DNA-DEPENDENT RNA POLYMERASES FROM THE FUNGUS *ASPERGILLUS NIDULANS*.

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DNA-dependent RNA polymerases from the fungus *Aspergillus nidulans*

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ter verkrijging van de graad van
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VOORWOORD

Een proefschrift wordt in het algemeen nauwelijks gelezen, behalve dan het voorwoord, de niet-wetenschappelijke stellingen en de levensloop van de schrijver. De redenen hiervoor liggen eigenlijk wel voor de hand. Ik heb daarom gemeend het hoe en waarom van het onderzoek te moeten uitleggen in min of meer begrijpbaar nederlands, zodat hopelijk een leek een indruk krijgt van wat ik nu eigenlijk heb gedaan.

Een organisme bestaat uit een groot aantal cellen, die allen dezelfde erfelijke informatie bevatten, maar verschillende funkties kunnen hebben in een organisme (bv. bloedcellen, spiercellen, zenuwcellen, etc.). Het voortkomen van deze verschillende celtypen uit één oorspronkelijke cel (bv. de bevruchte eicel) wordt differentiatie genoemd.

De erfelijke informatie ligt in de cel opgeslagen in een code, het DNA. Wordt een deel van de informatie opgevraagd, dan wordt het DNA eerst overgeschreven in een nieuwe code, het boodschapper-RNA. Het overschrijven van het DNA in het RNA, de transcriptie, wordt verzorgd door een eiwitcomplex, dat de DNA-afhankelijke RNA polymerase wordt genoemd. Het boodschapper-RNA wordt vertaald in een eindprodukt, het eiwit, de "bouwstenen" van een cel.

De transcriptie en vertaling van de erfelijke informatie ligt ten grondslag aan de ontwikkeling en differentiatie van een organisme. De controle en regulering van de transcriptie is één van de meest "duistere" processen in de moderne biologie. De regulerings-mechanismen moeten uitzonderlijk verfijnd en precies zijn, als men bedenkt, dat een hoger organisme, zoals de mens, uit een groot aantallen cellen en celtypes bestaat, die allen voortgekomen zijn vanuit een "niet-gespecialiseerde" cel. Omdat ontwikkeling en differentiatie van de hogere organismen zeer gecompliceerd zijn, kunnen relatief eenvoudige organismen, zoals de schimmel *Aspergillus nidulans* waarmee ik gewerkt heb, gebruikt worden als modelsystemen voor dit soort studies.

Tijdens de drie jaren van het promotie-onderzoek heb ik mij beziggehouden met de eiwitcomplexen, de RNA polymerases, die de transcriptie van het DNA

STELLINGEN

1. Een klassifikatie van eukaryote DNA-afhankelijke RNA polymerases op basis van differentiele remming door α-amanitine kan misleidend zijn: de DNA-afhankelijke RNA polymerase II uit *Aspergillus nidulans* wordt niet beinvloed door de remmer α-amanitine.
   Kedinger, C., Nuret, P. & Chambon, P. (1971) FEBS Letters 15: 169-175.
   Dit proefschrift.

2. De regulering van de RNA polymerase-activiteit door fosforylering-defosforylering van subeenheden van het eiwit-molecuul is als mechanisme voor de regulatie van de RNA synthese waarschijnlijker dan een regulering van het enzymniveau middels een gecontroleerde synthese en/of afbraak van het enzym of delen daarvan.
   Bell, G.I., Valenzuela, P. & Rutter, W.J. (1976) Nature 261: 429-431.

3. Op basis van het criterium, dat de poly(A)-staart covalent gebonden is aan boodschapper-RNA's van eukaryoten, mag geconcludeerd worden, dat het poly(A)-plus RNA uit *Aspergillus nidulans* bestaat uit boodschapper-RNA of een precursors hiervoor.
   Dit proefschrift.

4. De conclusie, dat de DNA-afhankelijke RNA polymerase II uit *Histoplasma capsulatum* ongevoelig is voor α-amanitine remming, is onvoldoende gefundeerd.
   Kumaf, B.V., McMillian, R.A., Medoff, G., Gutwein, M. & Kobayashi, G. (1980) Biochemistry 19: 1080-1087.

5. Het is onwaarschijnlijk, dat het "gaan drijven" van protoplasten uitsluitend door het zout magnesiumsulfaat wordt geïnduceerd.
   de Vries, O.M.H. & Wessels, J.G.H. (1975) Arch.Microbiol. 102: 209-218.
   Peberdy, J.F. & Isaac, S. (1976) Microbiol.Lett. 3: 7-9.

6. Het polyanion polymin P kan niet verwijderd worden door een selectieve precipitatie met ammoniumsulfaat, zoals wordt aangegeven door Valenzuela et al. (1976).
   Valenzuela, P., Weinberg, F., Bell, G. & Rutter, W.J. (1976) J.Biol. Chem. 251: 1464-1470.
   Dit proefschrift.

7. Sera, die bestaan uit monoclonale antibodies zullen geen rol gaan spelen in de serologische toetsen, die bij de keuring van pootaardappelen worden uitgevoerd.

8. Vrijdag de dertiende is alleen dan een ongeluksdag, als deze als zodanig wordt herkend.

9. Kunst geeft niet het zichtbare weer, ze maakt zichtbaar.
   W. Kandinsky.

10. Als de mensheid niet snel een eind maakt aan de kernwapens, zullen de kernwapens een eind maken aan de mensheid.

11. Hopelijk is er na reagan nog zonneschijn.

Proefschrift van H.G. Stunnenberg
DNA-dependent RNA-polymerases from the fungus *Aspergillus nidulans*.
Wageningen, 13 maart 1981.

verzorgen. Het eerste hoofdstuk in het proefschrift geeft een overzicht van de gegevens van de RNA polymerases, die tot nu toe bekend zijn. Evenals in andere hogere organismen komen drie klassen van RNA polymerases voor in de schimmel *Aspergillus nidulans*. In hoofdstuk 2 en 3 worden de zuiverings-procedures en de eigenschappen van twee van deze RNA polymerase klassen beschreven.Eén van deze twee RNA polymerases blijkt uitzonderlijk te zijn. Deze afwijkende eigenschap is daarom diepgaander bestudeerd (hoofdstuk 5). De voor deze laatste studies ontwikkelde "protoplast"-techniek is beschreven in hoofdstuk 4. De derde RNA polymerase klasse kon niet meer gezuiverd worden in de korte tijd, die voor het projekt beschikbaar was. De gegevens, die uit dit onderzoek zijn voortgekomen, vormen slechts één klein stukje van de grote puzzel. In Zürich hoop ik verder te kunnen werken aan dit voor mij zeer interessante onderwerp in de biologie.

Aan het tot stand komen van dit proefschrift hebben veel mensen meegewerkt. Deze wil ik hier gewoontetrouw, maar zeker niet met minder dankbaarheid, noemen.

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CONTENTS

Abbrevations 11

Chapter 1 12

General introduction 12

1.1. Opening remarks 12

1.2. Eukaryotic nuclear DNA-dependent RNA polymerases 14

1.2.1. Nomenclature 15

1.2.2. Chromatographic resolution 16

1.2.3. Subunit structure and function 18

1.2.4. Catalytic properties 22

1.2.5. α-Amanitin inhibition 24

1.2.6. Specificity of transcription 27

1.2.7. Heterogeneity of the enzyme classes 32

1.3. *Aspergillus nidulans* 34

1.4. Fungal protoplasts 37

References 40

Chapter 2 49

RNA polymerase from the fungus *Aspergillus nidulans*
Large-scale purification of DNA-dependent RNA polymerase I (or A)

H.G. Stunnenberg, L.M.J. Wennekes and H.W.J. van den Broek,
European Journal of Biochemistry (1979) 98: 107-119

Chapter 3 62

An α-amanitin resistant DNA- dependent RNA polymerase II from the fungus *Aspergillus nidulans*

H.G. Stunnenberg, L.M.J. Wennekes, T. Spierings and H.W.J. van den Broek, Submitted for publication

Chapter 4 89

Protoplasts from *Aspergillus nidulans*

H.W.J. van den Broek, H.G. Stunnenberg and L.M.J. Wennekes,
Microbios (1979) 26: 115-128

Chapter 5 103

The effect of α-amanitin and actinomycin D on the RNA synthesis in protoplasts from *Aspergillus nidulans*

H.G. Stunnenberg and H.W.J. van den Broek, Submitted for publication

Chapter 6 115

General discussion and summary

Samenvatting 124

Curriculum vitae 128

ABBREVATIONS

A280	absorbance at 280 nm
ATP	adenosine-5'-triphosphate
Ci	curie
cpm	counts per minute
CTP	cytidine-5'-triphosphate
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
GTP	guanosine-5'-triphosphate
M	molar
mRNA	messenger RNA
oligo(dT)	oligodeoxythymidylic acid
poly(A)	polyriboadenylic acid
polymin P	polyethylene imine
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SV_{40}	simian virus 40
TCA	trichloroacetic acid
TPNS	disodium-triisopropylnaphtalene sulphonate
Tris	tris(hydroxy)aminomethane
tRNA	transfer RNA
UTP	uridine-5'-triphosphate

CHAPTER 1 GENERAL INTRODUCTION

1.1. *Opening remarks*

The aim of the work presented here was the isolation and characterization of the DNA-dependent RNA polymerases from the fungus *Aspergillus nidulans*, which was a part of a project concerning the regulation of gene expression in this lower eukaryote.

The transcription of a genome and the regulation mechanisms involved are basic steps in the development and differentiation of an organism. The regulation mechanisms necessary for the development of a single fertilized egg cell into an organism like men, must be very precise and complicated, if one considers that the organism consists of dozens of different cell types, each having a specific function as part of the whole. The signals, which trigger a cell to develop into a highly specialized blood or brain cell, are largely unknown at the moment. Because the developmental and differentiation process in higher eukaryotes is so complex, relatively simple differentiating organisms, like *Aspergillus nidulans* may be more suitable for the study of the molecular mechanisms underlying the developmental regulation of gene expression. The limited knowledge of the biochemical organization of *Aspergillus*, as compared to a lower eukaryote like yeast, is certainly a disadvantage, but at the same time a challenge for the investigator. On the other hand, the genetics of *Aspergillus* has been extensively studied and this can be of great use in biochemical and developmental studies of this organism.

The transcription of coding sequences of the DNA into RNA, is one of the first processes of a complex chain of events underlying the expression of genetic information. The specific mechanisms involved in the regulation of transcription are largely unknown, but they must directly or indirectly affect the activity of the DNA-dependent RNA polymerases, responsible for the differential transcription of genetic information. Apart from the regu-

lation at the cellular level of the enzyme , other mechanisms must be responsible for the differential transcription of specific classes of genes, that are transcribed by a common enzyme. Structural modification of the chromatin could influence the accessibility of specific genes and hence their ability to be transcribed by an RNA polymerase. Transcription may also be controlled directly by regulators, altering the interaction between the enzyme and a specific gene or genes. It is therefore important to purify eukaryotic nuclear RNA polymerases and to study their structure and function. For understanding the actual transcription mechanism, the development and study of cell-free systems,supplemented with purified RNA polymerases and well characterized templates, will be required.

This thesis describes how the DNA-dependent RNA polymerases I and II from *Aspergillus nidulans* can be successfully purified and subsequently characterized with respect to their catalytic properties and subunit composition (Chapters 2 and 3). Preparation of protoplasts from *Aspergillus* (Chapter 4) was initally thought to be necessary for the isolation of the RNA polymerases, because desintegration of the rigid cell wall of *Aspergillus* was the first difficulty encountered. Although large amounts of protoplasts could be prepared, the procedure appeared to be rather time-consuming and impractical as a standard, large-scale procedure for the isolation of the RNA polymerases. Protoplasts, however, can be very useful when micro-assays or a gentle treatment to break open the cell wall are required. This is demonstrated in Chapter 5, where the effect of inhibitors of RNA synthesis has been studied *in vivo* in metabolically active protoplasts. The isolation and characterization of RNA polymerase III could not be achieved within the limited time available for the project.

1.2. *Eukaryotic nuclear DNA-dependent RNA polymerases*

The study on the regulation of gene expression in eukaryotes started about 20 years ago with the detection in a rat liver homogenate of an enzyme activity, responsible for the transfer of genetic information from DNA into RNA (Weiss & Gladstone, 1959). A spectacular progress in the study of gene expression in eukaryotes,as observed in prokaryotes,was not achieved however, because of the considerable larger size and complexity of the eukaryotic genome and the reduced practicality of the genetic approach, which was successfully used in the study of prokaryotes. Following the discovery of the mammalian nuclear RNA polymerase activity, a number of studies showed, that variations in ionic conditions effected both quantitative and qualitative changes in RNA synthesis in isolated nuclei (cf. Jacob, 1973). It was not clear at first, whether these results reflected selective ionic effects on different templates transcribed by a common enzyme or whether they were due to the presence of distinct RNA polymerases with different ionic requirements.

The method developed by Roeder and Rutter (1969, 1970) for the separation of the polymerase activity into three different forms, was the second important advance in the elucidation of gene expression. They showed, that RNA polymerase activity, solubilized from rat liver and sea urchin nuclei by a high-salt-sonication procedure, could be separated chromatographically into three different forms with distinct catalytic properties. Additional evidence for the existence of different forms of RNA polymerases was supplied by Kedinger et al. (1970) and Lindell et al. (1970). They were able to distinguish distinct classes of RNA polymerases according to the differential inhibitory effect of α-amanitin. Structural analysis and immunological properties of the purified enzymes have confirmed the multiplicity of the RNA polymerases (cf. Chambon, 1975; Roeder, 1976). Subsequently RNA polymerases have been purified from a wide range of lower as well as higher

eukaryotes, but specific transcription of DNA templates by the purified RNA polymerases could not be demonstrated. Only in isolated nuclei it was possible to achieve a differential transcription of the genome using selective concentrations of α-amanitin to inhibit specific classes of RNA polymerases. From analysis of the transcripts and subcellular localization of the RNA polymerase activity it was suggested, that the distinct classes of RNA polymerases were responsible for the transcription of different classes of genes.

Only recently, soluble cell-free systems have been developed in which DNA templates are accurately transcribed by endogenous RNA polymerases (Wu, 1977; Weil et al., 1979; Manley et al., 1980). These studies confirm the specificity of transcription of the different RNA polymerases. The regulation of transcription can now be studied with these *in vitro* cell-free transcription systems (Ford, 1980; Flavell, 1980), opening the way for the study of the developmental regulation of gene expression.

1.2.1. *Nomenclature*

The nomenclature of the multiple RNA polymerases is sometimes confusing since different criteria are used to classify the enzymes. The initial scheme of Roeder and Rutter (1969) numbered the enzyme species I, II and III according to the order in which the enzymes eluted from DEAE-Sephadex columns with salt-gradients. Chambon and coworkers (Kedinger et al., 1971) classified on the basis of the sensitivity of the RNA polymerases towards the toadstool toxin α-amanitin: form A (corresponding to type I) was resistant and form B (type II) was highly sensitive to the poison. The intermediate sensitive form C (type III) was originally not included in the nomenclature of Chambon. Subspecies of each main category were distinguished according to their relative elution position from ion-exchange columns and their α-amanitin sensitivity. Neither system, however, has escaped the need for modifications. Type III (C) RNA polymerase from

certain tissues, has been reported to be eluted from DEAE-Sephadex between form I (or A) and II (or B) (Hossenlop et al., 1975),and type II (or B) RNA polymerase from *Physarum* was reported to be eluted prior to type I (or A) (Hildebrandt & Sauer, 1973). Considerable variations in the relative and absolute sensitivity of RNA polymerase A (or I) and B (or II) to α-amanitin was reported for analogous RNA polymerases from different eukaryotes (Huet et al., 1975). Also considerable variations in α-amanitin sensitivity of the intermediate form C (or III), originally not included in the nomenclature, were observed (Sklar & Roeder, 1975). It is clear, that a definite classification of the purified enzymes should ultimately be based on known functions of the enzymes.

In this thesis the original nomenclature of Roeder and Rutter will be used.

1.2.2. *Chromatographic resolution*

Roeder and Rutter (1969, 1970) were able to separate the different classes of nuclear DNA-dependent RNA polymerases by chromatography under discriminating conditions on DEAE-Sephadex. Subsequently, several other laboratories confirmed these findings (cf. Roeder, 1976), although in most of these early studies only RNA polymerase I and II could be detected. From organism to organism great differences in the exact elution positions of the three RNA polymerases from several ion-exchange columns are reported. Therefore only the generally observed elution positions(summarized in Table 1) and some of the marked exception will be mentioned (cf. Roeder, 1976).

Table 1. Concentration of ammonium sulphate (M) by which eukaryotic RNA polymerases are eluted from different columns

	RNA polymerase		
Ion-exchange resins	I	II	III
DEAE-Sephadex	∿0.1	∿0.2-0.25	∿0.2-0.3
DEAE-cellulose	∿0.1	∿0.2	∿0.1
phosphocellulose	∿0.15-0.2	∿0.1	∿0.1-0.15
CM-Sephadex	∿0.1	<0.05	∿0.1

The elution positions (in M ammonium sulphate) are those generally found for the major forms of RNA polymerases I, II and III. The various heterogeneous forms of the three RNA polymerases are not included in the table.

Especially among lower eukaryotes markedly different elution positions can be observed, for example *Physarum* RNA polymerase II is eluted prior to RNA polymerase I on DEAE-Sephadex (Hildebrandt & Sauer, 1973). The elution position of RNA polymerase III can be markedly different and the enzyme is often eluted prior to or together with RNA polymerase II from DEAE-Sephadex (Hossenlopp et al., 1975; Sasaki et al., 1977).
Classification of the different RNA polymerases solely according to their order of elution from DEAE-Sephadex can therefore be misleading (Kumar et al., 1980).

Affinity chromatography on DNA-cellulose can also be used together with ion-exchange chromatography for separation and purification of the RNA polymerases (Hager et al., 1977). With DNA-cellulose RNA polymerase III can very well be separated from RNA polymerase I and II, the latter two being eluted at the same low salt position. Heparin, an analog for DNA, covalently attached to Sepharose can be used for purification of the RNA polymerases, but separation of the different classes can usually not be established (Teissere et al., 1977).

1.2.3. *Subunit structure and function*

All eukaryotic nuclear RNA polymerases are macromolecular multi-subunit enzymes with a molecular weight close to or in excess of 500,000 daltons. These molecular weights are determined by centrifugation of the enzymes in glycerol or sucrose gradients (Kedinger et al., 1974; Ponta et al., 1972; Broght & Planta, 1972).

The subunit composition of purified RNA polymerases has been investigated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulphate. In some cases, the molecular structure of the various enzymes has been further investigated by polyacrylamide gel electrophoresis in the presence of urea, since possible differences in charge cannot be detected with dodecylsulphate gel electrophoresis (Kedinger et al., 1974; Buhler et al., 1976; Jendrisak & Burgess, 1977). In one case the isoelectric point of the different subunits has been determined (Buhler et al., 1976). In all cases, the enzymes consist of two high-molecular-weight subunits (in excess of 100 000 daltons) and a variable number of smaller subunits (less than 100 000 daltons). There is, however, little resemblance in size and number of subunits between different species or at least between different analyses carried out in different laboratories (Buhler et al., 1974; Valenzuela et al., 1976; Huet et al., 1975). Discrepancies in molecular weight determination of the putative subunits, in particular due to the lack of sufficient marker polypeptide chains in the range of 120 000 - 200 000 daltons, explain most of the observed differences in molecular weights obtained for the various enzyme components in different laboratories.

The determination of the putative subunits belonging to the enzyme complex by means of gel electrophoresis under non-denaturing conditions and subsequent polyacrylamide gel electrophoresis in the presence of SDS in the second dimension, is complicated by the possibility of non-specific

binding of polypeptides to the complex. This electrophoresis technique cannot distinguish between a polypeptide that forms a fundamental part of the multimeric structure and non-specific protein interaction. The number of subunits can therefore be overestimated and too much significance may be attributed to small differences in macromolecular composition of the enzymes. As pointed out by Sentenac et al. (1978), the term "subunit" should be taken in its very broad sense; it refers to the various polypeptide chains, which remain tightly bound to the enzyme molecule. The co-purification of the polypeptides upon glycerol or sucrose gradient centrifugation at high ionic strength and during gel electrophoresis under non-denaturing conditions are used as the operational criteria (Kedinger et al., 1974; Schwartz & Roeder, 1974, 1975; Sklar & Roeder, 1976; Jendrisak & Burgess, 1977; Valenzuela et al., 1976). Another approach is the immunoprecipitation of the enzyme complex by antibodies against single subunits (Huet et al., 1975). A proper definition of the enzyme subunit structure should ultimately be based on more sound criteria, requiring biochemical and genetic evidence for the functional role of the different polypeptides. Up to now, dissociation-reconstitution experiments have failed to elucidate the subunit function in eukaryotic RNA polymerases (Sentenac et al., 1978).

It is almost impossible and certainly not illuminating, to give an extensive survey of the polypeptide composition of the distinct RNA polymerases isolated from a great variety of organisms and to discuss in detail the differences between the organisms, with respect to the polypeptide composition of their RNA polymerases. The polypeptide composition of yeast and mouse plasmacytoma RNA polymerases is shown in Table 2. These two organisms, representing the lower and higher eukaryotes, are well studied with respect to their RNA polymerases and are therefore chosen to illustrate the complexity of the RNA polymerases (Roeder, 1976; Sebastian, 1977).

Table 2. The polypeptide composition of RNA polymerase I, II and III, from mouse plasmacytoma and yeast

mouse plasmacytoma			yeast		
I	II	III	I	II	III
	240				
				220	
	205				
195					
		190	190		
				180	
	170				
					160
	140			150	
		138			
			135		
117					128
		89			
					82
61		70			
		53			
49		49			
			48		
			44		
		41			41
					40.5
			37		37
		33/32			34
				32	
29	*29*	*29*			
	27				28
			27	*27*	
					24
	22		*23*	*23*	
	19.5		*20*		*20*
19	*19*	*19*			
	16.5			17	
			14.5	*14.5*	*14.5*
			12.2	12.5	
			10		
				9	

The numbers indicate approximate molecular weights in daltons x 10^{-3}. The underlined numbers indicate the polypeptides which are different or lacking in the respective subforms. The numbers in italics indicate the polypeptides, which are common the to the different enzyme classes.

The distinct RNA polymerases cannot be recognized just on the basis of the polypeptide pattern,but some characteristics can nevertheless be used for their classification. RNA polymerase I can be identified on account of the different polypeptide patterns of the respective subforms. In most organisms, the class I subforms differ for only one polypeptide (60 000 - 70 000 daltons): subform I^B lacks this polypeptide, while it is present in the I^A subform. The I^B subform from yeast however, lacks two polypeptides of 48 000 and 37 000 daltons. Furthermore the two class I subforms of yeast differ in catalytic properties, while from all other organism they are indistinguishable in this respect. RNA polymerase II can be identified on account of the absence of polypeptides in the range of 50 000 - 100 000 daltons; furthermore the subforms of the class II enzyme differ in the molecular weight of the largest polypeptide. These rather generally observed differences indicate, that RNA polymerase II is probably more conserved than RNA polymerase I and III. Characteristic for RNA polymerase III is the presence of one or more polypeptides in the range of 70 000 - 100 000 daltons. The two high-molecular-weight subunits (in excess of 100 000 daltons) are smaller than the two comparable polypeptides of the class I and II enzymes. The phenomenon of the heterogeneity of the different RNA polymerases will be discussed in detail in section 1.2.7.

The structural studies of the polymerases indicate, that the three classes have a few low molecular weight subunits in common, belonging to the small polypeptides (Buhler et al., 1976; Hildebrandt & Sauer, 1973; Valenzuela et al., 1976). The existence of common subunits raises questions concerning the function of these subunits in the biosynthesis of the RNA chain. Part of the mechanism of transcription, for instance the elongation step, could be identical for the different RNA polymerases. The presence of common subunits also raises important problems concerning the synthesis, assembly and regulation of each of the RNA polymerases.

Preliminary information on a possible function of some RNA polymerase subunits has been obtained by more or less indirect methods. From experiments with pyridoxal 5'-phosphate it was suggested by Valenzuela et al. (1978), that the yeast RNA polymerase I subunits of 185 000 and 137 000 daltons are involved in both nucleotide and DNA binding and that the 48 000 and 37 000 daltons polypeptides also bind to DNA, although this interaction is not strictly required for polymerase activity. The possible involvement of the 48 000 and 37 000 polypeptides of RNA polymerase I^A from yeast (absent in the I^B form) in DNA binding was also suggested by Huet et al. (1976) and further substantiated by Cooper & Quincey (1979). A significant finding was the identification of the α-amanitin binding polypeptide in RNA polymerase II by affinity labeling experiments (Brodner & Wieland, 1976).

Several polypeptide components of the RNA polymerases are phosphorylated *in vivo* (Bell et al., 1976; Buhler et al., 1976; Bell et al., 1977). The significance of the phosphorylation *in vivo* is not clear, but in analogy to the regulation of enzyme activities involved in the glycogen metabolism through phosphorylation-dephosphorylation (Rubin & Rosen, 1975), it is tempting to suggest, that the RNA polymerase activities may also be regulated through phosphorylation (Bell et al., 1977). This possibility of control of the pre-existing RNA polymerase activities is more attractive, than alterations of the rate of synthesis or degradation of RNA polymerase molecules or components (Roeder, 1976).

1.2.4. *Catalytic properties*

It is possible to characterize the three classes of eukaryotic RNA polymerases, using an excess of native calf thymus DNA as a template, according to the preferential activation by either Mn^{2+} or Mg^{2+}, ionic strength optima and preferential transcription of native or denatured DNA. However, the catalytic properties should not be considered as invariant characteristics of the enzyme classes. The enzyme characteristics, in particular the optimal

ionic strength, are highly dependent on the nature of the template (synthetic or natural), the state of the DNA (native or denatured) and its concentration in the reaction mixture (cf. Chambon, 1974; Roeder, 1976).

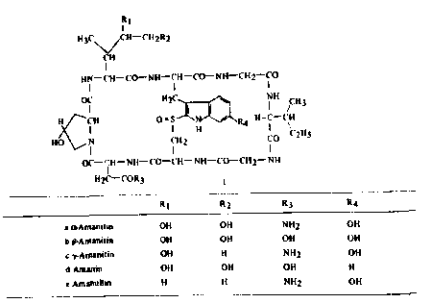
The most important difference in catalytic properties between the RNA polymerases I, II and III is the optimal ionic strength of the distinct enzyme activities under well defined conditions using an excess of native calf thymus DNA as a template. The salt activaty profiles of RNA polymerase I generally are sharp (optimal < 50 mM ammonium sulphate) and for RNA polymerase II more gradual between 50 and 100 mM ammonium sulphate. RNA polymerase III shows a very broad plateau or a biphasic curve within the range of 50-200 mM ammonium sulphate (cf. Roeder, 1976). The (characteristic) ammonium sulphate activaty profiles sometimes enable the investigator to distinguish clearly between the enzyme classes, where other properties (e.g. α-amanitin sensitivities) do not (Schultz & Hall, 1976).

Most RNA polymerases are more active with Mn^{2+} than with Mg^{2+} when assayed at their respective metal ion optima. RNA polymerase II is in general 5-10 fold more active with Mn^{2+} than with Mg^{2+}, while RNA polymerase I and III are only 1-2 fold more active with Mn^{2+} than with Mg^{2+}. With respect to the preferential transcription of a certain template, RNA polymerase II appears to be more active with denatured DNA than with native DNA, RNA polymerase III is almost equally active with either template and RNA polymerase I is in general more active with a native DNA template. These preferences should be considered with great caution, because single stranded regions (internal gaps or free ends) and nicks in the template can serve as initiation sites for the RNA polymerases and therefore not only influence the denatured *versus* native DNA activity ratios, but also the specific transcription by a particular RNA polymerase (Gissinger et al., 1974; Flint et al., 1974; van Keulen et al., 1975; Dynan et al., 1977). The enzymes often show markedly distinct activities with synthetic polynucleotide templates relative to their activities with native DNA (Blatti et al., 1971; Dezélée et al., 1974; Sasaki et al., 1976). Synthetic templates, like poly(dA-dT),

are very useful in detecting minor polymerase components, such as RNA polymerase III, which transcribes the synthetic template more efficiently than native DNA; the reverse is true for RNA polymerase I and II.

1.2.5. *α-Amanitin inhibition*

The amatoxins (α- and β-amanitin) are the lethal poisons from the toadstool *Amanita phalloides* (fig. 1). The amatoxins are cyclic octapeptides of which the ring is divided and held in compact shape by a sulfoxide bridge (Wieland, 1972) (fig. 1). The primary action of the poison, α-amanitin, is a reduction of liver and kidney function, resulting in a decreasing blood sugar level (Wieland, 1972).



	R_1	R_2	R_3	R_4
a α-Amanitin	OH	OH	NH_2	OH
b β-Amanitin	OH	OH	OH	OH
c γ-Amanitin	OH	H	NH_2	OH
d Amanin	OH	OH	OH	H
e Amaninin	H	H	NH_2	OH

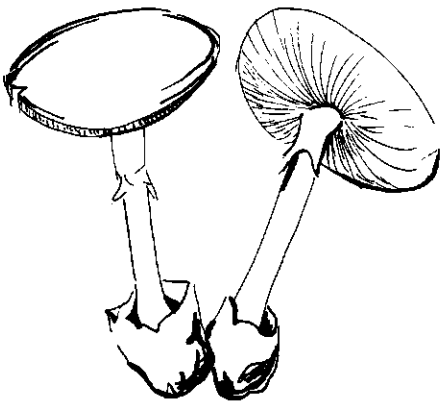


Fig. 1. *Amanita phalloides* and the chemical structure of amatoxins

It was shown by Fiume & Stirpe (1966) and Seifart & Sekeris (1969), that histological changes in the mammalian nucleus upon α-amanitin administration were related to an inhibition of RNA synthesis and that the DNA-dependent RNA polymerase activity was directly influenced by the poison α-amanitin. At a concentration of 10 ng/ml (10^{-8} M) of the toxin, the solubilized RNA

polymerase activity could be inhibited for 60-80%. Increasing the α-amanitin concentration did not result in a complete inhibition of the RNA polymerase activity. After separation of the solubilized RNA polymerase activity from rat liver into the forms I and II through DEAE-Sephadex chromatography, it could be demonstrated, that only RNA polymerase II is sensitive to α-amanitin inhibition (50% inhibition level at 10-50 ng/ml) and that RNA polymerase I was completely insensitive (Kedinger et al., 1970; Lindell et al., 1970). The mammalian RNA polymerase III was later shown to be intermediate sensitive to α-amanitin (50% inhibition level at 10-25 μg/ml) (Seifart et al., 1972; Weinmann & Roeder, 1974).

The early studies indicate that α-amanitin exerts its effect through interaction with the RNA polymerase and not with the template (Seifart & Sekeris,1969). More detailed studies have shown, that α-amanitin blocks the elongation step in the transcription by direct interaction with the RNA polymerase enzyme (Mandel & Chambon, 1971; Cochet-Meilhac & Chambon, 1974). The receptor site of α-amanitin was shown to be located on the 140 000 dalton subunit of RNA polymerase II from calf thymus (Brodner & Wieland, 1976).

