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Alkaline deoxyribonucleases released from Neurospora crassa mycelia: two activities not released by mutants with multiple sensitivities to mutagens

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ABSTRACT

Three major alkaline deoxyribonuclease (DNase) activities have been identified in sorbose-containing liquid culture medium in which wild-type Neurospora crassa were grown: DNase A, a Ca++-dependent endonuclease of molecular weight 65,000 daltons which has no specificity for single- or double-stranded DNA (ss-DNA or ds-DNA) and no activity with RNA; DNase B, a Mg++-dependent single-strand specific exonuclease of molecular weight 78,000 daltons active with both ss-DNA and RNA; DNase C, a divalent metal ion-dependent endo-exonuclease of molecular weight 65,000 having single-strand specific endonuclease activity with ss-DNA and RNA and exonuclease activity with ds-DNA. Three mutants which were shown previously to have wide spectra of sensitivities to mutagens, and which exhibited reduced release of DNase activity on sorbose-containing agar test plates (the Nuh phenotype), were deficient relative to the wild-type in the release of these major alkaline DNases into the liquid culture medium. 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) which is not mutagen sensitive relative to the wild-type released low levels of DNase B. On the other hand, an ultraviolet light-sensitive mutant (nuc-2) which does not have the Nuh phenotype was normal in the release of these DNases.

INTRODUCTION

Nuclease halo (nuh) mutants of Neurospora crassa fail to release normal amounts of deoxyribonuclease (DNase) activity when grown on commercial DNase test agar or on minimal medium overlaid with DNA-agar[1]. One of the nuh mutants isolated (nuh-4) was found to be sensitive to ultraviolet light, nitrosoguanidine and methylmethane sulfonate (see Table 1). In addition, two other mutants of Neurospora with wide spectra of mutagen sensitivities, uvs-3 and uvs-6 (see review by A.L. Schroeder, ref. 2), were also found to have the Nuh phenotype. The uvs-3 and uvs-6 mutants show abnormally high mitotic recombination and/or deletions. The three mutants described above have recently been found sensitive to mitomycin C[3], an agent which has been used to select recombination (rec) mutants in Escherichia coli[4] and to induce mitotic recombination in Ustilago maydis and in yeast[5]. In contrast, other DNA-repair defective

Table 1. Summary≠ of phenotypes of Neurospora crassa mutants relevant to this work.

MUTANT	Nuh phenotype	Sensitivity* to					Recombination		UV-induced Mutagenesis
		UV	IR	MMS	NG	mit C	Meiotic	Mitotic	
uvs-3	+	+	+	+	+	+	n.d.	HIGH	NONE
uvs-6	+	+	+	+	+	+	n.d.	HIGH	NORMAL
nuh-4	+	+	n.d.	+	+	+	n.d.	n.d.	n.d.
nuh-3	+	-	n.d.	n.d.	-	-	n.d.	n.d.	n.d.
uvs-2	-	+	+	+	+	-	NORMAL	NORMAL	HIGH
uvs-4	-	+	-	n.d.	+	-	NORMAL	NORMAL	LOW
upr-1	-	+	+	+	n.d.	-	NORMAL	n.d.	HIGH
nuc-2	-	+	-	n.d.	+	n.d.	NORMAL	n.d.	n.d.

≠The data are compiled from references 1-3 and unpublished work of E. Käfer (see Neurospora Newsletter 25, 19 (1978))

*Sensitivity to ultraviolet light (UV), ionizing radiations (IR), methyl-methane sulfonate (MMS), nitrosoguanidine (NG) and mitomycin C (omit C); n.d. means not determined.

mutants of Neurospora with narrower spectra of mutagen sensitivities[2] such as uvs-2, uvs-4 and upr-1, did not exhibit the Nuh phenotype[1] and were not sensitive to mitomycin C[3]. It thus seems possible that the Nuh phenotype, when found in conjunction with mutagen sensitivities, may reflect a deficiency in one or more DNase activities involved in DNA-repair and/or recombination. DNases have been shown to be involved in both processes in prokaryotes, in particular in Eschericia coli[4].

Very little is known about the involvement of DNases in DNA-repair and/or recombination in lower eukaryotes. A single-strand specific endonuclease has been implicated in mitotic recombination in the fungus, Ustilago maydis[6] and preliminary results have also implicated an endo-exonuclease of Neurospora in the same process[7]. The extracellular and intracellular DNase activities of wild-type Neurospora and mutagen-sensitive mutants with the Nuh phenotype have been investigated in an attempt to identify DNase activities that might be involved in DNA-repair and/or recombination. This report describes a preliminary characterization of the major extracellular alkaline DNase activities released by mycelia of the wild-type and some mutagen sensitive mutants with the Nuh pehnotype. A parallel study of the major intracellular alkaline DNases in these strains of Neurospora will be presented elsewhere[8].

