).

Fig. 3

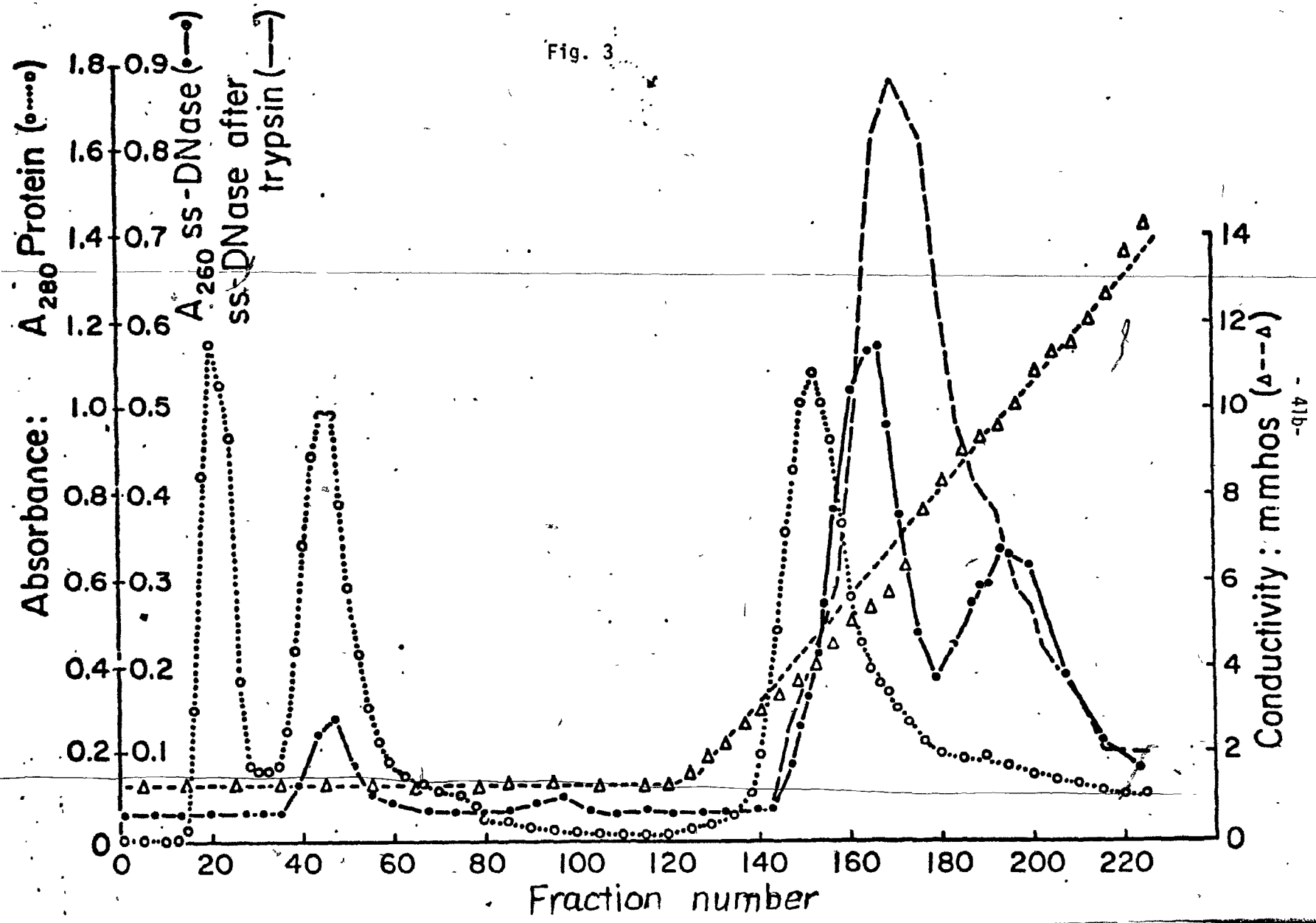
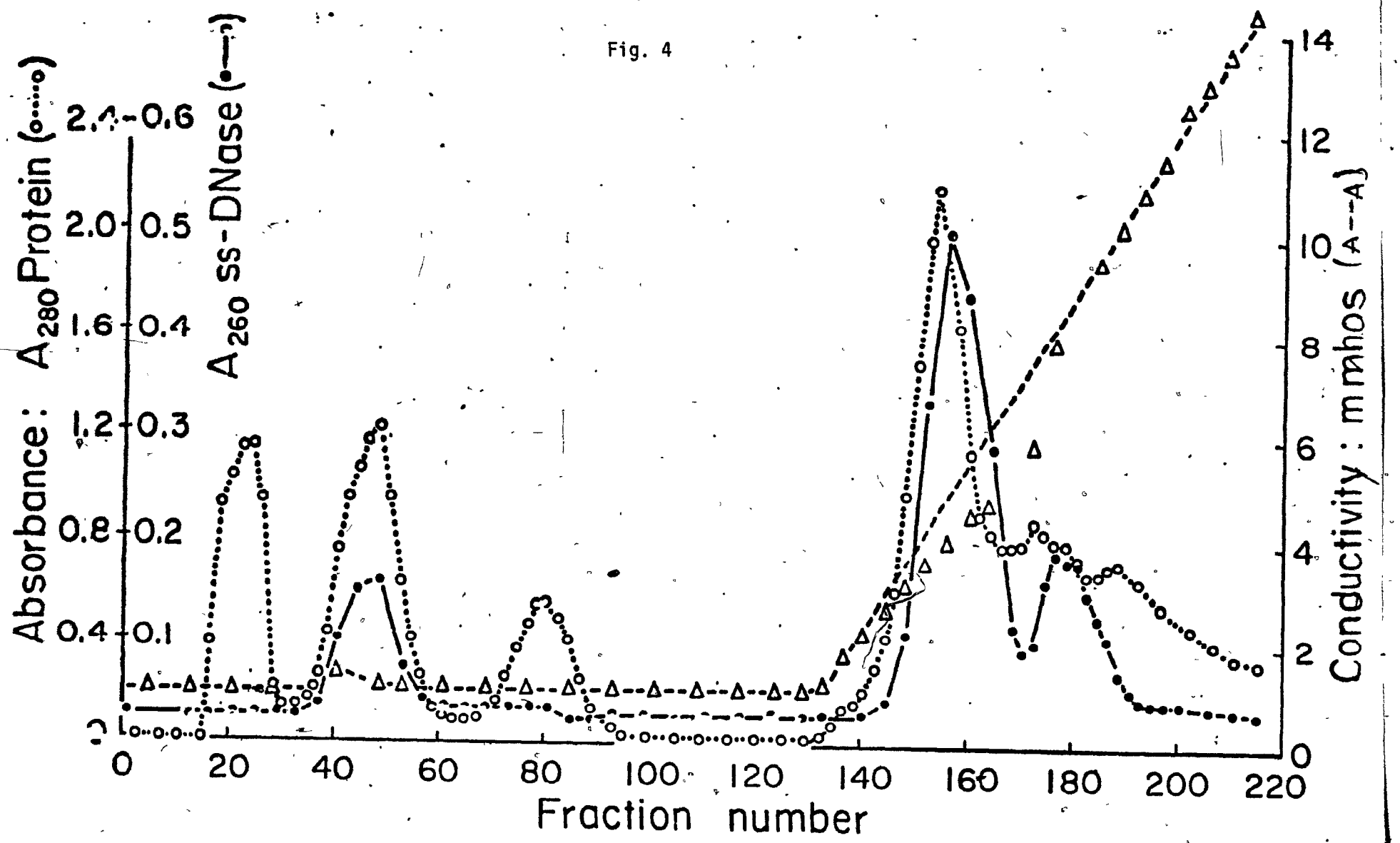

Absorbance: A_{280} Protein (o---o)
A_{260} ss-DNase (•—•)
ss-DNase after trypsin (—)
Conductivity: mmhos (Δ--Δ)
Fraction number
0
20
40
60
80
100
120
140
160
180
200
220
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
2
4
6
8
10
12
14

Fig 4: Chromatography on DEAE-Sepharose of a crude extract of 5 g (wet weight) uvs-3 mycelia grown for 2.5 days in liquid culture. For details see legend of fig. 3. Open circles represents protein (A_{280}), closed circles represents expressed ss-DNase activity (A_{260}), and open triangles represents conductivity (as measured with the conductivity meter).

Fig. 4


Absorbance: A_{280} Protein (o----o)
A_{260} ss-DNase (•—•)
Conductivity : m mhos (A--A)
Fraction number
0
20
40
60
80
100
120
140
160
180
200
220
2.4
2.0
1.6
1.2
0.8
0.4
0.6
0.5
0.4
0.3
0.2
0.1
14
12
10
8
6
4
2
0

Qualitatively, the same profiles were obtained for the mutants, but the third peak was much larger than wild-type. The separation of protein "peaks" in the pass-through fractions occurs presumably because of sieving of proteins in the gel. The second of these "peaks" (fractions 38 - 60) apparently contained ss-DNase activity (as determined by single time point assays on the fractions). These fractions, however, were very noticeably yellow in color, and on assay for ss-DNase activity over a 60 or 90 min incubation period, high optical blanks were observed (acid-soluble material absorbing at 260 nm), but no increase in the A_{260} occured with time. Thus, this "activity", which was also found in all extracts of mutants, was an artifact and will not be further discussed.

When a salt gradient was applied to the DEAE-Sepharose, protein was eluted nearly across the entire gradient, but an especially large $O.D._{280}$ absorbing "peak" was eluted with 0.04 to 0.11 M NaCl (fractions 138 -170, conductivity 3 - 5 m mhos). The elution of ss-DNase activity in the gradient occured in two distinct "peaks", which were assigned the notations D1 plus D2 (because it was subsequently resolved into two fractions D1 and D2) and D3, did not coincide with the major protein "peak". D1 plus D2 was eluted with 0.07 - 0.16 M NaCl (fractions 145 - 175, conductivity 4 - 8 m mhos) and D3 was eluted with 0.16 - 0.25 M NaCl (fractions 182 - 220, conductivity 8 - 12 m mhos). The elution of total wild-type ss-DNase activity, which is determined after trypsin activation of the fractions in gradient, was eluted out between D1 plus D2 and D3. This can be seen by subtracting the differences between ss-DNase activities in each fraction before and after trypsin

Table 6: Recoveries of ss-DNase activities are estimated by tracing the activity profiles from DEAE-Sepharose (cf. Fig 3 & 4) on card board. The appropriate areas are cut out of the cardboard and weighed. Corrections are made for the baselines (A_{260} of fractions with no ss-DNase activities). In no case does the degradation of substrate (ss-DNA) by enzyme in fractions making up the profile exceed 50% (A_{260} of 0.65 above the baseline).