It was suggested by Chambon and coworkers (Kedinger et al., 1971), that the classification of the distinct RNA polymerases should be based on the differential inhibition of the enzyme classes by α-amanitin. The class I, II and III (referred to as A, B and C, respectively) enzymes from animal cells could readily be distinguished with respect to their α-amanitin sensitivity as shown for the enzymes from mouse tissue (Schwartz et al., 1974), rat liver (Seifart & Benecke, 1975; Kedinger et al., 1971), calf thymus (Weil & Blatti, 1975) and human cells (Hossenlopp et al., 1975; Weil & Blatti, 1976). Similar RNA polymerases from different eukaryotes showed, however, considerable variation in their relative and absolute sensitivities towards α-amanitin. From the silkworm *Bombyx mori* and from the crustacean *Artemia salinea*, the class II enzymes showed α-amanitin

sensitivities equivalent to those of the animal class II enzymes, whereas RNA polymerase I and III were both completely resistant to α-amanitin concentrations as high as 1 mg/ml (Sklar & Roeder, 1975; Renart & Sebastian, 1976). Other insect class II enzymes showed similar α-amanitin sensitivities (Phillips & Forrest, 1973; Greenleaf & Bautz, 1975; Greenleaf et al., 1976). Striking differences in α-amanitin sensitivities have mainly been observed with enzymes from the lower eukaryotes (Table 3). In yeast, RNA polymerase II shows a moderate sensitivity to α-amanitin (1 µg/ml for 50% inhibition) more closely approximating the sensitivity of the animal class III enzymes than that of the class II enzymes (Sebastian, 1978). Similar moderate sensitivities of RNA polymerase II enzymes were observed with mushrooms from the *Amanita* species (Vaisius & Horgen, 1979; Johnson & Preston, 1979). Yeast RNA polymerase I is sensitive to very high concentrations of α-amanitin (0.2-0.6 mg/ml for 50% inhibition), whereas yeast RNA polymerase III is insensitive (Schultz & Hall, 1976; Valenzuela et al. 1976; van Keulen, 1979). (Table 3).

Table 3. α-Amanitin sensitivity of the eukaryotic RNA polymerases

Organisms	Concentration of α-amanitin causing 50% inhibition (µg/ml)		
	RNA polymerase I	RNA polymerase II	RNA polymerase III
Animal cells	1000	0.01-0.05	10-50
S. cerevisiae	200-600	1	1000
Bombyx mori	1000	0.01-0.05	1000
Amanita	-(1)	5-300	-(1)

(1) These values were not determined

Mutant animal cell lines containing an α-amanitin resistant RNA polymerase II have been an important tool for studying the regulation of RNA synthesis at the enzyme level (Chan et al., 1972; Amati et al., 1975; Ingles et al., 1976; Somers et al., 1975 a/b.; Wulf & Bautz, 1976; Bryant et al., 1977). The evidence, that these mutations may involve a structural alteration of RNA polymerase II,is based on: a) there is a

measurable decrease in the affinity of the enzyme to α-amanitin (Ingles et al., 1976); b) there can be a coexpression of wild type sensitive and resistant forms of RNA polymerase II when cells are grown in either the presence or absence of α-amanitin (Somers et al., 1975 a/b.; Wulf & Bautz, 1976); c) there is an increase in the thermolability of RNA polymerase II for the CHO cell line, which is α-amanitin resistant (Lobban et al., 1976). The α-amanitin resistant cell lines (Ingles, 1978) have been useful in studying several aspects of RNA polymerase biochemistry, including the regulation of biosynthesis of the enzyme (Somers et al., 1975 a/b.; Crerar & Pearsons, 1977; Guialis et al., 1977, 1979) and its role in viral gene expression (Amati et al., 1975; Ben-Ze'ev & Becker, 1977). Mutants of *Drosophila*, displaying amanitin resistant growth, were raised by Greenleaf et al. (1979) and it was shown, that these mutants contained an RNA polymerase II which is 250-times more resistant to inhibition by α-amanitin than the wild type enzyme. The α-amanitin resistance of the mutant appeared to be controlled by a locus on the X-chromosome, which might represent the structural gene for the α-amanitin binding subunit of RNA polymerase II.

It must be concluded, that a classification of eukaryotic RNA polymerases solely based on their α-amanitin sensitivity can be highly misleading and does not seem feasible.

1.2.6. *Specificity of transcription*

Different RNA polymerase activities could be observed when intact nuclei were incubated under distinct conditions (Widnell & Tata, 1966). At low ionic strength in the presence of Mg^{2+},(G+C) rich RNA similar to ribosomal precursor RNA was synthesized in rat liver nuclei. This RNA synthesis was localized in the nucleolus (Pogo et al., 1967) and appeared to be resistant to α-amanitin (Novello & Stirpe, 1969). At high ionic strength in the presence of Mn^{2+} however, the endogenous nuclear RNA polymerases synthesized mainly (A+U) rich RNA in the nucleoplasm (Widnell &

Tata, 1966; Pogo et al., 1967) in a reaction, which was highly sensitive to α-amanitin (Novello & Stirpe, 1969). Whether these results reflected the activity of distinct RNA polymerase enzymes or whether they were due to selective ionic effects on the template transcribed by one enzyme, was not clear at first. The demonstration of the eukaryotic RNA polymerase multiplicity (Roeder & Rutter, 1969) gave rise to the supposition, that distinct enzyme classes were responsible for the different transcription activities and thus for the expression of particular classes of genes.

Subcellular localization of the different enzyme classes supported the idea of enzyme specificity. It was demonstrated, that RNA polymerase I, which was not sensitive to α-amanitin (Lindell et al., 1970; Kedinger et al., 1970), was located exclusively in the nucleolar region (Roeder & Rutter, 1970; Sebastian et al., 1973; Hildebrandt & Sauer, 1973). This localization suggested, that RNA polymerase I was responsible for the synthesis of ribosomal RNA, as already indicated by the results of Widnell & Tata (1966). The highly sensitive RNA polymerase II activity was found in the nucleoplasmic fractions and was thought to be responsible for the synthesis of heterogeneous nuclear RNA, the precursor to messenger RNA (Widnell & Tata, 1966; cf. Jacob, 1973; Chambon, 1975; Roeder, 1976). Administration of α-amanitin *in vivo* inhibited the hnRNA synthesis and sometimes also the rRNA synthesis, probably as a secondary indirect effect (Hadjiolov et al., 1974), but left the tRNA synthesis unaffected (Shaaya & Sekeris, 1971). More detailed studies with isolated nuclei showed, that the synthesis of small RNA species, like tRNA and 5S RNA, was sensitive to very high concentrations of α-amanitin, which paralleled those concentrations needed to inhibit solubilized form III RNA polymerase (Weinmann & Roeder, 1974). This RNA polymerase III activity was found in the nucleoplasmic as well as in the cytoplasmic fractions (Seifart et al., 1972; Seifart & Benecke, 1975; Austoker et al., 1974). Although the storage and/or function of RNA poly-

merase III in the cytoplasm could not be ruled out, it seemed likely that the observed discrepancies in localization, reflected leakage of nuclear enzymes during cellular fractionation (Schwartz et al., 1974). The overall evidence clearly indicated, that RNA polymerase I was responsible for the synthesis of ribosomal RNA, polymerase II for that of messenger RNA and form III RNA polymerase for that of 5S and transfer RNA.

The complexity of the eukaryotic genome forced the different investigators to concentrate either on the transcript analysis from reiterated genes or to use simple templates such as viral DNAs. These viral DNAs, like SV_{40} and adenovirus-2 genomes, could readily be isolated in intact form and were transcribed by class II (SV_{40}) or class II and III (adenovirus-2) enzymes in infected cells. The function of specific host RNA polymerases in the transcription of viral genes was established by determination of α-amanitin sensitivities of specific viral RNA synthesis in isolated nuclei. It was demonstrated, that the class II RNA polymerases were involved in the synthesis of the major population of viral RNA, representing viral mRNA or precursors to viral mRNA (Price & Penman, 1972; Wallace & Kates, 1972; Weinmann et al., 1974, 1975). The RNA polymerase activity involved in the viral mRNA synthesis was demonstrated to be of host origin, since the respective α-amanitin sensitivities of viral mRNA synthesis and the host RNA polymerase II activity were indistinguishable (Weinmann et al., 1974, 1975; Amati et al., 1975). Class III RNA polymerases were shown to synthesize low-molecular weight viral RNA species in human cells lytically infected with adenovirus-2 (Roeder, 1976; Jaehning et al., 1976). These viral RNAs appeared to be localized in a small region of the adenovirus-2 genome and the RNAs were transcribed from the same strand of the viral DNA (Weinmann et al., 1976).

Initiation studies with soluble, purified enzymes initially did not provide support for the hypothesis of the enzyme specificity of transcription. Integrity of the template - no single-stranded breaks in the DNA - appeared to be critical for a bonafide initiation (Flint et al., 1974; Beebee & Butterworth, 1974; van Keulen et al., 1975). Several studies on

the transcription of specific genes *in vitro* by exogenous RNA polymerases were performed using isolated chromatin as a template. The use of chromatin templates was based on the premisses, that the template might be less damaged and that specific chromatin-associated proteins might be necessary cofactors for selective gene transcription (cf. Roeder, 1976). In no case however, were class I and II enzymes found to transcribe specific genes more accurately or efficiently than prokaryotic RNA polymerases (Honjo & Roeder, 1974; Wilson et al., 1975).

In similar studies, RNA polymerase III was shown to transcribe selectively and accurately the 5S genes in chromatin, but not in naked DNA (Parker et al., 1976; Parker & Roeder, 1979; Jaehning & Roeder, 1977). These findings were the first indication , that specific transcription events in living cells could be duplicated in reconstituted cell-free systems. It was shown that besides the RNA polymerase enzymes and a DNA template, other nuclear or chromatin-associated factors were necessary for the accurate transcription of these genes.

Rapid progress has been made in the study of gene transcription after this first report of accurate *in vitro* transcription of the 5S gene by RNA polymerase III. One of the reasons for this progress was the use of the recombinant DNA technique. Through this technique it was possible to isolate a specific gene or a set of genes from the bulk of the DNA and to replicate this DNA fragment independently in bacteria. Through sequence analysis it was possible to characterize the putative template and to define exactly the coding region. The integrity of the template could now be guaranteed and the difficulty of the complexity of the eukaryotic genome could now be avoided.

An important advance in the study of transcription regulation was made by Wu (1978), who established an *in vitro* system, in which the virus-associated (VA) RNA genes of purified adenovirus-2 DNA were selectively transcribed by RNA polymerase III. Subsequently, several other soluble

enzyme systems were developed from amphibian oocytes and cultured amphibian and mammalian cells, in which DNA templates, containing class III genes were accurately transcribed by endogenous class III RNA polymerases (Korn & Brown, 1978; Birkemeier et al., 1978; Schmidt et al., 1978). The various studies, reported thus far, show that in these systems a variety of class III genes (tRNA, 5S RNA or viral genes) are accurately transcribed. Even genes from simple eukaryotes like yeast are accurately transcribed in germinal vesicle (GV) extracts from *Xenopus oocytes* (Schmidt et al., 1978). Besides these *in vitro* transcription systems, a mechanical injection technique was developed, through which cloned and modified DNA probes, injected into the large nucleus of *Xenopus laevis* oocytes, could be accurately transcribed (Mertz & Gurdon, 1977; Kressmann et al., 1978). The results from *in vivo* and *in vitro* transcription studies suggest, that structural features of the transcription components of type III genes are highly conserved (Sakonju et al., 1980; Bogenhagen et al., 1980; Telford et al, 1979; Thimmappaya et al., 1979; Ng et al., 1979; Weil et al., 1979). An internal promoter site seems to exist in at least the 5S genes and probably in all RNA polymerase III transcribed genes; the polymerase binding and transcription appears to be dependent on this internal region (cf. Ford, 1980).

Recently, an *in vitro* transcription system for class II genes has been developed by Weil et al. (1979), existing of an S100 extract of mammalian cells in which adenovirus-2 DNA could be specifically transcribed by RNA polymerase II. A similar system has been developed by Manley et al. (1980) using a Hela nuclear extract. In both cases, the transcription of a major late adenovirus-2 pre-mRNA could be demonstrated. The *in vitro* system of Weil et al. (1980) has recently been used for the transcription of conalbumin and ovalbumin genes and has been compared with the adenovirus late and early regions (Wasylik et al., 1980). The authors showed, that the conalbumin gene and the adenovirus-2 late region are both efficiently transcribed in this *in vitro* system. However, the ovalbumin genes and the adenovirus-2 early regions are only poorly utilized. It is not clear,

whether these differences reflect weak and strong promoters. The promoter regions of most of the eukaryotic class II genes appear to consist of a highly conserved sequence (TATAAA), the Hogness box, which may be similar to the Pribnov box in prokaryotes (Pribnov, 1975; Proudfoot, 1979). It seems clear, that some of the sequences required for the transcription of structural genes by RNA polymerase II are localized in the DNA upstream of the coding region. Deletion of this region upstream the H_2A gene, including the Hogness box, did reduce, but not eliminate the transcription of the gene (Grosschedl & Birnstiel, 1980). The results from studies of the transcription of class II genes are in strong contrast to those obtained with the class III genes (Sakonju et al. , 1980; Bogenhagen et al., 1980; Kressmann et al., 1979). Some of the aspects of transcription appear to be regulated in a similar way as with prokaryotes.

It should be concluded, that specific transcription by the distinct RNA polymerase classes can be unequivocally demonstrated in the *in vitro* and *in vivo* transcription systems.

1.2.7. *Heterogeneity of the enzyme classes*

Heterogeneous forms of RNA polymerase I, II and III can be detected in enzyme preparations from various organisms by ion-exchange chromatography and by polyacrylamide gel electrophoresis under non-denaturing conditions. The polypeptide composition of the subforms within each class appear to differ only by one or two polypeptides. The heterogeneous subforms are in general indistinguishable with respect to catalytic properties such as metal-ion requirement, optimal ionic strength and α-amanitin sensitivity (Chesterton & Butterworth, 1971; Hossenlopp et al., 1975; Sklar & Roeder, 1976). Whether these subforms are physiologically significant is unknown and speculative. Most of the data suggest the loss or modification of specific polypeptides during the isolation procedure (Weaver et al., 1971; Dezélée et al., 1976; Osuna et al., 1977).

Although the significance of the heterogeneity of the different classes of RNA polymerases must be treated with great caution, a short survey will be presented considering the different classes independently.

Class I. Two chromatographically distinct forms of RNA polymerase I have been observed following DEAE-Sephadex, CM-Sephadex and phosphocellulose chromatography (Chesterton & Butterworth, 1971; Gissinger & Chambon, 1975; Huet et al., 1975). These distinct forms differ in having (I^A) or missing (I^B) one polypeptide in the range of 60 000-70 000 daltons, or as is the case with yeast in two small polypeptides of 48 000 and 37 000 daltons (Huet et al., 1975). Two electrophoretic forms have been reported for mouse plasmacytoma RNA polymerase I, as given in Table 2 (Schwartz & Roeder, 1974). The subforms of RNA polymerase I from *Artemia salina* larvae appeared to be produced by proteolysis during the extraction and solubilization of the RNA polymerases (Osuna et al., 1977). It was reported by Kellas et al. (1977), that through the high-salt-sonication technique, often used in RNA polymerase purification, one RNA polymerase I subform could be quantitatively converted into the other one.

Class II. At least three subforms of RNA polymerase II have been distinguished in *Xenopus* (Roeder, 1974) and mouse myeloma (Schwartz & Roeder, 1975) and probably four species in calf thymus (Kedinger et al., 1974). The occurrence of these forms, differing only in the size of the largest subunit, seems to be a fairly general observation. In wheat germ (Jendrisak & Burgess, 1975) and cauliflower (Goto et al., 1978) only one form could be detected. This could be due, however, to the isolation procedure, since Hodo and Blatti (1977) were able to isolate subforms of wheat germ RNA polymerase II by a slightly modified isolation procedure. In combination with the fact, that the subspecies of RNA polymerase II appear to be indistinguishable with respect to their catalytic properties, it seems likely, that the subforms are derived from one original form by proteolytic cleavage of the largest subunit during the isolation procedure, as suggested by Dezélée et al. (1976). Nevertheless, the occurrence of these polymerase II subforms

can be regarded as characteristic for the class II enzymes and is often used as an additional classification criterium.

Class III. RNA polymerase III from various organisms has been separated into two forms, designated III^A and III^B following DEAE-Sephadex chromatography or polyacrylamide gel electrophoresis (Benecke & Seifart, 1975; Weil & Blatti, 1976; Hossenlopp et al., 1975; Sklar et al., 1976).The two subforms differ only in one low-molecular-weight subunit which is present in the III^A form, but not in III^B. There are no apparent differences in the catalytic properties *in vitro*.

1.3. *Aspergillus nidulans*

Aspergillus nidulans is a sexually reproducing member of the Aspergillaceae which belong to the obligate aerobe *Ascomycetes*, a class of fungi having a vegetative and generative growth cycle, shown in fig. 2 (Raper & Fennel, 1965).

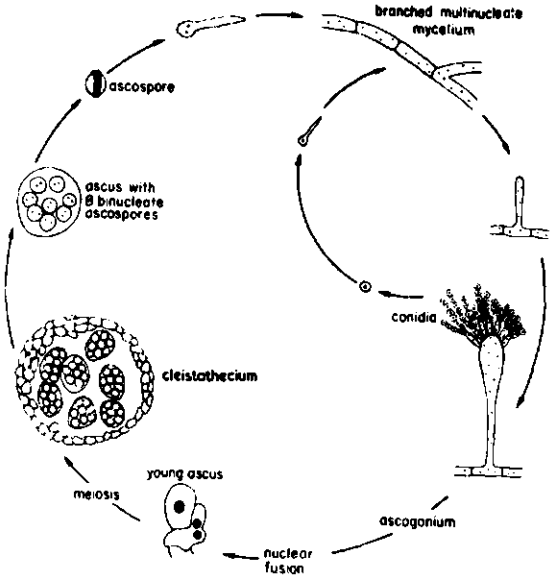


Fig. 2. Life cycle of *Aspergillus nidulans*

In the vegetative form, haploid conidiospores are uninucleated and have in general a green colour. The hyphae, arising from the haploid conidiospores, consists of a string of multinucleated cells surrounded by a rigid cell wall, consisting of chitin and glucan polymers. The hyphae have septea with a pore , which allow the exchange of nuclei and cytoplasm (Pontecorvo, 1953). Due to external signals, like nutrient exhaustion, condiogenesis takes place (Clutterbuck, 1976). The genes involved in the process of conidia formation, can be considered as those controlling inductive competence and those controlling the morphological competence. Several mutants are available affecting the regulation and development of conidia formation (Orr & Rosenburg, 1976; Morris, 1976; Clutterbuck, 1976). For example, the *bristle* mutants are altered in the genes controlling the inductive competence, resulting in the formation of only footcells (conidiophores), the first step in conidiogenesis.

A. nidulans was the main object in the study of the parasexual "cycle" in fungi (Pontecorvo, 1954), which has the sequence: (1) anastomosis between genetically different hyphae resulting in heterokaryon formation, (2) nuclear fusion giving diploid nuclei, (3) mitotic crossing over and haploidization. Among wild type strains of *A. nidulans* heterokaryon incompatibilities occur (Grindle, 1963), but among the frequently used Glasgow strains, failure to form heterokaryons is rare (da Cunha, 1970). Where diploid nuclei are present in the hyphae, also a number of diploid (uninucleated) conidia are formed. When placed on a selective .
medium, heterozygous (diploid) conidia will give rise to colonies. In such a selected heterozygous diploid strain,haploidization and mitotic crossing over result in recombination.These inter- and intra-chromosomal recombination processes facilitate the determination of linkage groups and the linear arrangement of genes relative to the centromere (Pontecorvo & Käfer, 1958).

The genetics and the metabolism of *Aspergillus* have been extensively studied but little is known about the biochemical organization of the nucleus. High-molecular-weight DNA has been prepared from nuclei of *A. nidulans*

(Morris, 1978) and was found to have a buoyant density in CsCl of 1.709 g/ml (unpublished results) corresponding to a (G+C) content of about 51% (Pontecorvo, 1957; Lopez-Peres & Turner, 1975). The genome size was calculated to be 2.6×10^7 base pairs or 0.028 pg per haploid nucleus (Timberlake, 1978; Brainbridge, 1971). From DNA-DNA reassociation studies it was concluded, that the genome of *Aspergillus* consists of approximately 97 to 98 percent unique and only 2 to 3 percent reiterated sequences. These reiterated DNA sequences were calculated to have a complexity of 11 000 base pairs and should be repeated about 60 times per haploid genome. Hybridization experiments indicated, that most of the reiterated DNA codes for ribosomal RNA (Timberlake, 1978). The existence of histones in *A. nidulans* and the organization of histones and DNA in nucleosomes has been reported by Morris et al. (1976). The DNA repeat length after micrococcal nuclease digestion appeared to be smaller in *A. nidulans* (154 bp) than in most higher eukaryotes (180-220 bp). The ribosomal RNAs has been isolated and the sedimentation values of the two ribosomal RNA were estimated to be 26 and 17S, respectively (Edelman et al., 1971; Verma et al., 1970). Together with the ribosomal RNAs a 5.8S RNA could be isolated from the ribosome fraction and from the post-ribosomal supernatant 5S rRNA and 4S tRNA could be purified (unpublished results).

The data indicate, that the biochemical organization of the chromatin in *Aspergillus nidulans* and in higher eukaryotes may be similar. The differences in the amount of repetitive sequences and, probably organization between the lower eukaryotes, like *A. nidulans* and *Neurospora* (Krumlauf & Marzluf, 1979) and more advanced systems,do not necessarily imply that they possess different molecular mechanisms for controlling gene expression, since it has not yet been fully established that the interspersed repetitive DNA plays a role in gene regulation in higher eukaryotes (Murray & Holliday, 1979). At present it is only possible to speculate on the selective value and function of these sequences.

1.4. *Fungal protoplasts*

Yeast and filamentous fungi have a rigid cell wall, consisting of chitin and glucan polymers, which can only be desintegrated with severe mechanical force. When cell-free extracts and intact cell organelles should be prepared, mechanical desintegration of the cell wall cannot be used. Through enzymatic degradation of the cell wall, it is possible to obtain protoplasts, which can be lysed in a gentle manner. Protoplasts, like cultured animal cells, are characterized by the (complete) absence of a cell wall and are therefore very useful for investigating particular biological problems, such as DNA and RNA synthesis. The last few years, new approaches to genetic manipulation of bacteria, fungi and plants, caused a renewed interest in the use of protoplasts. Removal of the cell wall and exposure of the protoplast membrane to specific biochemicals allowed manipulation involving the uptake of nucleic acids, being difficult to achieve or even impossible with intact cells. An example of fungal transformation was recently reported by Hinnen et al. (1978). These authors showed, that a bacterial plasmid (Col E_1), with a yeast gene incorporated, was taken up by the protoplasts of yeast. Evidence indicated, that the complete plasmid was integrated into the yeast genome, including the gene within the plasmid.

The first reports on the isolation of protoplasts by means of cell wall degrading enzymes were from Eddy & Williamson (1959) and Bachmann & Bonner (1959) using yeast and *Neurospora crassa*, respectively. Although mechanical and other non-enzymatic methods for protoplast isolation have been reported (Berliner, 1971; Carbonell et al., 1972), most investigators have favoured the use of lytic enzymes, generally being the most rapid procedure. The first lytic enzyme system used by Eddy & Williamson (1959) for the preparation of yeast protoplasts was a digestive juice of the snail *Helix pomatia*. Next to this enzyme system, which could be used for yeast as well as filamentous fungi (cf. Peberdy, 1978, 1979), several other lytic enzyme systems have been derived from a variety of organisms from both bacterial and fungal origin (Dziengel et al., 1977; Mann et al., 1972; Garcia & Villanueva,1962;

de Vries & Wessels, 1972; Musilkova & Fencl, 1968; Peberdy & Isaac, 1976). Considering the diversity in cell wall composition of especially the filamentous fungi (Bartnicki - Garcia, 1968), it is not surprising, that a wide range of lytic enzyme systems has been developed.

In most cases reported so far, the synthesis of the cell wall degrading enzymes by the different organisms required the presence of inducer substrates in the growth medium. In general, these inducers were supplied in the form of either homogenized mycelium (Musilkova et al., 1969) or semi-defined substrates. The latter were purified from cell walls (de Vries & Wessels, 1972) or commercially available substrates, similar to those found in the cell wall, such as chitin,laminarin and pustulan (Laborda et al., 1974). The enzyme composition of the lytic systems, involved in protoplast release, is known in only a few cases. The enzymes of *Trichoderma harzianum* were extensively studied with respect to protoplast release from *Schizophyllum commune* by de Vries & Wessels (1973). Three major enzyme components, S-glucanase, R-glucanase and chitinase, were shown to be necessary for protoplast release. The R-glucanase could be left out, since *S. commune* was able to produce this enzyme during the incubation of the cells with the other lytic enzymes. *Aspergillus nidulans* is an example of an organism producing exogenous enzymes, which are able to degrade their own cell wall. It was demonstrated by Zonneveld (1972 a/b), that under starvation conditions *A. nidulans* produced lytic enzymes, with α-1,3-glucanase as the main component. These extracellular lytic enzymes were able to degrade part of the mycelial cell wall mainly α-1,3-glucan, which obviously served as a reserve carbon and energy source for the cleistothecial formation.

The state of the mycelium, used for protoplast isolation, is very difficult to standardize, but is probably the most important factor determining protoplast yields. Two aspects of growth of the mycelium are important: the culture medium and the growth phase of the culture. It was shown by Musilkova & Fencl (1968), that protoplast formation from *A. nidulans* was dependent on both the carbon and nitrogen source as well as on their respective con-

centrations used in the medium. Similar effects have been reported for other fungi (cf. Peberdy, 1979), however, large differences were observed with respect to the optimal culture medium even when the same organism was used. This is obviously due to the fact, that each laboratory uses its own lytic system each with a different enzyme composition. Changes in the cell wall composition, caused by different amounts and varieties of carbon, nitrogen or phosphate sources in the growth medium (Zonneveld, 1972 a/b, 1975; San Blas & Cunningham, 1974 a/b), will directly affect the susceptibility of the mycelium to the lytic enzymes used. The addition of thiol compounds, Triton-X-100 or chelating agents to the growth medium or preincubating the mycelium with these chemicals prior to the exposure to the lytic enzymes, have been shown to alter the susceptibility to lysis. The mechanisms of action are largely unknown and speculative (cf. Peberdy, 1979). The state of the cells used for the protoplast production is also very important. In general, maximal yields of protoplasts are obtained with cultures in the early and mid-exponential growth phase (Peberdy et al., 1976; Anné et al., 1976). Cells in the stationary phase are poorly susceptible to the lytic enzymes, probably due to the formation of melanin in older cells (Carter & Bull, 1969; Bull, 1970 a/b).

An extensive range of inorganic salts, sugars and sugar alcohols have successfully been used to stabilize the protoplasts after the removal of the cell wall (Villanueva & Garcia Archa, 1971). In general, inorganic salts have proved to be more effective with filamentous fungi, while sugars and sugar alcohols were more effective with yeasts. These differences in effectiveness have not been explained, but they must somehow be related to unknown factors in the uptake and utilization of the particular compounds. A specific effect of high magnesium sulphate concentrations on the protoplasts and their release from the mycelium has been reported by de Vries & Wessels (1972). The mycelium of *Schizophyllum* became highly fragmentated in the early period of lytic digestion in the presence of 0.6 M magnesium sulphate and the protoplasts released from the mycelium appeared to float following

centrifugation. A similar effect of magnesium sulphate on protoplasts from *A. nidulans* has subsequently been found by Peberdy & Isaac (1976). The reason for this magnesium sulphate induced buoyancy remains to be explained.

When suspended in an osmotically stabilizing growth medium, part of the protoplast population demonstrates the capacity to return to the normal hyphal form of the organism. Two basic patterns of regeneration (sometimes indicated as reversion) can be identified. In *Rhizopus nigricans* (Gabriel, 1970) and in *Schizophyllum commune* (de Vries & Wessels, 1975), a cell wall is gradually formed and one or more hyphae emerge directly from the thick-walled spherical cell. Occasionally a few protoplasts develop a chain of bud-like cells and hyphae are formed from some of these. The second pattern of development is a more wide-spread phenomenon among different fungal species; the protoplast bulges out to one of more sides developing aberrant hyphae. From the latter structure normal hyphae develop, usually from the growing tip distal to the protoplast (Gibson & Peberdy, 1972; Anné et al., 1974; Benitez et al., 1975). This gradual regeneration of the protoplast into normal hyphae has made it possible to study more extensively the wall synthesis and ultrastructure during regeneration (de Vries & Wessels, 1975; Peberdy, 1978; Zonneveld, 1977).

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CHAPTER 2

RNA Polymerase from the Fungus *Aspergillus nidulans*

Large-Scale Purification of DNA-Dependent RNA Polymerase I (or A)

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The DNA-dependent RNA polymerase I (or A) from the lower eukaryote *Aspergillus nidulans* has been purified on a large scale to apparent homogeneity by homogenizing the fungal hyphae in liquid nitrogen, extraction of the enzyme at high salt concentration, precipitation of RNA polymerase activity with polymin P (a polyethylene imine), elution of the RNA polymerase from the polymin P precipitate, ammonium sulphate precipitation, molecular sieving on Bio-Gel A-1.5m, binding to ion-exchangers and DNA-cellulose affinity chromatography. By this procedure 1.6 mg of RNA polymerase I can be purified over 2000-fold from 500 g wet weight of starting material with a yield of 30–35%. The isolated RNA polymerase I is stable for several months at −20 °C.

The subunit composition has been resolved by polyacrylamide gel electrophoresis on two-dimensional gels, using either non-denaturing or 8 M urea (pH 8.7) cylindrical gels in the first dimension and sodium dodecyl sulphate slab gels in the second dimension. The putative subunits have molecular weights of 190000, 135000, 63000, 62000, 43000, 29000, 29000, (28000), 16000 and probably 13000 and 12000.

Two distinct forms of RNA polymerase I (Ia and Ib) have been resolved by DEAE-Sephadex A-25 chromatography showing ample differences in enzymatic properties and subunit pattern.

Additional information is given on RNA polymerase II (or B) which appears to be highly insensitive to α-amanitin at concentrations up to 400 μg/ml.

Aspergillus nidulans represents a well-defined genetic system within which mutations affecting regulation, development and mitosis [1] are available; furthermore, it has one of the smallest genomes of any differentiating eukaryote [2]. Since it has been suggested [3] that the biochemical organisation of the nucleus may be similar in *Aspergillus* and higher eukaryotes, the fungus *Aspergillus* may become an important model system for studying the molecular biology of the eukaryotic nucleus and the regulation of RNA synthesis at the transcriptional level. Because *A. nidulans* can be obtained in reasonably large quantities and the method presented allows the handling of large amounts of mycelia, it can be a good starting source for the purification of the fungal RNA polymerases.

Recently several large-scale procedures have been published on the isolation and purification of one or more DNA-dependent RNA polymerases from a wide range of organisms (cf. [4]) such as plants [5,6], animals [7,8] and yeast [9,10]. Up to now only one large-scale procedure with sufficient yields is known for the isolation of RNA polymerases from fungi [11]; all other procedures given for fungal systems [12–15] are hampered by low recoveries of enzymatic activity and instability or inhomogeneity of the isolated RNA polymerases. This omission, in general, is due to difficulties in cell disruption and most of all to the presence of high concentrations of RNase and protease activities in fungi [16].