MATERIALS AND METHODS

Strains. The wild-type (74-OR23-1A) and well-backcrossed mutant strains were described in a previous report[1].

DNA-agar plate tests for the Nuh phenotype. Two tests for the Nuh phenotype were applied[1] and are outlined here. One test was made by transferring dry conidia to agar plates containing denatured DNA at pH 7.3 (Bacto DNase Test Agar from Difco) and 1% to 2% sorbose plus 0.05% of each of glucose and fructose to maintain growth (at 34°C) in colonial form[9]. In the second test dry conidia were transferred to minimal agar plates containing the sugars listed above and, after an appropriate time for growth of the colonies, the plates were overlaid with a mixture of 300 µg/ml heat-denatured DNA, 0.1 M Tris-HCl buffer, pH 8.0 and 0.01 M $MgCl_2$ in 0.5% agar. The plates were then incubated overnight to develop the nuclease haloes (for details see ref. 1). In both tests the Nuh phenotype was detected by visualizing the clear haloes of DNA digestion around the colonies by flooding the plates with acid to precipitate the undigested DNA and thus render the background agar opaque. Whereas relatively large clear rings of digestion were seen around wild-type colonies, smaller and/or more opaque rings of digestion were seen around colonies of mutants with the Nuh phenotype indicating a reduced release of DNase activity. In different experiments to characterize the DNase activity released from wild-type colonies, the composition of the DNA-agar overlays was varied as indicated in the Results section, e.g. to replace denatured DNA (ss-DNA) with native DNA (ds-DNA), to alter the pH, buffer, ionic strength and divalent metal ion present and to add potential inhibitors.

Growth of mycelia in liquid shake cultures. Dry conidia were suspended in sterile distilled water at a density of 35 Klett units[10] (measured on a Klett-Summerson colorimeter with a blue 420 nm filter). Erlenmeyer flasks (250 ml) sucrose as the carbon source or Vogel's medium N with 1.5% sorbose and 0.05% each of glucose and fructose as carbon source[9], were innoculated with 0.5 ml conidial suspension. The cultures were grown for 5 days on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc.) operated at 150 r.p.m. The sucrose-containing cultures were grown at 30°C and the sorbose-containing culture at 34°C (to simulate more closely the growth on sorbose-containing agar plates[1]). Mycelia grew in the former medium as a thick tangled mat, but in the latter medium grew as individual colonies that looked like small "cotton balls" in suspension. Mycelia and culture filtrates were collected with the aid of Buchner funnels.

Fractionation of DNase activities in culture filtrates. Each culture filtrate (600-900 ml) was brought to 95% saturation with $(NH_4)_2SO_4$ by additions of the solid over a period of 1.5-2 hr in the cold. During this process the pH of the culture filtrate was adjusted to 6.5 by additions of 1N KOH. The mixture was allowed to stand 18 hr in the cold and then centrifuged in a Sorvall refrigerated centrifuge at 16,300 X g for 30 min. The $(NH_4)_2SO_4$ precipitate containing from 18 to 55 mg protein was dissolved in 7 ml buffer A (0.02 M potassium phosphate buffer, pH 6.5) and dialyzed 24 hr against two changes of 2 liters each of buffer A. In some experiments (e.g. see Fig. 1) 1,900 ml culture filtrate was used to prepare the $(NH_4)_2SO_4$ fraction. In this case a double-sized DEAE-Sepharose column (see below) was used in the next step.

The dialyzed $(NH_4)_2SO_4$ fraction was chromatographed in the cold on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden; a 2.2 cm diameter column containing either 10 or 20 ml packed volume). The column was pre-equilibrated with buffer A immediately before use. Samples were adsorbed at a flow rate of 0.5 ml/min and a flow rate of 1 ml/min was used in all other steps. Fractions of 2.5 ml were collected. The $(NH_4)_2SO_4$ fraction was loaded on the column and washed through with buffer A. Active fractions were combined and designated the "DEAE-wash". Adsorbed DNase activity was then eluted either stepwise with various concentrations of NaCl in buffer A (usually 0.1 M and 0.15 M, see Results) or with a linear 0-0.5 M gradient of NaCl in buffer A (checked by conductivity measurements). In some cases the DEAE-wash was further fractionated by chromatography on 10 ml (packed volume) columns of phosphocellulose (Whatman P11 obtained from Mandel Scientific Co. Montreal, Canada) or hydroxyapatite (Hypatite C, Clarkson Chemical Co.) pre-equilibrated with buffer A. The columns were eluted at a flow rate of 1 ml/min with linear 0.02-0.3 M gradients of potassium phosphate buffer, pH 6.5 (checked by conductivity measurements). In each case active fractions were pooled and dialyzed overnight in the cold against buffer A as described above. When necessary, pooled fractions were concentrated by applying dry Ficoll (Pharmacia) to the outside of the dialysis bags.