Table 6

Distribution of ss-DNase activities of extracts recovered in D1 plus D2 and D3 after chromatography on DEAE-Sepharose

Strains	Extract on DEAE-Sepharose	
	% Recovery in D1 plus D2	% Recovery in D3
wt-A	41,54,62	46,38,59
uvs-3	70,77	23,30
nuh-4	62	38
uvs-6	46,64	36,54
nuh-3	49,53	47,51
nuc-2	59	41

Fig. 5 (A and B): Chromatography of wild-type DEAE-Sepharose fractions D1 plus D2 (panel A) and D3 (Panel B) on phosphocellulose. D1 plus D2 and D3 are dialysed against buffer B and applied to 15 ml (packed volume) columns of phosphocellulose equilibrated with buffer B. After washing with more buffer B, the columns are eluted with linear gradients of 4 mM-0.3 M potassium phosphate buffer, pH 6.5. Proteins (open circles), express ss-DNase (close circles) and conductivity (open triangles) are determined as in Methods.

Fig. 5 A

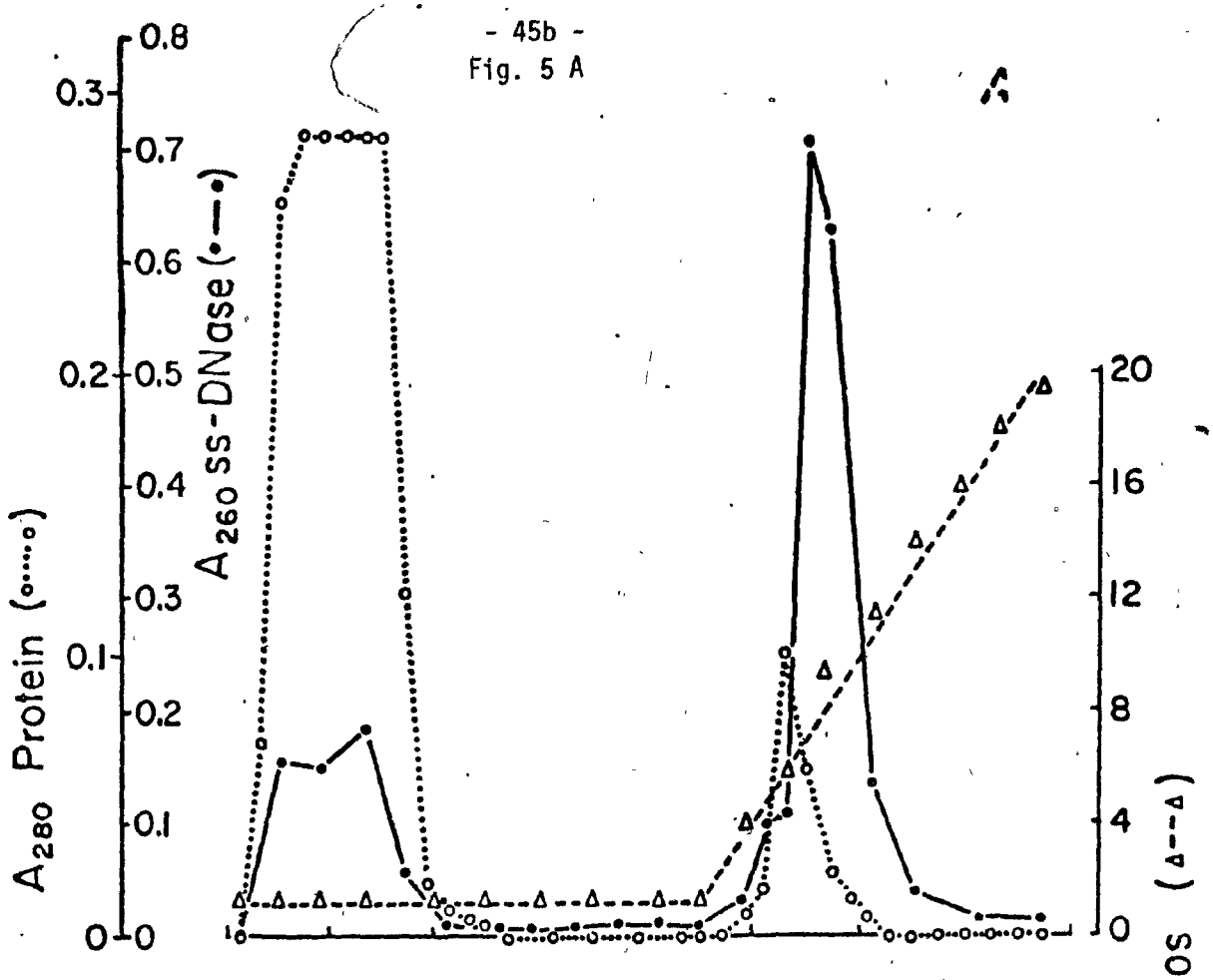

A280 Protein (•····•)
A260 ss-DNase (•—•)
Conductivity: mmhos (Δ--Δ)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.3
0.2
0.1
0
20
16
12
8
4
0

Fig. 5 B

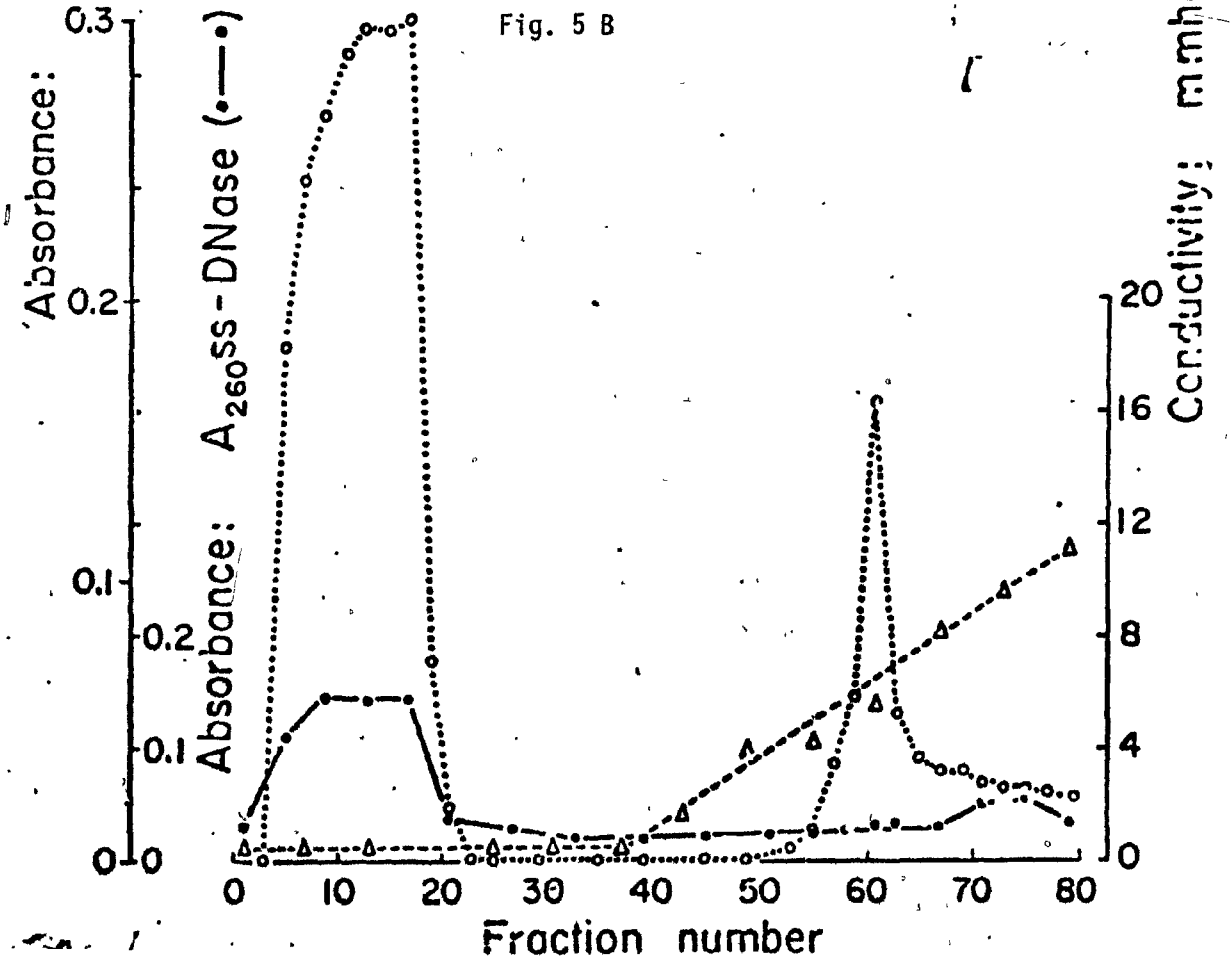

Absorbance:
A260ss-DNase (•—•)
Absorbance:
Conductivity: mmhos
0.3
0.2
0.1
0
20
16
12
8
4
0
0 10 20 30 40 50 60 70 80
Fraction number

Table 7: Recoveries of ss-DNase activities are estimated by tracing the activity profiles from phosphocellulose (cf. Fig 5A) on cardboard. The appropriate areas are cut out of the cardboard and weighed. Corrections are made for the baselines (A_{260} of fractions with no ss-DNase activities). In no case does the degradation of substrate (ss-DNA) by enzyme in fractions making up the profile exceed 50% (A_{260} of 0.65 above the baseline).

Table 7

ss-DNase activities recovered in D1 and D2 after chromatography of D1 plus D2 on phosphocellulose

Strains	D1 plus D2 on Phosphocellulose	
	% Recovery in D1	% Recovery in D2
wt-A	32	68
uvs-3	17	83
nuh-4	21,28	72,79
uvs-6	34	66
nuh-3	63	37
nuc-2	28	72

treatment. The results shows that endo-exonuclease precursor was eluted from DEAE-Sepharose with 0.09 - 0.18 M NaCl in fractions 165 - 190 (conductivity 5 - 9 m mhos).

The elution profiles for uvs-3 and all other mutants were very similar to that of the wild-type except for the presence of a fairly prominent third "peak" of unadsorbed protein (fractions 72 - 92, see Fig. 4).