In this report a method is presented for the large-scale isolation and purification of the DNA-dependent RNA polymerase I (A) from *A. nidulans*. The advantage of the procedure is the release of large amounts of enzyme activity of both RNA polymerase I (A), II (B) and III (C) by simply blending large quantities of the *Aspergillus* hyphal mass in liquid nitrogen, removal of nucleic acids by polymin P (a polyethylene imine) precipitation [17] and selective extraction of the polymin P pellet. Molecular sieving on Bio-Gel A-1.5m, followed by chromatography on DEAE-Sephadex A-25, phosphocellulose and DNA-cellulose results in a homogeneous preparation of RNA polymerase I (A). No time-consuming sonication or other cell disruption methods or high-speed centrifugation steps are necessary.

Enzyme. Ribonucleoside triphosphate : RNA-nucleotidyltransferase (EC 2.7.7.6).

By slightly changing and modifying the developed procedure, it should be possible to isolate the more labile RNA polymerases II (B) and III (C) with proper yields and to purify them to apparent homogeneity.

MATERIALS AND METHODS

Biochemicals

All chemicals used were reagent grade and were in general obtained from Merck, British Drug House or Difco. Ribonucleoside triphosphates (ATP, GTP, CTP and UTP), crystalline bovine serum albumin (fraction V) and calf thymus DNA (grade V) were purchased from Sigma; [5-^{3}H]UTP (25–50 Ci/mmol) from Amersham. The solutions for liquid scintillation counting (Lumasolve and Lipoluma) were obtained from Lumac. Electrophoresis reagents (acrylamide, *N,N'*-methylene bisacrylamide, *N,N,N',N'*-tetramethylethylene diamine and ammonium persulphate) were purchased from Serva; Coomassie brilliant blue R-250 from Merck and xylene brilliant cyanine G from Gurr. The molecular weight markers, e.g. β-galactosidase, phosphorylase *a*, *Escherichia coli* RNA polymerase, ovalbumin, bovine serum albumin, chymotrypsinogen, horse heart cytochrome *c* and aldolase from Boehringer. DEAE-Sephadex A-25 was obtained from Pharmacia, Bio-Gel A-1.5m from Bio-Rad, phosphocellulose P-11 and cellulose from Whatman. α-Amanitin was a kind gift from Dr M. Govindan (Heidelberg) or purchased from Boehringer. Polymin P was kindly donated by BASF (Ludwigshafen, F.R.G.). Partially purified RNA polymerase I from yeast was kindly provided by Dr H. van Keulen (Amsterdam).

Organism and Growth Conditions

A biA$_1$ (biotin-deficient) strain of *Aspergillus nidulans* of Glasgow origin was used throughout these studies. For both maintenance and the preparation of conidial suspensions *Aspergillus* was grown on complete medium [18] supplemented with 40 µg/l of D(+)-biotin. Conidiospores were induced by growth on 25 ml of agar-solidified medium in 100-ml infusion bottles for three days at 37 °C. Conidia for inoculation were collected by thoroughly shaking the sporulation flasks with 10 ml of a 0.8% (w/v) solution of NaCl containing 0.005% (v/v) of Tween 80. Cultures were inoculated with about 10^8 conidia/l medium, consisting of 0.5% (w/v) of yeast extract and 3% (w/v) of glucose, supplemented with 40 µg/l of biotin; cultures were grown in 1-l infusion bottles, containing 250 ml of medium, for 16 h at 37 °C with shaking in a home-made shaker-incubator. Approximately 500 g (wet weight) blotted mycelia (100 g dry weight) could be obtained from 80 l.

Buffers

Buffer A contained 50 mM Tris, pH 7.9 (adjusted with HCl at 4 °C), 10% (v/v) glycerol, 1 mM NaEDTA, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride; ammonium sulphate was added from a 3 M stock solution to a concentration as indicated. Buffer B was identical to buffer A, but with 0.5 mM $MgCl_2$ instead of 5 mM. Buffer C was identical to buffer A, except that the glycerol concentration was 50% (v/v) and the ammonium sulphate concentration 100 mM. Phenylmethylsulfonyl fluoride was added from a 200 mM stock solution in 96% ethanol immediately before use [19, 20]. All solutions were freshly made up in glass-double-distilled water.

RNA Polymerase Assay

The standard RNA polymerase assay mixture with a final volume of 0.2 ml contained: 50 mM Tris-HCl pH 7.9 (at 4 °C), 1 mM dithiothreitol, 1 mM ATP, GTP and CTP, 0.05 mM UTP, 1 µCi [5-^{3}H]UTP, 4 mM $MnCl_2$, 20 µg heat-denatured calf thymus DNA and 20 µl of sample; the final ammonium sulphate concentration was in general between 20 and 30 mM. In some experiments α-amanitin, in water, was added to the reaction mixture at the concentrations indicated.

The reaction was started by addition of the enzyme and the mixture was incubated for 30 min at 35 °C. The reaction was terminated by adding 4 ml of 5% (w/v) ice-cold trichloroacetic acid, containing 0.5% (w/v) sodium pyrophosphate. After 5–10 min at 0 °C the trichloroacetic-acid-insoluble material was collected by suction on GF/C (Whatman) glass-fiber filter discs, washed three times with 15 ml of the ice-cold trichloroacetic acid solution, and once with a mixture of cold ethanol/ether (1/1). The filters were dried and incubated for 30 min in 0.25 ml of Lumasolve at 60 °C. After addition of 2 ml of the scintillation liquid Lipoluma the samples were mixed thoroughly, cooled to 4 °C and counted in 6-ml polypropylene minivials in an MR300 Kontron liquid scintillation counter with an overall efficiency of 35–40%, as determined from calibration curves.

One unit of RNA polymerase activity is defined as the amount of enzyme activity necessary for the incorporation of 1 nmol of UMP into trichloroacetic-acid-insoluble material in 60 min under the standard assay conditions. Specific activity is defined as units/mg protein.

Preparation of Columns

Bio-Gel A-1.5m (100–200 mesh) was washed and equilibrated in buffer A containing 300 mM ammonium sulphate. To remove sticky materials the column was washed, in between several runs, with three volumes

of 5 M urea, 1 M NaCl and reequilibrated as described. DEAE-Sephadex A-25 (40–120 nm) was prepared as indicated by the manufacturer and equilibrated in buffer A. Phosphocellulose P-11 was prewashed as described by Burgess [21] and equilibrated with buffer B. DNA-cellulose was prepared as described by Alberts and Herrick [22] and modified by Hager et al. [23]. Irradiation of the ethanol-suspended DNA-cellulose was performed with a Hanau TQ 150 high-pressure mercury arc through a quartz filter under continuous stirring for 30 min (distance within 3 cm). The DNA-cellulose was dried by lyophilization and stored as dry powder at −20 C. The final preparation contained 3–5 mg of DNA/g cellulose.

Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate was carried out in 100 × 130 × 1-mm slabs using the discontinuous dodecylsulphate/Tris/glycine buffer system as described by Laemmli [24]. Samples were treated as described by Laemmli [24] and subjected to electrophoresis for 4–5 h at 40 mA, using bromophenol blue (0.004 %, w/v) as a dye marking the position of the front.

Polyacrylamide gel electrophoresis in the presence of 8 M urea (pH 8.7) was performed according to Jovin et al. [25]. Samples were dialysed against the Tris/glycine (pH 8.3) electrophoresis buffer, containing 8 M urea prior to electrophoresis and gels were run in glass tubes (70 × 2.5 mm) at a constant voltage of 15–20 V per tube until the marker dye had reached the bottom of the tube.

Gel electrophoresis under non-denaturing conditions was performed essentially as described by Ornstein [26] and Davis [27], and modified by Smith and Braun [11]. Gels were run at 4 °C in glass tubes (70 × 2.5 mm) at a constant voltage of 15 V per tube until the marker dye had reached the bottom of the tube.

Two-dimensional gel electrophoresis was performed as described by Jendrisak and Burgess [28]: either 8 M urea (pH 8.7) or native gels for the first dimension were run in glass tubes as described above until the marker dye was within 1 cm of the bottom of the gel. After electrophoresis the gels were immediately removed and equilibrated by soaking in the dodecylsulphate sample buffer of Laemmli [24] for 5 min at 80 °C. The gels were mounted horizontally on top of a slab gel (100 × 130 × 2 mm) containing 12.5 % acrylamide and electrophoresis in the second dimension in the discontinuous dodecylsulphate/Tris/glycine gel system was performed as described above. Gels were stained with Coomassie brilliant blue R-250 and destained as described by Burgess and Jendrisak [29].

Molecular weights of polypeptides were estimated by simultaneous electrophoresis of RNA polymerase with molecular weight markers in polyacrylamide gels containing 0.1 % (w v) sodium dodecylsulphate by the general method of Weber and Osborn [30]. The molecular weight markers used were: β-galactosidase (116000), phosphorylase *a* (92500), transferrin (80000), bovine serum albumin (68500), catalase (60000), ovalbumin (45000), chymotrypsinogen (25000), cytochrome *c* (12500), the two heavy subunits of RNA polymerase I from yeast [9] (190000, 135000) and *E. coli* RNA polymerase subunits [29] (165000, 155000, 87000 and 39000).

Molar ratios of the polypeptides were determined as described by Burgess and Jendrisak [29] by densitometric scanning of diapositives of stained slab gels or by scanning directly the stained cyclindrical gels at 550 nm in a Gilford recording spectrophotometer with scanning attachment.

General Procedures

Salt concentrations in solutions were determined with a Philips PW 9501 conductivity meter using standard curves prepared with buffers of known salt concentrations. Protein determination was performed as described by Bramhall et al. [31] with xylene brilliant cyanine G using bovine serum albumin as a standard. DNA and RNA concentrations were determined with diphenylamine [32] and orcinol [33], respectively. The 10 % (v v) stock solution of polymin P was prepared and neutralised as described by Jendrisak and Burgess [29].

Purification of RNA Polymerase I

All operations were carried out at 0–4 °C; all centrifugations were performed in an MSE 18 high-speed centrifuge.

Preparation of the Crude Extract and Solubilisation of the Enzyme. 500 g wet weight (100 g dry weight) of *Aspergillus* mycelia, harvested at log phase by filtration over cheesecloth, washed with distilled water and buffer A containing 300 mM ammonium sulphate, were suspended in 500 ml of buffer A containing 300 mM ammonium sulphate. The suspension was frozen rapidly in liquid nitrogen and disrupted into two portions in liquid nitrogen in a one-gallon (4.5-l) stainless steel Waring blendor at 'high' setting by blending nine times for 45 s at 160 V with intervals of 60 s at 90 V as described by Gealt et al. [34]. The frozen homogenate was rapidly thawed to 4 C, diluted with 1.5 l of buffer A containing 300 mM ammonium sulphate, homogenized in a 1-l stainless steel Waring blendor and centrifuged for 30 min at 15000 × *g*.

Polymin P Fractionation. The crude extract from the previous step was filtered through one layer of Miracloth (Calbiochem) and diluted with 5 vol. of buffer A. RNA polymerase was precipitated from this

fraction by slow addition of 70 ml of a 10% polymin P solution with continuous stirring. After 10 min the precipitate was collected by centrifugation at low speed. The polymin P pellet was washed by suspending it in 500 ml of buffer A containing 50 mM ammonium sulphate by means of a Potter-Elvehjem teflon-in-glass homogenizer and centrifuged at 26000 × g. RNA polymerase activity was extracted from the resulting pellet by homogenizing it in 500 ml of buffer A containing 300 mM ammonium sulphate, incubating on ice for 30 min and centrifuging for 20 min at 26000 × g.

Ammonium Sulphate Precipitation. RNA polymerase activity was precipitated from the clear yellow-brown supernatant by slowly adding 35 g of solid ammonium sulphate/100 ml solution. After centrifugation at 26000 × g for 30 min the pellet was resuspended in 100 ml of buffer A containing 300 mM ammonium sulphate and dialysed against the same buffer for 2 – 3 h to a final ammonium sulphate concentration of 300 mM as determined by conductivity measurement.

Bio-Gel A-1.5m Chromatography. The resulting clear yellow-brown solution from the previous step was loaded onto a Bio-Gel A-1.5m column and developed with buffer A containing 300 mM ammonium sulphate. Fractions containing the bulk of the RNA polymerase activity were pooled.

DEAE-Sephadex A-25 Chromatography. The Bio-Gel pool was diluted with buffer A until its conductivity was equal to that of 30 mM ammonium sulphate in the same buffer. DEAE-Sephadex A-25, equilibrated in buffer A containing 30 mM ammonium sulphate, was added (1 ml settled DEAE-Sephadex/2 mg of protein) and the mixture was slowly stirred for 15 – 20 min. The slurry was poured into a column, washed after packing with 1.5 column volumes of equilibration buffer and eluted with buffer A containing 500 mM ammonium sulphate. The fractions containing the RNA polymerase activity were pooled.

Phosphocellulose Chromatography. The DEAE-Sephadex pool was dialysed for 2 h against buffer B containing 30 mM ammonium sulphate and the dialysed RNA polymerase activity was batchwise bound to phosphocellulose, equilibrated with buffer B (1 ml settled bed volume/2 – 2.5 mg of protein) and adjusted to a conductivity equal to 30 mM ammonium sulphate in the same buffer. The slurry was stirred for 1 h, poured into a column, washed with two column volumes of equilibration buffer and developed with a linear salt gradient of 30 – 300 mM ammonium sulphate in buffer B. The RNA polymerase I activity eluting at 200 mM ammonium sulphate was pooled.

DEAE-Sephadex A-25 Rechromatography. The pooled fractions from the phosphocellulose column were diluted with buffer A to 50 mM ammonium sulphate and bound in batches to DEAE-Sephadex as described above. The enzyme activity could be recovered by applying a linear salt gradient of 50 – 200 mM ammonium sulphate in buffer A.

DNA-cellulose Chromatography. The pooled enzyme activity from the previous step was diluted with buffer A to 50 mM ammonium sulphate, as determined from the conductivity measurement. DNA-cellulose (1 g of dry DNA-cellulose/400 units of RNA polymerase I activity) was added and after stirring for 15 min the column was packed, washed with binding buffer and developed with a linear gradient of 50 – 200 mM ammonium sulphate in buffer A. Fractions containing RNA polymerase activity were pooled.

Storage. Peak fractions from the DNA-cellulose column were pooled and dialyzed against buffer C at – 20 °C and stored at – 80 °C.

RESULTS AND DISCUSSION

As confirmed by our studies and already indicated by Gealt et al. [34], the liquid nitrogen procedure has several advantages: (a) it increases mycelial breakage during homogenization, (b) it will stop enzymatic reactions instantaneously, thus minimizing the degradative effect of proteases and nucleases; furthermore; (c) the method allows handling of large amounts of mycelia in a very short time and (d) is highly reproducible due to its controllable conditions.

As judged from the DNA/RNA concentration in the crude extract obtained after centrifugation and filtration, at least 50 – 60% of the cell content was set free by the liquid nitrogen homogenization procedure. From 500 g (wet weight) of *Aspergillus* mycelia 10 – 12 g of protein an 2 g of nucleic acid (A_{280}/A_{260} = 0.62) could be extracted. The total RNA polymerase activity in the crude extract, as determined by the standard assay, corresponded very well with the total activity found at later stages of the purification procedure; however, no inhibition with α-amanitin at concentrations up to 10 µg/ml could be demonstrated in the crude extract.

Other methods of cell disruption were less successful. Disruption of mycelia with the French press, the Manton Gaulin press, the X-press, or a special grinding mill, or combining these methods with sonication were time-consuming, heat-denaturing and highly irreproducible. Using the French press at high pressure values resulted in massive cell disruption and maximal release of contaminating protease and nuclease activities, but the amounts of RNA polymerase activity released were highly variable and in general resulted in low recoveries and selective losses. The large-scale protoplast preparation procedure developed in our laboratory (method submitted for publication) could not be scaled up to these large amounts of cells needed for the isolation of RNA polymerases.

Table 1. *Purification of RNA polymerase I from* Aspergillus nidulans
The values given in the table are average values calculated from 3–6 different preparations. The values for RNA polymerase I are based on the assumption that 60–65% of the total RNA polymerase activity (given between the brackets) in the homogenate, Bio-Gel pool, DEAE-Sephadex eluate and dialysate can be ascribed to RNA polymerase I (see text). Units of activity are defined in the text

Fraction	Volume	Total protein	Total activity of polymerase I	Yield	Specific activity	Purification
	ml	mg	units	%	units/mg protein	-fold
Homogenate	2000	10000	7000 (11000)	100	0.7	–
Ammonium sulphate precipitate	100	1350		100	5.2	7.4
Bio-Gel pool	485	423	6500 (10250)	93	15.4	22
DEAE-Sephadex 500 mM eluate	110	251	5800 (9100)	83	23.1	33
Dialysate after DEAE-Sephadex	80	240	5530 (8700)	79	23.0	33
Phosphocellulose pooled fractions	29	18	3800 (5000)	54	212	303
DEAE-Sephadex rechromatography pooled fractions	21	5.2	2750	39	529	756
DNA-cellulose pool	14	1.6	2320	33	1450	2071

Polymin P Fractionation and Ammonium Sulphate Precipitation

Total enzyme activity was precipitated from the cleared homogenate with polymin P [17] by adding 14 ml of a 10% solution/100 g (wet weight) of starting material. Although it was stressed by Jendrisak and Burgess [29] that optimal conditions for the precipitation of RNA polymerase activity with polymin P and elution of the activity from the polymin P pellet should be derived from typical analysis curves as given by these authors, we were not able to produce similar precipitation and elution curves due to interference of residual polymin P. Neither in the supernatant after polymin P precipitation, nor in the eluate of the polymin P pellet, could reproducible values for the RNA polymerase activity or the protein content be obtained. Residual polymin P could be removed with cellulose and in this way reliable and reproducible elution curves could be obtained (Fig. 1). A decrease in polymerase activity could be observed at higher ammonium sulphate concentrations (> 400 mM), probably due to polymin P residues left in the solution or high RNase and protease activities (see also [7]). In combination with experiments using variable polymin P concentrations for precipitation and ammonium sulphate concentrations for extraction of the polymin P pellet, followed by partial purification through chromatography on Bio-Gel A-1.5m and DEAE-Sephadex A-25 or DNA-cellulose, it was experimentally derived that 14 ml of 10% polymin P/100 g (wet weight) of *Aspergillus* mycelia, resulting in a final concentration of 0.05% polymin P, were sufficient to precipitate all RNA polymerase activity. Also the elution of the polymin P pellet with 100 ml buffer A containing 300 mM ammonium sulphate/100 g of starting material resulted in complete recovery of the RNA polymerase activity without extracting contaminating amounts of RNA and DNA. These conditions gave highly reproducible results.

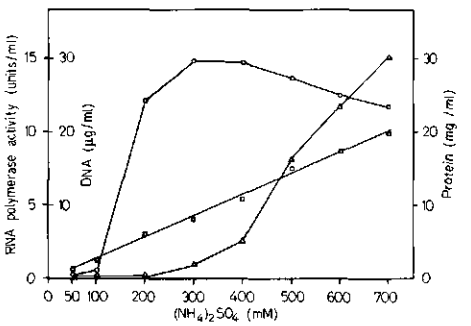

Fig. 1. *Elution of* Aspergillus *RNA polymerases from the polymin P pellet.* The polymin P pellet was prepared as described in the text and divided into seven portions. Each portion was extracted with buffer A containing increasing amounts of ammonium sulphate and then centrifuged. The supernatants were assayed for protein (□— □), DNA (△– –△) and RNA polymerase activity (○ ——○). In order to remove residual polymin P and to determine the amount of RNA polymerase extracted, the supernatant was either applied directly to a column of 4 g of cellulose, prewashed with buffer A containing the ammonium sulphate concentration used for elution, or treated in batches with cellulose; the flow-through of the columns or the supernatants after pelleting of the cellulose were assayed for RNA polymerase activity under the standard reaction conditions

In earlier experiments the majority of the nucleic acids was removed by precipitation with protamine sulphate as employed in purification procedures for both prokaryotic [35] and eukaryotic [36] RNA polymerases. Highly variable recoveries of enzyme activity and selective losses of RNA polymerase II and III were observed, variable from preparation to preparation and from batch to batch of protamine sulphate. As indicated by Hager et al. [23], the conditions under

which the precipitation is carried out might be very critical. The introduction of polymin P [5, 17] circumvented these problems.

In order to remove most of the residual polymin P [37] and to concentrate the enzyme preparation, the eluate from the polymin P pellet was precipitated with ammonium sulphate. The precipitated material was dissolved and dialyzed as described before. Solubilizing the drained ammonium sulphate pellets in buffer A containing 0 or 50 mM ammonium sulphate and adjusting the ammonium sulphate concentration to 300 mM resulted in variable degrees of irreversible aggregation of protein as deduced from molecular sieving on Bio-Gel. The ammonium sulphate fractionation resulted in a 5–10-fold concentration, an additional 2-fold purification and almost complete recovery of RNA polymerase activity. As judged from the ultraviolet absorption spectrum the RNA polymerase preparation through this step was nearly completely freed from nucleic acids ($A_{280}/A_{260} = 1.5-1.6$). At this stage the enzyme preparation can be stored at $-80\,^{\circ}C$ for several weeks without detectable loss of activity.

Bio-Gel A-1.5m Chromatography

Molecular sieving of the concentrated crude enzyme extract over a Bio-Gel A-1.5m column appeared to be the most critical step in the procedure for the isolation of the three classes of RNA polymerase from *Aspergillus*. If no optimal separation is obtained, the binding to DEAE-Sephadex in the following step of, in particular, RNA polymerase II and III will be disturbed, resulting in a complete loss of these enzyme activities. A typical elution pattern is given in Fig. 2. The relative high exclusion peak (fractions 80–95) consisted mainly of protein, as determined from the ultraviolet absorption spectrum and protein determination; it is probably due to complexation of protein with residual polymin P.

The type of column used and the conditions for developing the column should be chosen very carefully. Although RNA polymerase activity could be separated completely from contaminating inhibitory components, including RNase activity which eluted well behind the polymerase peak, on changing the dimensions of the column these substances may interfere very markedly. Secondly, this step of the purification procedure should be performed under high salt conditions, for lowering the ammonium sulphate concentration resulted in a strong, partially irreversible aggregation of protein. Removal of the aggregated material also means removal of a great deal of the RNA polymerase activity. Thirdly, if the ammonium sulphate fractionation was omitted the degree of aggregation (under the high salt conditions) was much higher (higher exclusion peak from the Bio-Gel),

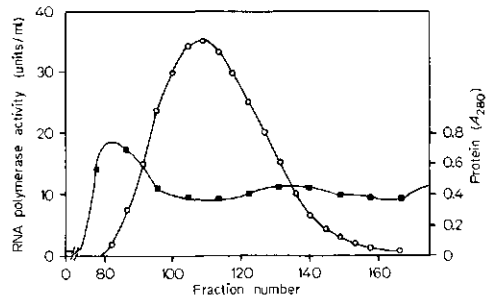

Fig. 2. *Chromatography of RNA polymerases of* Aspergillus *on Bio-Gel A-1.5m.* The crude RNA polymerase extract was chromatographed on a Bio-Gel column (55 × 9 cm) as described in the text. The column was run at a flow rate of 80 ml/h and fractions of 13 ml were collected; aliquots of 20 µl were assayed for RNA polymerase activity (■ —■). The absorbance at 280 nm (○ ○) was monitored with an Isco model UA-5 Absorbance monitor using a standard 1-cm flow cell. Fractions 95–135 were pooled

indicating that the ammonium sulphate precipitation might remove most, but definitely not all, polymin P from the eluate [37]. Some of the components causing inhibition or complex formation could be removed by subjecting the initial homogenate to high-speed centrifugation prior to polymin P fractionation. This suggests that proteins from ribosomal or mitochondrial origin might also be causing some of these disturbing effects apart from the polymin P residues.

The RNA polymerase through this step has been purified about 20-fold with 93% yield of the initial activity; this Bio-Gel pool which showed a typical protein absorption spectrum ($A_{280}/A_{260} \approx 1.7$) could be stored at $-80\,^{\circ}C$ for several weeks without detectable loss of activity. This fraction also was resistant to α-amanitin at a concentration of 10 µg/ml in the standard assay mixture.

DEAE-Sephadex A-25 Chromatography

The active fractions from the Bio-Gel column were pooled, diluted and bound in batches to DEAE-Sephadex as described. The batch procedure was chosen since the method is very quick and simple, making a long dialysis step unnecessary. Furthermore, at this stage no ammonium sulphate precipitation could be used since it resulted in extensive losses of enzyme activity, probably due to the low protein concentration.

After packing and washing the column, RNA polymerase activities were eluted from the column by applying a linear salt gradient of 30–500 mM ammonium sulphate in buffer A or, as routinely performed, by step elution with 500 mM ammonium sulphate in buffer A, resulting in an overall yield of polymerase activity of 80–85%. Using the linear

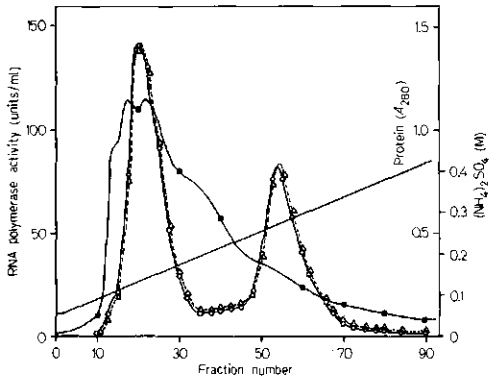

Fig. 3. DEAE-Sephadex A-25 chromatography of Aspergillus *RNA polymerases.* The Bio-Gel pool was diluted and bound in batches to DEAE-Sephadex A-25 as described. The column (20 × 4.2 cm) was developed at a flow rate of 150 ml h with a linear salt gradient of 30–500 mM ammonium sulphate in buffer A using the LKB 11300 Ultrograd gradient mixer. Fractions of 6 ml were collected and the absorbance at 280 nm (■ ■) was monitored. 20-µl aliquots were assayed for RNA polymerase activity in the presence (Δ– –Δ) or absence (O– –O) of 10 µg/ml of α-amanitin. The ammonium sulphate concentration is indicated by the solid line

salt gradient, two major peaks of activity were resolved (as illustrated in Fig. 3), one peak eluting at 130 mM ammonium sulphate, a second one at 260 mM. This salt elution profile is very similar to the ones obtained for higher eukaryotic systems and yeast [4]. Based on the chromatographic behavior of the Bio-Gel pool and the DEAE-Sephadex enzyme activities on different types of columns (DEAE-Sephadex, DEAE-cellulose, phosphocellulose, DNA-cellulose), the enzymatic properties (conditions for optimal activity of the enzyme with respect to salt concentration, Mn^{2+}/Mg^{2+} activity ratio, denatured/native DNA activity ratio) and the presence of the typical high-molecular-weight subunits (this report and unpublished observations) we have adopted the nomenclature of Roeder [4]. The first peak eluting at 130 mM was called RNA polymerase I or I(A) and the second eluting at 260 mM, RNA polymerase II or II(B). The third type of RNA polymerase III or III(C) was masked on this type of ion-exchanger, since by chromatography of the Bio-Gel pool over DNA-cellulose this activity could be separated at high salt concentration from the other two RNA polymerase activities without grossly changing the elution profile on DEAE-Sephadex thereafter (RNA polymerase III from *Aspergillus* constitutes only 5–10% of total activity). A similar separation pattern on DNA-cellulose was found for RNA polymerase III from yeast by Hager et al. [23], while a masking of RNA polymerase III on DEAE-Sephadex was described for cauliflower RNA polymerase [38].

In all eukaryotic systems known [4] the RNA polymerase activities can also be discriminated by their sensitivity towards the toxin α-amanitin. However, the activities eluted from the DEAE-Sephadex column were insensitive to this toxin at 10 µg/ml. Increasing the amount of α-amanitin to 400 µg/ml resulted only in a slightly detectable inhibition of enzyme activity. Although the enzyme activity eluting at 260 mM is exceptional in its sensitivity towards α-amanitin, we believe that it is a real RNA polymerase II. This is supported by the criteria given before, but also by preliminary binding studies of α-amanitin with RNA polamerase II from rat liver and RNA polymerase II from *Aspergillus* (unpublished observations).

Phosphocellulose Chromatography

The pooled activity from the DEAE-Sephadex, routinely eluted with buffer A containing 500 mM ammonium sulphate, was dialysed as described to lower the Mg^{2+} and salt concentration. It should be remarked that DEAE-Sephadex step elution was preferred to applying a salt gradient, since this resulted in higher protein concentrations and a smaller total volume. This was necessary for obtaining efficient binding to phosphocellulose. It was found that both prolonged dialysis and direct dilution of the pooled fractions to low salt concentrations resulted in considerable losses of enzyme activity. Concentrating the enzyme fraction at these stages of purification by ammonium sulphate precipitation or by membrane filtration as described for yeast RNA polymerase by Hager et al. [23] resulted in complete loss of activity.

The dialysed fraction was bound in batches to phosphocellulose and eluted from the column as described in Materials and Methods. A typical elution profile is given in Fig. 4. RNA polymerase I was eluted at 200 mM ammonium sulphate, well behind the activity peaks of RNA polymerase II and III. The positions of these polymerases had been derived from separate experiments where fractions from DEAE-Sephadex or DNA-cellulose columns had been applied to phosphocellulose under similar conditions (data not shown). Also these fractions were insensitive to α-amanitin at a concentration of 10 µg/ml.

Based on the assumption, derived from the use of several types of columns, that the total RNA polymerase activity in the crude extract consisted of 60–65% of RNA polymerase I, 25–30% of RNA polymerase II and 5–10% of RNA polymerase III, the recovery of RNA polymerase I from this column was 70%. With respect to the initial activity of RNA polymerase I the overall yield at this stage was 50–60% and a 400-fold purification had been achieved (Table 1). The observed loss in total activity on the phosphocellulose column was partly due to inactivation of RNA polymerase II and III.

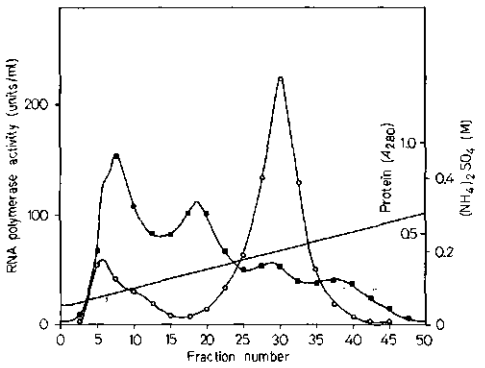

Fig. 4. *Phosphocellulose chromatography of* Aspergillus *RNA polymerases.* The active fractions from the DEAE-Sephadex column, stepwise eluted with buffer A containing 500 mM ammonium sulphate, were combined and after dialysis bound in batches to phosphocellulose. The column (20 × 2.4 cm) was developed at a flow rate of 50 ml/h with a linear salt gradient of 30 – 300 mM ammonium sulphate in buffer B. The absorbance at 280 nm (■——■) was monitored and fractions of 3 ml were collected. 20-µl aliquots were assayed for RNA polymerase activity (O——O). The ammonium sulphate concentration is indicated by the solid line. Fractions 25 – 35 were pooled

DEAE-Sephadex A-25 Rechromatography

By binding the phosphocellulose fractions containing the RNA polymerase I activity to DEAE-Sephadex A-25 as described before and developing the column with a shallow gradient of 30 – 200 mM ammonium sulphate in buffer A, two peaks of activity could be eluted from the column (Fig. 5). The enzyme activity eluting at 110 mM ammonium sulphate was designated RNA polymerase Ia, that at 130 mM RNA polymerase Ib, as suggested by Roeder [4]. Although several chromatographically distinct forms of RNA polymerases have been described in the literature, it cannot be excluded that form Ia might be a modification of form Ib as a result of the purification procedure, or might be due to a specific protease activity [39]. No significant differences could be observed if samples of the peak fractions were analyzed on sodium dodecylsulphate gels (Fig. 5, insert).