Nuclease and Protein Assays. Nuclease activities were routinely measured by following the release of cold acid-soluble material from either ss-DNA, ds-DNA or RNA as substrate at 37°C in reaction mixtures containing 0.1 M Tris-HCl buffer, pH 8.0 with (for DNA) or without (for RNA) 0.01 M $MgCl_2$, using perchloric acid (PCA) as a precipitant of undigested nucleic acid[11]. The PCA-soluble material was estimated by measuring the absorption at 260 nm (A_{260}). Two types of DNase assay were used, a single time point (60 min or 90 min)

assay to estimate semi-quantitatively the DNase activity in fractions derived from chromatographic columns and an assay in which 4 or 5 time points were used to determine accurately the rate of release of acid-soluble material. Only the latter type of assay was used for RNase activity. One unit of nuclease activity was defined as that amount of enzyme which released 1.0 A_{260} unit of PCA-soluble material in 30 min under the conditions of assay. For ss-DNase activity the unit is approximately equivalent to the release of 1.25 μmoles DNA nucleotides/min[12]. In addition, qualitative estimates of the relative amounts of endonuclease and exonuclease activities were made by comparing the rates of release of acid-soluble material with trichloroacetic acid (TCA) and uranyl acetate-trichloroacetic acid (TCA) as precipitants[11]. Protein was estimated by the method of Lowry et al[13].

RESULTS

The DNase activity released from wild-type colonies growing on solid media.

The commercial DNase test agar on which the Nuh phenotype of mutants was originally recognized is buffered at pH 7.3. Since Ryan et al.[14] had reported that Neurospora released acid when grown on media at pH 7.6, it was necessary to show that the pH in the halo region around the colonies remained buffered under the test conditions by staining DNase test plates with bromothymol blue. It was found that the pH of the agar was uniform (at 7.3) right up to the edges of the colonies. The use of DNA-agar overlays of different compositions on wild-type colonies grown on minimal medium at pH 5.7 permitted more extensive characterization of the released DNase activity. In the overlays with ss-DNA, haloes around wild-type colonies were relatively large at pH 8.0 under standard test conditions[1], but no haloes were seen when the pH was 6.0 in the absence of Mg^{++}, either in the absence or presence of 0.1 M EDTA. The latter conditions have been used for the in vitro assay of nuclease N_3[15]. When Mg^{++} was omitted from the ss-DNA overlay at pH 8.0, or when excess EDTA was present, no haloes were seen; Ca^{++} was as effective as Mg^{++} in supporting halo formation in the absence of EDTA. The DNase released from wild-type colonies was also found to be approximately the same when 0.1-0.2 M NaCl was added to the ss-DNA agar overlays and haloes also formed at tempertures up to 60°C. Finally, it was found that haloes also formed when ds-DNA replaced ss-DNA in the overlays, although their sizes were smaller than those seen with ss-DNA. The formation of haloes with ds-DNA was divalent metal ion-dependent. It was found that the reduced release of DNase activity seen for the Nuh mutants with ss-DNA agar

overlays was also seen for all Nuh mutants when ds-DNA replaced ss-DNA in the overlays. In summary, the above experiments indicate that Nuh mutants fail to release normal amounts of relatively stable divalent metal ion-dependent alkaline DNase activity which acts on both ss-DNA and ds-DNA. As might be expected, this activity is due to a mixture of DNases (see below).

Growth of mycelia and secretion of DNase in different liquid media. The yields of wild-type mycelia grown for 5 days in the sucrose-medium averaged 32 g wet weight per liter culture medium, but only 6 g per liter when grown in the sorbose medium. In contrast, the secretion of ss-DNase activity into the culture medium from wild-type mycelia averaged 14-fold higher in the sorbose medium, namely 3.2 U ss-DNase/ml culture medium versus 0.23 U/ml sucrose medium. Because of the higher yield of nuclease activity, most of the studies referred to below were performed on fractions derived from sorbose culture medium. The spectrum of DNase activities in both types of culture medium, as determined by chromatography of DEAE-Sepharose, was qualitatively the same (see below).

Fractionation of wild-type and uvs-3 culture filtrates. As expected from the results of the DNA-agar overlay tests on colonies growing on minimal agar (see above), no nuclease N_3^{15} activity was detected in $(NH_4)_2SO_4$ fractions derived from culture filtrates of the wild-type or the uvs-3 mutant. All of the DNase activity recovered was divalent metal ion-dependent. Preliminary experiments indicated that the same percentage yield (approximately 45%) of Mg^{++}-dependent ss-DNase activity was recovered from wild-type and uvs-3 culture filtrates in the $(NH_4)_2SO_4$ precipitation step, except when protein concentration in the culture filtrate was low. In those circumstances yields as low as 14% were seen. The data in Table 2 show the results of two separate experiments in

Table 2. Stepwise fractionation of DNase activities of wild-type and uvs-3 culture filtrates on DEAE-Sepharose.