The proportions of the ss-DNase activity in D1 plus D2 and D3 were estimated by determining the areas under the "peaks" of ss-DNase activity and are recorded in Table 6. It can be seen that the proportions of ss-DNase in D1 plus D2 and D3 were approximately 50% each for the wild-type and all other mutants except uvs-3. For uvs-3 the proportions of ss-DNase in D1 plus D2 and D3 averaged 74% and 26% respectively.

3.1.3.2 Fractionation on Phospho-cellulose Column

The ss-DNase activities in D1 plus D2 and D3 were further fractionated by chromatography on phosphocellulose. The chromatographic profiles for the wild-type D1 plus D2 and D3 are shown in Fig. 5 (panels A and B respectively). The ss-DNase activity in D1 plus D2 was resolved (Fig. 5A) into an unadsorbed ss-DNase (D1) and an adsorbed fraction (D2) which eluted from the column in middle of the gradient. The proportions of ss-DNase activity recovered from phosphocellulose in D1 and D2 are shown in Table 7. The amount of ss-DNase in D2 was much lower (37%) for nuh-3 mutant relative to wild-type (68%). In several cases of fractionation on D1 plus D2 of mutants on phosphocellulose, a second minor "peak" was eluted on

the leading edge of D2 in the gradient (Fig. 5A). The amounts of this minor fraction were highest for the nuh-4, uvs-6, and uvs-3 mutants, respectively 13%, 14% and 17% of the total ss-DNase recovered from phosphocellulose. However, this minor activity had the same distinctive properties as D2 (see below and Table 8). The recoveries of the minor ss-DNase have thus been included in the estimates of ss-DNase activities of D2 in all cases (Table 7).

Over 90% of the ss-DNase activity of D3 failed to adsorb on phosphocellulose and since the general properties of this fraction did not change appreciably at this step (Table 8), the designation D3' was assigned to this fraction. The small amount of ss-DNase activity which adsorbed on phosphocellulose from D3 was not further investigated.

RNase activity profiles for the D1, D2, D3' from phosphocellulose coincided with those for the ss-DNase profiles shown in Fig. 5. The ratio of the ss-DNase/RNase activity was very close to 1:1 for D1 and D3', but was 2.9 across the profile of D2 (gradient in Fig. 5A).

When aged D1 plus D2 fraction (1 week old) from DEAE-sepharose was applied to phosphocellulose, the D2 fraction was greatly reduced. Almost all the ss-DNase activity recovered (86% of ss-DNase applied) from phosphocellulose appeared as D1 (Fig. 6). When this D1 fraction was rechromatographed on DEAE-sepharose, two ss-DNase peaks were eluted in the gradient. These were assigned the names D1 (A) and D1(B). D1(B) eluted at the same salt concentration as D1 plus D2 (0.07 - 0.16 M) but D1(A) eluted very early in the gradient (Fig. 7) with 0.01 - 0.06 M NaCl. The proportions of ss-DNase activity recovered in D1(A) and

Fig. 6: Chromatography of 1 week old wild-type D1 plus D2 fraction from DEAE-Sepharose onto phosphocellulose. D1 plus D2 (in buffer B) was applied to 20ml (packed volume) columns of phosphocellulose eqilibrated with buffer B. After washing with buffer B, the columns were eluted with linear gradients of 4 mM-0.3 M KPB, pH 6.5. Protein (open circles), expressed ss-DNase (close circles) and conductivity (dashed line) are determined as in Methods.

Fig. 7: Chromatography of D1 from Fig. 6 onto DEAE-Sepharose. This D1 fraction was applied to 25 ml (packed volume) column of DEAE- Sepharose equilibrated with buffer A. After washing with more buffer A, the column was eluted with 0.0-0.5 M NaCl gradients in buffer A, pH 6.5. Protein (open circles), expressed ss-DNase (close circles) and conductivity (dashed line) are determined as in Methods.

Fig. 6

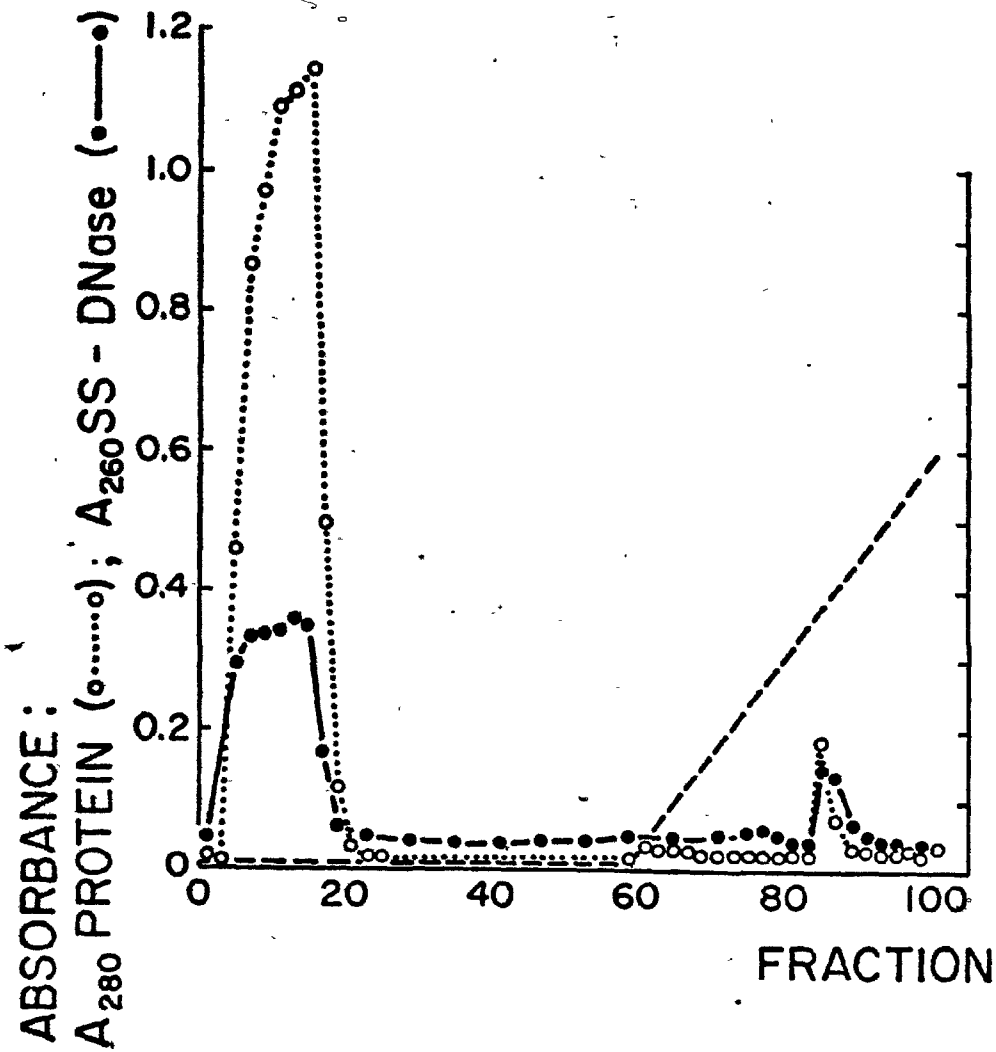
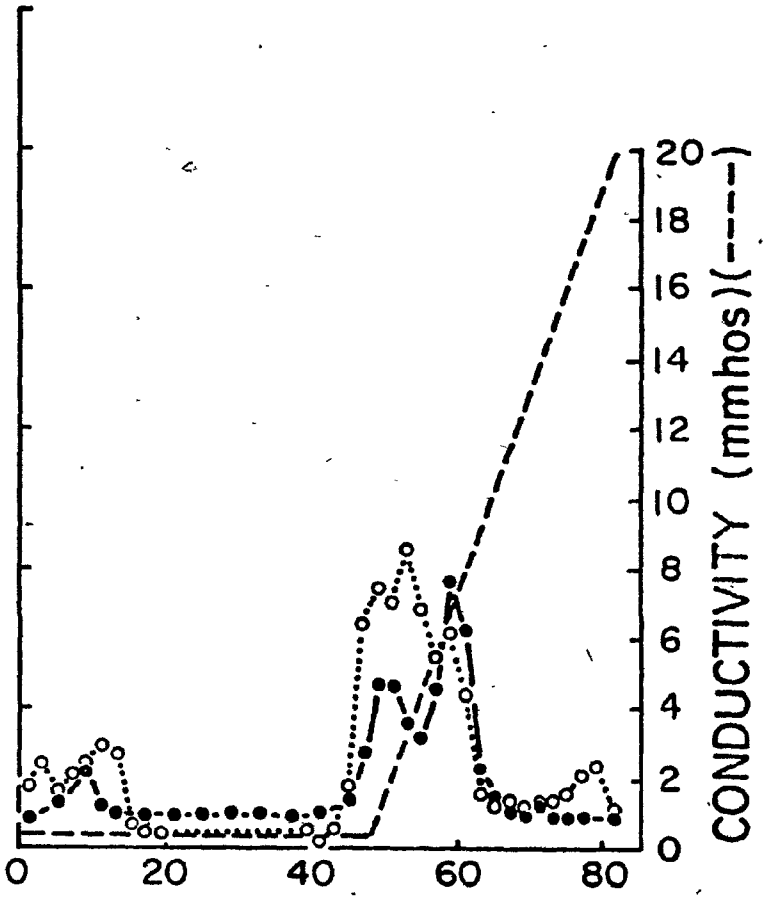

ABSORBANCE:
A280 PROTEIN (o.....o); A260SS - DNase (•——•)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
FRACTION NUMBER

Fig. 7


CONDUCTIVITY (mmhos)(---)
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80

D1(B) as in this step were 29% and 63% of the total respectively.

3.1.4 Properties of the DNases Eluted from DEAE-Sepharose and Phosphocellulose

Both the DEAE-sepharose gradient fractions (D1 plus D2 and D3) and the phosphocellulose fractions (D1, D2 and D3') of the wild-type and mutants were examined qualitatively for endonuclease and exonuclease activities using trichloroacetic acid (TCA) and uranyl acetate-trichloroacetic acid as precipitants of undigested DNA as described by Mills and Fraser (9). In addition, they were examined quantitatively (using perchloric acid (PCA) as a precipitant (8)) with respect to the following parameters: ratio of ss-DNase/ds-DNase activities (ss/ds), the inhibition of the ss-DNase activity by 0.5 mM ATP in the presence of 10 mM Mg^{++}, the ss-DNase activity in absence of 10 mM Mg^{++} in reaction medium, the inhibition of ss-DNase activity by EDTA (in absence of 10 mM Mg^{++} in reaction medium) and its restoration with Mg^{++} and Ca^{++}. The quantitative results for D1 plus D2 and D3 (from DEAE-sepharose) are presented in Table 8, and the results for D1, D2 and D3' (from phosphocellulose) are presented in Table 9.