The DEAE-Sephadex rechromatography step resulted in a 2.5 – fold purification with a recovery of about 70 – 80%.

DNA-cellulose Chromatography

The affinity chromatography step using denatured calf thymus DNA bound to cellulose removed the last traces of impurity very effectively. The amount of **DNA** bound to the cellulose strongly determined the capacity of the column. At this stage in the purification procedure, using the type of DNA-cellulose described in Materials and Methods, 400 units of RNA polymerase I could be bound/g cellulose. At earlier stages, smaller amounts of activity can be applied due to the presence of other DNA-binding proteins. Salt gradient development of the column resulted in the appearance of only one single activity peak eluting at 80 – 100 mM ammonium sulphate (Fig. 6). Another threefold purification with a recovery of 80 – 85% was achieved (Table 1). Across the elution peak a constant specific activity of the enzyme was measured, indicating a high degree of purity. Both native nondenaturing and dodecylsulphate-denaturing gel electrophoresis confirmed this result (see below).

Purity and Properties of the Enzyme

The purification of RNA polymerase I from 500 g of *Aspergillus nidulans* mycelia is summarized in Table 1. 1.6 mg of essentially pure RNA polymerase I is obtained with a specific activity of 1450 units/mg of protein as determined in the standard assay mixture. Based on the assumption that the initial homogenate consisted of about 60 – 65% of RNA polymerase I, the overall yield was 33% and the overall purification 2000-fold. Dialyzing the final preparation against buffer C which contained 50% of glycerol resulted in an enzyme preparation that was stable for several months at – 80 °C as well as – 20 °C; without this precaution a lower degree of stability was obtained at this stage or at earlier stages of purification.

The purified RNA polymerase I preparation has a high degree of purity as indicated by the constant specific activity of the DNA-cellulose peak fractions and the polyacrylamide gel patterns. The final preparation contained no detectable RNase and protease activities. No degradation of synthesized RNA was observed upon continued incubation of the reaction mixture after addition of EDTA at several temperatures for several hours. The absence of protease was indicated by the fact that RNA polymerase I preparations stored at – 20 °C for several months or at room temperature for several hours were identical in subunit pattern to freshly purified preparations.

The purified enzyme was further characterized by determining the conditions for optimal activity of the enzyme. The enzyme is completely dependent on added DNA, all four nucleoside triphosphates and a divalent cation for activity. The reaction at 30 ° or 35 °C is almost linear for the first 20 – 30 min; the product of the reaction is completely sensitive to ribonuclease digestion (data not shown). A monophasic salt titration curve was obtained with an optimum between 10 – 30 mM ammonium sulphate (Fig. 7A).

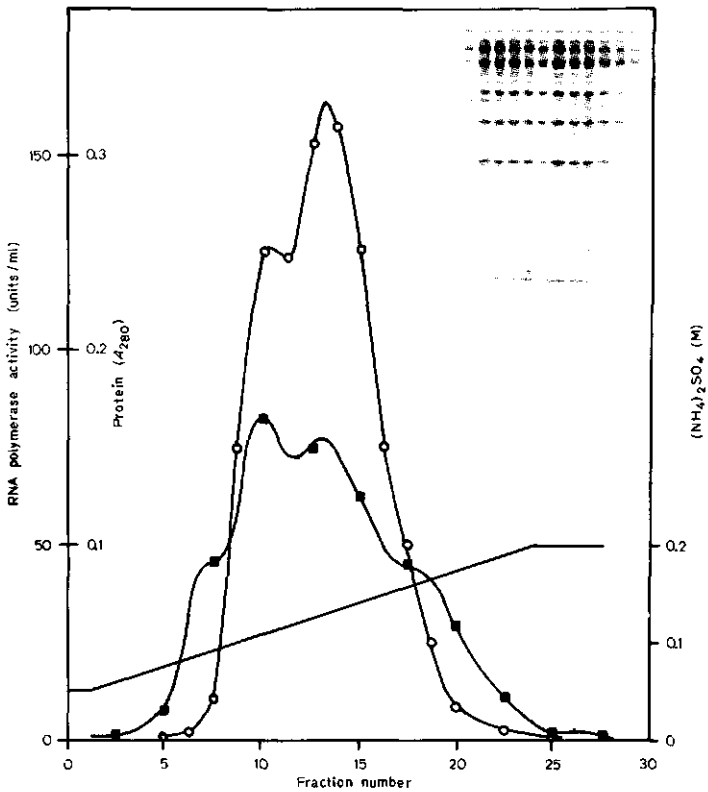

Fig. 5. *DEAE-Sephadex A-25 rechromatography of* Aspergillus *RNA polymerase I.* The fractions containing RNA polymerase I from the phosphocellulose column were bound in batches to DEAE-Sephadex A-25 as described. The column (10 × 1.6 cm) was developed at a flow rate of 10 ml h with a linear salt gradient of 50–200 mM ammonium sulphate in buffer A. Fractions of 2 ml were collected. The absorbance at 280 nm (■ —■) was monitored and 20-µl aliquots were assayed for RNA polymerase activity (O——O). The ammonium sulphate concentration is indicated by the solid line. Fractions 8–18 were pooled. Insert: aliquots of the peak fractions were analyzed on 12.5% dodecylsulphate/polyacrylamide gels as described; from left to right fractions 8–18, respectively

The divalent cation activity ratio was determined from the divalent metal titration curves. The effect of Mn^{2+} and Mg^{2+} was very clear as shown in Fig. 7B; a sharp optimum at low concentrations of Mn^{2+} (3–4 mM) and a broad optimum around 10 mM for Mg^{2+} were observed. The Mn^{2+}/Mg^{2+} activity ratio at optimal divalent cation concentration was 1.5, being very similar to the ones obtained for other lower and higher eukaryotic systems [5,7–9,11]. Upon testing the chromatographically separated forms Ia and Ib from the DEAE-Sephadex rechromatography (shown in Fig. 5) no differences could be observed with respect to salt and divalent metal cation conditions.

RNA polymerase I was not inhibited by α-amanitin, even at concentrations of 400 µg/ml; at α-amanitin concentrations of 300–400 µg/ml RNA polymerase A(I) from yeast showed 50% inhibition [40].

Subunit Composition

Enzyme purity and complexity were monitored by polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions. Native non-denaturing gels [26,27] showed only one single band, although chromatographically distinct forms were observed on DEAE-Sephadex. Denaturing acrylamide gels revealed a complex structure of the enzyme. For a better resolution of the polypeptides, two denaturing gel systems were used to analyze the subunit composition, these being polyacrylamide gels in the presence of dodecylsulphate [24] and 8 M urea, pH 8.7 [25], i.e. separation based on charge as well as on molecular weight. Complete resolution could be obtained by performing two-dimensional polyacrylamide gel electrophoresis using either native non-denaturing

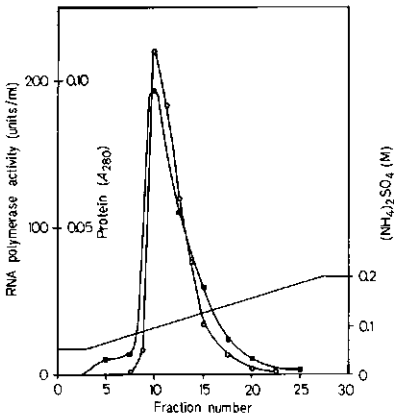

Fig. 6. *DNA-cellulose chromatography of* Aspergillus *RNA polymerase I.* Fractions from the DEAE-Sephadex A-25 rechromatography step containing RNA polymerase I (Ia and Ib) were pooled and chromatographed over DNA-cellulose. The column (10 × 1 cm) was developed at a flow rate of 8 ml/h with a linear salt gradient of 50–200 mM ammonium sulphate in buffer A and fractions of 2 ml were collected. The absorbance at 280 nm (■——■) was monitored and 20-µl aliquots were assayed for RNA polymerase activity (○——○). The ammonium sulphate concentration is indicated by the solid line. Fractions 9–15 were pooled

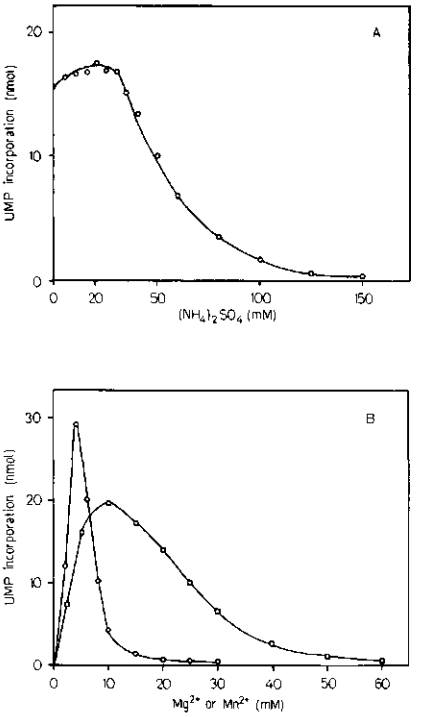

Fig. 7. *The effect of ammonium sulphate (A) and metal divalent cation (B) concentrations on the activity of RNA polymerase I from* Aspergillus. The RNA polymerase I pool from the DNA-cellulose column was diluted tenfold with buffer A lacking $MgCl_2$ in order to lower the concentration of Mg^{2+} as well as that of ammonium sulphate. RNA polymerase I activity was assayed in the standard reaction mixture (A) with increasing amounts of ammonium sulphate or (B) with increasing amounts of Mg^{2+} in the absence of Mn^{2+} (□——□) and increasing amounts of Mn^{2+} in the presence of 0.05 mM Mg^{2+} (○ ·—○)

gels or 8 M urea gels in the first dimension and dodecylsulphate gels in the second dimension.

As indicated by the 12.5% and 7.5% acrylamide gels shown in Fig. 8, the *Aspergillus* RNA polymerase I has a complex structure consisting of two high-molecular-weight polypeptides and several polypeptides with molecular weights lower than 63000, showing thus a striking similarity with other eukaryotic systems [4]. Through the DNA-cellulose peak fractions identical subunit patterns were observed (Fig. 8A), confirming the high degree of purity of the enzyme. Similar subunit patterns were observed after centrifuging RNA polymerase I over a glycerol or sucrose gradient, indicating that the observed subunits are associated with the polymerase activity. This correlation is also confirmed by the native-dodecylsulphate two-dimensional gel electrophoresis shown in Fig. 10B. A typical densitometric tracing of a sodium dodecylsulphate gel is shown in Fig. 9. The doublet in the 62000–63000-M_r region can only be seen in higher-resolution gels.

Complete resolution could only be obtained on the two-dimensional gels (Fig. 10), using different gel systems in the first dimension. From these analyses and using the molecular weight markers given in Materials and Methods, the molecular weights of the putative subunits of *Aspergillus* RNA polymerase I were estimated to be: 190000 (a), 135000 (b), 63000 (c), 62000 (d), 43000 (e), 29000 (f and g), 28000 (h) and 16000 (i), and probably also 13000 (j) and 12000 (k). The molecular weight estimations of the slowest and fastest moving subunits are uncertain due to (a) lack of reliable high-molecular-weight markers, (b) discrepancies in the literature with respect to the molecular weights of the different protein markers used (cf. β-galactosidase, M_r = 116000 [41] instead of 130000 [29]), and (c) deviation from linearity in these regions of the standard curve shown in Fig. 11.

The molar ratios of the polypeptides (Table 2) were determined from scans of one-dimensional and two-dimensional gels. The high-molecular-weight polypeptides (a and b) appeared in an almost 1 : 1 ratio. Greater differences, however, were observed for the other polypeptides, partly due to the fact that some of them could only be resolved on the two-dimensional gel. As shown in Fig. 10A the 29000-M_r polypeptide

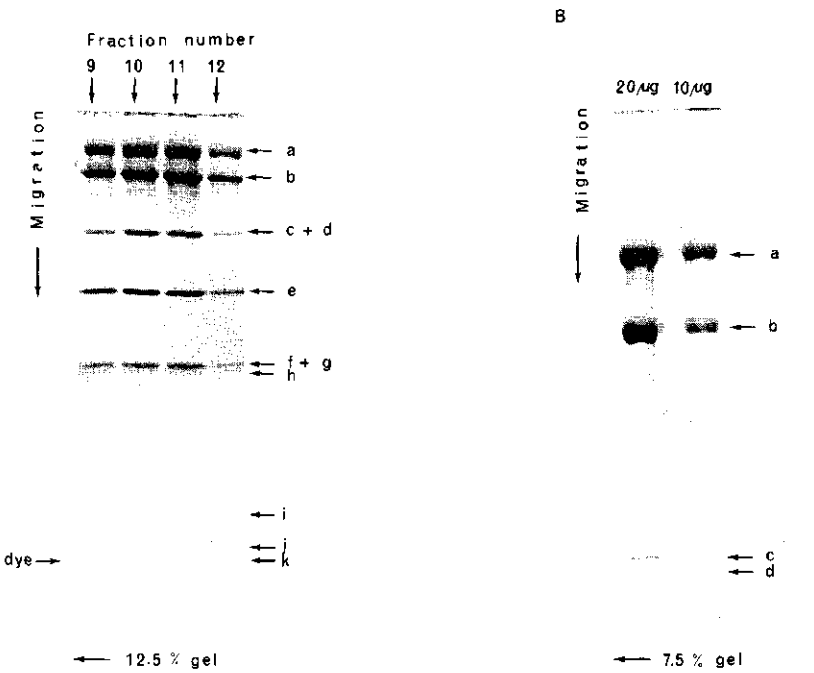

Fig. 8. *Polyacrylamide gel electrophoresis of* Aspergillus *RNA polymerase I under denaturing conditions.* Dodecylsulphate/polyacrylamide gel electrophoresis on 12.5% (A) and 7.5% (B) acrylamide was performed in a slab gel apparatus using the Tris/glycine discontinuous buffer system of Laemmli [24] as described in the text. The enzyme was purified as described and the fractions from the final DNA-cellulose column (shown in Fig. 6) were used. (A) Fractions 9–12 from the DNA-cellulose column containing 15, 20, 20 and 10 µg of protein, respectively. (B) Fraction 11 from the DNA-cellulose column 20 or 10 µg of protein. Gels were stained and destained as described

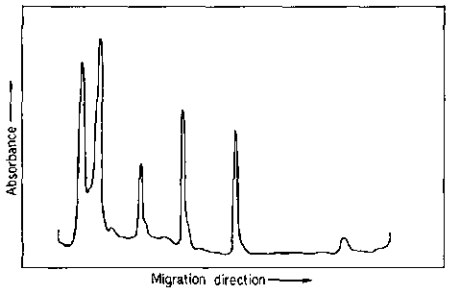

Fig. 9. *Densitometric tracing of* Aspergillus *RNA polymerase I as derived from a sodium dodecylsulphate/polyacrylamide gel.* 14 µg of DNA-cellulose-purified enzyme was applied to a 12.5% acrylamide gel containing dodecylsulphate and using the Tris/glycine-buffered system of Laemmli [24]. The gel was stained, destained and scanned as described in Materials and Methods. The direction of electrophoresis is indicated

could be resolved into two distinct forms based on their charge. The low-molecular-weight polypeptides of M_r 13000 (j) and 12000 (k) could not be detected with certainty in the gel system used, probably because they did not enter the 8 M urea stacking gel or they moved together with the dye front. This was also the case in the two-dimensional gel system using native-dodecylsulphate gel electrophoresis (Fig. 10B). The two high-molecular-weight components a and b, as well as polypeptides c and the one depicted as f, did not enter the 8 M urea separation gel (Fig. 10A) and remained at the interphase. Similar observations were done by Jendrisak and Burgess [5] for wheat germ RNA polymerase II and these authors suggested that these subunits might still be bound to each other under these conditions. Due to aberrations in the second dimension of the dodecylsulphate electrophoresis (Fig. 10A), it seems that subunit d has a higher position, thus a higher molecular weight, than subunit c but this is definitely not the case.

Several examples of electrophoretically and chromatographically distinct forms of RNA polymerase I are known [4]. The presence or absence of one or two subunits, as for instance observed for yeast [40] and mouse plasmacytoma [42], might cause the appearance of the two forms. However, samples taken from the DEAE-Sephadex gradient (Fig. 5, insert) did not show any difference in subunit pattern of RNA polymerase Ia

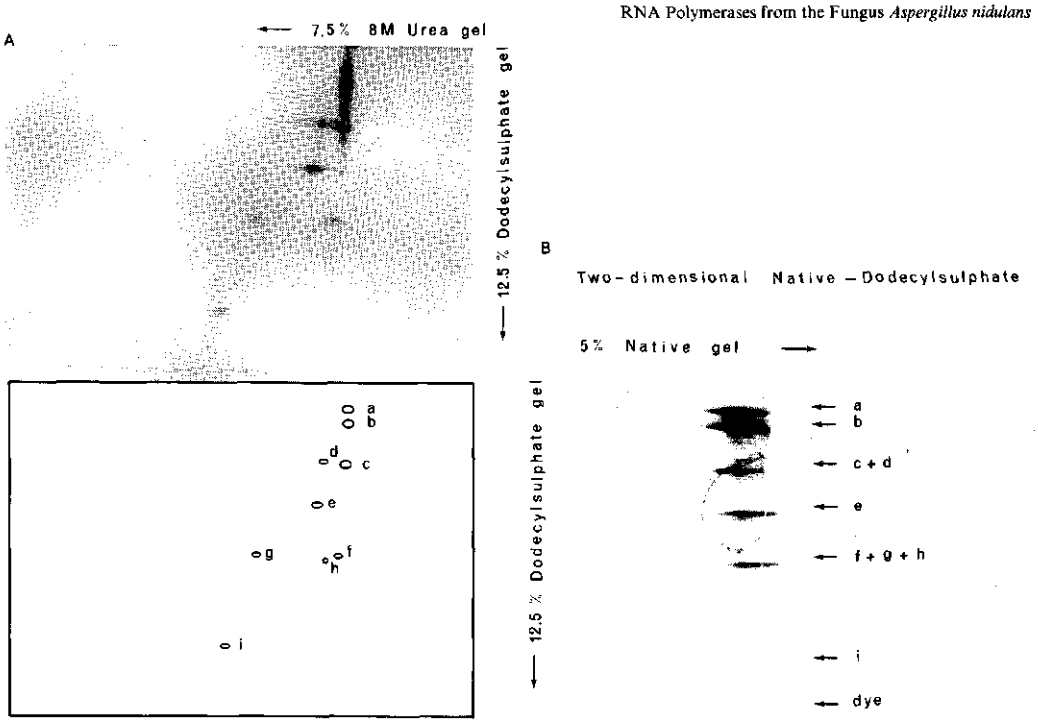

Fig. 10. *Two-dimensional polyacrylamide gel electrophoresis of* Aspergillus *RNA polymerase I.* The first dimension was either (A) run in the 8 M urea, pH 8.7 Tris-glycine-buffered system containing 7.5% acrylamide, or (B) in the non-denaturing gel system containing 5% acrylamide as described in Materials and Methods. The cylindrical gel was 7-cm long and the marker dye was run to within 1 cm of the bottom of the gel tube. After electrophoresis in the first dimension the gel was mounted on top of the slab gel for the second dimension which contained 12.5% acrylamide in dodecylsulphate and run in the Tris/glycine-buffered system of Laemmli [24]. The direction of migration is indicated by the arrows. (A) 4 µg and (B) 14 µg of the DNA-cellulose-purified RNA polymerase I

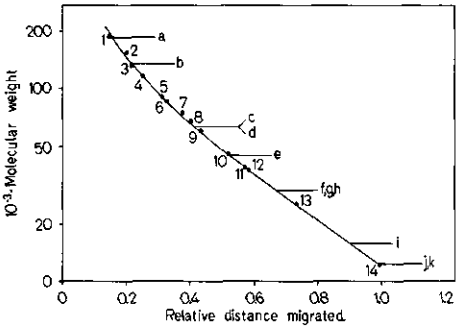

Fig. 11. *Molecular weight estimation of RNA polymerase I polypeptides in dodecylsulphate/polyacrylamide gels containing a linear gradient of 5–15% (w/v) acrylamide.* The position of the polypeptides are indicated by the horizontal lines. The standard curves were made from the migration of marker proteins of known molecular weight, indicated by number: (1) subunit of yeast RNA polymerase (190000); (2, 3) *E. coli* RNA polymerase subunits (165000 and 155000); (4) yeast RNA polymerase subunit (135000); (5) β-galactosidase (116000); (6) phosphorylase *a* (92500); (7) *E. coli* RNA polymerase subunit (87000); (8) transferrin (80000); (9) bovine serum albumin (68500); (10) catalase (60000); (11) ovalbumin (45000); (12) *E. coli* RNA polymerase subunit (39000); (13) chymotrypsinogen (25000); (14) cytochrome *c* (12500)

Table 2. *Polypeptide composition of* Aspergillus *RNA polymerase I*
Molecular weights of polypeptides were determined by simultaneous electrophoresis of RNA polymerase with marker protein standards (cf. Fig. 11). Molar ratios were determined by electrophoresis in Tris/glycine-buffered dodecylsulphate gels in the first or second dimension (in the latter case with 8 M urea gels in the first dimension) as described in the text and normalized to polypeptide a. The molar ratios of polypeptides f, g and h could only be determined from the two-dimensional gels, those of j and k could not be determined with certainty in our system

Polypeptide	Molecular weight	Stoichiometry in dodecylsulphate gels	
		– urea	+ urea
		mol/mol enzyme	
a	190000	1.0	1.0
b	135000	1.2	1.2
c	63000	0.9	0.9
d	62000	0.6	0.6
e	43000	2.0	2.0
f	29000	–	1.8
g	29000	3.0	–
h	(28000)	–	1.2
i	16000	1.2	1.2
j	(13000)	–	–
k	(12000)	–	–

and I b, using both 7.5% and 12.5% dodecylsulphate/acrylamide gels. The possibility that separation of subunits was masked due to the type of gel system used, cannot be excluded (e.g. polypeptides c and d); this needs further investigation.

General Conclusion

The purification procedure presented here is simple, rapid and highly reproducible, resulting in reasonable recoveries of homogeneous RNA polymerase I from the differentiatable lower eukaryote *Aspergillus nidulans*. With minor modifications similar procedures might be used to isolate and purify the other classes of RNA polymerases, although they seem to be more labile. Keeping in mind the exceptional character of RNA polymerase II towards α-amanitin, it will be of great interest to study the properties of this enzyme in different *Aspergillus* strains and to compare them with those of known systems.

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CHAPTER 3

AN α-AMANITIN RESISTANT DNA-DEPENDENT RNA POLYMERASE II FROM THE FUNGUS *ASPERGILLUS NIDULANS*

Summary

An α-amanitin resistant DNA-dependent RNA polymerase II has been purified from the lower eukaryote *Aspergillus nidulans* to apparent homogeneity by extraction of the enzyme at low salt concentration, polymin P (polyethylene imine) fractionation, binding to ion-exchangers and density gradient centrifugation. By this procedure 0.4 mg of RNA polymerase II can be purified over 6000-fold from 500 g of wet weight of starting material with a yield of 25% and a specific activity of 550 units/mg.

The subunit composition has been resolved by polyacrylamide gel electrophoresis in the presence of dodecylsulphate and by two-dimensional gel electrophoresis using a non-denaturing gel in the first dimension and a dodecylsulphate slab gel in the second dimension. The putative subunits have molecular weights of 170 000, 150 000, 33 000, 27 000, 24 000, 19 000, 18 000 and 16 000. Only one electrophoretical form of RNA polymerase II could be resolved. The chromatographic and catalytic properties and the subunit composition of the purified RNA polymerase II are clearly different from RNA polymerase I from *A.nidulans* but throughout comparable with other class II enzymes. It differs from all other class II enzymes by its insensitivity towards the toxin α-amanitin,even at concentrations upto 400 μg/ml,and appears to be unable to bind O-[^{14}C]-methyl-γ-amanitin at a concentration of 10 μg/ml of the toxin.

We conclude, that the purified RNA polymerase from *Aspergillus nidulans* is a real, but exceptional type of the class II RNA polymerases.

Introduction

DNA-dependent RNA polymerases have been purified and characterized from a variety of organisms (cf. Roeder (1)), belonging to the higher (2,3,4)

as well as the lower eukaryotes (6,7). It appeared to be more difficult to purify RNA polymerases from the lower eukaryotes, like fungi, than higher eukaryotes, due to the difficulties in desintegrating the cell wall. Up to now only a few useful procedures for lower eukaryotes other than yeast are reproducible by blending the hyphal mass in liquid nitrogen and to prepare a homogenate suitable for the isolation of RNA polymerase I (9). We now present the purification and characterization of RNA polymerase II from *A.nidulans*, using the same method of homogenization.

Sensitivity towards the toxin α-amanitin is often used as a classification criterion for eukaryotic RNA polymerases (10), although large differences in the sensitivity of especially RNA polymerase II from various organisms are observed. The 50% inhibition level of α-amanitin for mammalian RNA polymerase II is as low as 10-25 ng/ml, while the enzyme from the lower eukaryotes is far less sensitive, e.g. RNA polymerase II from yeast is 50% inhibited at 1 μg/ml (11) and the enzyme from the mushroom *Agaricus bisporus* at 6.8 μg/ml (12).

The isolated RNA polymerase from *A.nidulans* was classified as a class II enzyme, although it appeared to be insensitive through all stages of its purification towards the toxin α-amanitin, even at high concentrations upto 400 ug/ml. Furthermore,the enzyme was unable to bind O-[^{14}C]-methyl-γ-amanitin at 10 μg/ml. The insensitivity of RNA polymerase II from *Aspergillus* to 400 μg/ml of α-amanitin is much higher than that reported for all other class II enzymes; even class II RNA polymerases from mutants (*Drosophila*, CHO cell line), selected on their resistance towards α-amanitin (13,14) are completely inhibited at this toxin concentration.

Materials and Methods

Biochemicals

All biochemicals used were reagent grade and were in general obtained from Merck, British Drug House or Difco. Ribonucleoside triphosphates (ATP, UTP, GTP, CTP), crystalline bovine serum albumin (fraction V),calf

thymus DNA (type 1) and phosvitin were purchased from Sigma; [5-^{3}H]-UTP (25-50 Ci/mmol) and [^{32}P] -γ -ATP (>2000 Ci/mmol) from Amersham. The solutions for liquid scintillation counting (Lumasolve and Lipoluma) were obtained from Lumac. Electrophoresis reagents (acrylamide and N,N'-methylene bisacrylamide both recrystallized, N,N,N',N'-tetramethylethylene diamine and ammonium persulphate) were purchased from Serva; Coomassie brilliant blue R-250 from Merck and xylene brilliant cyanine G from Gurr. The molecular weight marker kits for gel electrophoresis, DEAE-Sephadex A-25, DEAE-Sepharose CL-6B were obtained from Pharmacia; Bio-Gel A-1.5m from Bio-Rad, phosphocellulose P-11 from Whatman and E.coli RNA polymerase from Boehringer. α-Amanitin was a kind gift of Dr. M. Govindan (Heidelberg) and O-[^{14}C]-methyl-γ-amanitin (63.4 Ci/mol) was a gift of Dr. C.E. Sekeris (Athens). Polymin P was kindly donated by BASF (Ludwigshafen, F.R.G.).

Organism and Growth Conditions

A biA$_1$ (biotin-deficient) strain of *Aspergillus nidulans* of Glasgow origin was used throughout this study. Growth conditions, maintenance and preparation of conidial suspensions were as described (9).

Buffers

Buffer A contained 50 mM Tris pH 7.9 (adjusted with HCl at 4^{o}C), 10% (v/v) glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and ammonium sulphate at various concentrations as indicated in the text. Buffer B and C were similar to buffer A, but with 25% and 50% (v/v) glycerol, respectively. Phenylmethylsulfonyl fluoride was added from a 200 mM stock solution in 96% ethanol, immediately before use (15,16). All solutions were freshly made up in glass-double-distilled water.

RNA polymerase assay

The standard RNA polymerase assay mixture (200 µl) contained: 50 mM Tris, pH 7.9, 1 mM dithiothreitol, 1 mM ATP, GTP and CTP, 0.05 mM UTP, 1 µCi [5-^{3}H] UTP, 4 mM $MnCl_2$, 20 µg heat-denatured calf thymus DNA and 20 µl of sample. The ammonium sulphate concentration in the samples, determined with a Philips PW 9501 conductivity meter, was adjusted if necessary with a neutralised ammonium sulphate stock solution to 50-80 mM with RNA polymerase II and 10-30 mM with RNA polymerase I in final concentration in the reaction mixture.

Heat-denatured calf thymus DNA was replaced in some experiments by either 40 µg of native calf thymus DNA or 2 µg of poly(dA-dT) for the determination of template specificity of RNA polymerase I and II. The reaction was started by addition of the enzyme and the mixture was incubated for 30 min at 35°C. The reaction was stopped by addition of 4 ml of 5% (w/v) ice-cold TCA, the precipitate collected on GF/C filters (Whatman), washed and counted as described (9).

One unit of RNA polymerase activity is defined as the amount of enzyme activity necessary for the incorporation of 1 nmole of UMP into TCA-insoluble material in 60 min under standard conditions. The specific activity is defined as units/mg protein.

Partial Purification of Rat Liver RNA Polymerase II

Rat liver nuclei were isolated according to Chauveau (17) from male rats (U strain) and the polymerase activity was extracted from the nuclei by sonication in a high salt buffer as described by Chesterton & Butterworth (18). RNA polymerase II activity was separated from RNA polymerase I activity through DEAE-Sephadex A-25 chromatography (18). The peak fractions containing RNA polymerase II were pooled and used as a control in the amanitin binding experiments.

Amanitin binding assay

Amanitin binding assay with O-[^{14}C]-methyl-γ-amanitin (63.4 Ci/mol) was performed according to the method of Cochet-Meilhac et al. (19) with either purified RNA polymerase II from *Aspergillus* or partially purified rat liver RNA polymerase II. Simultaneous measurement of the polymerase activity under standard conditions was as described above.

Preparation of Columns

Bio-Gel A-1.5m, DEAE-Sephadex A-25, DEAE-Sepharose CL-6B and phosphocellulose P-11 were prewashed and equilibrated as described before (9).

Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate was carried out in 100 x 130 x 1 -mm slabs using a modification of the discontinuous dodecylsulphate Tris/glycine gel system of Laemmli (20); the concentration of N,N'-methylene bisacrylamide was lowered, compared to the Laemmli system, to 0.085%, 0.10%, 0.135% for a 15%, 12.5% and 10% acrylamide gel, respectively. Samples were treated as described by Laemmli (20) and subjected to electrophoresis for 3-4 h at 150 V, using bromophenol blue (0.004% w/v) as a dye marking the position of the front.

Gel electrophoresis under non-denaturing conditions was performed as described by Smith and Braun (8) using a slab gel (100 x 130 x 1 -mm). Two dimensional gel electrophoresis was performed as described before (9). Molecular weights of the polypeptides were estimated by simultaneous electrophoresis of RNA polymerase with molecular weight markers in the acrylamide gels in the presence of dodecylsulphate by the general method of Weber and Osborn (21). The molecular weight markers used were:

thyroglobulin (330 000), ferritin (220 000 and 18 500), phosphatase (92 500), bovine serum albumin (68 500), catalase (60 000), ovalbumin (45 000), lactate dehydrogenase (36 000), carbonic anhydrase (30 000), trypsin inhibitor (20 000), α-lactalbumin (14 400) and the E.coli RNA polymerase subunits (22) (165 000, 155 000, 87 000 and 39 000).