Fraction derived from culture filtrate	Total units DNase recovered from 1 liter of culture filtrate of:			
	wild-type		uvs-3*	
	ss-DNase	ds-DNase	ss-DNase	ds-DNase
$(NH_4)_2SO_4$ fraction	1720,2260	1460,1980	380, 740	93, 170
DEAE-wash	530, 480	770, 640	0, 0	0, 0
DEAE eluate (0.15 M NaCl)	770,1310	0, 0	270, 620	13, 0

*The yields of uvs-3 mycelia in the two experiments were respectively 25% and 30% of the wild-type in spite of the larger innoculum of uvs-3 conidia (see Materials and Methods).

stepwise fractionation of wild-type and uvs-3 culture filtrates. The ss-DNase activities in the two $(NH_4)_2SO_4$ fractions derived from uvs-3 were 22% and 33% respectively of that from the wild-type, i.e. approximately the same per gram wet weight of mycelia. However, the ds-DNase activities from uvs-3 were much lower, respectively 6% and 9% of wild-type. When the $(NH_4)_2SO_4$ fractions were chromatographed on DEAE-Sepharose, no ss-DNase or ds-DNase activity was recovered in the DEAE-wash fraction and only a small amount of ds-DNase was eluted from DEAE-Sepharose with 0.15 M NaCl from the uvs-3 $(NH_4)_2SO_4$ fraction in one experiment. Only 40% of the ds-DNase activity was recovered from the wild-type $(NH_4)_2SO_4$ fraction and this was found entirely in the DEAE-wash fraction. The ratio of activities with ss-DNA/ds-DNA (ss/ds) for this fraction was about 0.7 in each experiment (calculated from data in Table 2). For convenience this DEAE-Sepharose fraction is referred to as DNase A. The activity eluted from DEAE-Sepharose with relatively low concentrations of NaCl is referred to as DNase B. This activity was virtually specific for ss-DNA in both the wild-type and uvs-3 DEAE-eluates.

A second series of experiments was undertaken using large amounts of culture filtrate (1900 ml) to prepare the wild-type and uvs-3 $(NH_4)_2SO_4$ fractions. When the wild-type $(NH_4)_2SO_4$ fraction was eluted from DEAE-Sepharose with a 0-0.5 M NaCl gradient in buffer A (see Fig. 1a), more DNase activity was eluted at higher salt concentrations. This can be seen as a shoulder on the trailing edge of the chromatographic profile. The activity is referred to below as DNase C. In this experiment, the material eluted at the leading edge of the profile (DNase B) was found to be nearly single-strand specific (ss/ds of 21) as expected, but the active fractions eluted above 0.15 M NaCl (DNase C), contained ds-DNase as well as ss-DNase activity (ss/ds of 2.4). A small amount of DNase A activity was recovered in this series of experiments from the uvs-3 $(NH_4)_2SO_4$ fraction (13% of the total ss-DNase activity recovered) and, in addition, a small amount of DNase C was eluted from DEAe-Sepharose (also about 13% of the total ss-DNase activity recovered) (see gradient profile in Fig. 1b). The ss/ds ratio for the DNase B activity of uvs-3 eluted from DEAE-Sepharose was 19 close to that for the wild-type DNase B. The ss/ds for the DNase C component eluted with 0.2 M NaCl was approximately 4.

Characterization of the DNase activities secreted by wild-type Neurospora.

(a) DNase A. The DNase A activity of wild-type chromatographed as a single component on phosphocellulose (Fig. 2) and on hydroxyapatite (data not shown). In the experiment described in Fig. 2, ds-DNase activity was determined in each chromatographic fraction. The activity recovered (90% of the total) from

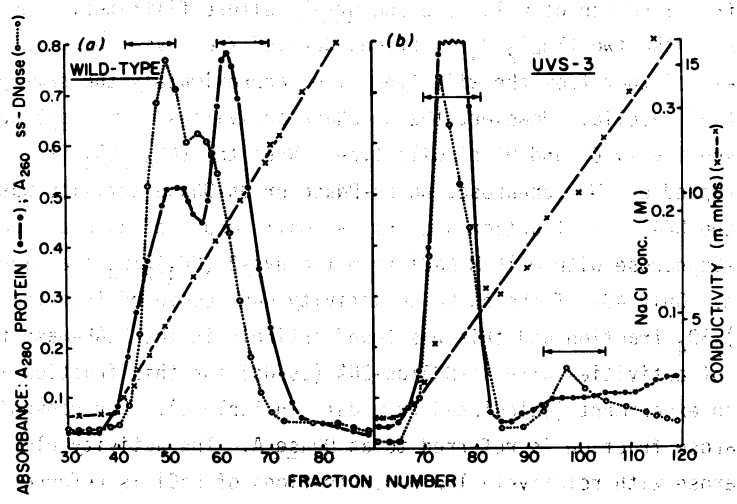

FIG. 1: Chromatography of dialyzed $(NH_4)_2SO_4$ fractions of wild-type (a) and uvs-3 (b) culture filtrates on DEAE-Sepharose. Only the gradients are shown here. The bars in each panel indicate the fractions which were pooled and analyzed qualitatively for single- and double-strand endo- and exonuclease activities as in Fig. 3.