3.1.5 Properties of the DEAE-Sepharose fractions: D1 plus D2 and D3

It can be seen from the data in Table 8 that even though wide variations in the properties of D1 plus D2 and D3 of the wild-type and mutants occurred, in general, the properties of D1 plus D2 and D3 were different. There was little or no ATP inhibition of D1 plus D2 ss-DNase activity, but substantial inhibition was obtained for D3 ss-DNase activity. The values for ATP inhibition of D3 ranged from 17% to 87%

Table 8: The controls for dependence on Mg^{++} (no Mg^{++} in assay mixture) and for ATP inhibition were performed with regular routine assays (i.e. in presence of 10 mM Mg^{++} in assay mixture). The controls for EDTA inhibition, restoration of activity with Mg^{++} and Ca^{++} was ss-DNase assay in absence of metal ions.

Table 8

Properties of D1 plus D2 and D3 from DEAE-Sepharose chromatography

Strains		% ss-DNase Activity in presence of:				
	ss/ds*	$-Mg^{++}$	EDTA	EDTA+Mg^{++}	EDTA+Ca^{++}	ATP
D1 plus D2						
wt-A	7.7	68	14	93	14	100
uvs-6	35,5.4	65,37	42,26	47,200	0,44	80,81
uvs-3	16,4.2	34,49	25,12	185,139	35,6	100,78
nuc-2	3.6	43	19	131	46	100
nuh-4	5.6	40	12	140	0	79
nuh-3	12.5	34	28	174	22	77
D3						
wt-A	11.4	81	31	51	6	66
uvs-6	28,31	104,81	25,29	8,66	4,42	83,69
uvs-3	144+,21	110,60	45,47	54,52	28,4	70,57
nuc-2	27	60	36	46	32	64
nuh-4	21	102	37	34	0	76
nuh-3	25	58	34	52	9	13

* ss/ds= single-strand DNase activity to double-strand DNase activity ratio, it is not in percent activity as the case of other values.

with majority of them between 24% to 43%, thus gives an average of 33% inhibition or 67% activity remained. Both D1 plus D2 and D3 were partly inhibited by EDTA, but D3 was less sensitive than D1 plus D2. The restoration of ss-DNase activity by Mg^{++} after EDTA treatment for D1 plus D2 is complete, and in most of the cases, the restoration was greater than 100%. Incomplete restoration of D3 ss-DNase activity was obtained with Mg^{++}. Ca^{++} was ineffective in reversing the EDTA inhibition of ss-DNase activity of both D1 plus D2 and D3, and the presence of Ca^{++} further lowered the activity in most cases.

The ss-DNase activity of untreated D1 plus D2 was also partially dependent on divalent metal ions. In absence of divalent metal ions, the activity of D1 plus D2 was about 45% of the activity observed with 10 mM Mg^{++}. The ss-DNase activity of untreated D3, however, was only slightly dependent on added Mg^{++} ions. The average ss-DNase activity in absence of Mg^{++} was about 82%.

The ss/ds ratios of D1 plus D2 and D3 were also different. The ss/ds ratios of D1 plus D2 were lower than D3 within the strain measured (Table 8). The ss/ds ratios of D1 plus D2 between strains were variable. They ranged from 3.6 in nuc-2 to 35 in one case of uvs-6. (see discussion p.79). The ss/ds ratio of D3 between strains, however, were more limited, in the range of 21 to 28 (table 8). The D3 ss/ds of mutants in average was 25, which is higher than an average of 11 for the wild-type. The qualitative examination of D1 plus D2 and D3 (TCA and UTCA as precipitant) from wild-type indicated that both enzyme behaved like endo-exonuclease (7, 10, 11).

3.1.6 Phosphocellulose fractions D1, D2 and D3'

During chromatography on phosphocellulose, fresh D1 plus D2 was resolved into D1 and D2, while most of the ss-DNase activity of D3 was recovered as D3' in the unadsorbed fraction. Although wide variations in the properties of D1 and D3 of the wild-type and mutants occured (even within the same strain in different preparations), in general, the properties of D1 and D3' had some common properties (Table 9). All D1 and D3' fractions showed inhibition by ATP, but the variations in the inhibitions were wider for D1 than for D3'. In the latter case, the ss-DNase activity in the presence of ATP ranged from 35 - 68% (Table 9) with most values falling close to the average of 54% activity or 46% inhibition. This average is very close to that observed previously for the inhibition by ATP of purified ss-endonuclease (12) and of purified endo-exonuclease (10). Both D1 and D3' were only partially inhibited by EDTA and restorations of ss-DNase activity were incomplete with Mg^{++} and even lower with Ca^{++}.

The ss/ds ratios for the wild-type D1 and D3' were both relatively low, averaging 2.9 and 5.9 respectively and both enzymes behaved qualitatively like endo-exonuclease (7, 10, 11). In contrast, the ss/ds ratios for all of the mutant D1 and D3' preparations fell within considerably higher ranges, respectively 11 - 50 for mutant D1 and 19 - 50 for mutant D3' preparations (Table 9). These high ratios proved to be due to the presence of ds-DNase inhibitor in the preparations (see section 5).

The properties of DNase in D2 of the wild-type and mutants were very similar and distinctly different from the properties of the DNases

Table 9: The control for dependence of Mg^{++} (no metal ions in assay mixture) and ATP inhibition were the routine assays (i.e. in presence of 10 mM Mg^{++} in assay mixture). The control for EDTA inhibition, restoration of activity with Mg^{++} and Ca^{++} were ss-DNase assay in absence of metal ions.

Table 9

Properties of D1, and D2 from phosphocellulose chromatography

Strains		% ss-DNase Activity in presence of				
	ss/ds*	$-Mg^{++}$	EDTA	EDTA+Mg^{++}	EDTA+Ca^{++}	ATP
D1						
wt-A	2.5,3.3	91	12,3	39,35	50,6	70,18
uvs-6	50,23	52	22,19	40,67	30,31	70,52
uvs-3	27	29,52	31	58	0	36
nuc-2	17	31	4	27	0	87
nuh-4	50,12	63	17,15	39,39	31,6	61,42
nuh-3	11	29	24	24	0	81
D2						
wt-A	1.9,2.7	61	2,0	163,146	4,0	103,-
uvs-6	4.4	32	18	204	9	100
uvs-3	4.2	12,3	28	99	37	119
nuc-2	1.7	24	0	185	0	88
nuh-4	2.1,2.9	33	0,0	323,275	0,0	112,86
nuh-3	2.8	25	11	90	12	91

* ss/ds= single-strand DNase activity to double-strand DNase activity ratios; these are not expressed as percentages of the wild-type values as is the case for all other values.

Table 9 (continued)

Properties of D3' from phosphocellulose chromatography

Strains		% ss-DNase Activity in presence of				
	ss/ds*	$-Mg^{++}$	EDTA	$EDTA+Mg^{++}$	$EDTA+Ca^{++}$	ATP
wt-A	5.1,6.7	100	9,16	8,62	7,1	62,57
uvs-6	19,40	85	26,33	46,45	15,50	56,54
uvs-3	47	50,87	21	21	3	52
nuc-2	30	47	45	33	0	53
nuh-4	23,27	116	48,27	53,27	30,36	67,35
nuh-3	50	34	36	33	0	68

* ss/ds= single-strand DNase activity to double-atrand DNase activity ratios; these are not expressed as percentages of the wild-type values as is the case for all other values.

in D1 and D3' (Table 9). Qualitatively the DNase activity of D2 behaved as an endonuclease with very little strand specificity for DNA. The ss/ds ratios were all very low (Table 9) and averaged 2.5. The ss-DNase activity of D2 was not appreciably affected by 0.5 mM ATP, unlike that in D1 and D3'. The ss-DNase activity of D2 was found to be very sensitive to EDTA and its activity was restored with Mg^{++} to higher than control (no EDTA, no divalent metal ions) levels, but no activity was restored with Ca^{++} (Table 9).

The dependence of ss-DNase activity of D1, D2 and D3' on Mg^{++} was quite variable from strain to strain, but in spite of the variations, a general trend of difference can be seen between D1, D2 and D3'. Of the three nuclease, D3' has the lowest dependence on Mg^{++}, while D2 has the highest requirement for Mg^{++}. D1 has a dependency which is intermediate between D2 and D3' (Table 9).

None of the fractions derived from DEAE-Sepharose or phosphocellulose contained detectable nuclease N_3 activity (13), presumably because the mycelia were all grown on high phosphate medium.

3.1.7 Characterization of D1(A) and D1(B) from Wild-type

As the results in Table 10 indicated both D1(A) and D1(B) were similar in character with respect to EDTA inhibition, restoration of ss-DNase activity with Mg^{++} and Ca^{++}, and ATP inhibition. The ss-DNase activity was quite dependent on Mg^{++}. The ss-DNase activity in the absence of Mg^{++} were 19% and 30% of the activities in the presence of Mg^{++} for D1(A) and D1(B) respectively (Table 10). Qualitatively, both D1(A) and D1(B) exhibited endonucleolytic towards ss- DNA, and mixed endonucleolytic and exonucleolytic activity with ds-DNA. The ss/ds

Table 10: The control for dependence of Mg^{++} (no metal ions in assay mixture) and ATP inhibition were the routine assays (i.e. in presence of 10 mM Mg^{++} in assay mixture). The control for EDTA inhibition, restoration of activity with Mg^{++} and Ca^{++} were ss-DNase assay in absence of metal ions.

Table 10

Properties of D1(A) and D1(B) from DEAE-Sepharose chromatography

Nuclease fractions	% ss-DNase Activity in presence of					
	ss/ds (ratio)	$-Mg^{++}$	EDTA	$EDTA+Mg^{++}$	$EDTA+Ca^{++}$	ATP
D1(A)	5.0	19	0	333	48	102
D1(B)	2.0	30	13	363	25	102

Table 11: Equal volumes of PHMB (1 mM) and enzyme are preincubated for 30 minutes at room temperature before assaying for ss-DNase activity.