The gels were stained with Coomassie brilliant blue R-250 and destained as described by Burgess and Jendrisak (23). Molar ratios of the polypeptides were determined as described by Burgess and Jendrisak (23) by densitometric scanning of diapositives of stained slab gels.

General procedures

DNA, RNA and protein concentrations were determined with diphenylamine (24), orcinol (25) and xylene brilliant cyanine G (26), respectively. The 10% (v/v) stock solution of polymin P was prepared and neutralised as described by Jendrisak and Burgess (2).

Standard Purification Procedure of RNA Polymerase II

All operations were carried out at 0-4^{0}C; centrifugations were performed in a MSE 18 high speed and 65 ultracentrifuge.

Solubilization of the enzyme. The hyphal mass (500 g wet weight) was harvested, washed and disrupted in liquid nitrogen as described (9), with the exception, that the extraction buffer A contained only 25 mM ammonium sulphate. The homogenate was diluted after rapid thawing with 1.5 l buffer A (25 mM), homogenized in a 1 l Waring blendor and centrifuged for 30 min at 15,000 x g.

Polymin P Fractionation. The crude extract was filtered through one layer of Miracloth (Calbiochem) and diluted with one volume of buffer A (75 mM). The RNA polymerases were precipitated by slowly adding 70 ml of a 10% (v/v) polymin P/100 g wet weight mycelia with continuous stirring. After 10 min the precipitate was collected by centrifugation, washed and

extracted with buffer A (300 mM) as described before (9). The polymin P extract was precipitated by slow addition of solid ammonium sulphate (0.35 g/ml). The precipitate was collected by centrifugation, dissolved in buffer A (300 mM) and dialysed against the same buffer as described before (9).

Bio-Gel A-1.5m Chromatography. The dialysed crude enzyme preparation was loaded onto a 55 x 9 cm Bio-Gel A-1.5m column and developed with buffer A (300 mM) at a flow rate of 80 ml/h. Fractions containing the bulk of RNA polymerase activity were pooled.

DEAE-Sephadex A-25 Chromatography. The Bio-Gel pool was diluted with one volume buffer A to give a final ammonium sulphate concentration of 150 mM. DEAE-Sephadex A-25 equilibrated with buffer A (150 mM) was added (1 ml settled bed volume of DEAE-Sephadex/3 mg of protein) and the mixture was slowly stirred for 30 min. The slurry was poured into a column and washed with one volume of equilibration buffer. The flow through contained RNA polymerase I. The bound polymerase activity was eluted by applying a 150-400 mM ammonium sulphate gradient in buffer A. The polymerase activity eluting at 260 mM ammonium sulphate was pooled.

DEAE-Sepharose CL-6B Chromatography. The pooled fractions from the DEAE-Sephadex were diluted or dialysed against buffer B to give a final concentration of 25% glycerol and 150 mM ammonium sulphate. The dialysate was loaded, with one column volume/h, onto a DEAE-Sepharose CL-6B column equilibrated with buffer B (150 mM), (1 ml DEAE-Sepharose/mg protein). After washing the column with equilibration buffer, the RNA polymerase activity was eluted with buffer B (400 mM). The fractions containing the RNA polymerase activity were pooled.

Sucrose Gradient Centrifugation. The DEAE-Sepharose pool was loaded onto a 5-20% (w/v) sucrose gradient in buffer A (300 mM) containing 30% (v/v) glycerol. Two ml containing maximum 500 units of RNA polymerase activity were loaded onto a 10 ml gradient and centrifuged at 155,000 x g_{av} for

68-72 h at 4°C in a MSE 65 ultracentrifuge. The gradients were fractionated from the bottom of the tube into 0.5 ml fractions with a MSE gradient fractionator. The fractions were tested for RNA polymerase activity.

Phosphocellulose P-11 Chromatography. The fractions from the density gradient containing the RNA polymerase activity were dialysed twice against buffer B without Mg^{2+} to give a final concentration of 15-20 mM ammonium sulphate. The dialysate was loaded, with one column volume/h onto a five ml phosphocellulose P-11 column equilibrated with buffer B (20 mM) without Mg^{2+}. The column was washed with 2 volumes of equilibration buffer and developed with a 20-300 mM ammonium sulphate gradient in buffer B without Mg^{2+}. The RNA polymerase activity was pooled.

Storage. Storage of impure enzyme fractions at either -80°C or -20°C should be avoided to prevent loss of activity, but if necessary, the salt and protein concentration should be as high as possible. At two stages of purification, after the ammonium sulphate precipitation step and after sucrose gradient centrifugation, the partially purified enzyme can be stored at -80°C without loss of activity for several months. The purified enzyme can be stored at either -80°C or -20°C without loss of activity for prolonged periods, provided it is dialysed against buffer C (100 mM) containing a high concentration of stabilizing glycerol.

Results and discussion

Aspergillus nidulans mycelium was harvested at log phase (16-18 h) and the cell wall was disrupted by blending the hyphal mass in liquid nitrogen. This method of breaking up the cells, also used for the isolation of RNA polymerase I (9), appeared to be highly reproducible with respect to the amount of polymerase activity solubilized. The cell content was solubilized with a low salt buffer and after removal of cell wall debris, the total RNA polymerase activity could be measured in the homogenate; no inhibition with α-amanitin at 10 µg/ml could be measured.

The RNA polymerase activity could be extracted from the hyphal mass with either a low or high salt buffer. No differences in the total polymerase activity could be measured if the extraction was performed with a buffer containing either 25 mM or 300 mM ammonium sulphate. Extraction with 300 mM ammonium sulphate resulted at a later stage in an aggregation of part of the polymerase activity (mainly influencing the purification of RNA polymerase II). Aggregation was strongly reduced, if the solubilization was done with 25 mM ammonium sulphate. Solubilization at low ionic strength was also successfully used for the isolation of RNA polymerase II from wheat germ (2), calf thymus (3) and *Acanthamoeba* (7).

Polymin P Fractionation and Ammonium Sulphate Precipitation.

The cleared homogenate was diluted with buffer A (75 mM) to give a final concentration of 50 mM ammonium sulphate. Nucleic acids were removed through precipitation with polymin P (2,27) and polymerase activity was extracted selectively with 300 mM ammonium sulphate (9). The polymin P released into the extract could only be partially removed by ammonium sulphate precipitation, therefore only variable values for RNA polymerase activity could be obtained at this stage. As indicated in our earlier publication on RNA polymerase I, the presence of trace amounts of polymin P resulted in a very weak binding of polymerase activity to DEAE-Sephadex, if loaded directly onto this column (9). Under these conditions all RNA polymerase activities eluted from the column at an ammonium sulphate concentration lower than 100 mM.

Selective binding of RNA polymerase II to DEAE-cellulose has been used after polymin P fractionation and ammonium sulphate precipitation as the first chromatographic step (2,3). If at this stage the crude enzyme extract

of *Aspergillus* was loaded onto a DEAE-cellulose column at 150 mM ammonium sulphate, the retention of RNA polymerase activity by this type of column was very low and irreproducible.

DEAE-Sephadex A-25 Chromatography

When the crude enzyme extract from *Aspergillus* was sieved on Bio-Gel A-1.5 m at high ionic strength, as described earlier (9), it was possible to bind the RNA polymerase activity reproducible to DEAE-Sephadex A-25 and to obtain a separation into two different RNA polymerase activity peaks. The first peak eluting from DEAE-Sephadex A-25 at 110-130 mM ammonium sulphate was classified as RNA polymerase I and the second peak eluting at 260 mM ammonium sulphate was classified as RNA polymerase II according to the Roeder classification, based on the order of elution of the different RNA polymerases from DEAE-Sephadex (28). The purification of the first RNA polymerase activity peak to apparent homogeneity confirmed that it consisted of RNA polymerase I (or A) (9).

The Bio-Gel pool could also be loaded at 150 mM instead of 50 mM ammonium sulphate onto the DEAE-Sephadex in this purification procedure. This selective binding of the RNA polymerase activity (eluting at 260 mM ammonium sulphate) onto the column appeared to be more reproducible. Under these conditions RNA polymerase I was found in the flow-through. After applying a 150-400 mM ammonium sulphate gradient, two peaks eluted from the column (fig. 1). The first minor peak elutes at 150-170 mM ammonium sulphate and consists probably of residual RNA polymerase I. The major RNA polymerase activity peak elutes at 260 mM ammonium sulphate and has to be regarded s a RNA polymerase II, although it is not sensitive to α-amanitin even to concentrations upto 400 μg/ml of the toxin. Because it is impossible to measure or to estimate the exact amount of RNA polymerase II activity in the homogenate or Bio-Gel pool (no α-amanitin sensitivity), the amount of RNA polymerase II eluting from the DEAE-Sephadex is taken as 100% to calcu-

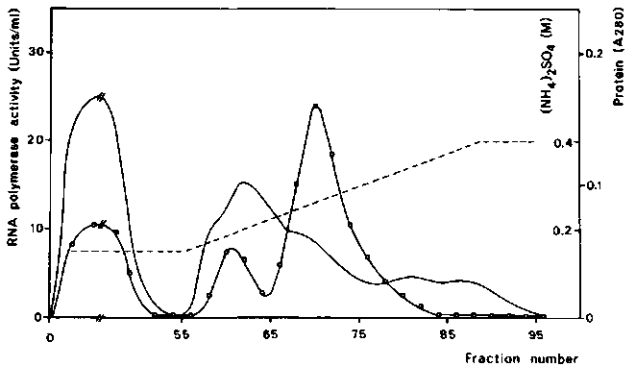

Fig. 1. *DEAE-Sephadex A-25 chromatography of Aspergillus RNA polymerase*
The Bio-Gel pool was diluted and bound in batches to DEAE-Sephadex A-25 at 150 mM ammonium sulphate as described. The column (26 x 2.6 cm) was developed at a flow rate of 25 ml/h with a linear salt gradient of 150-500 mM ammonium sulphate in buffer A (----) using the LKB 11300 ultragrad gradient mixer. Fractions of 6 ml were collected and the absorbance at 280 nm (——) was monitored with an Isco model UA-5 Absorbance monitor using a standard 1-cm flow cell. 20 µl aliquots were assayed for RNA polymerase activity (□ - □) and fractions 66-77 were pooled.

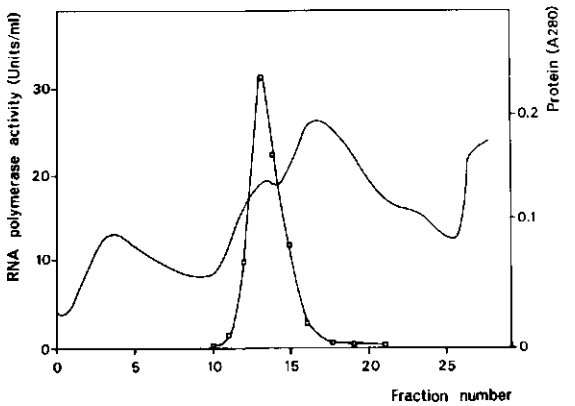

Fig. 2. *Sucrose gradient centrifugation of Aspergillus RNA polymerase II*
The pooled fractions from the DEAE-Sepharose CL-6B column, containing the RNA polymerase activity, were loaded onto a 5-20% (w/v) sucrose gradient in buffer A (300 mM) containing 30% (v/v) glycerol as described. The gradient was centrifuged at 155,000 x g_{av} for 68-72 h at 4 °C and fractionated from the bottom of the tube. Fractions of 0.5 ml were collected and the absorbance at 280 nm (——) was monitored. 20 µl aliquots were assayed for RNA polymerase activity (□ - □). Fractions 12-15 were pooled.

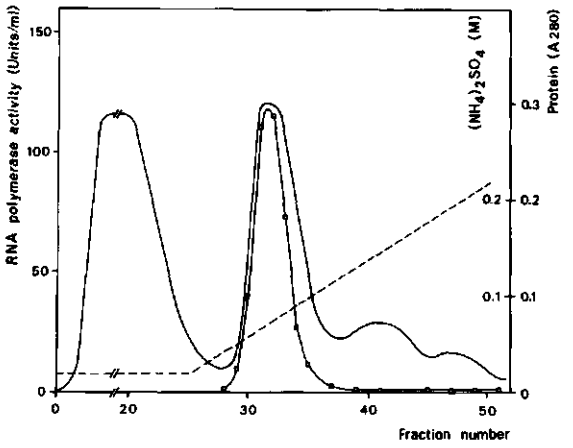

Fig. 3. *Phosphocellulose P-11 chromatography of Aspergillus RNA polymerase II.* The pooled fractions from the sucrose gradient were loaded onto a phosphocellulose P-11 column (5 x 1 cm) as described. A linear salt gradient of 20-300 mM ammonium sulphate in buffer B minus Mg^{2+} (----) was applied. The absorbance at 280 nm (——) was monitored and fractions of 0.6 ml were collected. 20 µl aliquots were assayed for RNA polymerase activity (▫ - ▫). Fractions 30-34 were pooled.

Table 1. *Purification of RNA polymerase II from Aspergillus nidulans*

The values given in the table are average values calculated from 3 different preparations. The amount of RNA polymerase II activity in the DEAE-Sephadex pool is taken as 100% in calculating the yield, and was used to calculate the specific activity in the homogenate and Bio-Gel pool. These values were then used as the basis for the calculation of the yield and the purification at each stage.

Fraction	Volume	Total protein	Total activity polymerase II	Yield	Spec. act.	Purification
	ml	mg	Units	%	U/mg	-fold
Homogenate	2 000	10 000	890(11 000)*	-	0.09	-
Bio-Gel A-1,5 m	420	450	890(10 000)*	-	2.1	23
DEAE-Sephadex A-25	72	11	890	100	81	900
DEAE-Sepharose CL-6B	12	5.5	841	94	153	1700
Sucrose gradient	11.5	1.2	577	65	480	5340
Phosphocellulose P-11	2.4	0.4	219	25	550	6090

* Total RNA polymerase activity

late the specific activity in the homogenate (Table 1).

The specific activity after DEAE-Sephadex chromatography is 81 units/mg. The amount of RNA polymerase II activity is very low (~ 900 units) at this stage of purification compared with that RNA polymerase I (6 000 units). In general RNA polymerase II from other systems represents more than 50% of the total RNA polymerase activity (29).

DEAE-Sepharose CL-6B Chromatography

The pooled RNA polymerase activity eluting at 260 mM ammonium sulphate from the DEAE-Sephadex, was diluted or dialysed and loaded at 150 mM ammonium sulphate onto a DEAE-Sepharose CL-6B column (1 mg protein/ml settled bed volume). Step elution of the polymerase activity with 400 mM ammonium sulphate was preferred since applying a salt gradient resulted in a broad elution of the RNA polymerase activity without improving its specific activity. Through this step an almost 2-fold purification was achieved with a 90% yield of activity.

Sucrose Gradient Centrifugation

Since the average molecular weight of the proteins loaded onto the sucrose gradient was high (300 000-400 000 daltons), due to the molecular sieving on Bio-Gel A-1.5 m, it was necessary to use a 5-20% (w/v) sucrose gradient containing 30% (v/v)glycerol (fig. 2). Because of the high density in the gradient, a 2-3 fold higher initial protein concentration could be loaded onto this sucrose gradient, than onto the normally used 15-30% (v/v) glycerol gradient, resulting in a similar purification, but a 2-fold better recovery of the polymerase activity. This is in agreement with the findings of Goldberg et al. (4), that a higher initial protein concentration and a higher glycerol concentration gives a better recovery of the polymerase activity using a gradient centrifugation step. In contrast to the results of Goldberg et al.(4), the presence or absence of Mg^{2+} had no influence on

the yield of activity. The high ionic strength in the gradient was necessary to avoid aggregation of the polymerase activity.

The sucrose gradient centrifugation resulted in a 3-fold purification with a 70-80% yield of activity. The pooled enzyme could be stored at -80°C for several months without loss of activity, because of the stabilizing effects of the glycerol and sucrose concentration.

Phosphocellulose P-11 Chromatography

Fractions from the preceeding sucrose gradient containing the polymerase activity were loaded onto a phosphocellulose column. The column was developed with a 20-300 mM ammonium sulphate gradient and the RNA polymerase activity eluted at approximately 60 mM ammonium sulphate (fig. 3). The recovery of RNA polymerase activity from the phosphocellulose column was low and variable (40-60%) and appeared to be dependent on the initial protein concentration of the fraction loaded onto the column; a higher initial protein concentration resulted in a better recovery. Similar low recoveries and a dependency on the protein concentration have also been reported by Hodo and Blatti (3).

A change in the preference of RNA polymerase II for a certain DNA template through phosphocellulose chromatography has often been reported (30,31). We could not detect a change in preference for native or heat-denatured calf thymus DNA of this *Aspergillus* RNA polymerase through phosphocellulose chromatography. Although more than 50% of the contaminating proteins were removed through phosphocellulose chromatography, only a 1.1-1.2 fold purification was achieved, because of the decrease in the enzyme activity (Table 1). The specific activity of the enzyme was 550 units/mg at this stage.

Subunit Composition

The polypeptide composition of the purified RNA polymerase was determined by polyacrylamide gel electrophoresis under denaturing conditions in the presence of sodium dodecylsulphate. It appeared, that a modification of the Laemmli polyacrylamide gel system (20), using a 3-5 fold lower concentration of N,N'-methylene bisacrylamide, resulted in a better resolution of the polypeptides. The range in which a linear correlation exists between the log of the molecular weight of the polypeptides and their relative distance migrated is larger in these modified acrylamide gels than is the case with the Laemmli system (not shown). Based on the better resolution the molecular weights of the subunits of RNA polymerase I, from *Aspergillus*, as reported in our earlier publication (9), have been revised and are listed in Table 2. The polypeptide pattern of the purified RNA polymerase, together with that of a partially purified RNA polymerase I from *Aspergillus* are shown in fig. 4^{A}. The purified RNA polymerase (lane 1) consists of two heavy polypeptides with molecular weights higher than 100 000 daltons and 6 or 7 polypeptides smaller than 100 000 daltons; this is characteristic for all RNA polymerases. The two heaviest polypeptides (a+b) are clearly different in molecular weight from those of RNA polymerase I from *Aspergillus* (lane 7). The polypeptides of the purified RNA polymerase smaller than 100 000 in molecular weight are also different from those of RNA polymerase I, although some polypeptides seem to be present in both RNA polymerases (e.g. the polypeptides of 33 000 (c) comigrates with a subunit of RNA polymerase I). Characteristic of the purified RNA polymerase is also the absence of polypeptides in the range of 50 000-100 000 daltons. The molecular weights of the putative subunits of the purified RNA polymerase complex were estimated to be: 17000 (a), 150 000 (b), 33 000 (c), 27 000 (d), 24 000 (e), 19 000 (f) 18 000 (h) and 16 000 (l). That these polypeptides are the subunits of the polymerase complex,

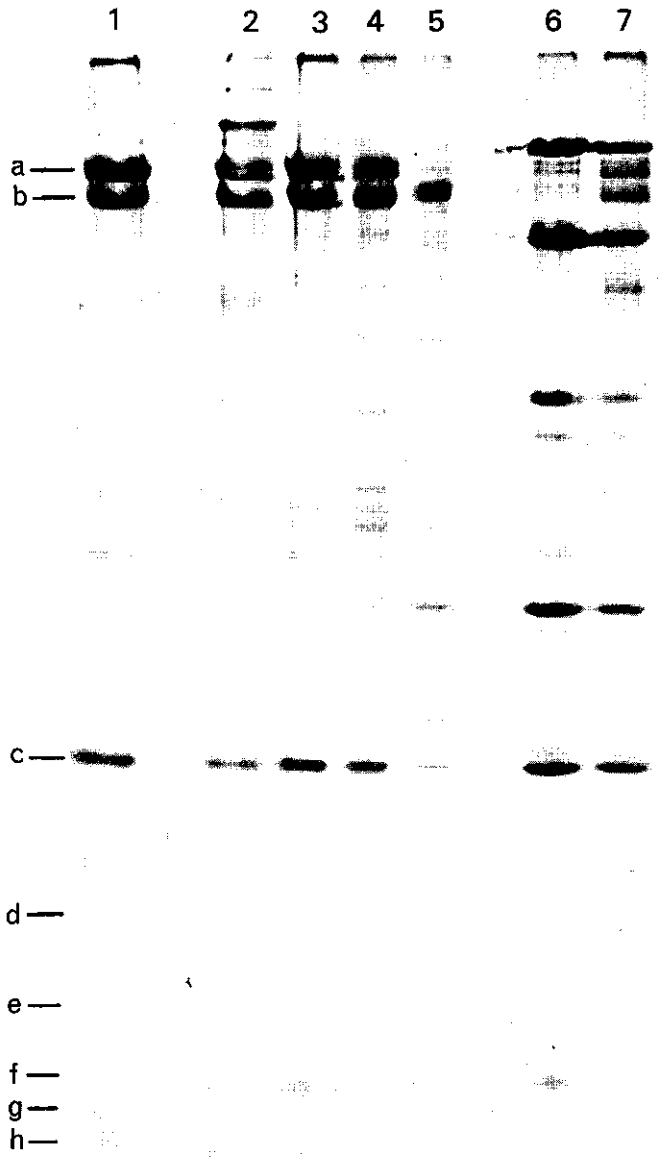

Fig. 4[A]. *Polyacrylamide gel electrophoresis of RNA polymerase II under denaturing conditions.* Dodecylsulphate/polyacrylamide gel electrophoresis on 15% acrylamide slab gels was performed as described in *Materials and Methods*. Essentially pure RNA polymerase II (20 µg), pooled after P-cellulose chromatography, is shown in lane 1. Lanes 2-5 contain respectively the fractions 29, 34, 35 and 36 from the phosphocellulose column (fig. 3). Lane 6 contains 10 µg of partially purified *Aspergillus* RNA polymerase I. Lane 7 contains 5 µg of partially purified RNA polymerase I and 5 µg of pure RNA polymerase II from *Aspergillus*.

Table 2. *Polypeptide composition of Aspergillus RNA polymerase I and II*

Molecular weights of the polypeptides were determined by simultaneous electrophoresis of RNA polymerase with marker protein standards (*Materials and Methods*). Molar ratios were determined by electrophoresis in Tris/glycine-buffered dodecylsulphate gels in the first or second dimension as described and normalized to the largest polypeptide a in the case of RNA polymerase I and to the second polypeptide of RNA polymerase II.

Molecular weight		Stoichiometry	
I	II	I	II
190 000(a)		1.0	
	170 000(a)		0.9-1.0
	150 000(b)		1.0
115 000(b)		1.2	
70 000 (c+d)		1.5	
69 000			
46 000(e)		2.0	
33 000 (f+g+h)	33 000(c)	3.0	2.0
32 000	27 000(d)		0.7
	24 000(e)		0.9
19 500(i)		1.2	
	19 000(f)		1.2
	18 000(g)		0.9
	16 000(h)		0.5
15 000(j)		0.9	

was concluded from their unchanged stoichiometry throughout the phosphocellulose (partly shown in fig. 4^A). This was confirmed by two dimensional gel electrophoresis of the purified enzyme using a 5 % (w/v) polyacrylamide gel under non-denaturing conditions in the first dimension and 12.5 % (w/v) modified polyacrylamide gel in the second dimension (fig. 4^B). It is not clear, whether the 47 000 MW polypeptide (fig. 4^A lane 1) belongs to the polymerase complex. The molar ratios of the polypeptides of the purified RNA polymerase (Table 2) were determined from densitometric scans of stained polyacrylamide gels in the presence of dodecylsulphate (fig. 5). The high-molecular-weight polypeptides appear in an almost 1:1 ratio. In both RNA polymerases, the polypeptide of 33 000 is present more than once in the complex (Table 2). The subunit composition of the RNA polymerases from class II is first of all characterized by the absence of subunits in the range of 50 000-100 000 daltons in which they differ clearly from the class I and III RNA polymerases (cf. 1). The subunit composition of the purified RNA polymerase is in this regard comparable with the class II RNA polymerases from higher eukaryotes like mouse plasmacytoma (1), calf thymus (3,32) and wheat germ (2) as well as lower eukaryotes, like yeast (33,34), *Physarum* (8) and *Acanthamoeba* (7).

The second characteristic of the class II enzymes is the presence of subforms of RNA polymerase II, differing in the molecular weight of the largest subunit. A magnification of the scan of the polypeptide pattern of the purified RNA polymerase, reveals a shoulder in the peak of the largest subunit and two minor peaks, migrating slower, which may indicate that subforms exist (insert fig. 5). We were not able to reveal these subforms of the purified RNA polymerase from *Aspergillus* by two dimensional gel electrophoresis. In wheat germ, as described by Jendrisak and Burgess (2) and cauliflower as described by Goto et al. (35), RNA polymerase II enzymes were also present in only one form. This could be due to the iso-

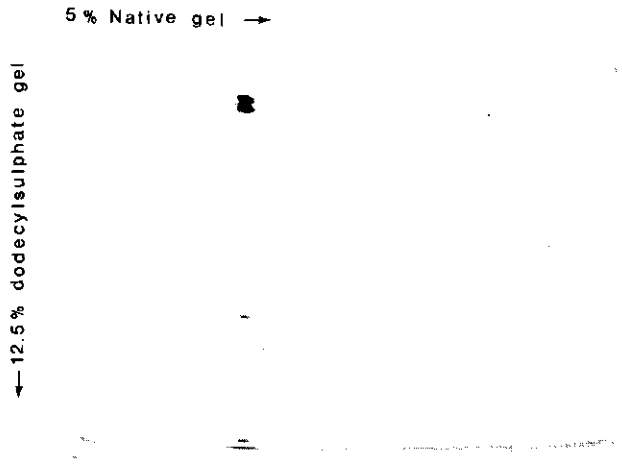

Fig. 4[B]. *Two dimensional polyacrylamide gel electrophoresis of Aspergillus RNA polymerase II.* Pure RNA Polymerase II (15 µg) was run in the first dimension under non-denaturing conditions in a 5% acrylamide slab gel as described in *Materials and Methods*. After electrophoresis the lane containing RNA polymerase II was cut out, equilibrated with Laemmli sample buffer and mounted on top of a 12.5% polyacrylamide gel in the presence of dodecylsulphate as described in *Materials and Methods*. The direction of migration is indicated by the arrows.

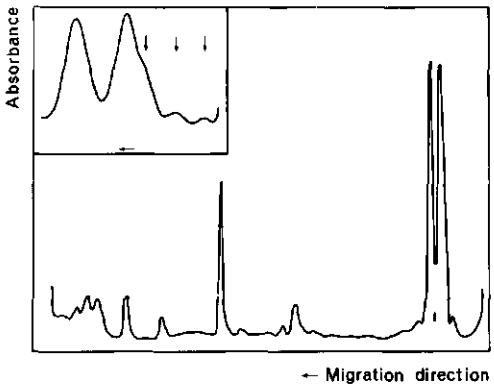

Fig. 5. *Densitometric tracing of Aspergillus RNA polymerase II as derived from a sodium dodecylsulphate polyacrylamide gel.* 20 µg of P-cellulose purified enzyme was applied to a 15% acrylamide gel (fig. 8, lane 1). The gel was stained, destained and scanned as described. The insert shows a magnification of the scan from the upper part of the gel, containing the largest subunits. The vertical arrows indicate the minor polypeptides preceeding the 170 000 dalton subunit. The direction of migration is indicated by the horizontal arrows.

lation procedure, because Hodo and Blatti (3) reported two subforms of wheat germ RNA polymerase II isolated with a slightly modified procedure of Jendrisak and Burgess (2). It is possible, that subforms of RNA polymerase II from *Aspergillus* can also be revealed after modifications of the isolation procedure.

We conclude, that the subunit pattern of the purified RNA polymerase is throughout comparable with class II RNA polymerases and is clearly different from class I and III RNA polymerases.

Properties of the Enzyme

The purified enzyme remained stable for prolonged periods at -80 ^{o}C as well as -20 ^{o}C when dialysed against buffer C (100 mM) containing 50% (v/v) glycerol. The final enzyme preparation did not contain detectable RNase or protease activity. The RNase activity was measured through continuous incubation of the enzyme in the standard polymerase assay mixture after addition of EDTA to stop the incorporation of ^{3}H-UMP into TCA-insoluble material; no loss of TCA-insoluble ^{3}H-UMP could be measured after several hours. Protease activity was monitored by incubation of the enzyme at room temperature for several hours, followed by polyacrylamide gel electrophoresis in the presence of dodecylsulphate. No changes in the stoichiometry of the polypeptides were observed. The purified enzyme was further characterized by determining the optimal conditions for activity. The purified RNA polymerase was completely dependent for its activity on the addition of DNA, as a template, all four nucleotides and divalent cations. The rate of incorporation of ^{3}H-UMP into TCA-insoluble material was almost linear for 20 min under standard reaction conditions at 35 ^{o}C using heat-denatured calf thymus DNA as a template; the product was sensitive to alkali and RNase digestion (not shown).

The optimal salt concentration appeared to be dependent on the type of template (added in excess) used. With heat-denatured calf thymus DNA, the purified RNA polymerase had a broad salt optimum between 50-100 mM

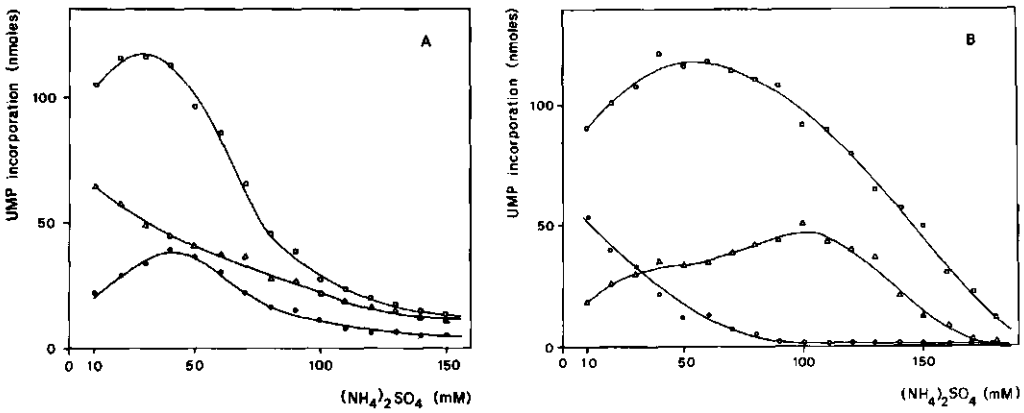

Fig. 6. *The effect of the ammonium sulphate concentration on the activity of RNA polymerase I and II with different templates.* RNA polymerase I, isolated as described (9), and essentially pure RNA polymerase II, both in storage buffer, were assayed for their RNA synthesizing capacity with increasing amounts of ammonium sulphate in the presence of 20 µg heat-denaturing calf thymus DNA (□ - □), 40 µg native calf thymus DNA (Δ - Δ) or 2 mg poly(dA-dT) (o-o) per assay. (A) RNA polymerase I and (B) RNA polymerase II.