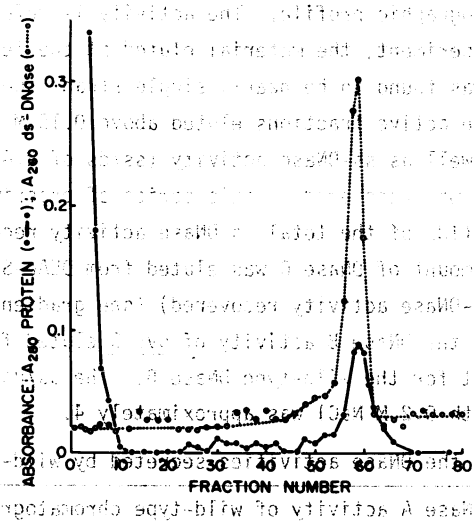

FIG. 2: Chromatography of the wild-type pass-through fraction ("DEAE-wash" fraction) from DEAE-Sepharose on phosphocellulose. Note that ds-DNase activity was determined in each fraction.

phosphocellulose had the same ss/ds ratio (about 0.7) as that washed through DEAE-Sepharose. The phosphocellulose fraction also had no activity with RNA, even though the DEAE-wash fraction from which it was derived had a high RNase activity. Both the DEAE-Sepharose and phosphocellulose fractions had endonucleolytic activity with ss-DNA and ds-DNA (Fig. 3a). There was no DNase activity with either substrate in the absence of divalent metal ions (Table 3). When DNase A was pre-treated with 5 mM EDTA at 37°C for 30 min, the activity was restored with Ca^{++}, but not with Mg^{++}, although Mg^{++} stimulated the activity in the absence of EDTA (Table 2). Neither the ss-DNase nor the ds-DNase activity was inhibited by 0.5 M ATP. DNase A was found to be quite sensitive to ionic strength. The presence of 0.1 M and 0.2 M NaCl in the assay reduced the ds-DNase activity to 10% and 3% respectively. DNase A also behaved as a single component during sedimentation in linear 5-20% sucrose density gradients (data not shown). Two sedimentation experiments were carried out with two different marker proteins (bovine serum albumin and bovine hemoglobin). Both experiments gave a value of 4.3 S for the sedimentation constant of DNase A. Assuming that DNase A activity is associated with a single globular protein of normal partial specific volume, the molecular weight[16] was estimated to be about 65,000 daltons.

(b) DNase B. Both the wild-type and uvs-3 DNase B activity which eluted from DEAE-Sepharose at relatively low NaCl concentrations in the experiments described in Table 2 and Fig. 1 had identical properties. There was only a very low level of activity with ds-DNA (ss/ds of 19-21). The ss-DNase activity was found to be exonucleolytic in character (Fig. 3b) and was inhibited completely

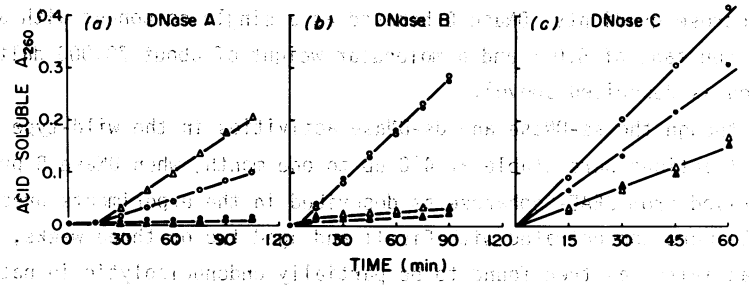

FIG. 3: Qualitative analysis of wild-type DNase (a), DNase B (b) and DNase C (c) fractions for single- and double-strand endo- and exonuclease activities. Open circles, ss-DNase activity with TCA as precipitant; closed circles, ss-DNase activity with UTCA as precipitant; open triangles, ds-DNase activity with TCA as precipitant; close triangles, ds-DNase activity with UTCA as precipitant.

Table 3. Effects of divalent metal ions on DNase activities secreted from wild-type Neurospora crassa mycelia grown in sorbose medium.