Table 11

PHMB inhibition on the Nucleases

Nuclease fraction	Units of activity (ss-DNase)		
	-PHMB	+PHMB	% inhibition
D1 plus D2(wt-A)	11.6	7.9	32
D1 (wt-A)	25.6	16.8	34
D3 (wt-A)	61.4	38.9	37
D1 (nuh-3 aged)	50.4	9.2	82
D3 (nuh-3)	109.2	8.4	92
D3' (nuh-3)	112.9	93.5	17
D1(A) (wt-A)	15.4	1.4	91
D1(B) (wt-A)	98.7	20.2	80

ratios were low, 5.0 for D1(A) and 2.0 for D1(B).

3.1.8 PHMB inhibition of ss-DNase Activities

When treated with equal volumes of saturated PHMB solutions (1 mM), the isolated intracellular nucleases exhibited different degrees of inhibition (Table 11). D3' was the least sensitive to PHMB, while D2 exhibited the greatest sensitivity. In addition, a change in PHMB inhibition apparently occurred for D1 as it aged. In a fresh preparation D1 (from the wild-type) showed an inhibition of 34%, but an aged preparation (from nuh-3) showed an inhibition of 82%. The ss-DNase activities of D1(A) and D1(B) were very sensitive to PHMB. D1(A) was inhibited by 91% and D1(B) was inhibited by 80%.

3.1.9 Evidence for ds-DNase Inhibitor

The presence of ds-DNase inhibitor(s) in D1 plus D2, D1, and D3 preparations with high ss/ds ratios was shown by three types of experiments: (i) relief of the ds-DNase inhibition on passing the preparations through DEAE-cellulose, (ii) differential activations of the ss-DNase abd ds-DNase activities of the preparations with trypsin and (iii) isolation of an inhibitory protein from an "aged" preparation of D1 plus D2. The data in Table 12 indicate that passage through DEAE-cellulose and trypsin treatment had essentially the same effect on preparations with high ss/ds ratios: the ss/ds ratios were dramatically lowered. In the case of trypsin treatments, the effects of lowering the ss/ds ratios were paralleled by preferential activations of ds-DNase activities versus ss-DNase activities in different preparations. All of these results are consistent with the removal of ds-DNase inhibitor(s) from preparations with high ss/ds ratios. The presence of ds-DNase inhibitor has been confirmed more directly by chromatographing

Table 12: DNase was activated by pre-treatment with trypsin (30 min, 23-24°C) at a concentration of 10 µg of trypsin per ml of activation mixture. The activation was stopped with SBI (3 times the amount of trypsin added (30 µg/ml)) before assaying for DNase activity.

Table 12

Evidence for the presence of ds-DNase inhibitor(s) in preparations from DEAE-Sepharose and phosphocellulose with high ratios of ss-DNase/ds-DNase (ss/ds)

Preparation	ss/ds Ratios		Trypsin activation (-fold)	
	Before	After	ss-DNase	ds-DNase
I. Chromatography on DEAE-cellulose				
wt-A D1				
plus D2	50	2.9	-	-
uvs-3 D1	7.0	2.5	-	-
uvs-3 D3	25	5.2	-	-
nuh-4 D1	14	11	-	-
nuh-4 D3	33	6.6	-	-
II. Treatment with trypsin				
wt-A D1				
plus D2	50	6.7	6.0	45
uvs-3 D1	7.0	5.3	1.8	3.1
uns-3 D3	25	8.6	2.1	6.0
nuh-4 D1	14	3.1	3.9	18
nuh-4 D3	33	18	1.0	1.7

Table 13: The amount of trypsin used to activate the crude fractions was 100 µg/ml and for the D1 plus D2 was 10 µg/ml. Conditions for activation were 30 minutes at 23-24°C. Activation was stopped with SBI, in 3 times the amount of trypsin added.

Table 13

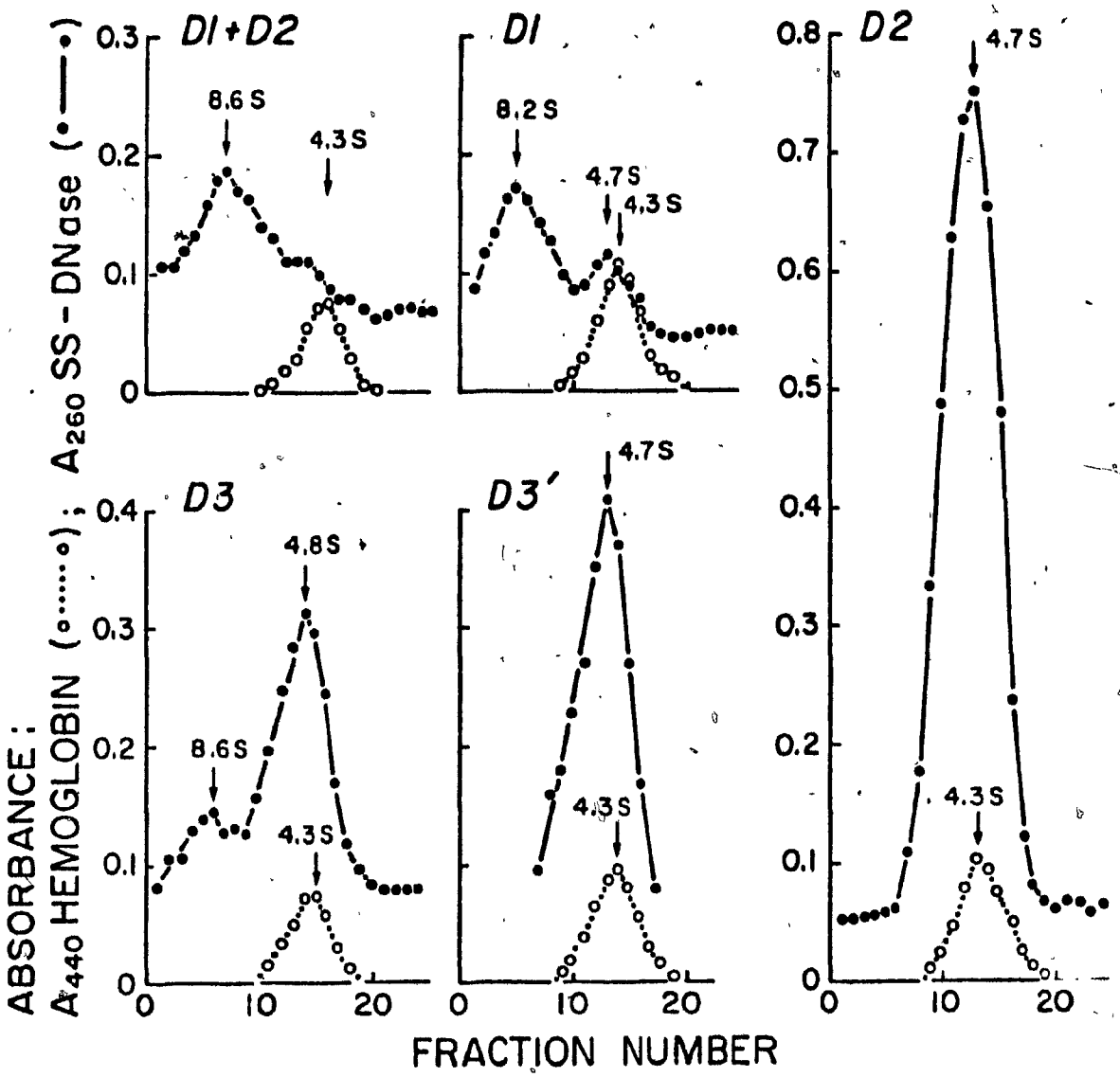
Differential trypsin activation of crude extract, D1 plus D2, and DEAE-strip fraction of wt-A (mid-log)

Preparations	Crude Extract						D1 plus D2						DEAE-cellulose					
	ss-DNase activity		ds-DNase activity		+T/-T		ss-DNase activity		ds-DNase activity		+T/-T		ss-DNase activity		ds-DNase activity		+T/-T	
	-T	+T	-T	+T	ss	ds	-T	+T	-T	+T	ss	ds	-T	+T	-T	+T	ss	ds
I	68	201	2	16	3.0	7.9	-	-	-	-	-	-	7.7	23	.93	1.2	3.0	1.3
II	72	189	3	18	2.6	6.0	-	-	-	-	-	-	8.1	24	.93	2.2	3.0	2.4
III	144	467	6.4	44	3.2	6.8	-	-	-	-	-	-	8.6	17.3	.90	2.4	2.0	2.7
IV	-	-	-	-	-	-	54.4	330	1.1	49	6	45	11	34.4	.50	1.8	3.1	3.6

a 14-day old preparation of wild-type D1 plus D2 (stored at 0 - 4°C) with a ss/ds ratio of 50 through DEAE-cellulose. Unexpectedly, a substantial amount of protein (with no DNase activity) failed to absorb on DEAE-cellulose. When this protein was added to the DNase eluted in the gradient from the same column (a mixture of D1 and D2 with a ss/ds of 3.4), no inhibition of ss-DNase activity was observed, but a 33% inhibition of ds-DNase activity occurred. It is not known whether this inhibition could have been pushed to completion by adding more protein or whether the inhibition was preferential for the ds-DNase activity of D1 or of D2. In this experiment, no differential trypsin activation of ss-DNase and ds-DNase was detected (Table 13), indicating that no ds-DNase inhibitor was present in the fraction derived from DEAE-cellulose, although the 3-fold activations of ss- and ds-DNase activities indicate that endo-exonuclease precursor was present. This result is in contrast to the huge differences in activation of ss- and ds-DNase activities (6-fold and 45-fold) before DEAE-cellulose chromatography and for the crude extracts (Table 13). Attempts to recover ds-DNase inhibitor from fresh (1-day old) preparations of D1 plus D2 have so far failed, in spite of the fact that the ss/ds ratios were lowered in the expected manner by passage through the DEAE-cellulose (Table 12). These results may indicate that limited proteolysis is required to release the inhibitor(s) from the enzyme-inhibitor complexes.

Fig. 8: Sedimentation velocity centrifugation of nuclease fractions in linear 5-20% sucrose gradients. Hemoglobin was used as a marker. It has a sedimentation coefficient of 4.3S. Hemoglobin concentration was determined by A_{440} (open circles), and ss-DNase activity (close circles) was determined by "point" assay.