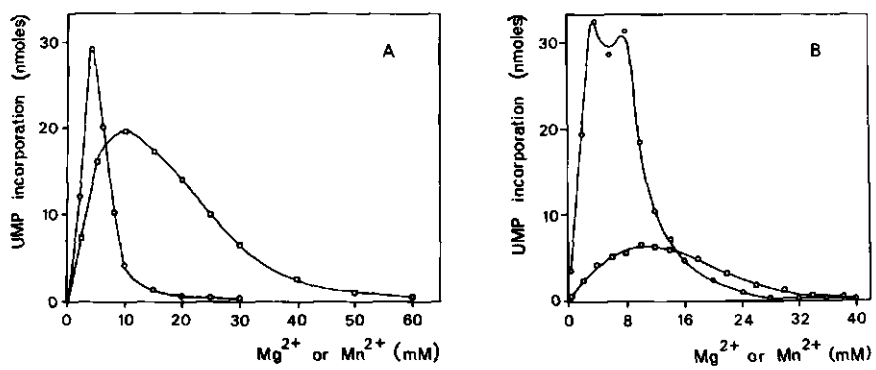

Fig. 7. *The effect of metal divalent cation concentration on the activity of RNA polymerase I and II.* RNA polymerase I and II activity were assayed in the standard reaction mixture, containing 25 mM and 60 mM ammonium sulphate respectively with increasing amounts of Mg^{2+} in the absence of Mn^{2+} (□ - □) or increasing amounts of Mn^{2+} in the presence of 0.5 mM Mg^{2+} (o-o). (A) RNA polymerase I and (B) RNA polymerase II.

ammonium sulphate. In contrast, RNA polymerase I activity was optimal at a salt concentration of 10-30 mM ammonium sulphate (fig. 6). Native calf thymus DNA as a template appeared to be less efficiently transcribed by RNA polymerase I as well as by the purified RNA polymerase. The denatured versus native calf thymus DNA activity ratio was 2 for RNA polymerase I and 3 for the purified RNA polymerase II. For both RNA polymerase activities the optimal salt conditions with native calf thymus DNA as template were similar compared to denatured calf thymus DNA as a template (fig. 6).

The transcription of the synthetic template poly(dA-dT) by the purified RNA polymerase was optimal at low salt conditions, when tested at a low template concentration (2 µg/assay) and was not transcribed at all at 80-100 mM ammonium sulphate. Under the same conditions RNA polymerase I was optimal at 30-50 mM ammonium sulphate with poly(dA-dT) as a template (fig. 6). The divalent cation conditions were determined by incubation of the RNA polymerase activity with an increasing Mn^{2+} or Mg^{2+} concentration. The purified RNA polymerase was optimal with Mn^{2+} at 4-8 mM; the activity was 7-8 fold higher than with Mg^{2+} (fig. 7). RNA polymerase I from *Aspergillus* was optimal at 3-4 mM Mn^{2+} and the activity with Mn^{2+} was only 1.5 fold higher than with Mg^{2+} (9) (fig. 7). The catalytic properties of the purified RNA polymerase are clearly different from those of RNA polymerase I from *Aspergillus* (9) and are throughout comparable with class II RNA polymerases (cf. Roeder (1) and Chambon (29)).

α-amanitin insensitivity

The purified RNA polymerase from *Aspergillus* was classified as a class II enzyme because of its chromatographical behaviour,subunit composition and catalytic properties. In contrast to other class II enzymes the RNA polymerase II from *Aspergillus* appeared to be insensitive

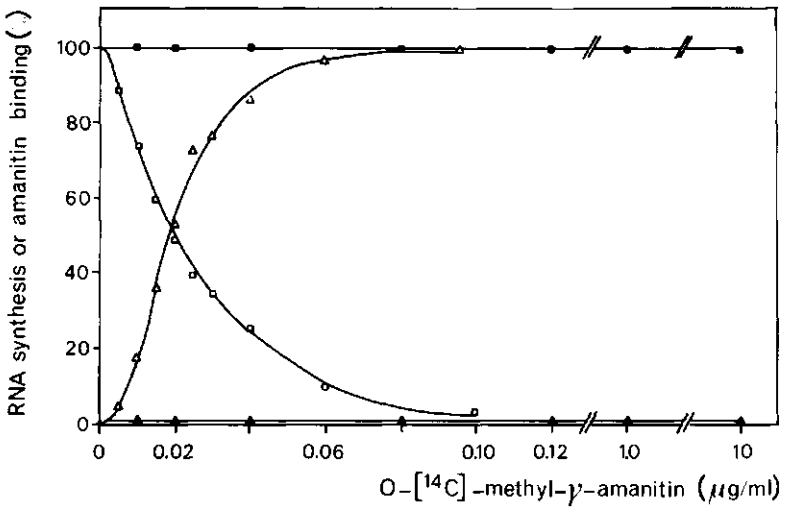

Fig. 8. *Inhibition of RNA synthesis and binding of amanitin to RNA polymerase II, as a function of O-[^{14}C]-methyl-γ-amanitin concentration.* The incubation mixture (0.5 ml) was as described by Cochet-Meilhac et al. (19) and contained either 8 μg *Aspergillus* RNA polymerase II (closed symbols) or partially purified rat liver RNA polymerase II (8 μg) (open symbols) and various concentrations of O-[^{14}C]-methyl-γ-amanitin as indicated. After 10 min at 37 °C, 100 μl aliquots were removed and processed for determination of enzyme-bound amanitin as described (19). 100 μl aliquots were added to a mixture (100 μl) containing 20 μg heat-denatured calf thymus DNA, 2 mM ATP, CTP and GTP, 0.10 mM UTP, 1 μCi [5-^{3}H]-UTP, 100 mM Tris-HCl pH 7.9, 2 mM dithiothreitol, 8 mM $MnCl_2$ for RNA synthesis determination (30 min, 35 °C). 100% amanitin binding to rat liver RNA polymerase II was 1620 dpm. (□ - ○, ■ - ●) RNA synthesis; (△ - △ , ▲ - ▲) amanitin binding.

to α-amanitin at concentrations upto 400 µg/ml, tested throughout the purification procedure. Binding experiments with ^{14}C labeled methyl-γ-amanitin, performed as described by Cochet-Meilhac et al. (19), showed, that RNA polymerase II from *A.nidulans* was unable to bind the toxin at 10 µg/ml, the highest concentration tested (fig. 8). Rat liver RNA polymerase II, tested in a similar way, was inhibited by O-[^{14}C]-methyl-γ-amanitin (50% at 18 ng/ml) and simultaneous binding of the labeled toxin could be measured (fig. 8).

The values obtained for rat liver RNA polymerase II are comparable with those of Cochet-Meilhac et al. (19). The class II RNA polymerases from lower eukaryotes are in general less sensitive to inhibition with α-amanitin (e.g. 50% inhibition of RNA polymerase II from yeast at 1 µg/ml (12) and *Agaricus bisporus* at 7 µg/ml (13) than from higher eukaryotes (50% inhibition of RNA polymerase II from mammalians and insects at 0.01-0.05 µg/ml (1) and plants at 0.1-0.3 µg/ml (3,35)), but they are all completely inhibited at 400 ug/ml of α-amanitin. Even the 50% inhibition level of α-amanitin "resistant" mutants of RNA polymerase II, is importantly lower (14,15). Although the purified RNA polymerase from *Aspergillus* is insensitive to inhibition with the toxin α-amanitin, we regard the enzyme as a real, but exceptional class II RNA polymerase.

Concluding Remarks

We conclude, that the purified DNA-dependent RNA polymerase from *A. nidulans* is an exceptional type of the class II enzymes. No crucial differences concerning chromatographic and catalytic properties or subunit composition were found, when compared to RNA polymerases of the class II from higher or lower eukaryotes. It is therefore very interesting and unexpected, that no inhibition with α-amanitin could be measured either in the crude extract or in the final purified form

even at concentrations upto 400 µg/ml of the toxin. Several wild types of *A.nidulans*, belonging to different incompatability groups, were tested in an *in vivo* ^{3}H-uridine incorporation study in the presence of α-amanitin; they also appeared to be insensitive towards the toxin α-amanitin (in preparation). The insensitivity may be a more widespread phenomenon among fungi.

It will be of interest to study the transcription of lower eukaryotes, like *A.nidulans in vitro* in S-100 cell-free transcription systems, which perhaps can be derived from protoplasts of *A.nidulans*. It may also be possible to study the purified RNA polymerase II from *Aspergillus* in a well-defined heterologous cell-free transcription system, like Hela S-100 extract supplemented with DNA as described by Weil et al. (36) and Manley et al. (37) and to compare the mechanism of gene transcription between higher and lower eukaryotes. It should be kept in mind however, that highly purified RNA polymerases may have lost specific subunits or factors necessary for bona fide transcription of specific genes.

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CHAPTER 4

Protoplasts from *Aspergillus nidulans*

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Abstract

A very effective lytic enzyme system for massive micro/macro-scale production of protoplasts from the filamentous fungus *Aspergillus nidulans* is described. A striking coincidence was observed between maximal lytic activity towards *Aspergillus* mycelium and the presence of both chitinase and α-(1 → 3)-glucanase activities.

The release of protoplasts was greatly enhanced by preincubating the mycelium with 2-deoxy-D-glucose. Furthermore, protoplast formation was influenced by fungal age, culture conditions, pH of incubation and the osmotic stabilizer used. From 40 mg of fresh mycelium, grown for 14–16 h on 1% glucose in a low phosphate-citrate medium, preincubated with 2-deoxy-D-glucose for 45 min, and then incubated with the lytic enzyme mixture at pH 6.5 in the presence of 0.3–0.4 M $(NH_4)_2SO_4$, 2.5×10^8 stable protoplasts were produced within 3 h of incubation at 30°C. Comparable results were obtained with 40–50 g of mycelium. At low osmotic stabilizer concentrations a peculiar type of regeneration was observed in the presence of the lytic enzyme system; within 12 h of incubation aberrant hyphal structures emerged from the large vacuolated protoplasts.

Introduction

The isolation and characterization of cell components of filamentous fungi has, in general, been hampered by the drastic mechanical procedures used to rupture the tough fungal cell wall. Although the liquid nitrogen procedure of Gealt *et al.* (1976) has proven that for *Aspergillus nidulans* some of these problems can be overcome, the preparation of protoplasts may provide another reasonable and useful alternative for the isolation and the study of intact organelles and their biochemical constituents, provided a simple and general procedure is available for obtaining protoplasts in large quantities. Cells and organelles obtained in this gentle way may be very useful for biochemical and metabolic studies.

Suitable lytic enzymes should be able to degrade the complex cell wall of *A. nidulans* (Bull, 1970a; Zonneveld, 1971), and this should result in a massive release of protoplasts. Since it has been shown (Villanueva and Garcia Acha, 1971) that several micro-organisms are able to induce the formation of extra-cellular lytic enzymes when grown on cell walls or semi-defined media containing substrates such as chitin, laminarin and pustulan, a great diversity of organisms has been introduced for the production of lytic enzymes. In the case of *A. nidulans*, good protoplast preparations have been obtained by Peberdy and coworkers (Peberdy and Gibson, 1971; Gibson and Peberdy, 1972; Peberdy and Buckley, 1973; Peberdy *et al.*, 1976; Peberdy and Isaac, 1976), and Ferenczy *et al.* (1975a,b) using the lytic enzymes produced by *Streptomyces venezuela* and *Trichoderma harzanium*. These micro-scale procedures were satisfactory in the study of cell wall synthesis, protoplast regeneration and cell fusion. Efforts to prepare protoplasts on a scale sufficiently great to generate large amounts of nuclei failed (Gealt *et al.*, 1976), although very recently the preparation of small amounts of DNA from protoplasts was described by Morris (1978).

In this report a method is given for the production of large amounts of protoplasts

from *A. nidulans*, based upon a combination of extra-cellular lytic enzyme extracts from *A. nidulans* in a cleistothecium-producing stage and *Oerskovia xanthineolytica* grown on *Aspergillus* cell walls. The effective release of protoplasts from *Aspergillus* mycelium is independent of strain and stage of growth.

Materials and methods

Organisms and growth conditions

A biA_1 (biotin deficient) strain of *A. nidulans* (Glasgow origin) was used in most experiments. Some experiments were performed with wild type isolates, kindly provided by Dr J. H. Croft. The fungus was maintained on complete medium (Pontecorvo *et al.*, 1953), supplemented with D(+)-biotin (40 μg/l) if necessary. For the preparation of protoplasts *Aspergillus* was grown in a shaker-incubator at 37°C on a citrate-phosphate medium containing (g/l): glucose, 10; Na-citrate, 5.9; Na-pyruvate, 1.0; $NaNO_3$, 6.0; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; and adjusted to pH 6.0 before sterilization. Cultures were inoculated with conidial suspensions to a final concentration of 10^6 conidia/ml of medium. For the preparation of cell wall fractions, and α-D-(1 → 3)-glucan according to Zonneveld (1971), *Aspergillus* was grown at 37°C for 24–30 h in a 3% (w/v) glucose-containing mineral salts medium. For the induction of lytic enzymes *Aspergillus* was grown from thick conidial suspensions on agar plates (Zonneveld, 1971) and incubated at 37°C for 5–6 days.

O. xanthineolytica (strain G-62, ATCC 27402), provided by Dr M. P. Lechevalier, was maintained at 30°C on an agar-solidified mineral salts medium in phosphate buffer (pH 7.0) (Mann *et al.*, 1972), supplemented with 0.1% (w/v) Trypton (Difco) and 0.5% (w/v) yeast extract (Difco). For the induction of lytic enzymes *Oerskovia* was grown at 28–30°C for 28 h in a liquid mineral salts medium with 0.5% (w/v) of *Aspergillus* cell walls; cells grown in a similar medium containing in addition 0.1% (w/v) Trypton were used as inoculum.

S. violaceus MR and *S. venezuela* RA, provided by Dr M. V. Elorza, were maintained and used for induction of lytic enzyme, by growing either on chitin or chitin-laminarin medium as described (Elorza *et al.*, 1966, 1969; Laborda *et al.*, 1974) or on *Aspergillus* cell wall fractions (0.5%, w/v) in a similar medium.

Bacillus circulans, kindly donated by Dr F. Rombouts, and *T. viride* G.A.G., provided by Dr J. A. Schellart, were maintained and used for induction of lytic enzymes on *Aspergillus* cell wall fractions according to published procedures (Fleet and Phaff, 1974a; de Vries and Wessels, 1972).

Preparation of lytic enzyme extracts

The *Aspergillus* lytic enzyme system was prepared according to a slightly modified procedure of Zonneveld (1972a). *Aspergillus* cultures (5–6 -day-old) on agar plates were homogenized in the cold in a Waring Blendor for 10 min at maximum speed after addition of an equal volume of 0.05 M Na-citrate-phosphate buffer, pH 6.2. The homogenate was centrifuged for 10 min at 4°C at 15,000 x *g*. The supernatant was saved and the pellet was re-extracted once more. Two volumes of cold acetone (−18°C) were added to the combined supernatants and after 2–4 h at −18°C the resulting precipitate was collected by centrifugation. The pellet was resuspended in 0.05 M citrate-phosphate buffer (pH 6.2), and dialysed at 4°C overnight versus the same buffer. After dialysis insoluble material was removed by centrifugation. The clear brown super-

natant contained the *Aspergillus* lytic enzyme system; it was either stored at −80°C or lyophilized and stored at 4°C. From 1 litre of medium at least 80 ml of a highly active and stable enzyme preparation was obtained.

The *Oerskovia* lytic enzyme system was prepared according to a modified procedure of Mann *et al.* (1972). Freshly grown *Oerskovia* was subcultured in a liquid mineral salts medium supplemented with 0.1% (w/v) Trypton and 0.4% (w/v) of *Aspergillus* cell walls. After 18–20 h of incubation at 28°C in a shaking bath (foaming should be prevented), the seed culture (30 ml) was used to inoculate 270 ml of medium containing 0.5% of *Aspergillus* cell walls without Trypton and incubated at 28°C for 28 h under careful shaking. At the end of the incubation period the *Oerskovia* cells and the residual cell wall fragments were removed by centrifugation at 4°C (30 min, 20,000 x *g*). The cleared yellowish culture medium was saturated with solid ammonium sulphate, the pH being adjusted to 7.0 with NH_4OH, and after 2 h at 0°C the resulting precipitate was collected by centrifugation. The pellets were solubilized in 0.2 M K-phosphate buffer (pH 6.5), containing 0.5 mM $CaCl_2$ and dialysed for 5–6 h at 0–4°C versus the same buffer. The dialysed material was cleared by centrifugation and either stored at −80°C or lyophilized and stored at 4°C. From 1 litre of medium 25–30 ml of highly active and stable *Oerskovia* lytic enzyme preparation was obtained.

Similar procedures were used to obtain lytic extra-cellular enzyme preparations from *Streptomyces, Bacillus* and *Trichoderma.*

Preparation of protoplasts

In general protoplasts were prepared as follows: *A. nidulans* mycelium was grown for 14–16 h at 37°C in the citrate-phosphate medium containing 1% (w/v) of glucose, harvested by filtration and washed several times with 0.2 M K-phosphate buffer (pH 6.5) containing 0.4 M $(NH_4)_2SO_4$ and 0.5 mM $CaCl_2$. The washed mycelium was suspended in the same buffer (1 g wet weight/20 ml), containing 100 µg/ml of 2-deoxy-D-glucose and incubated for 45 min at 37°C under mild agitation. The hyphae were filtered again, washed with buffer without 2-deoxy-D-glucose and resuspended in the same buffer [40–50 mg wet weight (5–6 mg dry weight)/ml]. The lytic enzyme systems were added (in general 0.1 ml each of both lytic enzyme systems per ml) and the mixture was incubated at 30°C in a shaker-incubator (130 osc/min). Release of protoplasts was followed with the light microscope by counting the total number of protoplasts using a haemocytometer.

Enzyme assays

Chitinase activity with crustacean chitin (Skujiņš *et al.*, 1970) as a substrate, was determined according to the method of Reissig *et al.* (1955); glucanase activities were determined as described (Fleet and Phaff, 1974a,b) using defined glucan substrates.

Results and discussion

In the course of our studies on the isolation of nuclei, chromatin, DNA, RNA and RNA polymerases from the filamentous fungus *A. nidulans* (Stunnenberg *et al.*, 1979), we investigated the use of different lytic enzyme systems for the large scale production of protoplasts. From the literature it was known that release of protoplasts from *Aspergillus* could be obtained by using either freeze-dried gastric juice of *Helix pomatia*

(Ferenczy *et al.*, 1976) or extra-cellular lytic enzymes as produced by *Streptomyces* sp (Peberdy and Gibson, 1971; Peberdy *et al.*, 1976), or *T. harzanium* (ex *T. viride*) (Peberdy and Isaac, 1976, Kevei and Peberdy, 1977; Morris, 1978). These lytic enzymes were induced by growth of the organisms on mineral salts medium containing *Aspergillus* cell walls or chitin-laminarin as sole carbon source. In contrast to such results, these or similar systems, including the use of the commercial enzyme preparation Driselase (Schafrick and Horgen, 1978), resulted in our experiments in relatively low yields of protoplasts after rather long incubation periods. Because it was not possible to reduce the incubation periods needed or to scale up the amounts of mycelium to be digested we investigated the use of other lytic enzyme systems.

From the work of Mann *et al.* (1972) it became evident that the actinomycete *O. xanthineolytica* was able to grow very effectively on yeast cell walls, producing a lytic enzyme system specific for yeast. When *Oerskovia* was grown on *Aspergillus* cell walls, the concentrated culture extract was more effective in producing protoplasts than the other lytic systems tested. Since the glucanase/chitinase-containing preparation Onozuka 10 R cellulase (Wakasa, 1973) was able to promote protoplast formation, extracts from agar-grown *Aspergillus*, which also produce lytic activities to supply the carbon and energy for cleistothecium development (Zonneveld, 1971), were tested. These extracts on their own, however, were found to have a poor ability to produce protoplasts, but by combining the extracts with the concentrated *Oerskovia* extract, an optimal release of protoplasts could be obtained. From 40 mg of fresh weight mycelium ($\approx$ 5 mg of dry weight) at least 2.5×10^8 protoplasts could be obtained within 3 h of incubation under our standard conditions (Figure 1). Similar results were obtained by scaling up to 40–50 g of mycelium. In general, the release of protoplasts followed an S-shaped curve (Figure 2), the lag being dependent on the state of the cells and the incubation conditions (see below). During this lag-period small, non-vacuolated protoplasts were released, while during the exponential period much larger protoplasts with large vacuoles were produced (Figure 1,B–E). On prolonged incubation they even increased to sizes of 25 μm, depending on the concentration of the osmotic stabilizer and the carbon source used in the growth medium.

Optimal production of the lytic enzyme activities produced by *Aspergillus* and *Oerskovia* was determined from combination of extracts of both cultures, harvested at different times and concentrated as described. From Figure 3 it can be concluded that optimal production of *Aspergillus* lytic enzyme extract was obtained after 5–6 days of incubation at 37°C, and that of *Oerskovia* after 28 h at 28°C. Optimal production of the *Oerskovia* lytic system was strongly promoted by using fresh and dense sub- or seed-cultures adapted to *Aspergillus* cell walls. Although *Oerskovia* was able to grow very effectively on the semi-defined media containing chitin or chitin-laminarin, these concentrated culture extracts were far from effective in producing protoplasts; α-(1 $\rightarrow$ 3)-glucan from *Aspergillus* was a poor substrate for growth.

The crude culture extracts of both micro-organisms, harvested at different times after induction, were also analysed for different enzyme activities being lytic towards amylose, laminarin, α-(1 $\rightarrow$ 3)-glucan, pustulan and chitin. As shown in Figures 4A and 4B a striking coincidence of maximal release of protoplasts and the presence of α-(1 $\rightarrow$ 3)-glucanase and chitinase activities, was observed, indicating that these enzyme activities might be rate-limiting factors in the formation of protoplasts. This observation is in good agreement with the results of Bull (1970b) and Zonneveld (1971),

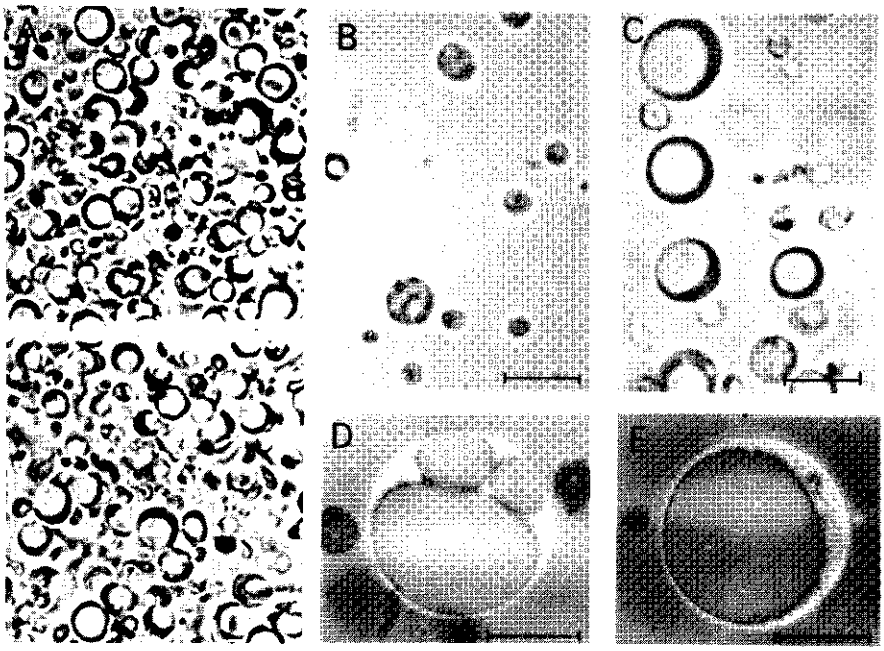

Figure 1 Protoplasts from *Aspergillus nidulans*. Fresh mycelium, grown and pretreated with 2-deoxy-D-glucose under standard conditions, was incubated at 30° C with the combined extracellular lytic enzyme systems from *Aspergillus* and *Oerskovia*. Photographs were taken at the times indicated using a Zeiss photomicroscope. Bars represent 10 μm. (A) Massive release of protoplasts after 3 h of incubation. (B) Small non-vacuolated protoplasts in the first h of incubation. (C) Vacuolated protoplasts after 2–3 h of incubation. (D–E) Large protoplasts after 3–4 h of incubation.

on the composition of the *Aspergillus* cell wall, although differences were observed in our curves relating enzyme activity and incubation time. Since the addition of commercial chitinase to lytic preparations with low chitinase activity did not promote the release of protoplasts, the possibility exists that other lytic enzyme activities might be even more essential for lysis. Due to the complexity of these lytic enzyme systems, purification of the different lytic enzyme activities and reconstitution to an active complex has not been achieved until now.

As already indicated by several authors and summarized by Peberdy (1976), release of protoplasts might be influenced by several factors such as type of mycelium used for digestion, nature and concentration of osmotic stabilizer, pH of incubation mixture, and pretreatment of mycelium. In order to determine the conditions for optimal release of protoplasts with our lytic enzyme systems some of these factors were investigated.

The state of the cells used for the production of protoplasts is most important. Maximal yields of protoplasts were obtained from homogeneous cultures in exponential growth phase under our culture conditions for this biA$_1$-strain, 14–16 h after inoculation. With older mycelia slightly lower final levels were obtained and longer incubation periods were needed due to an increase in the length of the lag period of the S-shaped release curve (cf. Figure 2). Highly branched irregularly grown hyphae,

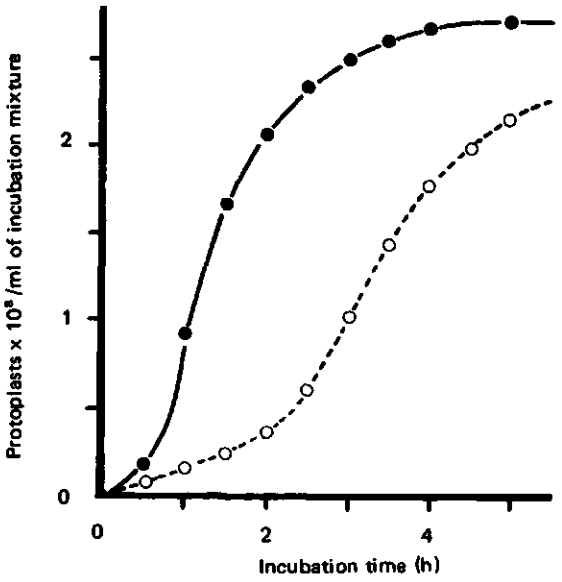

Figure 2 Effect of mycelial age on the time course of protoplast release from *Aspergillus nidulans*. Mycelium, grown on citrate-phosphate medium containing 1% (w/v) of glucose, and harvested at 16 h (●– ●) and 24 h (○– ○) after inoculation, was pretreated with 2-deoxy-D-glucose and incubated with the combined extra-cellular lytic enzyme systems under standard conditions. Release of protoplasts was monitored by light microscopy; numbers of protoplasts were counted as described.

normally obtained when old conidiospores were used for inoculation, were very insensitive. Upon testing some wild type isolates of *Aspergillus* a close correlation was found between the growth rate of the strain and the sensitivity of the mycelium of different ages, towards the lytic system, pointing to the importance of the physiological age of the cells.

The medium used to culture *Aspergillus* mycelium also had a marked effect on the release of protoplasts. By lowering the phosphate concentration and introducing a chelating agent such as citrate the yield of protoplasts could be greatly enhanced. Cells grown on glucose/mineral salts medium supplemented with yeast-extract or trypton were less susceptible. Also mycelium grown on concentrations of glucose $> 2\%$ (w/v) appeared to be less sensitive. Substituting glucose by other growth promoting carbon sources did not greatly influence the formation of protoplasts, although differences could be observed in the dimensions of the protoplasts, e.g. mycelium grown on sucrose released very large protoplasts (25 μm).

Although melanization of the cell wall has been implicated in the action of lytic enzymes on the hyphal wall (Bull, 1970a,b; Peberdy, 1976), other factors such as changes in the glucan or chitin components in the cell wall may be much more important: (1) melanization starts after complete exhaustion of the glucose in the medium (Bull, 1970a; Rowley and Pirt, 1972), and higher glucose concentrations, which prevent the formation of melanin but support the formation of α-(1 → 3)-glucan (Zonneveld, 1972b; 1974), result in a mycelium that is less susceptible to the

action of the lytic system. (2) The glucose concentration can greatly influence the relative synthesis of alkali soluble and insoluble glucan (Zonneveld, 1972b), thus drastically changing the cell wall composition. (3) The low phosphate concentration affects the amount and composition of certain wall components such as phosphomannan in yeast (San Blas and Cunningham, 1974a,b). A comparable situation might exist in *Aspergillus*. (4) The presence of a chelating agent such as citrate might cause a deficiency in trace elements resulting in a decrease in the amount of α-(1 → 3)-glucan (Zonneveld, 1972b; 1975), although this might also result in a decrease in the amount of melanin (Kuo and Alexander, 1967; Rowley and Pirt, 1972). All these observations strongly support the suggestion that a rather complex enzyme system is necessary to

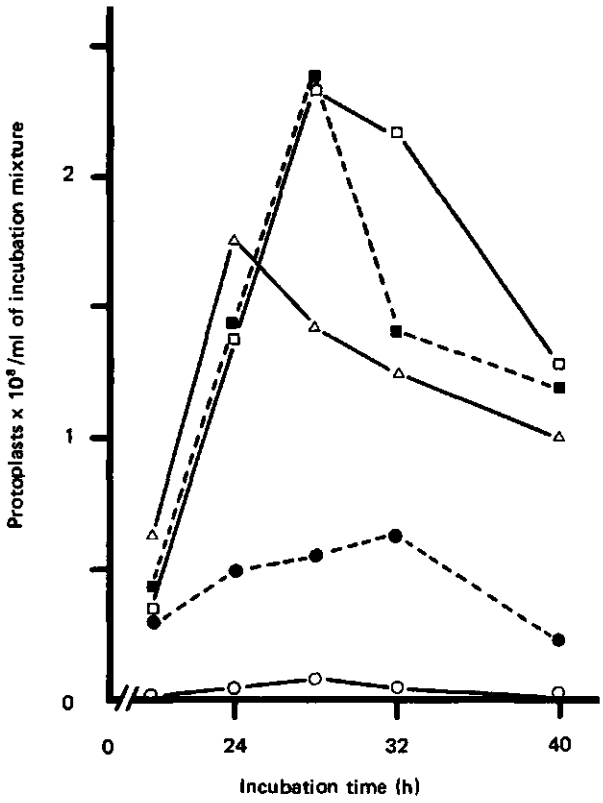

Figure 3 Production of lytic enzyme activities by cleistothecia developing *Aspergillus nidulans* and *Oerskovia xanthineolytica* grown on *Aspergillus* cell walls. At the times indicated, extracellular lytic enzymes produced by *Oerskovia* were isolated and concentrated. Combinations of these extracts with extracts from *Aspergillus* isolated at different days after inoculation were used to release protoplasts from *Aspergillus* mycelium under standard conditions. The number of protoplasts was counted after an incubation period of 3 to 3.5 h. Symbols represent extracts used from respectively 3 (○– ○), 4 (●– ●), 5 (□– □), 6 (■ – ■) and 7 (△– △) days old *Aspergillus* cultures.