CONDITIONS	SUBSTRATE	Activity (% of control)* DNase A	DNase B	DNase C
no EDTA, no metal ion	ss-DNA	0	18	41
" 5 mM Mg++	"	70	85	89
" 10 mM Mg++	"	100	100	100
" 5 mM Ca++	"	286	0	10
" 10 mM Ca++	"	195	0	5
5 mM EDTA, no metal ion	"	0	0	19
" 10 mM Mg++	"	0	42	24
" 10 mM Ca++	"	315	0	15
no EDTA, no metal ion	ds-DNA	0	—	39
" 5 mM Mg++	"	80	—	94
" 10 mM Mg++	"	100	—	100
" 5 mM Ca++	"	280	—	2
" 10 mM Ca++	"	223	—	—
5 mM EDTA, no metal ion	"	0	—	27
" 10 mM Mg++	"	0	—	62
" 10 mM Ca++	"	324	—	48

*Controls taken as 100% activity under standard assay conditions i.e. in 0.1 M Tris-HCl buffer, pH 8.0 with 10 mM Mg++.

by EDTA (Table 3). The activity of the EDTA-inhibited enzyme was restored by Mg++, but not by Ca++. DNase B also had a high RNase activity (data not shown). The ss-DNase activity was not inhibited by 0.5 mM ATP. On sedimentation in sucrose gradients, DNase B behaved as a single component with a sedimentation constant of 5.0 S and a molecular weight of about 78,000 daltons (calculated as described above).

Even though the ss-DNase and ds-DNase activities in the wild-type $(NH_4)_2S)_4$ fractions were stable at 4°C up to one month, when DNase B preparations (derived from DEAE-Sepharose as described in the experiments presented in Table 2) were concentrated with Ficoll and aged two or three weeks, the ss-DNase activity was then found to be partially endonucleolytic in nature and ds-DNase activity had appeared (see Discussion).

(c) DNase C. When the ss-DNase and ds-DNase activities of wild-type DNase C (ss/ds of 2.4) were measured with TCA and UTCA as precipitants, the ss-DNase activity was found to be partially endonucleolytic, but the ds-DNase activity was exonucleolytic in character (Fig. 3c). The ss- and ds-DNase acti-

vity were both inhibited approximately 45% by 0.5 mM ATP (data not shown). Both DNase activities of DNase C were partially inhibited by 10 mM EDTA and partially restored with Mg++ and Ca++ (Table 3). The untreated DNase C had a much lower activity with Ca++ than with Mg++. DNase C also had a high RNase activity and the ss-DNase activity sedimented as a single component in sucrose density gradients. The sedimentation constant and estimated molecular weight, determined as described above, were 4.3 S and 65,000 daltons respectively.

Comparison of DNase activities secreted from the wild-type and some mutants. A comparison of the release of DNase activities by the wild-type and some mutants into sorbose medium is shown in Table 4. Here the activities of culture filtrates were assayed directly without prior dialysis against buffer A. The yields of mycelia in this series of experiments varied over a 2-fold range, although the growth rates of all strains in sucrose medium are the same[8]. Also, the yield of wild-type mycelia in this series of experiments was only one-third of that seen in the experiments described in Table 2, while the yields of uvs-3 mycelia in the two experiments were the same. The reason for these variations is not known but, as a result, quantative comparisons of the yields of DNase activities in culture filtrates, and the $(NH_4)_2SO_4$ fractions derived from them, are difficult to evaluate.

Obvious differences in the relative amounts of DNases A, B and C in the $(NH_4)_2SO_4$ precipitates of culture filtrates of the wild-type and mutants were seen, however, when the activities were resolved by chromatography on DEAE-Sepharose. For the sake of simplicity, only the ss-DNase profiles (single time-point assays) are compared in Fig. 4. In each case, the "peaks" of DNase

Table 4. Comparison of the release of total ss-DNase and ds-DNase activities from wild-type Neurospora mycelia and from various mutant mycelia.

STRAIN	Wet weight mycelia (g)	Activity (total units)* in Culture Filtrate			Activity (total units)* in $(NH_4)_2SO_4$ fraction		
		ss-DNase	ds-DNase	ss/ds	ss-DNase	ds-DNase	ss/ds
wild-type	1.5	3200	1300	2.5	780	190	4.1
uvs-3	1.6	3400	530	6.4	480	52	9.2
uvs-6	1.4	1600	520	3.0	700	270	2.6
nuh-4	0.9	900	530	1.2	370	69	5.3
nuh-3	1.7	5000	1260	4.0	2690	1180	2.3
nuc-2	1.4	1900	790	2.4	850	490	1.7

*In 1 liter culture filtrate and in the $(NH_4)_2SO_4$ fraction derived from this.