Fig. 8


DI+D2
8.6 S
4.3 S
DI
8.2 S
4.7 S
4.3 S
D2
4.7 S
4.3 S
D3
4.8 S
8.6 S
4.3 S
D3'
4.7 S
4.3 S
ABSORBANCE:
A440 HEMOGLOBIN (o.....o); A260 SS-DNase (•——•)
FRACTION NUMBER

3.1.10 Molecular Weight Determination by Sedimentation Velocity Centrifugation on Sucrose Density Gradients

The method of Martin and Ames (4) was employed to determine the apparent molecular weights of all nuclease fractions isolated here. A linear relationship was found to occur between distance sedimented from the meniscus and the sedimentation coefficient of the protein. In these experiments, the nucleases were all sedimented with bovine hemoglobin as a marker (molecular weight 63,000 daltons). The D1 plus D2, D1, D2, D3 and D3' fractions were sedimented individually and also in various different combinations with each other (Fig. 8). D1 plus D2, D1, D2, D3 and D3' had sedimentation constants of 8.6 S 8.2 S, 4.7 S, 4.8 S, and 4.7 S respectively. These values corresponded to apparent molecular weights respectively of 184,000; 170,000; 75,000; 77,000; and 75,000 (Table 14). A second minor component in D1 had a sedimentation constant of 4.7 S (apparent molecular weight of 75,000) (Fig. 8). A mixture of D1 and D2 sedimented as a single component of 5.2 S (85,000 daltons) rather than two components as expected. A mixture of D2 and D3' sedimented as a single component of 4.7 S as expected (molecular weight 75,000).

CHAPTER IV

4.1 Discussion

4.1.1 Histidine and Mitomycin C Sensitivities of wild-type and mutants

Based on the results of tests for sensitivity to histidine and mitomycin C (Table 3), the mutants can be divided into three groups: (1) those which sensitive to both agents, (2) those which sensitive to either one or the other agent, and (3) those which are not sensitive to either of the two agents. Mutants which have been found to be sensitive to a wide spectra of mutagens, uvs-3 and uvs-6 (78), mms(04) and nuh-4 (E. Kafer, unpublished results), and mei-3 (56) were found to be sensitive to both histidine and mitomycin C . The meiotic UV-sensitive mei-3 mutant which causes deletions of duplications and uvs-3 had been found previously to be histidine sensitive (79). All histidine and mitomycin C sensitive mutants above were also found to have Nuh phenotype i.e. they fail to secrete normal amounts of deoxyribonuclease (55). Analogies can be drawn between these histidine and mitomycin C sensitive mutants with the rec A and lex A mutants of E. coli. The rec A and lex A mutants in E. coli show a set of startling pleiotropic effects, which include high sensitivity to a wide spectra of mutagens (80). The sensitivity of these N. crassa mutants toward histidine and mitomycin C as well as to other mutagens like UV, and ionized irradiation strengthens the possibility that the Neurospora mutants are rec mutants. In addition, two of these mutants, uvs-3 and uvs-6 have been found to show high mitotic recombination and/or deletions (56). They are thus phenotyically closer to E. coli lex A mutants than to rec A mutants which are deficient in recombination. The effects of the nuh-4 and mms(04) mutations on recombination have not yet been

assessed.

The results for strains which do not have the Nuh phenotype but which have narrow spectra of mutagen sensitivities, uvs-4 and the two pyrimidine dimer excision-deficient strains, uvs-2 and upr-1, confirm those of Newmeyer, Schroeder and Galeazzi (56) for histidine sensitivity. Athough uvs-4 was sensitive to histidine, it was not sensitive to mitomycin C. On the other hand, uvs-2 and upr-1 were not sensitive to either agent. The uvs-4 mutant was found previously to be sensitive to UV but not to ionizing radiation, unlike uvs-3 and uvs-6 which are sensitive to both agents (21). The insensitivity of these three mutants toward mitomycin C, and to histidine in the cases of uvs-2 and and upr-1, correlates with the lack of any effects on recombination. Nuh-3 was found previously not to be sensitive to UV or to the chemical mutagens, nitrosoguanidine (NG) and methylmethanesulfonate (MMS), (E. Kafer, unpublished results) and here it is shown not to be sensitive to histidine and mitomycin C. Is these respects, it is representative of other nuh mutants which do not show sensitivity to either UV or chemical mutagens e.g. nuh-1, nuh-8, nuh-5, and nuh-6. In conclusion, nuh mutants which fail to secrete normal amounts of DNase fall into two classes, mutagen-sensitive and mutagen-insensitive. The former class appears to be Rec-like, based on the wide spectra of sensitivities to mutagens and in two cases (uvs-3 and uvs-6) are known to have altered recombination. It thus becomes of interest to determine whether or not a given pattern of intracellular DNases correlates with the Rec-like phenotype. Of special interest is the Neurospora endo-exonuclease which has a number of enzymological properties in common with the major recombination

nuclease of bacteria e.g. exonuclease V of E. coli (37).

4.1.2 Identity of the Major Intracellular Alkaline of Log-phase Mycelia.

The expressed ds-DNase activity of extracts of wild-type log phase N. crassa mycelia is low (Table 5). The ds-DNase specific activity is 0.6 units/mg protein while expressed ss-DNase specific activity is 18 units/mg protein. These values give a single to double strand ratio (ss/ds) of activities of 30. The expressed ss-DNase activity was followed during fractionation, and expressed ds-DNase was determine only on isolated fractions.

The expressed ss-DNase activity of extracts of mid-log wild-type mycelia resolved into three fractions by chromatography in turn on DEAE-Sepharose and on phosphocellulose. The proportions of these three fractions, D1, D2, and D3', can be calculated from table 6 to comprise respectively 16%, 34%, and 50% of the total ss-DNase activity recovered. The isolated fractions also expressed substantial ds-DNase activity in spite of the fact that the expressed ds-DNase activity in crude extracts was extremely low (see above). Two of the three isolated fractions have properties very similar to two nucleases which have been previously described. D3'is very similiar in properties to endo-exonuclease (10, 7, 11) and D2 is very similar in properties to mitochondrial nuclease (50, 51). The properties of the minor component, D1, appear to be time dependent (see below).

D3'exhibited qualitatively endonuclease activity with ss-DNA and exonuclease activity with ds-DNA when assayed with TCA and UTCA as precipitants of undigested DNA (10). D3' also had a high RNase activity. In addition, the ss-DNase activity of D3' in the presence of 0.5 mM

ATP was 54% of the control, and it was relatively resistant to inhibition by EDTA. Mg^{++} only restored the ss-DNase activity of the EDTA-inhibited enzyme to a small extent and Ca^{++} was even less effective in restoring the activity (Table 9). The apparent molecular weight of native D3' was 75000 (Fig. 8 and Table 8), which is only slightly higher than that of 61000 found for the molecular weight of the trypsin-activated endo-exonuclease precursor (6), but lower than that of 88000 found for the inactive precursor (7, 81). The endo-exonuclease and its inactive precursor were both found previously to be single polypeptides (7). The highest molecular weight determined for the active endo-exonuclease by electrophoresis on denaturing gels, however, was 53000. This discrepancy was explained (7) by the observation that the enzyme was very susceptible to proteolysis by endogenous proteinases during purification (7, 10). The proteolysis reduced the exonuclease activity while exhibiting little effect on the endonuclease activity. Thus, the apparent molecular weight of 75000 for D3 may indicate that little proteolysis had occurred during the isolation process, or, alternatively it could indicate that the enzyme may be associated with other protein(s). The first possibility was supported by the fact that the inactive endo-exonuclease precursor had a large hydrophobic portion (6, 7) and the secreted endo-exonuclease had an apparent molecular weight of 65000 (11). The properties of the secreted endo-exonuclease was similar to those of D3'. However, some evidence for the latter possibility was also found. This is the possibility of association of the enzyme with a ds-DNase inhibitor which will be discussed below.

The properties of D2 were very similar to those of the previously described mitochondrial nuclease (50, 51). It exhibited qualitatively endonuclease activity with both ss-DNA and ds-DNA. It also had RNase activity. Its activity was very sensitive to EDTA and was restored to greater than 100% of the control on addition of Mg^{++}. Ca^{++}, however, was not effective in reversal of the EDTA inhibition. Although D2 was derived from a fraction (D1 plus D2) which adsorbed on DEAE Sepharose, the isolated D2 fail to adsorb when rechromatographed on DEAE-Sepharose with 20mM potassium phosphate buffer, pH 6.5. This chromatographic behavior is very similar to that of the major fraction of mitochondrial nuclease on DEAE-cellulose (50). The apparent molecular weight found here for D2 was 75000, which is substantially lower than that of 120000 reported for mitochondrial nuclease (51). However, it seems likely, from the apparent conversion of D1 to D2 (see below), that a higher molecular weight D2 does esists in the form of D1 (apparent molecular weight of 180000), but the discrepancy in molecular weights would still remain unexplained. D2 is clearly a different nuclease from the extracellular non-strand specific endonuclease (DNase A) which had been described by Fraser (11). Five differences in properties exist between these two nucleases: (i) DNase A is a Ca^{++} dependent enzyme, whereas D2 is Mg^{++} dependent and has no activity with Ca^{++}; (ii) Although both enzymes were classed as a "non-specific" endonucleases, DNase A degrades ds-DNA faster than ss-DNA (ss/ds of 0.6), while D2 hydrolyzes ss-DNA faster (ss/ds of 2.5) under optimal conditions for both enzymes; (iii) The native molecular weight of DNase A is 65000 as compared 75000 for D2; (iv) DNase A is a much

more stable enzyme than D2 (11); (v) DNase A exhibits no activity with RNA whereas D2 hydrolyzes RNA.

The properties of freshly isolated D1 preparations were quite variable (Table 5) from preparation to preparation, and in time of storage at low temperature. When characterization of fresh preparations was carried out, their properties resembled those of D3' in some instances. These properties include the inhibition of ss-DNase activity with ATP, relative resistance to inhibition by EDTA, and partial restoration of activity with either Mg^{++} or Ca^{++}. The p-hydroxymercuribenzoate (PHMB) inhibition of D1 and D3 were also similar (a relatively low level of inhibition), and were clearly different from that of D2 (Table 11). However, after D1 was "aged" one week at 0-4°C, the properties of D1 had converted to those of D2 with concomitant loss of inhibition by ATP, and increase of EDTA sensitivity, a Mg^{++} reversal of EDTA inhibition ability which restored over 100% that of control, and no restoration of activity with Ca^{++}. There was also an increase in sensitivity to PHMB. When D1 was sedimented in a linear sucrose density gradient, a more slowly sedimenting component of apparent molecular weight of 75000 appeared in addition to the original one of 180000. Finally, the chromatographic properties of D1 changed on ageing into those of D2. Similar to the isolated D2, aged D1 preparations did not adsorb or only weakly adsorbed on DEAE-Sepharose. The cause of this conversion of D1 to D2 with time is not known, but it is strongly suspected that endogenous proteinase(s) are involved. It seems quite likely that all of the D2 exists initially in the high molecular weight form, since all of the ss-DNase activity in fresh preparations of D1 plus D2 (from DEAE-Sepharose) sedimented with an apparent molecular weight of 180000.