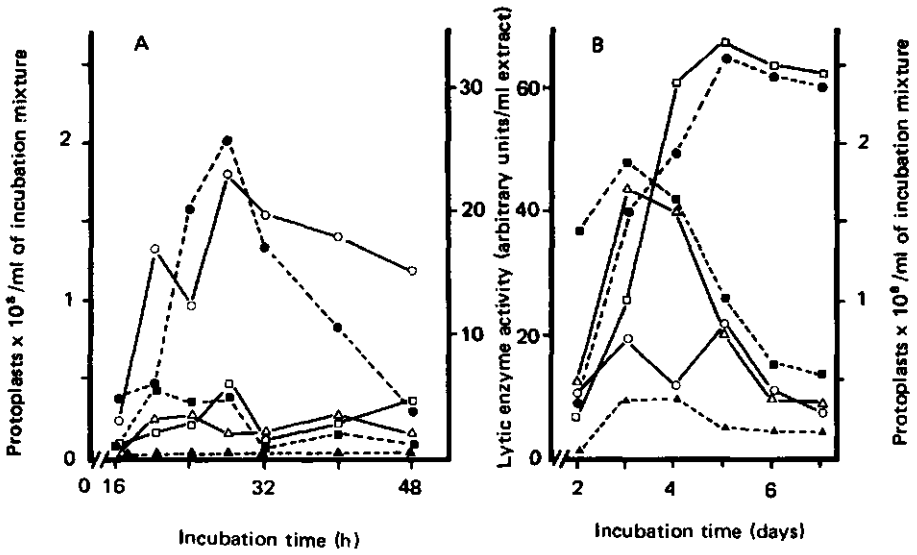

Figure 4 Relation between incubation time of extra-cellular lytic enzyme producing *Oerskovia* (Figure 4A) and *Aspergillus* (Figure 4B), and the presence of specific lytic enzyme activities in their concentrated extracellular extracts. Cultures of *Oerskovia* and *Aspergillus* were grown and the extracellular lytic enzyme activities were isolated and concentrated at the times indicated, as described in Materials and methods. Enzymatic activities lytic towards α-(1 → 3)-glucan (○—○), chitin (□—□), laminarin (△—△), amylose (■ — ■), and pustulan (▲—▲), were measured. Protoplast producing capacity of these extracts under standard conditions (●—●) was determined by combining the different *Oerskovia* extracts with a lytic extract from a 5-day-old *Aspergillus* culture (Figure 4A) and by combining the different *Aspergillus* extracts with a lytic extract from a 28-h-old *Oerskovia* culture (Figure 4B). Numbers of protoplasts were counted after 3 h of incubation.

solubilize the *Aspergillus* cell wall effectively. This is further substantiated by the fact that selective induction of extra-cellular lytic enzymes on single cell wall components does not result in a similar digestion of the cell wall.

Pretreatment of mycelium with various agents has been used with some organisms. In several cases thiol compounds or Triton X-100 were found to support protoplast release. In our system, however, preincubation of washed mycelium with 2-mercaptoethanol, 2-mercaptoethylamine, thioglycol, cystein, dithiothreitol, Triton X-100 or EDTA was not effective, sometimes being even highly inhibitory. However, preincubation with 2-deoxy-D-glucose promoted the formation of protoplasts, being maximal at 100 μg/ml of incubation mixture containing 50 mg of fresh mycelium (Figure 5). Similar promoting effects could be obtained by introducing this compound at low concentrations (5–10 μg/ml) in the culture medium during growth of the mycelium. It is clear from the studies of Zonneveld (1973) that 2-deoxy-D-glucose might interfere with the incorporation of glucose in the cell wall, and thus change the cell wall composition by decreasing the α-(1 → 3)-glucan synthesis. Therefore, the stimulating effect of a relatively short preincubation with this glucose analogue cannot only be explained in terms of making the cells more fragile (Foury and Goffeau, 1973). It is

more likely that lytic activities, endogenously derived from the fungus are activated by this preincubation. This suggestion is substantiated by the effect of 2-deoxy-D-glucose on the formation of protoplasts from agar-grown conidiospores (C. J. Bos and S. M. Slakhorst-Wandel, manuscript in preparation). It is also in agreement with the observations of Moore and Peberdy (1976) that conidia produced in liquid cultures are more sensitive to lytic enzymes than agar-slant grown conidia due to differences in their cell wall structure.

With 0.4 M $(NH_4)_2SO_4$ as osmotic stabilizer, optimal release of protoplasts was obtained within a relative wide pH range of 5.5 to 6.5 in 0.2 M K-phosphate buffer; higher and lower pH values resulted in lower yields of protoplasts probably due to inactivation of the lytic enzyme system during incubation.

Several osmotic stabilizers were tested for their ability to promote the release of stable protoplasts from *Aspergillus*. In contrast to the results of Peberdy *et al.* (1976) obtained with the *Streptomyces* extracts, in our system 0.3–0.4 M $(NH_4)_2SO_4$ gave the highest yields of stable protoplasts. Sugars or sugar alcohols were very ineffective. Although similar high yields could be obtained with 0.5 M KCl, the total number of protoplasts decreased upon prolonged incubation (Figure 6A). Lower yields of protoplasts were also obtained with NH_4Cl or NaCl and were probably due to instability.

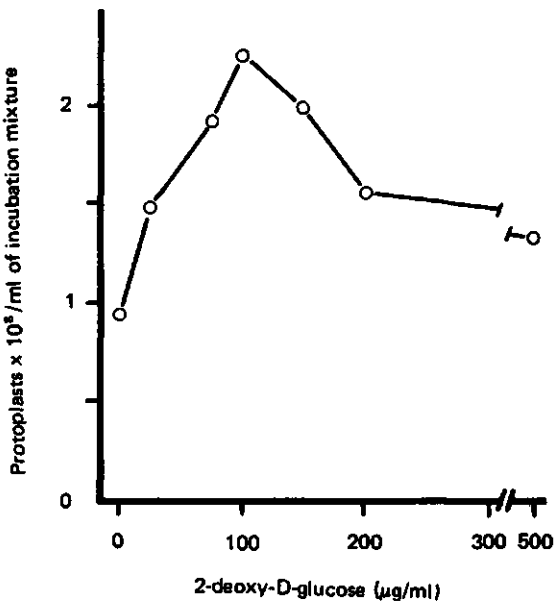

Figure 5 Effect of 2-deoxy-D-glucose pretreatment on the formation of protoplasts from *Aspergillus*. Fresh *Aspergillus* mycelium from a 16 h culture was preincubated for 45 min at 37° C with different concentrations of 2-deoxy-D-glucose. The pretreated mycelium was washed and incubated with the combined lytic enzyme system under standard conditions. Numbers of protoplasts were counted after 3 h of incubation.

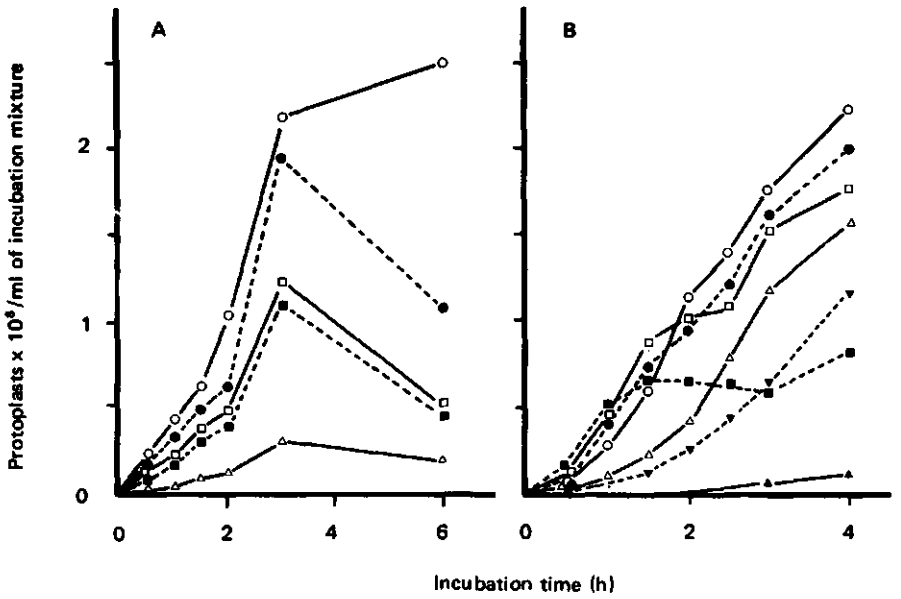

Figure 6A Effect of different osmotic stabilizers of similar osmotic potentials on the formation of protoplasts. Fresh *Aspergillus* mycelium, pretreated with 2-deoxy-D-glucose, was incubated under standard conditions in the presence of 0.4 M $(NH_4)_2SO_4$ (○–○), 0.45 M KCl (●–●), 0.47 M NH_4Cl (□–□), 0.43 M NaCl (■–■) or 0.72 M sorbitol (△–△). The formation of protoplasts was monitored with the microscope and numbers of protoplasts were counted at the times indicated.

Figure 6B Effect of different concentrations of the osmotic stabilizer $(NH_4)_2SO_4$ on the release of protoplasts. Conditions were as described in Figure 6A. Incubations were performed in 0.1 M (■–■), 0.2 M (□–□), 0.3 M (●–●), 0.4 M (○–○), 0.5 M (△–△), 0.6 M (▼–▼) and 0.8 M (▲–▲) $(NH_4)_2SO_4$ respectively.

Higher concentrations of the $(NH_4)_2SO_4$ stabilizer were strongly inhibitory. At low concentrations, i.e. 0.1–0.2 M $(NH_4)_2SO_4$, slightly larger amounts of protoplasts were released initially, but after 1.5 to 2 h of incubation a levelling off was visible (Figure 6B). In general, protoplasts formed at lower osmotic stabilizer concentrations had very large vacuoles and were larger in size than at the higher concentrations, indicating that the osmotic pressure can greatly influence the dimension and the degree of vacuolization.

At the lowest $(NH_4)_2SO_4$ concentrations it was also observed that in the presence of the lytic enzyme system regeneration of protoplasts could occur within 12 h of incubation. Increasing the $(NH_4)_2SO_4$ concentration greatly delayed this development and above 0.5 M it was even completely prevented, although sometimes clustering (fusion ?) of protoplasts could be seen (Figures 7A and 7B). The regeneration observed was completely different from that described by Peberdy and Gibson (1971). It started with the formation of a specific germ tube-like outgrowth from a protoplast followed by development of a mycelium-like structure with a dimension twice that of normal hyphae (Figures 7C–7G); no lengthening of a chain of cells was visible. Since septation in the hyphae was observed and a mycelium-like structure was obtained, the

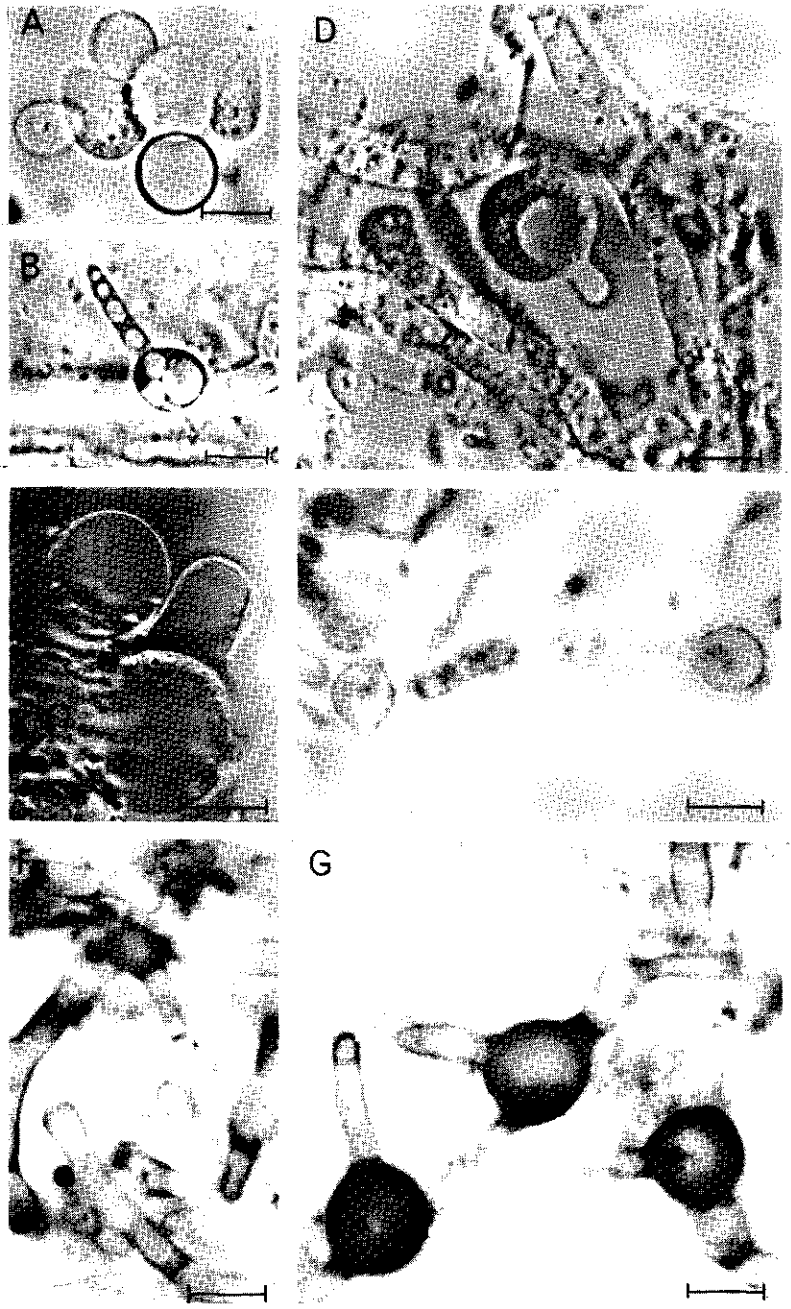

Figure 7 Regeneration of protoplasts from *Aspergillus* nidulans in the presence of the combined lytic enzyme systems. Fresh mycelium was pretreated with 2-deoxy-D-glucose and incubated at 30° C with lytic enzymes. Samples were taken at the times indicated and photographs were made with a Zeiss photomicroscope. Bars represent 10 μm. (A) Clustering of protoplasts in 0.6 M $(NH_4)_2SO_4$, t = 24 h. (B) Limited regeneration in 0.4 M $(NH_4)_2SO_4$, t = 24 h. (C) Early stage of regeneration in 0.1 M $(NH_4)_2SO_4$, t = 10–12 h. (D–G) Regenerating structures in 0.1 M $(NH_4)_2SO_4$, t = 20–24 h; in (E–G) the preparation was stained on the slide with 0.2% naphthalene blue black.

presence of the lytic system does not seem to prevent the formation of cell wall-like structures. However, due to their dimensions and their high degree of vacuolization these structures were completely different from the normal hyphae.

The procedure described above can result in large amounts of protoplasts both on micro- and macro-scale, and seems to be applicable to different stages (from conidia up to older mycelium) and different strains (cf C. J. Bos and S. M. Slakhorst-Wandel, manuscript submitted). The protoplasts obtained can be purified by differential centrifugation or filter filtration, but not by flotation. They can be used for further studies, such as uptake of labelled compounds in the study of the regulation of transcription *in vivo* and the isolation of highly polymerized (labelled) DNA and chromatin (van den Broek *et al.*, in preparation).

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CHAPTER 5

THE EFFECT OF α-AMANITIN AND ACTINOMYCIN D ON THE RNA SYNTHESIS IN PROTOPLASTS FROM *ASPERGILLUS NIDULANS*

Summary

RNA synthesis has been studied in protoplasts from *Aspergillus nidulans* , which remained metabolically active for several days under the appropriate conditions. From the results it is concluded, that at certain actinomycin D concentrations the ribosomal RNA synthesis can be selectively inhibited. However, α-amanitin at concentrations upto 400 µg/ml did neither influence the total nor the RNA polymerase II directed RNA synthesis. It was possible to discriminate between the RNA polymerase I or II directed RNA synthesis due to the presence of poly (A) tails attached to at least part of the RNA, presumably messenger RNAs or their precursors.

Introduction

Three distinct classes of nuclear DNA-dependent RNA polymerases can be purified from lower as well as higher eukaryotes (cf. Roeder, 1976). As suggested by Roeder and Rutter (1969) they can be classified by their order of elution from DEAE-Sephadex as RNA polymerase I, II and III, respectively. Later Chambon and coworkers (Kedinger et al., 1971) introduced the nomenclature RNA polymerase A, B and C, according to their sensitivity towards the toxin α-amanitin. This latter classification was based on their findings, that from animal sources, RNA polymerase A was insensitive towards α-amanitin, B highly sensitive (10-50 ng/ml for 50% inhibition) and C sensitive at high concentrations (10-25 µg/ml for 50% inhibition). Other enzyme characteristics, like salt-optimum, Mn^{2+}/Mg^{2+} activity ratio or preference for certain natural or synthetic templates, were merely used to confirm these classifications.

Subcellular distribution of the different enzyme classes and analysis of the gene transcripts (cf. Chambon, 1975) led to the assumption, that RNA polymerase I (or A) synthesizes r-RNA (Blatti et al., 1971), RNA polymerase II (or B) hn-RNA, the precursor of m-RNA (Zylber and Penman, 1971) and RNA polymerase III (or C) t-RNA and 5S RNA (Weinman and Roeder, 1974). The specificity of the distinct RNA polymerases for certain genes was recently confirmed in studies using an *in vitro* cell-free transcription system (Manley et al., 1980; Weil et al., 1979). It was demonstrated, that for example genes coding for 5S RNA were only transcribed by RNA polymerase III and not by RNA polymerase I or II (Weil et al., 1979).

The mammalian nuclear RNA polymerases are readily distinghuisable with respect to their sensitivity towards α-amanitin (Kedinger et al., 1971). Differences are observed with insects, where RNA polymerase III (or C) is insensitive to α-amanitin (Sklar et al., 1975) and with lower eukaryotes, where RNA polymerase I is sensitive at high concentrations, e.g. from yeast at 300-600 μg/ml for 50% inhibition (Huet et al., 1975), and RNA polymerase II only sensitive at higher concentrations, e.g. from yeast at 1 μg/ml (Schultz and Hall, 1976) and from the mushroom *Agaricus bisporus* at 7 μg/ml for 50% inhibition (Vaisus and Horgen, 1979). Also mutant cell-lines (e.g. CHO-line) have been developed, containing a RNA polymerase II, which is 100-800 fold less sensitive to α-amanitin inhibition than the wild type enzyme (Chan et al., 1972; Ingles, 1978). Adult mutants of *Drosophila melanogaster*, grown at concentrations of α-amanitin lethal to the wild types, contain a RNA polymerase II, which is 250-fold less sensitive than the wild type enzyme. The RNA polymerase II acitivities isolated from these α-amanitin mutants, differ in their sensitivity towards inhibition with α-amanitin, but their catalytic and chromatographic properties and their subunit composition are indistinguishable from the wild type polymerase enzyme complex (Greanleaf et al., 1979).

Although variations in the α-amanitin sensitivity of the RNA polymerases occur, it is generally accepted, that RNA polymerase II is the most sensitive class of the RNA polymerases. However, we have isolated a DNA-dependent RNA polymerase II from the fungus *A. nidulans*, which is not sensitive to α-amanitin even at concentrations upto 400 μg/ml. Nevertheless, we have concluded that it belongs unequivocally to the class II RNA polymerases, because of its chromatographic and catalytic properties and its subunit composition (Chapter 3). In this chapter, we will give additional evidence, that α-amanitin has no influence on the transcription activity of RNA polymerase II from *A. nidulans* when tested *in vivo* in protoplasts from *A. nidulans*. At selective concentrations of the antibiotic actinomycin D, the ribosomal RNA synthesis is reduced compared to the overall RNA synthesis.

Materials and Methods

Biochemicals

All chemicals used were reagent grade and in general obtained from Merck, British Drug House or Difco. Actinomycin D was purchased from Sigma, [5,6-^{3}H] -uridine (43 Ci/mmol) from Amersham, D(+)-biotin from Merck. The solutions for liquid scintillation counting (Lipoluma and Lumasolve) were purchased from Lumac; electrophoresis reagents (acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylene diamine and ammonium per-

sulphate) from Serva; Oligo(dT)-cellulose was obtained from P.L. Biochemicals Inc. α-Amanitin was a kind gift of Dr. M. Govindan (Heidelberg). Wild type isolates from *Aspergillus nidulans* were kindly provided by Dr. J.H. Croft (Leicester).

Organism and Growth conditions

A biA_1 (biotin deficient) strain of *Aspergillus nidulans* from Glasgow origin was used in most experiments; some experiments were performed with wild type isolates. Maintenance and preparation of conidial suspensions were performed as described (van den Broek et al., 1979). For preparation of the protoplasts, *Aspergillus* was grown on a citrate-phosphate medium for 12-14 h at 37 °C (van den Broek et al., 1979).

Preparation and purification of protoplasts

Mycelium of *Aspergillus* was harvested, washed and pre-incubated with 2-deoxy-D-glucose as described (van den Broek et al., 1979). The mycelium was suspended in a K-phosphate/ammonium sulphate buffer 0.2 M K-phosphate buffer pH 6.5 , 0.4 M $(NH_4)_2SO_4$, 0.5 M $CaCl_2$, the lytic enzyme systems (derived from *Oerskovia xanthineolytica* and *Aspergillus nidulans*) were added (van den Broek et al., 1979) and the mycelium suspension was incubated for 12-16 h at 30 °C.

Cell wall debris and residual mycelium were separated from the protoplasts by repeated centrifugation of the suspension through a 30% (w/v) sucrose cushion for 20 min at 600 x g (Bos and Slakhorst, in prep.).The protoplasts remained on the top of the cushion, while mycelium and cell wall debris were found at the bottom of the tube. The protoplasts were removed from the tube and diluted, if necessary, with the K-phosphate buffer to obtain a concentration of 0.5-1 x 10^8 protoplasts/ml.

Measurements of ^{3}H-UMP incorportation

The purified protoplasts (0.5-1 x 10^8/ml K-phosphate buffer) were pre-incubated for 5 hours at 30 °C in the presence or absence of either α-amanitin or actinomycin D at the concentrations indicated. Biotin (40 µg/l) was added, when the biA_1 strain of *Aspergillus* was used. After 5 hours 40 µCi/ml of [^{3}H]-uridine was added and the protoplasts were further incubated at 30 °C for the times indicated.

To determine the incorporation of radioactivity, samples (100 µl) of the incubated protoplast suspension were taken and the reaction was stopped by the addition of 5 ml of 10% (w/v) ice-cold TCA-1% (w/v) pyrophosphate. After 20 min on ice, the precipitate was collected on Whatmann 3 MM filters. The filters were washed twice with 5% TCA, three times with 1 M HCl containing 0.1 M pyrophosphate, to remove acid-soluble nuleic acids, and twice with 80% (v/v) ethanol, successively. All washings were done for 10-15 min on ice. The dried filters were treated with 0.5 ml of Lumasolve for 2 hours at 60 °C and counted after the addition of 5 ml of Lipoluma in a Kontron liquid scintillation counter

For the isolation of RNA, the incubation of protoplasts was stopped by transfering the samples into centrifuge tubes and collecting the protoplasts at 4 °C by centrifugation at 1 000 x g for 20 min. The pellets were stored at -20 °C.

Isolation and analysis of RNA

RNA was isolated from protoplasts by a modification of the method of Glisin et al. (1974). A protoplast pellet, obtained after incubation of 1 x 10^8 protoplasts as described above, was dissolved in 3 ml 50 mM Tris-HCl pH 8.2, 0.1 M NaCl and 10 mM EDTA containing 2% (w/v) para-amino-salicylate, 1% (w/v) sodium deoxycholate, 0.5% (w/v) TPNS and 2% (w/v) sarcosyl (the latter four reagents were added immediately before use). This mixture was incubated for 5 min at 60 °C. To the solution solid CsCl (2.7 g/3 ml) was added, solubilized and layered on a CsCl-containing cushion (1.0 ml of 5.7 M CsCl, 20 mM Tris-HCl pH 8.2, 0.1 M EDTA) in tubes for the SW 50.1 Beckman rotor. The tubes were centrifuged for 16 hours at 35,000 rpm and 18 °C. The supernatant was pipetted off and the pellets were dissolved in 200 µl 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The RNA was precipitated overnight at -20 °C after addition of ammonium acetate to a concentration of 0.24 M (Osterburg et al., 1975) and two volumes of ethanol. The precipitate was collected by centrifugation at 10,000 x g for 10 min, dried and solubilized in 100 µl Loening sample buffer, supplemented with 10% (w/v) Ficoll. Aliquots (10 µl) were subjected to electrophoresis according to the method of Loening (Loening and Ingle, 1967).

After electrophoresis (3-4 hours at 100 V) the gel was stained with 0.01% (w/v) toluidine blue in 40% (v/v) methanol and destained with water. For autoradiography, the gels were soaked in 1 M Na-salicylate for 1 hour

at room temperature, dried on Whatmann 3 MM paper and exposed to Kodak XRP-1 film. Fractionation of RNA into poly(A)-plus and poly(A)-minus species, by oligo(dT)-cellulose chromatography was carried out according to the method of Pemberton et al. (1975).

Results

RNA polymerase II, isolated from *A. nidulans* (Chapter 3), appeared to be insensitive towards the toxin α-amanitin when tested either in the homogenate or in the final purified form. In order to investigate, whether this *in vitro* insensitivity reflects the actual situation *in vivo* in the cell, we looked for a system to test the *in vivo* inhibition by α-amanitin.

The cell wall of *Aspergillus* is very impermeable and prevents the uptake of antibiotics, like actinomycin D (unpublished observations). It was reported by Cheung et al. (1974), that the conidia of *Neurospora crassa* were impermeable to α-amanitin and actinomycin D, but the vegetative mycelium was not. Because Aspergillus medium was impermeable to actinomycin D, we took it for granted, that α-amanitin would also not be taken up into the mycelium.

Inhibition of the RNA synthesis can be studied with isolated nuclei (Zylber and Penman, 1971; Price and Penman, 1972;Hadjiolov et al., 1974), but the results are often ambiguous, due to leakage and instability of the nuclei especially after long incubation times. This problem can be avoided by preparing protoplasts, which remain stable and metabolically active for several days at 30 ^{o}C. As described in an earlier publication (Chapter 4), large amounts of protoplasts can be prepared from *A. nidulans* rather easily (van den Broek et al., 1979). Depending on the incubation condition, the protoplasts are able to bud and form aberrant hyphae and after 24-36 hours normal hyphae (unpublished observations). These protoplasts can therefore be considered as metabolically active.

Since intact cells or protoplasts may not be freely permeable to α-amanitin, long incubation times are necessary. Therefore the freshly prepared and purified protoplasts from *Aspergillus* were pre-incubated with the inhibitors of RNA synthesis for 5 hours before addition of the labeled uridine. The RNA synthesis was measured as the incorporation of ^{3}H-UMP into TCA-insoluble material. After a short lag-time, the rate of incorporation of ^{3}H-UMP into TCA-insoluble material was constant during

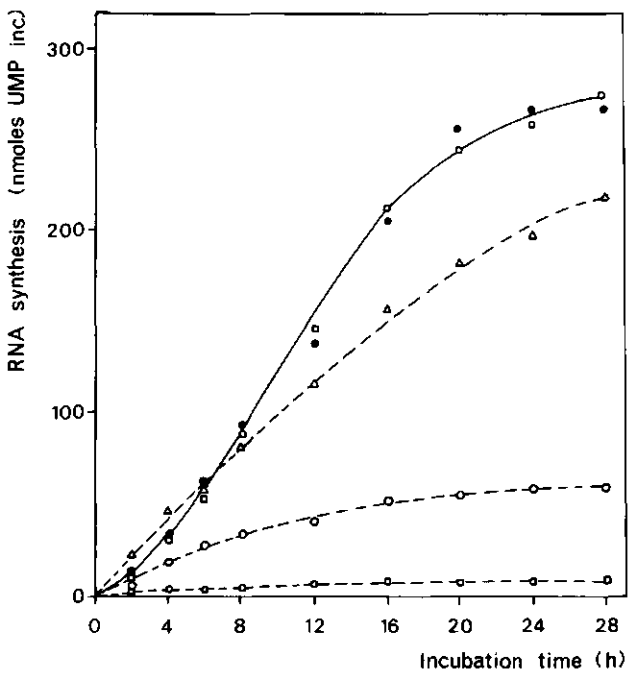

Fig. 1. *The time-course of ^{3}H-UMP incorporation into RNA of Aspergillus nidulans protoplasts.* Freshly prepared protoplasts from *Aspergillus nidulans* strain biA_1 were purified and incubated with or without either α-amanitin or actinomycin D for 5 h at 30 °C as described. Then [^{3}H]-uridine (40 µCi/ml) was added to the protoplast suspension and the incubation at 30 °C was continued. Samples (100 µl) were taken at various times and the label incorporation into RNA was determined as described.
Incorporation into RNA of protoplasts without (o - o) and with either α-amanitin at a concentration of 400 µg/ml (● - ●) or actinomycin D at a concentration of 5 µg/ml (Δ -- Δ), 15 µg/ml (o -- o) and 45 µg/ml(o -- o), respectively.

the first 12-18 hours and levelled subsequently (fig 1).

Pre-incubation of the protoplasts with actinomycin D at a concentration of 45 µg/ml resulted in a more than 90% inhibition of RNA synthesis, whereas only a partial inhibition of ^{3}H-UMP incorporation was observed at 5 and 15 µg/ml of actinomycin D (fig. 1). Incubation of the protoplasts with α-amanitin did not result in a decrease of ^{3}H-UMP incorporation into TCA-insoluble material, even at concentrations of 400 µg/ml of the toxin (fig. 1). This could be due however, to a limited contribution of the α-amanitin senstive RNA synthesis to the overall RNA synthesis (Horgen and Key, 1973; Chambon, 1975). In order to investigate this possibility, we purified the RNA from control and treated protoplasts by a modified method of Glisin et al. (1974), 12 hours after the addition of the label. The poly (A)-plus RNA was separated from the poly(A) - minus RNA by repeated chromatography on oligo(dT)-cellulose in the presence of dodecyl sulphate (Pemberton et al., 1975).

The stained polyacrylamide gel and the autoradiogram of the purified total RNA from control and α-amanitin or actinomycin D treated protoplasts are shown in fig. 2. No differences in the RNA pattern of control (lane 1) and α-amanitin treated (lane 2) protoplasts can be detected. The poly(A) minus RNA from protoplasts incubated with 400 µg/ml of α-amanitin, is shown in lane 3. The rRNA is not visibly degraded through the oligo(dT)-cellulose chromatography fractionation. The effect of increasing concentrations of actinomycin D on the incorporation of radioactivity into the RNA of the protoplasts is shown in lane 4, 5 and 6. It is interesting, that besides an overall reduction of label incorporation into the RNA with increasing amounts of actinomycin D, a marked reduction of the incorporation of radioactivity into the large ribosomal RNA component can be observed compared to the small ribosomal RNA component. It is not clear whether this reflects a more rapid degradation or a reduced synthesis of the large rRNA component compared to the small rRNA component.

Separation of the total RNA through oligo(dT)-cellulose chromatography into a poly(A) - plus and poly(A) - minus RNA fraction showed no difference in the amount of radioactivity incorporated into the poly(A) - plus RNA between control (∿ 14%) and α-amanitin treated (∿ 14%) protoplasts (Table 1). These results indicate, that under these conditions, RNA polymerase II directed RNA synthesis was not influenced by α-amanitin even at a concentration of 400 µg/ml.