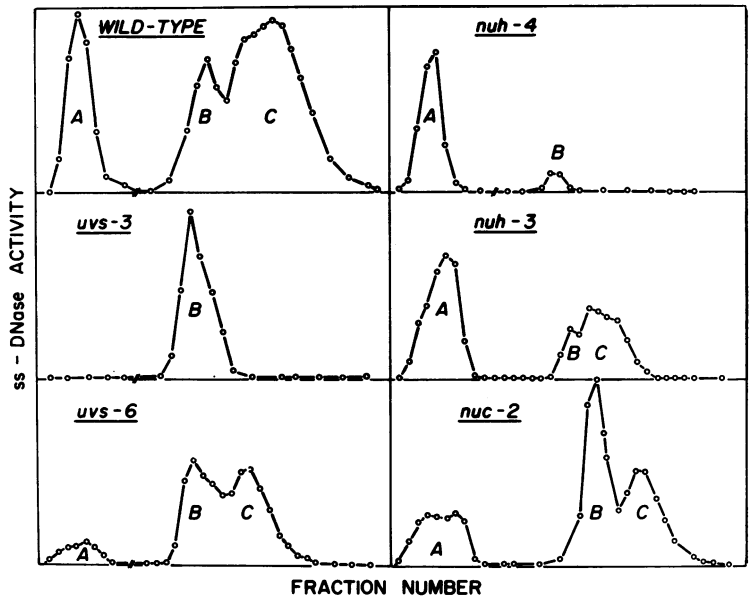

FIG. 4: Comparison of chromatographic profiles of wild-type and mutant $(NH_4)_2SO_4$ fractions of culture filtrates on DEAE-Sepharose. The letters A, B, C indicate that chromatographic fractions in these regions of the profile were shown to have DNase A, DNase B or DNase C activities as described in Fig. 3.

B and DNase C activity were eluted in the gradients with 0.10-0.12 M NaCl and 0.18-0.22 M NaCl respectively. The "peak" fractions (or fractions taken from the leading or trailing edges of peaks not well resolved) were pooled, dialyzed against buffer A and analyzed quantitatively for ss- and ds-DNase activities (PCA as precipitant) and qualitatively for endonucleolytic and exonucleolytic activities (TCA and UTCA as precipitants, cf. Fig. 3) in order to confirm the identities of DNases A, B and C. The percentage recoveries of ss-DNase activity (which all three DNases possess) in each component are summarized in Table 5. These were estimated by measuring the areas under the ss-DNase activity "peaks" in the elution profiles of Figs. 1 and 4. It can be seen from Fig. 4 and Table 5 that the presence of all three DNases in the wild-type $(NH_4)_2SO_4$ fraction was confirmed. The presence of only very small amounts of DNases A and C (no appreciable acid-soluble material above the blank in one experiment) in the

Table 5. Recoveries of ss-DNase activity in the DNase A, B and C components after chromatography of the $(NH_4)_2SO_4$ fraction on DEAE-Sepharose.

STRAIN	Relative total ss-DNase recovered	% of ss-DNase activity recovered from DEAE-Sepharose in DNase component		
		DNase A	DNase B	DNase C
wild-type*	approx. 2.0	31	46	23
wild-type	1.0	22	19	59
uvs-3*	approx. 0.6	13	74	13
uvs-3	0.3	0	100	0
uvs-6	0.6	10	43	47
nuh-4	0.4	90	10	0
nuh-3	0.4	58	9	33
nuc-2	0.7	25	41	34

*Data from the experiments reported in Fig. 1. All other data derived from the experiments described in Table 3.

uvs-3 fraction was also confirmed. The $(NH_4)_2SO_4$ fraction derived from the uvs-6 mutant contained only a small amount of DNase A, that of the nuh-4 mutant contained no detectable DNase C and only a very small amount of DNase B, while that of the nuh-3 mutant contained only a relatively small amount of DNase B, but a relatively high amount of DNases A and C. The distribution of DNases in the nuc-2 fraction was similar to that of the wild-type.

DISCUSSION

It is possible to identify two of the three major alkaline DNases secreted by wild-type Neurospora by comparing their properties with the properties of previously isolated and characterized nucleases of Neurospora. DNase B is a Mg++-dependent, single-strand specific, alkaline exonuclease activity which is not activated by Ca++ and not inhibited by ATP. If the RNase activity of DNase B is an intrinsic activity rather than a contaminating RNase, then all of the properties of DNase B coincide with those of a single-strand specific exonuclease which was previously purified[17] from Neurospora conidia and characterized[18,19] in this laboratory. The estimated molecular weight of the native DNase B (78,000 daltons) agrees quite closely with the molecular weight of the purified conidial exonuclease of 72,000 determined by SDS-gel electrophoresis[18]. Single-strand specific exonuclease activity has not been detected in extracts of mycelia[8,11] and so may be strictly an extracellular enzyme released by

mycelia.