When the ss-DNase activity in the D1 plus D2 fraction was characterized (Table 8), it behaved in the expected manner, like a mixture of D1 and D2. The reason for the rapid sedimentation of D2 in the D1 plus D2 preparations is not yet clear. What is clear is that the passage of D1 plus D2 through phosphocellulose resolved the two components, and on standing more D2 is produced from D1.

The D1 plus D2 fraction can be considered to consist of two forms of mitochondrial nuclease which are separable by chromatography on phosphocellulose. Two forms of mitochondrial nuclease were also reported by Linn and Lehman (50) and by Martin and Wagner (51), but in each case, the two forms were distinguished on a different basis, the former by different affinities for DEAE-cellulose and the latter by different solubilizations in the presence of Triton X-100. However, the possibility that the conversion of D1 to D2 with time may result from artifacts of extraction can not be dismissed. This conversion occurred in vitro, where the environment for the enzyme is quite different from that in vivo, e.g. passage through DEAE-Sepharose may result in activation of various proteases (94). The properties of fresh D1 may be closer to the properties in vivo and in such a case, D1 would behave more like another form of endo-exonuclease than it would behave like D2 (see above). Thus, in spite of the results obtained in vitro indicating that D1 converts to D2, it can not be absolutely ruled out that D1 is another form of endo-exonuclease (D3). One possibility that may reconcile these two apparently very different interpretations of the relationship between D1 and the other two major intracellular nucleases is that both endo-exonuclease and the mitochondrial nuclease arise from the same inactive precursor via different routes of proteolysis. This might explain the pleiotropic effects of various mutations on both

intracellular enzymes (see below). One way to examine the interrelationships between these enzymes and the endo-exonuclease precursor would be to test their antigenic cross-reactivity. This will require the isolation in quantity of at least one of these entities in highly purified form.

4.1.3 DNase Deficiencies in Mutants

Three of the mutants, uvs-3, nuh-4, and nuh-3, show clear deficiencies in the levels of expressed ss-DNase activity throughout log phase growth (Table 4). The levels of total ss-DNase (measured after trypsin activation), however, were not significantly different from the wild-type. The low expressed levels of DNase of these mutants are consistent with their Nuh phenotype, i.e. the production of smaller nuclease haloes as compared to wild-type when grown on DNA contained miminal agar plates (55). They are also consistent with the observations that these mutants are deficient in the release of extracellular DNases into the liquid culture medium, including endo-exonuclease (11). The normal levels of total DNase activity in these mutants, together with the isolation of an inactive endoexonuclease precursor (6, 7), suggests that the low levels of expressed DNase may be due to a deficiency in proteases in which participated in the processing of endo-exonuclease precursor.

There were no significant differences expressed and total ds-DNase observed between the mutants (uvs-3, nuh-4, nuh-3) and the wild-type. However, the expressed ds-DNase activity is all but completely supressed in all strains by a ds-DNase inhibitor (see 4.1.4 of Discussion). This eliminates any differences in ds-DNase activity which may exist between mutants and the wild-type. The activation to ds-DNase

activity with trypsin was consistently greater than the activation of ss-DNase due both to the destruction of this inhibitor and the conversion of endo-exonuclease precursor to active enzyme.

The data in Table 6 indicate that the deficiency in expressed ss-DNase activity is shared in each case between the mitochondrial nuclease (assumed for the purposes of Discussion to be D1 plus D2) and endo-exonuclease (D3). It can be calculated from the proportions of mitochondrial nuclease and endo-exonuclease in the mutants, that the levels of mitochondrial nuclease in uvs-3, nuh-4, and nuh-3 are respectively 56%, 76%, and 40% of the wild-type, and that the levels of endo-exonuclease are respectively 18%, 46%, and 38% of the wild-type. Although both nucleases are present at lower levels in the mutants than in the wild-type, neither of the two nucleases is completely absent in the mutants. These three mutants, therefore, appear to exert pleiotropic effects on the levels in the intracellular DNases as they do on the secretion of DNases (11). The secretion of endo-exonuclease is, however, reduced only in the Rec-like mutants, uvs-3 and nuh-4 , and is normal in the mutagen insensitive mutant, nuh-3. Another Rec-like mutant, uvs-6, did not show any deficiency of expressed intracellular endo-exonucliase, but did had deficiency of DNase C which was the secreted form of endo-exonuclease (11).

Since the levels of total ss-DNase activity (after trypsin activation) in the uvs-3 and nuh-4 mutants are the same as in the wild-type, it is possible to calculate that the ratios of the inactive precursor to the active endo-exonuclease. The ratios calculated for uvs-3 and nuh-4 are 30:1 and 12:1 as compared to 5:1 for wild-type. These high

ratios suggest that these mutants, especially uvs-3 , may be defective in protease(s) involved in the processing and perhaps the secretion of endo-exonuclease. There are no clear correlations between mutagen sensitivities (DNA-repair deficiencies) and deficiencies of expressed endo-exonuclease activity or other striking differences between the mutagen sensitive mutants and the wild-type. Thus, the implication of endo-exonuclease in DNA-repair and/or recombination (10, 81) awaits the isolation of a conditional mutants which alter directly the structure and functions of the enzyme.

The mutant nuh-3 shows a definite deficiency of expressed ss-DNase activity (Table 4) which is shared equally between the mitochondrial nuclease and endo-exonuclease, and it also exhibits a Nuh phenotype. In spite of this, it is not sensitive to mutagens relative to the wild-type. A lesion in the nuh-3 mutant may possibly involve defects in the processing of the mitochondrial nuclease, and the secretion of ss-exonuclease. The process by which D1 is converted to D2 appears to be impaired in this mutant. Nuh-3 is the only mutant examined in which the ratio of D1 to D2 was higher than that for the wild-type (Table 7). The appearance of a 75000 dalton molecular weight "peak" in aged D1 preparations examined by Sucrose density gradient centrifugation suggests that endogenous protease(s) may be involved in this process. The nuh-3 mutant appears to secrete normal amounts of DNase A and endoexonuclease, but secretes only low levels of ss-exonuclease (11). This enzyme was not found intracellularly in mycelia (9), and may be largely periplasmic in conidia since large amounts of activity were released from conidia only by freezing and thawing. The ss-exonuclease has a number of properties

in common with the D2 (mitochondrial nuclease) described here: size (about 70-75000 daltons), RNase activity, Mg^{++}-dependence, inhibition by Ca^{++} and lack of inhibition by ATP. It differs in specificity for DNA however. Whereas D2 hydrolyzes ss- and ds-DNA at almost equal rates in an endonucleolytic manner, the ss-exonuclease was found to degrade ss-DNA exonucleolytically in a quite specific manner (52). However, a small amount of endonuclease activity (which destroyed the infectivity of ϕX-174 viral DNA) was found in association with highly purified enzyme. This was originally believed (52) to be contamination from the ss-endonuclease (now known to be endo-exonuclease), but could be an intrinsic activity. Both ss-exonuclease and the mitochondrial nuclease hydrolyze DNA to produce 3'-OH and 5'-phosphoryl termini (50, 52).

4.1.4 The Expression of ds-DNase Activity

The appearance of ds-DNase activity in the isolated fractions of mitochondrial nuclease and endo-exonuclease indicated that this ds-DNase activity was masked in less purified fractions. In crude extracts, the ds-DNase activity was extremely low, less than 5% of that seen after trypsin activation. However, when the crude extracts were treated with trypsin, the ds-DNase activities increases 7 times while the ss-DNase activities only increased about 3 times (Table 13). The activation by endogenous protease(s), however, increased the ds-DNase activity by 20 times but only increased the ss-DNase activity by 3 times, same as that for the trypsin activation. The differential activation of ds-DNase activity with trypsin indicates that the increase of activities was not merely due to conversion of inactive endo-exonuclease to active

enzyme (both the ss- and ds-DNase activities of partially purified precursor are activated to the same extent), but involves also the proteolytic destruction of a ds-DNase inhibitor. The spontaneous differential activation of ss- and ds-DNase activities in crude extracts (78) was shown to be strongly inhibited by PMSF and therefore likely due to the action of endogenous protease(s). Activation of ds-DNase activity also occurred when the various fractions were passed through DEAE- cellulose (Table 12), a step which turns on protease activities in crude extracts (94) and/or remove a ds-DNase inhibitor.

Direct evidence for protein inhibitor(s) of ds-DNase activities was obtained by isolating an inhibitory activity from the D1 plus D2 fraction. This protein fraction inhibited the ds-DNase activity, but not the ss-DNase activity, of DEAE-cellulose activated D1 plus D2 (mitochondrial nuclease). Trypsin activation of this DEAE-cellulose activated D1 plus D2 fraction increased both ss-DNase and ds-DNase activities (in the absence of inhibitor) 3-fold, indicating that inactive endo-exonuclease precursor was still present and not activated by passage through DEAE-cellulose. This confirms previous observations (6). The same D1 plus D2 fraction, before fractionated on the DEAE-cellulose column, however, showed an activation of ds-DNase activity with trypsin of 45-fold and an activation of ss-DNase activity with trypsin of 6-fold (Table 13). Recent attempt to repeat the isolation of ds-DNase inhibitor with a fresh preparation of D1 plus D2 on DEAE-cellulose have failed. However, since the previous isolation was from an "aged" D1 plus D2 fraction (2 weeks at $0^{o}C$), partial proteolysis may be necessary for the release of the ds-DNase inhibitor from the enzyme. Possibly

it is very tightly bound or even covalently linked to the enzyme and released as part of the processing mechanism.