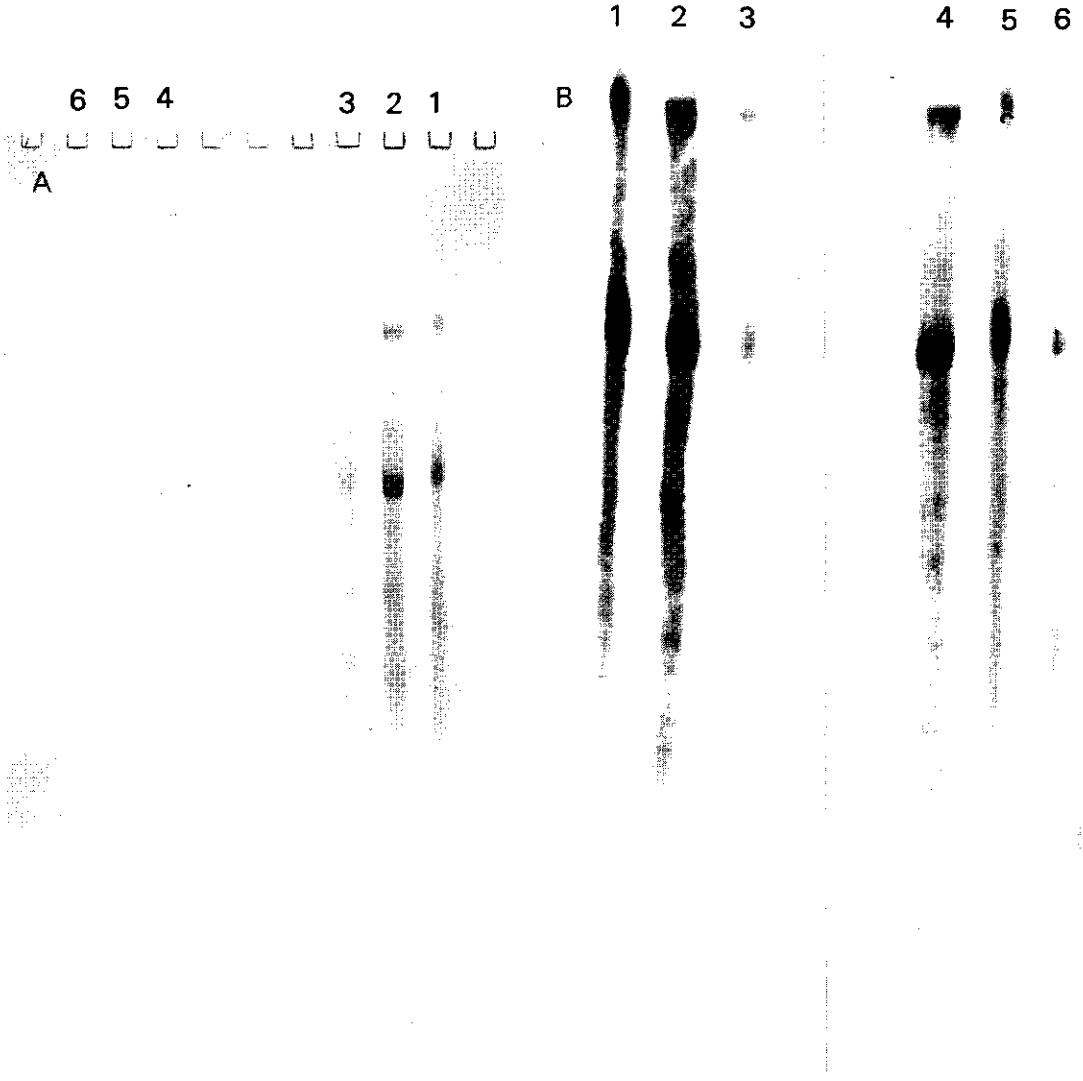

Fig. 2. *Analysis of RNA from control and either α-amanitin or actinomycin D treated protoplasts from Aspergillus nidulans.* Purified protoplasts were pre-incubated with or without either α-amanitin or actinomycin D for 5 h; the incubation was continued for 12 h at 30 °C after the addition of [^{3}H]-uridine (40 μCi/ml). The RNA was isolated from the protoplasts and samples were analyzed on a 3% polyacrylamide gel as described. The gel was stained and destained, soaked in 1 M Na-salicylate, dried and exposed to Kodak XRP-1 film.

(A): polyacrylamide gel stained with toluidine blue, (B): autoradiogram of the stained gel.

Lane (1): RNA from control protoplasts

Lane (2): RNA from protoplasts incubated with 400 μg/ml of α-amanitin

Lane (3): Poly(A)-minus RNA, separated from poly(A)plus RNA as described, from protoplasts incubated with 400 μg/ml of α-amanitin

Lane (4): RNA from protoplasts incubated with 5 μg/ml of actinomycin D

Lane (5): RNA from protoplasts incubated with 15 μg/ml of actinomycin D

Lane (6): RNA from protoplasts incubated with 45 μg/ml of actinomycin D.

A relative increase in the percentage of radioactivity incorporated into poly(A)-plus RNA, can be observed, when the protoplasts are treated with 5 or 15 µg/ml of actinomycin D (Table 1). This can be explained as a selective inhibition of rRNA synthesis by actinomycin D at these concentrations. As the RNA polymerase transcribed rRNA genes are more sensitive to actinomycin D than the RNA polymerase II transcribed (pre)-mRNA genes, it should be concluded that the (G+C) content of the ribosomal genes or part of the genes is importantly higher than the overall (G+C) content of total DNA from *Aspergillus nidulans*, which is reported to be ∿ 51% (Pontecorvo, 1967; Lopez-Peres and Turner, 1975). At 45 µg/ml of actinomycin D both the synthesis of rRNA and (pre)-mRNA is more than 90% inhibited. The actinomycin D concentration necessary to inhibit the RNA synthesis completely is 4-5 fold higher than appeared to be necessary for complete inhibition of RNA synthesis of *cowpea* mesophyl protoplasts (Rottier, 1980). In that case, more than 90% of RNA synthesis was inhibited at 10 µg/ml of actinomycin D, compared to 40-50% with *Aspergillus*, and 10 µg/ml of α-amanitin resulted in a 6% inhibition of RNA synthesis, derived from inhibition of (pre)-mRNA synthesis by RNA polymerase II.

Data on a possible inhibition of the RNA polymerase III directed RNA synthesis could not be derived from the experiments described here, because tRNA and 5S RNA are not isolated together with rRNA and mRNA by the method of Glisin et al. (1974).

Wild types of *Aspergillus nidulans* belonging to different incompatability groups (Pontecorvo et al., 1953), were also tested for their sensitivity towards inhibition with α-amanitin and actinomycin D. The results were identical to those obtained with the biA_1 strain, although differences in the rate of ^{3}H-UMP incorporation into TCA-insoluble material were observed (not shown). The insensitivity towards α-amanitin inhibition appears to be common for the different *Aspergillus nidulans* strains and is perhaps a more widespread phenomenon among fungi.

Discussion

We have shown that the RNA synthesis in protoplasts from different strains of *Aspergillus nidulans* is not inhibited by α-amanitin even at a concentration of 400 µg/ml. This *in vivo* result confirms our *in vitro*

Table 1. *The effect of α-amanitin and actinomycin D on the RNA synthesis in protoplasts from Aspergillus nidulans.*

Antibiotic	Concentration (μg/ml)	Incorporation into RNA (% of control)	Incorporation into poly(A)-plus RNA (% of total RNA)
α-amanitin	10	101	13.8
	100	96	14.1
	400	103	15.3
actinomycin D	5	74	45.4
	15	31	70.1
	45	9	18.8
control	-	100	14.1

Protoplasts were incubated with or without the inhibitor for 5 h. Then [^{3}H]-uridine (40 μCi/ml) was added and the incubation was continued for 12 h. Protoplasts were harvested, RNA was isolated and fractionated into poly(A)-plus and poly(A)-minus RNA through oligo(dT)-cellulose chromatography as described.

findings, that the purified RNA polymerase II from *Aspergillus* strain biA_1 is not inhibited at all at 400 µg/ml of α-amanitin and that the polymerase complex is unable to bind [O-^{14}C]-methyl-γ-amanitin at a concentration of the amanitin derivate of 10 µg/ml (Chapter 3). Some restrictions should be made concerning the interpretation of the *in vivo* inhibition experiments as described in this Chapter. It can not be excluded, that the protoplasts of *Aspergillus* may be impermeable to α-amanitin and not to actinomycin D. As already indicated, Cheung et al. (1974) have reported that the vegatative mycelium of *Neurospora* is permeable to both α-amanitin and actinomycin D, in spite of the presence of the cell wall. It seems unlikely, that *Aspergillus* protoplasts, lacking the cell wall, are specifically impermeable to α-amanitin. α-Amanitin inhibition experiments using fused protoplasts from *A. nidulans* and a fungus, which is known to be α-amanitin sensitive, may give the answer, whether impermeability of *Aspergillus nidulans* protoplasts to α-amanitin is causing the *in vivo* insensitivity towards this toxin.

An important result from the studies described here, is the demonstration of the presence of a poly(A)-tail attached to a part of the (newly synthesized) RNA of *Aspergillus nidulans*. Following the finding from Darnell et al. (1971) and many others, that only messengers are known to have a poly(A)-sequence (at the 3' end of the molecule), we conclude, that the poly(A)-plus RNA from *Aspergillus nidulans*, separated from the bulk RNA through oligo(dT)-cellulose chromatography, consists of mesenger RNAs or its precursor.

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CHAPTER 6 GENERAL DISCUSSION AND SUMMARY

The investigations of the biochemical organization and the regulation of transcription in filamentous fungi have been delayed by the presence of a rigid cell wall, which can only be desintegrated with severe mechanical force. Various procedures have been developed for the mechanical desintegration of the fungal cell wall, such as the French press or the X-press. These methods are often poorly reproducible and suffer from a relatively low yield in terms of release of the cell content. The method for the preparation of nuclei from *Aspergillus nidulans* developed by Gealt et al. (1976) appeared to be highly reproducible. Through blending large amounts of hyphal mass in liquid nitrogen, 50-60% of the DNA content could be released. We used a similar desintegration procedure to prepare a homogenate suitable for the purification of DNA-dependent RNA polymerases from *Aspergillus*.

It is also possible to prepare protoplasts from the filamentous fungi by an enzymatic method, which can be used to investigate DNA, RNA or protein synthesis *in vivo*. The preparation of protoplasts from yeast was first reported by Eddy & Williamson (1959) who used *Helix pomatia* digestive juice as the lytic enzymes degrading the cell wall. Several different lytic enzyme systems have been developed since, but these procedures could only be used at a micro-scale. Furthermore, protoplasts could only be released from the mycelium at a certain state of growth under very defined conditions. We have developed a more widely applicable method for the release of protoplasts from mycelium in both the exponential and stationary phase of growth. With a slightly modified procedure even protoplasts from conidiospores of *A. nidulans* can be obtained (Bos and Slakhorst, in prep.). The cell wall degrading enzymes were a mixture of enzymes produced by *A. nidulans* in the cleistothecium-producing stage and by *Oerskovia xanthineolytica* grown on *Aspergillus* cell walls. This mixture of extra-cellular lytic enzymes was able to effectively degrade the *Aspergillus* cell wall, independent of the strain used and the phase of growth (Chapter 4). The protoplasts appeared

to be metabolically active and could be used to study the RNA synthesis *in vivo* and to purify intact RNA (Chapter 5). The protoplasts can also be used for the isolation of high-molecular-weight DNA (unpublished results). It should also be possible to prepare cell-free extracts from these protoplasts, which can be used as an *in vitro* system for accurate transcription or RNA processing, in analogy to the cell-free extracts derived from mammalian cells (Weil et al., 1979; Manley et al., 1980). The procedure for the protoplast release from *Aspergillus* was however rather time-consuming and was therefore not used for the isolation of DNA-dependent RNA polymerases.

For the purification of *Aspergillus* RNA polymerases, the mycelium was desintegrated through blending the hyphal mass in liquid nitrogen. The RNA polymerase activity could be extracted from the mycelium with either a low or a high salt buffer, both resulting in a similar yield of RNA polymerase activity in the extract. Extraction of the desintegrated mycelium with a high ionic strength buffer resulted in a homogenate suitable for the purification of RNA polymerase I (Chapter 2), but not for that of RNA polymerase II. In the latter case it was necessary to extract the hyphal mass with a low ionic strength buffer instead of a high ionic strength buffer, in order to avoid instability and inactivation of the RNA polymerase II enzymes during purification (Chapter 3). The reason for this instability and inactivation has not been revealed.

Nucleic acids were removed from the homogenate upon precipitation with polymin P and selective extraction of the polymerase activity from the polymin P pellet (Zillig et al., 1970; Jendrisak & Burgess, 1975). Residual polymin P in the crude polymerase extract appeared to affect the binding of the RNA polymerases to DEAE-Sephadex and DEAE-Sepharose. As a consequence the separation of the different enzyme classes could not be achieved using these columns. The residual polymin P could not entirely be removed by precipitation of the RNA polymerases with ammonium sulphate as suggested by Valenzuela et al. (1976). Subsequent molecular sieving on Bio-Gel A-1.5 m appeared to be suitable to remove the polymin P, since it enabled correct

binding and elution of the different enzyme classes from DEAE-Sephadex and DEAE-Sepharose columns.

In Chapter 2 the purification and characterization of RNA polymerase I (or A) from *A. nidulans* is described. The chromatographic and catalytic properties of the *Aspergillus* class I enzyme are comparable with those of the class I enzymes from both lower and higher eukaryotes. The subunit composition of the *Aspergillus* RNA polymerase I resembles that of class I enzymes from higher eukaryotes. The presence of polypeptides in the range of 60 000 - 70 000 daltons, typical for the mammalian class I enzymes, distinguishes *Aspergillus* RNA polymerase I from class I enzymes from some lower eukaryotes, like yeast and *Acanthamoeba* (Table 1). Class I enzymes generally are characterized by their occurrence in two subforms, which can be separated chromatographically or by polyacrylamide gel electrophoresis under non-denaturing conditions. The mammalian class I subforms differ for only one polypeptide in the range of 60 000 - 70 000 daltons, which is present in subform I^A, but is lacking in subform I^B. The corresponding enzyme from yeast differs in two polypeptides of 48 000 and 37 000 daltons, which are lacking in the I^B subform. These subforms are usually indistinguishable with respect to the catalytic properties and the transcription specificity *in vitro*. No physiological significance, at all, could be attributed to these subforms, so far. By means of DEAE-Sephadex chromatography two subforms of *Aspergillus* RNA polymerase I could be distinguished, but no differences in polypeptide composition were observed after polyacrylamide gel electrophoresis in the presence of dodecylsulphate. Furthermore, *Aspergillus* RNA polymerase I could not be separated into two subforms through polyacrylamide gel electrophoresis under non-denaturing conditions. However, two dimensional urea-dodecylsulphate polyacrylamide gel electrophoresis revealed, that the subunit of 33 000 (*29 000**) daltons

* The number in italics represents the molecular weight of the polypeptide as reported in Chapter 2. The molecular weights have been revised in Chapter 3 and are indicated in roman.

Table 1. Subunit composition of class I RNA polymerases from various organisms

Aspergillus nidulans (1)	Yeast (2)	*Acanthamoeba* (3)	*Physarum* (4)	Soybean (5)	Mouse (MOPC) (6)
190	190	185	200	183	195
117	135	133	135	136	117
70					
69			85		61
46	48	41.5	45	50	49
33	40	37	29	46	29
32	37	35	17	40	19
19.5	27	22.5		33	
15	23	17.5		28	
	20	15.5			
	14.5	13.3			
	12.2	< 10			
	10				

The numbers indicate the molecular weights of the subunits in daltons x 10^{-3}.

(1) This thesis; (2) Sebastian, 1977; (3) Spindler et al., 1978a; (4) Smith & Braun, 1978; (5) Guilfoyle et al., 1976; (6) Roeder, 1976.

could be separated into two polypeptides, which may represent two differently charged forms of one polypeptide. A correlation between these two polypeptides of 33 000 (*29 000*) daltons and the occurrence of *Aspergillus* RNA polymerase I in two subforms seemed very likely,but could not be proved so far.

Aspergillus RNA polymerase II also contains a polypeptide of 33 000 daltons, comigrating with the respective polypeptide of RNA polymerase I. The 33 000 daltons subunit is present twice in both enzyme complexes. With various organisms,the existence of polypeptides common to the three RNA polymerases has been demonstrated through immunology and tryptic mapping (Buhler et al., 1976). It is striking, that the common subunits can be phosphorylated *in vivo* and *in vitro*, as reported by Bell et al. (1976) for the 24 000 daltons polypeptide of yeast. These authors have suggested, that the RNA polymerase activity is regulated *in vivo* through phosphorylation-dephosphorylation. We assume, that the subforms of *Aspergillus* RNA polymerase I reflect the *in vivo* active and inactive forms of the enzyme and that one subform can be converted into the other through phosphorylation-dephosphorylation of the 33 000 (*29 000*) daltons subunit. A similar regulation of the enzyme activity may occur with RNA polymerase II, which also contains a 33 000 daltons polypeptide.

The purification and characterization of *Aspergillus* RNA polymerase II is described in Chapter 3. It is demonstrated, that the purified RNA polymerase is clearly different from RNA polymerase I with respect to its chromatographic properties, its subunit composition as well as its catalytic properties, although both enzymes are insensitive to α-amanitin. Although it is not unequivocally demonstrated, that the purified RNA polymerase is not a class III enzyme, circumstantial evidence strongly indicates, that we are dealing with a class II enzyme, which is not sensitive to α-amanitin inhibition. First of all, the subunit composition of the purified RNA polymerase is characterized by the absence of polypeptides in the range of

Table 2. Subunit composition of class II and III RNA polymerases from various organisms

Class II RNA polymerases					Class III RNA polymerases			
Aspergillus nidulans (1)	Yeast (2)	*Acanthamoeba* (3)	Wheat germ (4)	Mouse (MOPC)(5)	*Acanthamoeba* (6)	Yeast (2)	*Xenopus* (7)	Mouse (MOPC)(5)
				240				
	220	193	220	205				
170	180	178		170	169	160	155	155
150	150	152	140	140	138	128	137	138
							92	89
					82	82	68	70
					52	53	52	53
33	45	38.5-40	40	41	37	41	42	49
27	32	22.5	40	29	34	40.5	33	41
24	27	18	27	27	30	37	29	33/32
19	23	15.5	25	22	28.5	34	19	29
18	17	14	21	19.5	22.5	28		19
16	14.5	13.3	20	19	17.5	24		
	12.5	12.5	17.8	16.5	15.5	20		
	9	12	17		13.3	14.5		
		< 10	16.3		< 12.0	11		
			16					
			14					

The numbers indicate the molecular weights of the subunits in daltons x 10^{-3}.

(1) This thesis; (2) Sebastian, 1977; (3)D'Allessio et al., 1979; (4) Jendrisak & Burgess, 1977; (5) Roeder, 1976; (6) Spindler et al., 1978b; (7) Sklar et al., 1975.

50 000 - 100 000 daltons, which can be regarded as characteristic for the class II enzymes. The class III enzymes, in contrast, possess two or more polypeptides in this molecular weight range(Table 2). Secondly, densitometric scans of stained polyacrylamide gels of purified RNA polymerase II show minor polypeptides larger than 180 000 daltons, which might indicate subforms of the RNA polymerase II enzyme. Thirdly, the purified RNA polymerase II elutes from a DNA-cellulose column at 80-100 mM ammonium sulphate (not shown), while class III RNA polymerases are reported to be eluted at 200-400 mM ammonium sulphate (Hager et al., 1975). An *Aspergillus* RNA polymerase activity could be detected at the elution position expected for an RNA polymerase III enzyme. The latter enzyme appears to be more active with native DNA as template than with denatured DNA, which is characteristic for a class III RNA polymerase. In contrast, RNA polymerase II is more active with denatured DNA as template than with native DNA. We therefore conclude, that the purified enzyme is a class II RNA polymerase.

The purified RNA polymerase II appeared to be insensitive to inhibition with α-amanitin, even at concentrations upto 400 µg/ml of the toxin. Furthermore, *Aspergillus* RNA polymerase II was unable to bind O-[^{14}C]-methyl-γ-amanitin at 10 µg/ml, the highest concentration tested. Additional evidence for the α-amanitin insensitivity is given in Chapter 5. In this chapter, it is demonstrated, that the RNA polymerase II directed RNA synthesis in metabolically active protoplasts from *A. nidulans* is not affected by α-amanitin at concentrations upto 400 µg/ml. It could not be excluded, however, that the protoplasts were impermeable to α-amanitin. The α-amanitin sensitivity of the class II enzymes is often regarded as the most important characteristic of the enzyme, although large differences in absolute and relative sensitivities are observed (section 1.2.5). We have concluded, that the purified α-amanitin insensitive *Aspergillus* RNA polymerase should be classified as a class II enzyme. The final evidence can only be provided from *in vitro* cell-free transcription studies as reported by Weil et al.

(1979) and Manley et al. (1980).

Incubation of *Aspergillus* protoplasts with increasing concentrations of actinomycin D showed, that the poly(A)-minus RNA synthesis could be inhibited at a concentration of the antibiotic by which the poly(A)-plus RNA synthesis remained unaffected. It seems likely, that the poly(A)-plus RNA, separated from the poly(A)-minus RNA through oligo(dT)-cellulose chromatography, represented the messenger RNAs or their precursors, in analogy to the poly(A)-tails attached to the (pre)messenger RNA species in most eukaryotes. Because at selective actinomycin D concentrations only the poly(A)-minus RNA, representing mainly ribosomal RNA, was inhibited, we have concluded, that the (G+C) content of the ribosomal genes or part of the genes should be significantly higher than the overall (G+C) content of 51% of the DNA (Lopez-Peres & Turner, 1975).

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SAMENVATTING

Omdat ontwikkeling en differentiatie van de hogere organismen zeer gecompliceerd zijn, kunnen relatief eenvoudige organismen, zoals de schimmels *Aspergillus nidulans*, gebruikt worden als modelsystemen voor de bestudering van deelprocessen. In dit proefschrift is een aanvang gemaakt met het onderzoek naar het overschrijven, de transcriptie, van de genetische informatie in deze schimmel door middel van DNA-afhankelijke RNA polymerases. Eén van de aspecten van de transcriptie-studies is het zuiveren en karakteriseren van deze RNA polymerases. Het grootste gedeelte van het onderzoek in dit proefschrift is hieraan gewijd.

In hoofdstuk 1 is een overzicht gegeven van de DNA-afhankelijke RNA polymerases, van eukaryoten. De eigenschappen van de verschillende klassen van de RNA polymerases, die gebruikt kunnen worden bij hun indeling in de klassen I, II en III, zijn beschreven. De functies van de drie RNA polymeraseklassen met betrekking tot hun specifieke transcriptie van de verschillende genen zijn aangegeven. Uit *in vitro* transcriptiestudies aan celvrije systemen kan geconcludeerd worden, dat RNA polymerase I de ribosomale genen overschrijft, RNA polymerase II de boodschapper-RNA genen en RNA polymerase III de 5S genen, de transport-RNA genen en enkele virus-geassocieerde VA-genen. De verschillende categorieën van genen blijken zeer uiteenlopend georganiseerd te zijn, waardoor de transcriptie slechts door één bepaalde RNA polymeraseklasse verzorgd kan worden. Binnen één categorie blijken de genen echter een grote mate van overeenkomst te vertonen in opbouw en organisatie, zowel binnen één organisme als tussen niet verwante organismen.

De zuivering en karakterisering van de DNA-afhankelijke RNA polymerase I van *Aspergillus* is beschreven in hoofdstuk 2. De catalytische en chromatografische eigenschappen van *Aspergillus* RNA polymerase I blijken overeen te stemmen met die van andere klasse I RNA polymerases uit lagere en hogere eukaryoten. De subunit samenstelling van het gezuiverde enzym is te vergelijken met de subunit samenstelling van RNA polymerase I uit zoogdieren, maar wijkt af van die van lagere eukaryoten, zoals gist en *Acanthamoeba*.

Klasse I RNA polymerases worden gekarakteriseerd door de twee subvormen waarin het enzym kan voorkomen.Bij zoogdieren verschillen de twee RNA polymerase I subvormen in één polypeptide met een molecuulgewicht van 60 000 - 70 000 daltons; deze is aanwezig in subvorm I^A doch afwezig in subvorm I^B. RNA polymerase I^B uit gist mist twee polypeptiden namelijk die met een molecuulgewicht van 48 000 en 37 000 daltons; deze zijn wel aanwezig in subvorm I^A. De fysiologische betekenis van deze subvormen is onduidelijk aangezien geen verschillen van betekenis in de transcriptie-specificiteit van de subvormen is waargenomen. *Aspergillus* RNA polymerase I blijkt eveneens gescheiden te kunnen worden in twee subvormen, die echter niet verschillen in subunit samenstelling. Met behulp van twee-dimensionale ureum-dodecylsulfaat gel electroforese kan de subunit van 33 000 daltons gescheiden worden in twee polypeptiden, welke mogelijk twee ladingsvormen van één polypeptide voorstellen. Een verband tussen de twee subvormen van RNA polymerase I en de twee polypeptiden van 33 000 daltons lijkt zeer waarschijnlijk, maar kon nog niet worden aangetoond. Wij veronderstellen, dat de twee subvormen van *Aspergillus* RNA polymerase I de *in vivo* actieve en inactieve vorm van het enzym vertegenwoordigen en dat de enzym-activiteit gereguleerd wordt door middel van fosforylering-defosforylering van de 33 000 daltons subunit.

In hoofdstuk 3 is de zuivering en karakterisering van de DNA-afhankelijke RNA polymerase II uit *Aspergillus* beschreven. De catalytische en chromatografische eigenschappen van het gezuiverde enzym zijn te vergelijken met die van andere klasse II RNA polymerases. De gezuiverde *Aspergillus* RNA polymerase blijkt echter niet gevoelig te zijn voor een remming door α-amanitine in concentraties tot 400 µg/ml en blijkt niet in staat te zijn 0-[^{14}C]-methyl-γ-amanitine in een concentratie van 10 µg/ml te binden. In het algemeen worden de klasse II enzymen juist gekarakteriseerd door hun grote gevoeligheid voor de remmer α-amanitine, in tegenstelling tot de enzymen uit de klasse I en III. Op grond van indirecte bewijzen is toch geconcludeerd, dat het gezuiverde enzym behoort tot de klasse II RNA polymerases en niet tot de

klasse III RNA polymerases.

- de subunit samenstelling van ons gezuiverde RNA polymerase is vergelijkbaar met klasse II enzymen uit andere organismen. Allen worden gekarakteriseerd door het ontbreken van polypeptiden in het gebied van 50 000 - 100 000 daltons. RNA polymerase III enzymen daarentegen bezitten twee of drie polypeptiden in dat gebied,
- klasse II enzymen worden tevens gekenmerkt door de aanwezigheid van twee of meer subvormen, die verschillen in het molecuulgewicht van het grootste polypeptide. Ons gezuiverde RNA polymerase heeft eveneens kleine hoeveelheden polypeptiden, die groter zijn dan 170 000 daltons. Dit kan duiden op de aanwezigheid van subvormen,
- een gedeeltelijk gezuiverd RNA polymerasemengsel uit *Aspergillus* kan gebonden worden aan een DNA-cellulose kolom en de RNA polymerase-activiteiten kunnen van deze kolom geëlueerd worden met een ammonium sulfaat gradient. Ons gezuiverde RNA polymerase wordt geëlueerd bij 80-100 mM ammonium sulfaat tegelijk met RNA polymerase I terwijl een derde RNA polymerase-activiteit bij 200-400 mM ammonium sulfaat van de kolom geëlueerd kan worden. Deze laatste RNA polymerase-activiteit prefereert natief DNA boven gedenatureerd DNA als matrijs voor de RNA-synthese, wat als kenmerkend beschouwd wordt voor een klasse III RNA polymerase. Ons gezuiverde RNA polymerase, daarentegen, prefereert gedenatureerd DNA als matrijs, evenals andere klasse II enzymen,
- de RNA polymerase II geleide RNA-synthese in *Aspergillus* protoplasten wordt niet geremd, als protoplasten geïncubeerd worden in aanwezigheid van 400 µg/ml α-amanitine. Dat de protoplasten niet doorlatend zouden zijn voor α-amanitine kon echter niet uitgesloten worden.

Het uiteindelijk bewijs, dat de gezuiverde RNA polymerase-activiteit een α-amanitine ongevoelige klasse II enzym is, kan alleen geleverd worden met behulp van *in vitro* celvrije transcriptiesystemen, zoals die recentelijk

voor eukaryoten ontwikkeld zijn.

De bereiding van protoplasten van *Aspergillus nidulans* is beschreven in hoofdstuk 4. Met behulp van lytische enzymen is het mogelijk gebleken de taaie celwand van *Aspergillus* zodanig aan te tasten, dat protoplasten vrijkomen. Een mengsel van lytische enzymen, geproduceerd door *Aspergillus nidulans* tijdens de vorming van cleistothecia en door *Oerskovia xanthineolytica* gekweekt op *Aspergillus* celwanden, blijkt in staat de celwand van *Aspergillus nidulans* af te breken, onafhankelijk van het groeistadium en van de gebruikte stam. De bereiding van protoplasten op grote schaal bleek te arbeids- en tijdsintensief te zijn om gebruikt te kunnen worden voor de zuivering van RNA polymerases, waarvoor de procedure in eerste instantie was ontwikkeld. De RNA-synthese *in vivo* kon echter goed bestudeerd worden in deze metabolisch actieve protoplasten (hoofdstuk 5).

Het effect van remmers op de RNA-synthese in protoplasten is beschreven in hoofdstuk 5.Zoals reeds aangegeven heeft α-amanitine geen enkele invloed op de RNA-synthese *in vivo*. Incubatie van *Aspergillus* protoplasten met toenemende concentraties actinomycine D resulteert in een remming van de poly(A)-min RNA-synthese bij een concentratie van het antibioticum waarbij de poly(A)-plus RNA-synthese niet beïnvloed wordt.Op basis van het criterium dat de poly(A)-staart covalent gebonden is aan de boodschapper-RNAs van de meeste eukaryoten, werd geconcludeerd, dat het poly(A)-plus RNA uit *Aspergillus* bestaat uit boodschapper RNA of hun precursors. Omdat bij bepaalde actinomycine D concentraties alleen de poly(A)-min RNA-synthese (hoofdzakelijk ribosomale RNA-synthese) werd geremd, kon hieruit geconcludeerd worden, dat het (G+C) gehalte van de ribosomale genen of delen daarvan beduidend hoger moest zijn dan het (G+C) gehalte van 51% van het totale DNA.

CURRICULUM VITAE

De auteur werd op 14 juni 1954 als Hendrik Gerard Stunnenberg te Renkum geboren. Hij behaalde in 1971 het HBS-B diploma aan het Christelijk Streeklyceum te Ede. In dat zelfde jaar werd de studie biologie aan de Landbouwhogeschool te Wageningen aangevangen. Het ingenieursexamen in de (cel)biologie werd in april 1972 afgelegd,met (biochemische) genetica als verzwaard hoofdvak en (molekulaire) virologie als 2e hoofdvak. In het kader van de praktijktijd was hij werkzaam aan het Deutsche Krebsforschungs Zentrum te Heidelberg (W-Duitsland) bij Prof.dr. C.E. Sekeris.

Op 4 april 1977 werd een begin gemaakt met een promotie-onderzoek aan de afdeling Erfelijkheidsleer van de Landbouwhogeschool onder (bege)leiding van Dr.ir. H.W.J. van den Broek en Prof.dr. A. van Kammen. Het betreffende 3-jarige projekt had als titel: "De isolatie en karakterisering van de DNA-afhankelijke RNA polymerases van de schimmel *Aspergillus nidulans*".

Vanaf april 1981 zal hij werkzaam zijn als "post-doctoral fellow" van de Europesche Molekulair Biologische Organisatie aan het Institut für Molekular Biologie van de Unversität Zürich bij Prof.dr. M.L. Birnstiel, waar hij zal werken aan de regulatie van de genexpressie in *Xenopus laevis*.