It is not clear whether DNase B is related to the phosphate repressible extracellular alkaline DNase activity described by Hasunama[20] (which is supposedly not de-repressed in nuc-2). That DNase, like DNase B, is very specific for ss-DNA at pH 9.0, it also has an associated RNase activity and it is activated by Mg++ and inhibited by Ca++. DNase B, however, is present in culture filtrates of nuc-2 grown on sorbose medium which contains a high concentration of phosphate. Sorbose may possibly interfere in some way with the repression of this enzyme, but it did not relieve the repression of nuclease N_3 in this high phosphate medium. Nuclease N_3 activity was not detected in any of the $(NH_4)_2SO_4$ fractions derived from culture filtrates.

DNase C is a divalent metal ion-dependent alkaline DNase that is activated by both Mg++ and Ca++ and also has an associated RNase activity. The mode of degradation of ss-DNA is partially endonucleolytic, but with ds-DNA it is fully exonucleolytic (Fig. 3c). Both DNase activities were inhibited by ATP. These properties coincide with those of another nuclease that has been purified[21] and characterized[21,22] in this laboratory, Neurospora endo-exonuclease[12,22]. It occurs intracellularly in mycelia in an inactive precursor form which is activated by protease(s)[22]. The trypsin-activated precursor was found to have a molecular weight for the native protein of 61,000 daltons[23], which corresponds closely to that found for DNase C (65,000 daltons). It is possible that the inactive form of endo-exonuclease is released into sucrose and sorbose culture media (at least from wild-type mycelia) since DNase C-like activity developed in DNase B preparations on standing. However, in preliminary experiments, treatment of $(NH_4)_2SO_4$ fractions from wild-type culture filtrates with trypsin failed to unmask DNase C activity suggesting that no endo-exonuclease precursor was present. At least two "peaks" of endopeptidase activity (with the p-nitrophenyl ester of t-BOC-methionine) were eluted from DEAE-Sepharose in the region of the gradient where DNases B and C eluted. A high level of aminopeptidase activity (with the p-nitroanilide derivative of lysine, but not with the corresponding derivatives of leucine or glycine) has also been detected in these fractions. These observations suggest another possibility for explaining the appearance of DNase C activity, namely a direct conversion of DNase B to DNase C by limited proteolysis. Even the purest preparations of the conidial ss-exonuclease (likely the same enzyme as DNase B) had a low level of endonucleolytic activity[18]. The activation of DNase C in DEAE-eluate fractions from culture filtrates is currently under investigation.

The DNase A activity described here does not correspond to any of the

known nucleases of Neurospora. It is a Ca^{++}-dependent alkaline DNase with no strand specificity and no RNase activity. Although it behaved as a single protein during chromatography on phosphocellulose and on hydroxyapatite and during sedimentation (see Results), it could nevertheless be a mixture of DNases. Further work will be necessary to fully characterize DNase A.

The deficiencies of DNase A and/or DNase C probably account for the Nuh phenotypes of uvs-3, uvs-6 and nuh-4 seen on solid media and the low recovery of DNase B from the culture filtrate of the nuh-3 mutant may account for the Nuh phenotype of that mutant. However, it is not obvious how these deficiencies account for the Nuh phenotypes when the total ss- and ds-DNase activities of 5-day culture filtrates are examined (Table 4). Several explanations for the apparent discrepancies are possible, but the most likely concerns the critical timing of the tests for the Nuh phenotype[1]. If the colonies are allowed to grow on solid medium longer than the standard test time (2 days), differences in the DNase release by the wild-type and mutants are no longer apparent. It seems possible that, as the colonies continue to grow, more DNase activity is released from both wild-type and mutants resulting in a "swamping out" of the differences seen on the DNA-agar test plates. It can be seen from the data in Table 4 that the ss-DNase activity (an activity of all three alkaline DNases described here) released into the culture filtrates increased sharply as the weight of the mycelia increased. The observations are at least consistent with the above hypothesis.

The low relative recoveries of DNases A and C from culture filtrates of the uvs-3, uvs-6 and nuh-4 mutants as compared with their recoveries from the wild-type and other mutants may indicate that these two DNases are somehow involved in DNA-repair and/or recombination. However, in order to prove a direct involvement, it will be necessary to obtain conditional mutants which directly affect the polypeptides comprising these DNases. The fact that the uvs-3 and nuh-4 mutants yielded dramatically different spectra of alkaline DNases (see Fig. 4) likely indicates that these two mutants, which map very close to one another, are not allelic after all, as suggested earlier[1]. Thus, at least three genes affect the release of DNases A and C from Neurospora mycelia. The release process itself may be affected by these genes rather than the mutations having direct effects on the nucleases e.g. proteases may be involved in the secretion of these enzymes. In addition, the expression of at least one of these intracellular activities (that of endo-exonuclease or DNase C) appears to be regulated by proteases[8].

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