The presence of proteinaceous DNase inhibitors has been reported for many organism. These inhibitors include the DNase inhibitors of mammalian tissue (71, 72) and Actinomyces levoris (73). However, for nucleases, which degrade both DNA and RNA, only two such DNase inhibitors have been reported, the nuclease 0 inhibitor of Aspergillus oryzae (77), and the nuclease N_3 inhibitor of Neurospora crassa (57). Nuclease 0 is an enzyme with some properties in common with Neurospora mitochondrial nuclease. The two enzymes behaved like a basic protein in chromatographic properties. Mitochondrial nuclease did not adsorb on DEAE-cellulose and nuclease 0 adsorbed on CM-cellulose. Both enzyme also activated by Mg^{++} and inactive in presence of EDTA (95). The nuclease 0 inhibitor is an acidic protein, it adsorbed to DEAE-Sephadex (77). The inhibitor has a molecular weight of 22000 dalton and is highly specific for nuclease 0. The inactive enzyme-inhibitor complex is reactivated by treatment with chymotrypsin. Another protease sensitive DNase inhibitor found in eukaryotes is actin (74, 75). Actin forms a very tight inactive complex with pancreatic DNase I which can be activated by treating with trypsin (96). Recently Neurospora has been shown to contain actin (76), but it is not known whether it can inhibit Neurospora DNases and there are no reports of differential inhibitions by actin of ds-DNase versus ss-DNase activity. Although the nature of the inhibition of the Neurospora nuclease and identity of the ds-DNase inhibitor(s) have not yet been elucidated, all of the evidence accumulated here points to their existence.

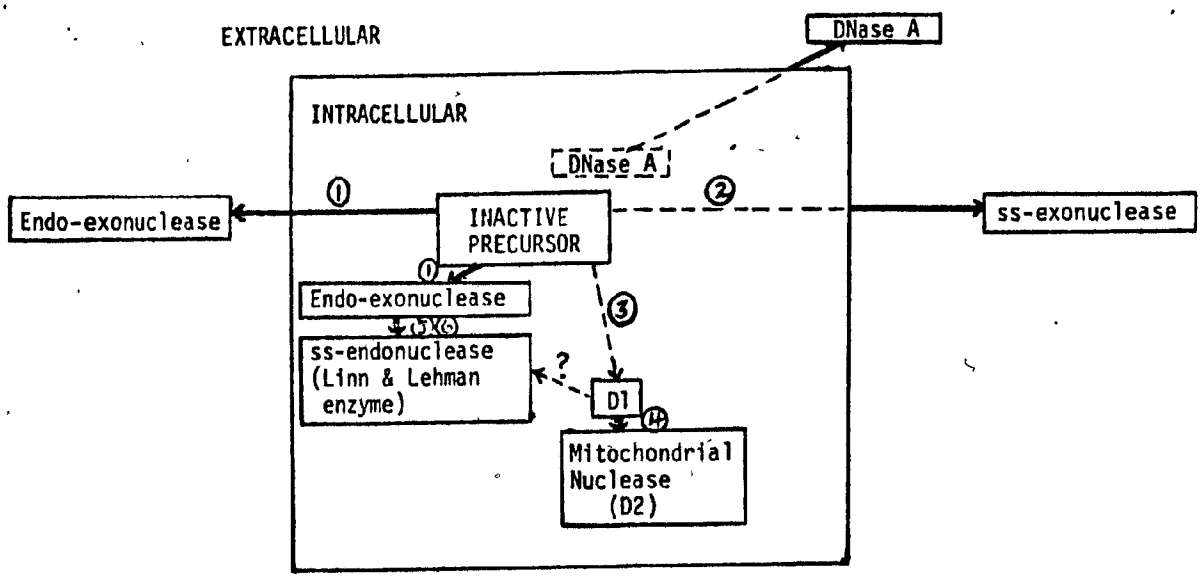
4.1.5 Unanswered Problems and Prospects

Several questions have been raised during the course of this research. Two questions concern the apparent relationship between fresh D1 and endo-exonuclease (D3) and the conversion of D1 to mitochondrial nuclease as it ages in vitro (see Fig. 9). There is no direct evidence for conversion of D1 to D3, but the inhibition by ATP, resistance to EDTA and lack of stimulation by Mg^{++} as well the ds-exonuclease activity associated with some of the D1 preparations indicated that the two enzymes may be related. (D1 vs D3) The changes in physical and chemical properties of D1 that occurred on ageing clearly indicated a conversion to D2 (see Results). The mechanism for the conversion is not known, but it is suspected that proteolysis may be involved. However, whether this conversion of D1 to D2 occurred only in vitro, i.e. whether it is an artifact of extraction, or whether the conversion reflects the true situation in vivo is unknown. If the in vitro data do reflect the true state in vivo, then the question arises as to which protease(s) are responsible for this conversion. There is no evidence at present that D1 originates from an inactive precursor. A secreted DNase (11), ss-exonuclease (DNase B), which was not found in extracts of mycelia (9) differs from the mitochondrial nuclease (D2) only in the mode of degradation and the strand specificity. D2, the mitochondrial nuclease has an endonucleolytic activity toward ss-DNA and ds-DNA with ss/ds ratio of about 3, while ss-exonuclease has an exonucleolytic activity toward ss-DNA and a high single-strand specificity (52). A low level of endo-nucleolytic activity was also detected. Other properties of these two nucleases such as a high Mg^{++} dependence, high sensitivity to EDTA, lack of inhibition by ATP, RNase

Fig. 9: Schematic representation of the possible relationships between five Neurospora nucleases (ss-endonuclease, mitochondrial nuclease (D2), ss-exonuclease, endo-exonuclease (D3) and D1) and how they may derive from a single inactive precursor.

Fig. 9:

POSSIBLE MODEL FOR THE PROCESSING AND SECRETION OF NEUROSPORA NUCLEASES



STRONG EVIDENCE FOR

SPECULATION

① Plasma membrane protease I (PMSF-sensitive)?

② Plasma membrane protease II ?

③ Microsomal membrane protease?

④ Mitochondrial membrane protease?

⑤,⑥ Lysosomal (vesicular) protease?

activity were very similar. Thus, it is possible that a relationship may exist between these two DNases. One possibility is that the two nucleases are derived from the same precursor (see Fig. 9). DNase C, the secreted form of endo-exonuclease (11), was identical in properties to the intracellular endo-exonuclease (D3) except that it was smaller in size, 65000 daltons as compared to 75000 daltons for D3. This difference in molecular weights may not be real. Since both endogenous protease(s) in extracts and exogenous proteases such as trypsin convert endo-exonuclease precursor to active enzyme (6,7), it may postulated that plasma membrane protease converts the precursor to active enzyme during secretion (see Fig. 9). Possibly the same protease associated with microsome membranes may catalyze the intracellular conversion of precursor to active enzyme, via a pathway involving D1 (see Fig. 9).

The above discussion raises the possibility that D1 may be an active precursor of both D2 and D3 and that D2 (the mitochondrial nuclease) might be related to the extracellular ss-exonuclease. This would accounted for the variations in Table 8. The characteristics of D1+D2 would depend on pleiotropic effects the fraction of D1 which converted to D2 or D3. Thus, it is possible that the inactive precursor is a precursor of no less that 5 nucleases in Neurospora: endo-exonuclease (D3), ss-endonuclease, D1, the mitochondrial nuclease (D2), and ss-exonuclease. The possibility that these nucleases arise from the same precursor may thus explain the pleiotropic effects of various mutations on the nucleases.

The postulated endogenous protease(s) involved in the D1 to D2 conversion may be identified by incubating D1 with either mitochondrial or microsome membrane fractions in the presence of various protease inhibitors. Protease(s) which are involved in other postulated conversions could be identified in a similar manner. Five intracellular proteases

of Neurospora had been isolated by Siepen et al (82). They include an acid endopeptidase, an alkaline endopeptidase, a carboxypeptidase, and two aminopeptidases. Any of these five proteases may be involved in the above conversions, or some highly specific minor proteases could be involved in these conversions. Once protease(s) were identified, then the mutants (e.g. uvs-3, nuh-3) would be examined for deficiencies in them.

Proof of the relationships between the intracellular nucleases, D1, D2 and endo-exonuclease (D3), may require the isolation of pure inactive endo-exonuclease precursor. This could be used to generate specific antibodies by immunological technques. Similarities between D1, D2 and endo-exonuclease (D3) with the precursor would show up as cross-reactivity of the nucleases with the antibody to the pure precursor. A hypothetical model to summarize the possible interelationships between the major intracellular nucleases, extracellular DNases and the protease(s) involved is proposed in Fig. 9.

The existence of ds-DNase inhibitor(s) also raises some questions. The inhibitor(s) appear to mask completely the ds-DNase activities in crude extracts and these activities are completely suppressed throughout growth. The functions that these inhibitors have are not understood, and why the ds-DNase activity, as opposed to the ss-DNase, is specifically suppressed is also not understood. The isolation of the inhibitor from the D1 plus D2 fraction seems to require limited proteolysis. Endogenous trypsin-like protease(s) may be involved in unmasking the ds-DNase activity in vitro, and exogenous proteases such as trypsin, chymotrypsin, elastase, and thermolysin have also been found to activate the ds-DNase activities of extracts as well (data not shown). One question is: Are there

any physiological conditions under which protease(s) "turn on" the ds-DNase activity *in vivo*? If so, then which intracellular protease(s) are involved? The proteolytic activation of ds-DNase activity *in vitro* suggests either that the inhibitor may be covalently linked to the enzyme, or may be very tightly bound to the enzyme. In either case limited proteolytic action would be necessary to unmask the ds-DNase activity.

Covalently linked versus tightly bound inhibitor could be differentiated by using extreme denaturing conditions. A covalently linked inhibitor would not be released in the absence of protease activity whereas a tightly bounded inhibitor should be recoverable. If such an inhibitor is also heat-stable, as in the case of nuclease 0 inhibitor of *Aspergillus oryzae* (77), heat denaturation could be rised to release the ds-DNase inhibitor. The effects of purified actin, on the intracellular *Neurospora* nucleases should also be tested, even though there is apparently no intracellular DNase in *Neurospora* analogous to pancreatic DNase I.

Although the lower levels of endo-exonuclease in the *Rec*-like mutants may indicate some role for this enzyme in DNA-repair and/or recombination, there is no direct correlation between mutagen sensitivity and levels of this enzyme. The *uvs-3*, *nuh-4* and *uvs-6* mutants are all approximately three times as sensitive to U.V.-light as the wild-type (28, 55), but level of endo-exonuclease in these mutants varies from 18%-66% of the wild-type and the level of this enzyme in a mutant which has wild-type sensitivity to U.V.-light (*nuh-3*) is 38% of that in the wild-type. Therefore, the isolation of a temperature-sensitive mutants which directly affect this nuclease is necessary to permit conclusions about any involvement in DNA-repair and/or recombination.

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